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**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1533571> since 2017-05-11T22:21:39Z

*Published version:*

DOI:10.1111/jam.12852

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(Article begins on next page)

This is the author's final version of the contribution published as:

Spadaro D. ; Bustos Lopez, M. del Pilar; Gullino, M.L.; Piano, S.; Tabacco, E.; Borreani, G.. Evolution of fungal populations in corn silage conserved under polyethylene or biodegradable films. JOURNAL OF APPLIED MICROBIOLOGY. 119 (2) pp: 510-520.  
DOI: 10.1111/jam.12852

The publisher's version is available at:

<http://doi.wiley.com/10.1111/jam.12852>

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<http://hdl.handle.net/2318/1533571>

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

2

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17 **Running Head:** Dominant fungi in corn silage

18

19 **Abstract**

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

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

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

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

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

34

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

36

## 37 **Introduction**

38

39 Food and feed losses due to mold growth and mycotoxin contamination greatly affect animal and human health, and the  
40 food safety. Whole corn silage is used throughout North America and Europe as a feed source for cattle, and it is stored  
41 in horizontal silos for long periods (Wilkinson and Toivonen 2003). Corn silage is particularly susceptible to aerobic  
42 deterioration, initiated by yeasts and molds, when exposed to oxygen or in the feed bunk (Ashbell and Weinberg 1992;  
43 Borreani and Tabacco 2010). The preservative effect against the growth of detrimental microorganisms, including  
44 fungi, is a result of anaerobiosis and acidification of ensiled forages by lactic acid-producing bacteria (Pahlow et al.  
45 2003). Therefore, if good quality silage is prepared under anaerobic conditions and oxygen is successfully excluded,  
46 further mold growth is unlikely to occur (Scudamore and Livesey 1998). Poor storage conditions during ensiling or  
47 feed-out could lead to the entry of oxygen into the silage, with undesirable dry matter (DM) and nutritional losses, and  
48 could increase the risk of the growth of toxigenic fungi (Nout et al. 1993; Cavallarin et al. 2011; Cheli et al. 2013). If  
49 airtight sealing of the silo is not obtained, air penetrates the silage, and yeasts and molds multiply, resulting in aerobic  
50 deterioration; the DM losses in the top 0.5 m can occasionally exceed 35% (Borreani et al. 2007). Moreover, the  
51 microbiological quality of the whole silage mass could be negatively affected (Borreani and Tabacco 2014).  
52 Filamentous fungi often occur in silage as ball-sized lumps or as layers at 200 to 500 mm from the surface (Nout et al.  
53 1993; Storm et al. 2010). Several efforts have been made to prevent air penetration during silage conservation, and  
54 plastic films utilized to seal the silo have shown to play a key role (Borreani and Tabacco 2014). In recent years, the  
55 standard polyethylene film has sometimes been replaced by oxygen barrier films, and these have been shown to prevent  
56 the growth of pathogenic and mycotoxigenic molds during conservation and to delay their growth after silage exposure  
57 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  
58 for cost-effective alternatives to replace petroleum-based commodity plastic films to cover silages, such as  
59 biodegradable materials with competitive mechanical properties (Borreani and Tabacco 2015). Biodegradable plastic  
60 films have proved to work well for conservation periods longer than 5 months in indoor conditions (Borreani and  
61 Tabacco 2015), but they still need to be tested in farm conditions to evaluate their ability to protect silage from the  
62 detrimental effects of molds during conservation.

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

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

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

78

## 79 **Material and Methods**

80

### 81 *Crop and Ensiling*

82

83 Two trials were carried out at the experimental farm of the University of Turin in Carmagnola (TO), Piedmont, northern  
84 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  
85 2, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) were sown on 14 May 2010 at an intended  
86 planting density of 75,000 seeds ha<sup>-1</sup>. Whole corn crops were harvested at around 3/5 and 5/6 milk-line stages for  
87 PR36B08 and Eleonora, respectively. Fresh forage was chopped using a precision forage harvester (Claas Jaguar 950  
88 equipped with an 8-row Orbis head, Claas, Harsewinkel, Germany) to a theoretical cut length of 12 mm and ensiled  
89 within 2 hours in drive-over piles (1.2 m height, 4 m width, and 6 m length), with three replications per film treatment  
90 (6 piles for each cultivar). The effects of two types of plastic sheet used to seal the silos (standard polyethylene vs. a  
91 new biodegradable film) were studied. The two sealing materials were: a) a single 200 µm thick (6 m width) black-on-  
92 white polyethylene, UV protected film (**PE**); b) a single 120 µm thick (4 m width) light green Mater-Bi® (Novamont  
93 SpA, Novara, Italy) biodegradable plastic film (**MB**). The oxygen permeability of the plastic films, determined on the  
94 basis of the American Society for Testing and Materials (ASTM) D 3985-81 standard method (ASTM, 1981), was  
95 1,196 cm<sup>3</sup> m<sup>-2</sup> per 24 h at 100 kPa at 23°C, 90% relative humidity (RH) for PE, and 500 cm<sup>3</sup> m<sup>-2</sup> per 24 h for MB. The  
96 water vapor transmission rate (WVTR) was determined on the basis of the ASTM F1249-06 standard method (ATSM,  
97 2011) and was 1.05 g m<sup>-2</sup> (for 24 h at 38°C and 90% RH) for PE and 17.4 g m<sup>-2</sup> for MB. Furthermore, the effect on the  
98 silage quality of the distance from the plastic film in the upper part of the silos was assessed in two zones: close to the

99 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  
100 the biodegradable film (MB-CF), silage far from the biodegradable film (MB-FF), silage close to the polyethylene film  
101 (PE-CF), and silage far from the polyethylene film (PE-FF). Each pile was alternatively covered with PE (three piles) or  
102 MB film (three piles), in each trial. The plastic sheets were held in place by putting gravel bags around the silage.  
103 During silo pile filling, plastic net bags (6 for each treatment) with well mixed fresh material (around 7 kg per bag)  
104 were sub-sampled (around 400 g) for pre-ensiling analyses and then the bags were closed, weighed and buried in the  
105 pile. Four bags were placed at the two depths (two for CF and two for FF) in each pile. The silos were uncovered and  
106 the bags dug from the piles after 146 d of conservation.

107 After sampling, the silages were subjected to an aerobic stability test, which involved by monitoring the temperature  
108 increases due to the microbial activity of the samples exposed to air. About three kilograms of each bag were allowed to  
109 aerobically deteriorate at room temperature ( $22 \pm 1.6^\circ\text{C}$ ) in 17 L polystyrene boxes (290 mm diameter and 260 mm  
110 height) for 14 days. A single layer of aluminum foil was placed over each box to prevent drying and dust  
111 contamination, but also to allow air penetration. The room and silage temperatures were measured hourly by a data  
112 logger. Aerobic stability was defined as the number of hours the silage remained stable before its temperature increased  
113 by  $2^\circ\text{C}$  above room temperature. The maximum temperature rise ( $^\circ\text{C}$ ) was also reported as another index of aerobic  
114 stability. The silage was sampled after 7 d and 14 d aerobic exposure in order to quantify the microbial changes in the  
115 silage during exposure to air.

116

#### 117 *Sample Preparation and Analyses*

118

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

127 The third subsample (about 300 g) was extracted as a wet sample, using a Stomacher blender (Seward Ltd.,  
128 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  $\text{H}_2\text{SO}_4$   
129 at an acid-to-sample material (fresh weight) ratio of 5:1. The water extract was immediately analysed for the nitrate

130 (NO<sub>3</sub>) content, through semi-quantitative analysis, using Merckoquant test strips (detection limit 100 mg NO<sub>3</sub>/kg of  
131 fresh matter), pH and ammonia nitrogen (NH<sub>3</sub>-N) contents, determined using a specific electrode. The water activity  
132 (aw) was measured at 25°C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA),  
133 which adopted the chilled-mirror dew point technique. An aliquot of 40 ml of silage acid extract was filtered with a  
134 0.20-µm syringe filter and used for quantification of lactic and monocarboxylic acids (acetic, propionic, and butyric),  
135 ethanol and 1,2-propanediol by HPLC (Agilent Technologies, Santa Clara, CA) coupled to a refractive index detector,  
136 on an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA) (Canale et al. 1984). The analyses were  
137 performed isocratically under the following conditions: mobile phase 0.0025M H<sub>2</sub>SO<sub>4</sub>, flow rate 0.5 ml min<sup>-1</sup>, column  
138 temperature 37°C, injection volume 100 µl. Duplicate analyses were performed for all the determined parameters. The  
139 duplicates were averaged and the six means (replicated silage bags) were considered as six observations in the statistical  
140 analysis.

141

#### 142 *Fungal isolation and morphological identification*

143

144 The fourth subsample was used for microbiological analyses. A 30-g sample was transferred into sterile  
145 homogenization bags, suspended in 270 g peptone salt solution (1 g bacteriological peptone and 9 g NaCl per liter), and  
146 homogenized in a laboratory Stomacher blender for 4 min for the microbial counts. The colony-forming units (CFU) of  
147 yeasts and molds were counted using the serial plate dilution technique with 40.0 g l<sup>-1</sup> of Yeast Extract Glucose  
148 Chloramphenicol Agar (YGC agar, DIFCO, West Molesey, UK) after incubation at 25°C for 3 d for the yeasts and for  
149 5, 7 to 14 d for the molds. Mold and yeast CFUs were counted separately. During incubation, the plates were examined  
150 daily and fungal colonies were transferred on first observation on YGC agar. The direct plating method was used to  
151 isolate molds on the YGC agar. Colonies of filamentous fungi were collected from dilutions on Petri dishes with 1 to 50  
152 CFU. Isolates growing on YGC agar were examined visually and microscopically for each silage sample.

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

157

#### 158 *Molecular identification*

159

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

165 Genomic DNA was amplified using universal primers ITS1 and ITS4 (White et al. 1990). PCR reactions were  
166 performed using a TGradient thermal cycler (Biometra, Göttingen, Germany). Each 20 µl PCR reaction contained 1 µl  
167 of DNA template (50 ng), 200 mM of deoxynucleotide triphosphate, 2 µl of 10X buffer (Taq DNA Polymerase, Qiagen,  
168 Chatsworth, CA, USA), 0.7 mM of primer, and 1.0 U Taq DNA Polymerase (Qiagen). PCR program followed: 95°C, 3  
169 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  
170 PCR products from each reaction was electrophoresed in 2.0% agarose gel and then stained with SYBR SAFE  
171 (Invitrogen, Eugene, OR, USA). Gel images were acquired using a Gel Doc 1000 System (Bio-Rad Laboratories,  
172 Hercules, CA, USA). PCR amplification products were cloned into the PCR4 TOPO vector (Invitrogen) using the  
173 TOPO TA cloning kit following the manufacturers' protocol and sequenced by Genome Express (Padova, Italy) using  
174 an ABI PRISM 3730XL DNA Sequencer. For some isolates belonging to the *Gibberella fujikuroi* species complex, the  
175 DNA was also amplified by using specific primers and sequenced in the portion of the coding region and introns of the  
176 translation elongation factor 1-alpha (TEF) gene (Geiser et al. 2004, Amatulli et al. 2012), to confirm the result of the  
177 ITS sequence.

178

#### 179 *Sequence analysis*

180

181 ITS and EF-1 $\alpha$  amplicon sequences were deposited in GenBank and comparison with available sequences was made  
182 using the BLAST program ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Multiple sequence alignment of nucleotide (nt) and amino acid  
183 (aa) sequences and identification of open reading frames were done by using the program AlignX (Vector NTI Suite V  
184 5.5, InforMax, North Bethesda, Maryland, USA) with the Clustal W algorithm (Thompson et al. 1994).

185

#### 186 *Statistical analysis*

187

188 Yeast and mold counts were log<sub>10</sub> transformed and were presented on a fresh weight basis. The chemical compositional  
189 data and microbial counts were analyzed for their statistical significance via analysis of variance, with their significance  
190 reported at a 0.05 probability level using the general linear model of the Statistical Package for Social Science (v 17.0,

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

194

## 195 **Results**

196

### 197 *Herbage at ensiling*

198

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

203

### 204 *Silage and aerobic deterioration*

205

206 The fermentative and microbiological characteristics of the silages stored under two plastic films in two silo layers  
207 (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  
208 MB film and the distance from the film affected most of the parameters, with the exception of acetic acid, nitrate,  
209 ammonia and butyric acid content, which was always below the detection limit ( $0.01 \text{ g kg}^{-1} \text{ DM}$ ). The DM content was  
210 affected by the film type and sampling zone. The pH was altered in the MB-CF silage and this led to lower lactic and  
211 acetic acid contents, higher pH and higher yeast and mold counts. On the other hand, in the inner layer MB-FF, where  
212 anaerobiosis was maintained, the pH was below 4 and the lactic and acetic acid contents were higher than in the  
213 peripheral areas, with similar values to those observed below the PE film. The yeast count in the MB-FF zone was  
214 around  $6 \log_{10} \text{ cfu g}^{-1}$  and the mold count was around  $3 \log_{10} \text{ cfu g}^{-1}$ , indicating the beginning of aerobic microbial  
215 activity, whereas both the yeast and mold counts in the PE-FF silage were around  $2 \log_{10} \text{ cfu g}^{-1}$ . The PE silages showed  
216 similar value of pH, lactic and acetic acid for the PE-CF and PE-FF zones, whereas the yeast count was higher in the  
217 PE-CF layer. Ethanol content also was influenced by the film and the sampling zone. Aerobic stability was higher in the  
218 PE silages than in MB, and higher values were observed in the FF zones.

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

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

223 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  
224 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  
225 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<sub>10</sub> cfu g<sup>-1</sup>,  
226 respectively. After 14 days aerobic exposure, the mold count exceeded 8.0 log<sub>10</sub> cfu g<sup>-1</sup>, except for the MB-CF in Trial 2  
227 (7.74 log<sub>10</sub> cfu g<sup>-1</sup> of molds).

228

### 229 *Identification of filamentous fungi*

230

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

238 The prevalent species of filamentous fungi found in the two corn forages before ensiling are reported in Figure 1. The  
239 fungal populations in the two trials were similar both in number and share of identified species. The main species were  
240 *Fusarium verticillioides* and *F proliferatum*, which accounted for 62% and 78% in Trials 1 and 2, respectively. After  
241 ensiling, both the sampling zone and the film type significantly modified the prevalent fungal population in Trials 1 and  
242 2 (Figure 2 and 3). The silage far from the films showed the presence of *Penicillium* spp., *A. fumigatus* and  
243 *Galactomyces geotrichum*, which were always present in PE-FF and MB-FF in both trials. The zones close to the films,  
244 which are more prone to air penetration due to the permeability of the films, were characterized by the dominance of *G.*  
245 *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-  
246 FF silage showed a 66% and 75% presence of *G. geotrichum* out of the isolated colonies in Trials 1 and 2, respectively.  
247 In Trial 2, the two films showed different dominant fungal population, with the MB film showing a dominance of *G.*  
248 *geotrichum* both in the CF and FF zones; while the PE film showed a more diversified population both in the CF and FF  
249 zones.

250

## 251 **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

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

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

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

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

314 *fumigatus* has frequently been isolated from silages throughout the world, both in temperate and warm climates (Cole et  
315 al. 1977; Pereyra et al. 2008; Dolci et al. 2011), particularly in silages where the microbial heat generated by organic  
316 matter degradation selected heat-tolerant species. Dolci et al. (2011) observed mold counts of 1.74 and 1.41 log<sub>10</sub> cfu g<sup>-1</sup>  
317 silage after 110 d of anaerobic conservation below PE and oxygen barrier films, and found a dominance of *A. fumigatus*  
318 after 14 days air exposure, with a mold count greater than 8 log<sub>10</sub> cfu g<sup>-1</sup> silage. In the present experiment, after 7 d air  
319 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  
320 saprophytic, thermophilic, microaerophilic, low pH growing fungus (Mansfield and Kuldau 2007), capable of growing  
321 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  
322 on aerobic deteriorating silage (Gisi 2014). *A. fumigatus* resulted to be detectable after silage opening, indicating that  
323 the anaerobic and acidic environment did not affected its presence in silage. Probably it was present since the harvesting  
324 phase, but at a very low count that was masked by other potential plant pathogenic fungi present at higher counts. This  
325 is in agreement with Dolci et al. (2011) which, with culture-independent technique, found similar results with *A.*  
326 *fumigatus* appearing only after ensiling, when a great reduction of the mold counts was observed compared to the pre-  
327 ensiling phase. The presence of the thermophilic species *A. fumigatus* is one of the main problems in silage, as many  
328 strains can produce several mycotoxins, such as gliotoxin, fumagillin, helvolic acid, verruculogen, sphingofungin and  
329 kojic acid (Kosalec and Pepeljnjak 2005; Pereyra et al. 2008) that can affect the health of dairy cattle. In addition, the  
330 fungus is a well-known human and animal pathogen that causes aspergillosis in immuno-compromised humans (dos  
331 Santos et al. 2002; Dagenais and Keller 2009).

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

336 The present work highlighted the need of coupling molecular methods to traditional plate count to identify, enumerate  
337 and monitor the evolution of the fungal population when the mold count is lower than 5 log<sub>10</sub> cfu g<sup>-1</sup> (i.e. at forage  
338 harvesting, at silo opening, and in the first phases of aerobic deterioration). Future studies will involve also a  
339 microbiome analysis approach, to include also the unculturable component of the fungal population.

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

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

350

351 **Acknowledgements.** The authors wish to thank: Sara Guerrini and Roberto Ponti (Novamont SpA, Novara, Italy) for  
352 providing the biodegradable plastic films utilized in the experiment; Mauro Gilli and Mario Gilardi (DISAFA -  
353 University of Turin, Italy) for the technical assistance in the field. This work was funded by the Regione Piemonte -  
354 POR. FESR 07-13-ASSE I.1.1 - Agroalimentare, Years 2011–2013 project “F&F BIOPACK: FEED & FOOD  
355 PACKAGING - Film biodegradabili per la sostenibilità ambientale della filiera agro- alimentare”. The authors  
356 contributed equally to the work described in this paper.

357

358 **Conflict of Interest:** No conflict of interest declared.

359

## 360 **References**

361

362 Ashbell, G., and Weinberg, Z.G. (1992) Top silage losses in horizontal silos. *Can J Eng* 34, 171-175.

363 Amatulli, M.T., Spadaro, D., Gullino, M.L., and Garibaldi, A. (2010) Molecular identification of *Fusarium* spp.  
364 associated with bakanae disease of rice in Italy and assessment of their pathogenicity. *Plant Pathol* 59, 839-844.

365 Amatulli, M.T., Spadaro, D., Gullino, M.L., and Garibaldi, A. (2012) Conventional and real-time PCR for the  
366 identification of *Fusarium fujikuroi* and *Fusarium proliferatum* from diseased rice tissues and seeds. *Eur J Plant*  
367 *Pathol* 134, 401-408.

368 ASTM (1981) ASTM Standard method D 3985-81. In Annual Book of Standards. American Society for Testing  
369 Materials. Philadelphia, PA.

370 ASTM (2011) ASTM Standard method F1249-06. Standard test method for water vapor transmission rate through  
371 plastic film and sheeting using a modulated infrared sensor. In Annual Book of Standards. American Society for  
372 Testing Materials. Philadelphia, PA.

373 AOAC International. (2005) Official Methods of Analysis. 18th ed. Gaithersburg, MD: AOAC International.

374 Auerbach, H., Oldenburg, E., and Weissbach, F. (1998) Incidence of *Penicillium roqueforti* and roquefortine C in  
375 silages. *J Sci Food Agric* 76, 565-572.

376 Borreani, G., and Tabacco, E. (2010) The relationship of silage temperature with the microbiological status of the face  
377 of corn silage bunkers. *J Dairy Sci* 93, 2620-2629.

378 Borreani, G., and Tabacco, E. (2014) Improving corn silage quality in the top layer of farm bunker silos through the use  
379 of a next generation barrier film with high impermeability to oxygen. *J Dairy Sci* 97, 2415-2426.

380 Borreani, G., and Tabacco, E. (2015) Bio-based biodegradable film to replace the standard polyethylene cover for silage  
381 conservation. *J Dairy Sci* 98, 386-394.

382 Borreani, G., Tabacco, E., and Cavallarin, L. (2007) A new oxygen barrier film reduces aerobic deterioration in farm-  
383 scale corn silage. *J Dairy Sci* 90, 4701-4706.

384 Canale, A., Valente, M.E., and Ciotti, A. (1984) Determination of volatile carboxylic acids (C1-C5) and lactic acid in  
385 aqueous acid extracts of silage by high performance liquid chromatography. *J Sci Food Agric* 35, 1178-1182.

386 Cavallarin, L., Tabacco, E., Antoniazzi, S., and Borreani, G. (2011) Aflatoxin accumulation in whole crop maize silage  
387 as a result of aerobic exposure. *J Sci Food Agric* 91, 2419-2425.

388 Cheli, F., Campagnoli, A., and Dell'Orto, V. (2013) Fungal populations and mycotoxins in silages: From occurrence to  
389 analysis. *Anim Feed Sci Technol* 183, 1-16.

390 Cole, R.J., Kirksey, J.W., Dorned, J.W., Wilson, D.M., Johnson, J. Jr., Bedell, D., Springer, J.P., Chexal, K.K., Clardy,  
391 J., and Cox, R.H. (1977) Mycotoxins produced by *Aspergillus fumigatus* isolated from silage. *Ann Nutr Alim* 31,  
392 685-691.

393 Dagenais, T.R.T., and Keller, N.P. (2009) Pathogenesis of *Aspergillus fumigatus* in invasive Aspergillosis. *Clin*  
394 *Microbiol Rev* 22, 447-465.

395 De Hoog, G., and Smith, M. (2004) Ribosomal gene phylogeny and species delimitation in *Geotrichum* and its  
396 teleomorphs. *Studies Mycol* 50, 489-515.

397 Dolci, P., Tabacco, E., Cocolin, L., and Borreani, G. (2011) Microbial dynamics during aerobic exposure of corn silage  
398 stored under oxygen barrier or polyethylene films. *Appl Environ Microbiol* 79, 7499-7507.

399 dos Santos, V.M., Dorner, J.W., and Carreira, F. (2002) Isolation and toxigenicity of *Aspergillus fumigatus* from moldy  
400 silage. *Mycopathologia* 156, 133-138.

401 Driehuis, F., and Oude Elferink, S.J.W.H. (2000) The impact of the quality of silage on animal health and food safety: a  
402 review. *Vet Quart* 22, 212-216.

403 Fink-Gremmels, J. (2008) Mycotoxins in cattle feeds and carryover to dairy milk: a review. *Food Add Contam Part A*  
404 25, 172-180.

405 Garon, D., Richard, E., Sage, L., Bouchart, V., Pottier, D., and Lebailly, P. (2006) Mycoflora and multimycotoxin  
406 detection in corn silage: experimental study. *J Agric Food Chem* 54, 3479-3484.

407 Geiser, D.M., Jimenez-Gasco, M.D., Kang, S.C., Makalowska I., Veeraraghavan N., Ward T.J., Zhang, N., Kuldau,  
408 G.A., and O'Donnell, K. (2004) FUSARIUM-ID v. 1.0: a DNA sequence database for identifying *Fusarium*. *Eur J*  
409 *Plant Pathol* 110, 473-479.

410 Gisi, U. (2014) Assessment of selection and resistance risk for demethylation inhibitor fungicides in *Aspergillus*  
411 *fumigatus* in agriculture and medicine: a critical review. *Pest Manag Sci* 70, 352-364.

412 Jensen, H., Aalbaek, B., Basse, A., and Shoenheyder, H. (1992) The occurrence of fungi in bovine tissues in relation to  
413 portals of entry and environmental factors. *J Comp Pathol* 107, 127-140.

414 Kosalec, I., and Pepeljnjak, S. (2005) Mycotoxigenicity of clinical and environmental *Aspergillus fumigatus* and *A.*  
415 *flavus* isolates. *Acta Pharm* 55, 365-375.

416 Lepom, P., Baath, H., and Knabe, O. (1988) Occurrence of *Fusarium* species and their mycotoxins in maize. 3. The  
417 influence of silaging on the zearalenone content of CCM maize. *Arch Anim Nutr* 38, 817-823.

418 Mansfield, M.A., and Kuldau, G.A. (2007) Microbiological and molecular determination of mycobiota in fresh and  
419 ensiled maize silage. *Mycologia* 99, 269-278.

420 Marcellino, N., Beuvier, E., Grappin, R., Guéguen, M., and Benson, D.R. (2001) Diversity of *Geotrichum candidum*  
421 strains isolated from traditional cheesemaking fabrications in France. *Appl Environ Microbiol* 67, 4752-4759.

422 Marin, S., Sanchis, V., and Magan, N. (1995) Water activity, temperature, and pH effects on growth of *Fusarium*  
423 *moniliforme* and *Fusarium proliferatum* isolates from maize. *Can J Microbiol* 41, 1063-1070.

424 Matic, S., Spadaro, D., Prella, A., Gullino, M.L., and Garibaldi, A. (2013) Light affects fumonisin production in strains  
425 of *Fusarium fujikuroi*, *Fusarium proliferatum*, and *Fusarium verticillioides* isolated from rice. *Int J Food Microbiol*  
426 166, 515-523.

427 Middelhoven, W.J., and van Baalen, A.H.M. (1988) Development of the yeast flora of whole-crop maize during  
428 ensiling and during subsequent aerobiosis. *J Sci Food Agric* 42, 199-207.

429 Munkvold, G.P., McGee, D.C., and Carlton, W.M. (1997) Importance of different pathways for maize kernel infection  
430 by *Fusarium moniliforme*. *Phytopathology* 87, 209-217.

431 Nout, M.J.R., Bouwmeester, H.M., Haaksma, J., and Vandijk, H. (1993) Fungal growth in silages of sugar-beet press  
432 pulp and maize. *J Agric Sci* 121, 323-326.

433 Pahlow, G., Muck, R.E., Driehuis, F., Oude Elferink, S.J.W.H., and Spoelstra, S.F. (2003) Microbiology of ensiling. In  
434 *Silage Science and Technology*. Vol. 42. eds Buxton, D.R., Muck, R.E. and Harrison J.H. (ed.) pp. 31-93. Madison,  
435 WI: ASA, CSSA, SSSA.

- 436 Pereyra, C.M., Alonso, V.A., Rosa, C.A.R., Chiacchiera, S.M., Dalcero, A.M., and Cavaglieri, L.R. (2008) Gliotoxin  
437 natural incidence and toxigenicity of *Aspergillus fumigatus* isolated from corn silage and ready dairy cattle feed.  
438 *World Mycotoxin J* 1, 457-462.
- 439 Richard, E., Heutte, N., Bouchart, V., and Garon, D. (2009) Evaluation of fungal contamination and mycotoxin  
440 production in maize silage. *Anim Feed Sci Technol* 148, 309-320.
- 441 Robertson, J.B., and Van Soest, P.J. (1981) The detergent system of analysis and its application to human foods. In *The*  
442 *Analysis of Dietary Fiber in Food* eds James, W.P.T. and Theander, O. pp. 123–158. New York, NY: Marcel  
443 Dekker.
- 444 Scudamore, K.A., and Livesey, C.T. (1998) Occurrence and significance of mycotoxins in forage crops and silage: a  
445 review. *J Sci Food Agric* 77, 1-17.
- 446 Shimshoni, J.A., Cuneah, O., Sulyok, M., Krska, R., Galon, N., Sharir, B., and Shlosberg, A. (2013) Mycotoxins in corn  
447 and wheat silage in Israel. *Food Add Contam: Part A* 30, 1614-1625.
- 448 Stevenson, D.M., Muck, R.E., Shinnars, K.J., and Weimer, P.J. (2006) Use of real time PCR to determine population  
449 profiles of individual species of lactic acid bacteria in alfalfa silage and stored corn stover. *Appl Microbiol*  
450 *Biotechnol* 71, 329-338.
- 451 Storm, I.M.L.D., Kristensen, N.B., Raun, B.M.L., Smedsgaard, J., and Thrane, U. (2010) Dynamics in the microbiology  
452 of maize silage during whole-season storage. *J Appl Microbiol* 109, 1017-1026.
- 453 Storm, I.M.L.D., Sørensen, J.L., Rasmussen, R.R., Nielsen, K.F., and Thrane, U. (2008) Mycotoxins in silage. *Stewart*  
454 *Postharv Rev* 4, 1-12.
- 455 Teller, R.S., Schmidt, R.J., Whitlow, L.W., and Kung, L.Jr. (2012) Effect of physical damage to ears of corn before  
456 harvest and treatment with various additives on the concentration of mycotoxins, silage fermentation, and aerobic  
457 stability of corn silage. *J Dairy Sci* 95, 1428-1436.
- 458 Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: Improving the sensitivity of progressive  
459 multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice.  
460 *Nucleic Acids Res* 22, 4673-4680.
- 461 White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990) Amplification and direct sequencing of fungi ribosomal RNA  
462 genes for phylogenetics, in: Innis MA, Gelfand, D.H., Sninsky, J.J., and White, T.J. (Eds.), PCR Protocols. A Guide  
463 to Methods and Applications. Academic Press, San Diego, USA, pp. 315–322.
- 464 Wicklow, D.T., Roth, S., Deyrup, S.T., and Gloer, J.B. (2005) A protective endophyte of maize: *Acremonium zeae*  
465 antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*. *Mycol Res* 109, 610-618.

- 466 Wilkinson, J.M., and Toivonen, M.I. (2003) World Silage: a survey of forage conservation around the world. Lincoln,  
467 UK: Chalcombe Publications.
- 468 Woolford, M.K. (1990) The detrimental effect of air on silage. *J Appl Bacteriol.* 68, 101-116.
- 469

470 **Figure legends**

471

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

475

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

481

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

487

488 **Tables**

489  
490 **Table 1.** Chemical and microbiological characteristics of the herbage prior to ensiling.

491

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

492

493 \* Values represent the average of three replications and the standard deviation. ADF = acid detergent fiber; CP = crude  
494 protein; DM = dry matter; NDF = neutral detergent fiber; NH<sub>3</sub>-N = ammonia nitrogen.

495

496 **Table 2.** Fermentation and microbiological characteristics of the silages produced from corn cv PR36B08 (Trial 1)  
 497 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)  
 498 after 146 days of conservation.  
 499

Parameters*	PE		MB		SEM	F	Z	FxZ
	PE-CF	PE-FF	MB-CF	MB-FF				
DM, g kg <sup>-1</sup>	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 <sub>w</sub>	0.988	0.989	0.991	0.988	0.0004	0.031	0.035	<0.001
Lactic acid, g kg <sup>-1</sup> DM	50.3	59.2	6.6	45.6	5.58	<0.001	<0.001	0.006
Acetic acid, g kg <sup>-1</sup> DM	15.2	21.2	10.7	29.4	2.07	0.419	<0.001	0.014
Butyric acid, g kg <sup>-1</sup> DM	< 0.01	< 0.01	< 0.01	< 0.01	-	-	-	-
Propionic acid, g kg <sup>-1</sup> DM	3.01	< 0.01	1.01	2.60	-	-	-	-
1,2-Propanediol, g kg <sup>-1</sup> DM	0.62	1.33	< 0.01	0.33	0.137	<0.001	0.001	0.118
Ethanol, g kg <sup>-1</sup> 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 <sup>-1</sup> silage	0	86	165	85	21.7	0.036	0.940	0.035
NH <sub>3</sub> -N, g kg <sup>-1</sup> DM	6.59	6.57	7.04	7.32	0.47	0.571	0.901	0.887
Yeast, log <sub>10</sub> cfu g <sup>-1</sup> silage	4.48	2.74	7.55	5.99	0.479	<0.001	<0.001	0.664
Mold, log <sub>10</sub> cfu g <sup>-1</sup> 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

500  
 501 \* a<sub>w</sub> = water activity; LAB = lactic acid bacteria; NH<sub>3</sub>-N = ammonia nitrogen; PE = commercial plastic film with  
 502 polyethylene; F = film type; Z = sampling zone; FxZ= interaction between film and zone effects; MB-CF = zone close  
 503 to biodegradable film; film; MB-FF = zone far from biodegradable film; PE-CF = zone close to polyethylene film; PE-  
 504 FF = zone far from polyethylene film.  
 505

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		SEM	F	Z	FxZ
	PE-CF	PE-FF	MB-CF	MB-FF				
DM, g kg <sup>-1</sup> 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 <sub>w</sub>	0.991	0.989	0.994	0.990	0.0005	<0.001	<0.001	0.002
Lactic acid, g kg <sup>-1</sup> DM	48.1	69.3	20.4	61.4	5.46	0.010	<0.001	0.117
Acetic acid, g kg <sup>-1</sup> DM	16.8	24.9	8.9	29.8	2.30	0.524	<0.001	0.018
Butyric acid, g kg <sup>-1</sup> DM	< 0.01	< 0.01	< 0.01	< 0.01	-	-	-	-
Propionic acid, g kg <sup>-1</sup> DM	3.13	< 0.01	2.55	0.75	0.390	0.854	< 0.001	0.178
1,2-Propanediol, g kg <sup>-1</sup> DM	0.40	1.77	< 0.01	0.46	0.176	<0.001	<0.001	<0.001
Ethanol, g kg <sup>-1</sup> 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 <sup>-1</sup> silage	979	1414	1219	1815	160	0.324	0.124	0.801
NH <sub>3</sub> -N, g kg <sup>-1</sup> DM	8.00	7.43	5.65	7.15	0.44	0.148	0.596	0.247
Yeast, log <sub>10</sub> cfu g <sup>-1</sup> silage	6.58	5.11	7.61	5.03	0.351	0.343	0.001	0.266
Mold, log <sub>10</sub> cfu g <sup>-1</sup> 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<sub>w</sub> = water activity; LAB = lactic acid bacteria; NH<sub>3</sub>-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

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

518

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 <sub>10</sub> cfu g <sup>-1</sup> silage)	8.02	7.25	6.80	5.53	0.254	<0.001	0.001	0.302
Mold (log <sub>10</sub> cfu g <sup>-1</sup> 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 <sub>10</sub> cfu g <sup>-1</sup> silage)	7.81	6.69	6.94	6.99	0.158	0.290	0.057	0.040
Mold (log <sub>10</sub> cfu g <sup>-1</sup> 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

519

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

523

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

526

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 <sub>10</sub> cfu g <sup>-1</sup> silage)	7.70	6.10	5.47	6.75	0.253	0.024	0.611	<0.001
Mold (log <sub>10</sub> cfu g <sup>-1</sup> 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 <sub>10</sub> cfu g <sup>-1</sup> silage)	7.19	7.00	7.08	6.54	0.204	0.525	0.413	0.687
Mold (log <sub>10</sub> cfu g <sup>-1</sup> 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

527

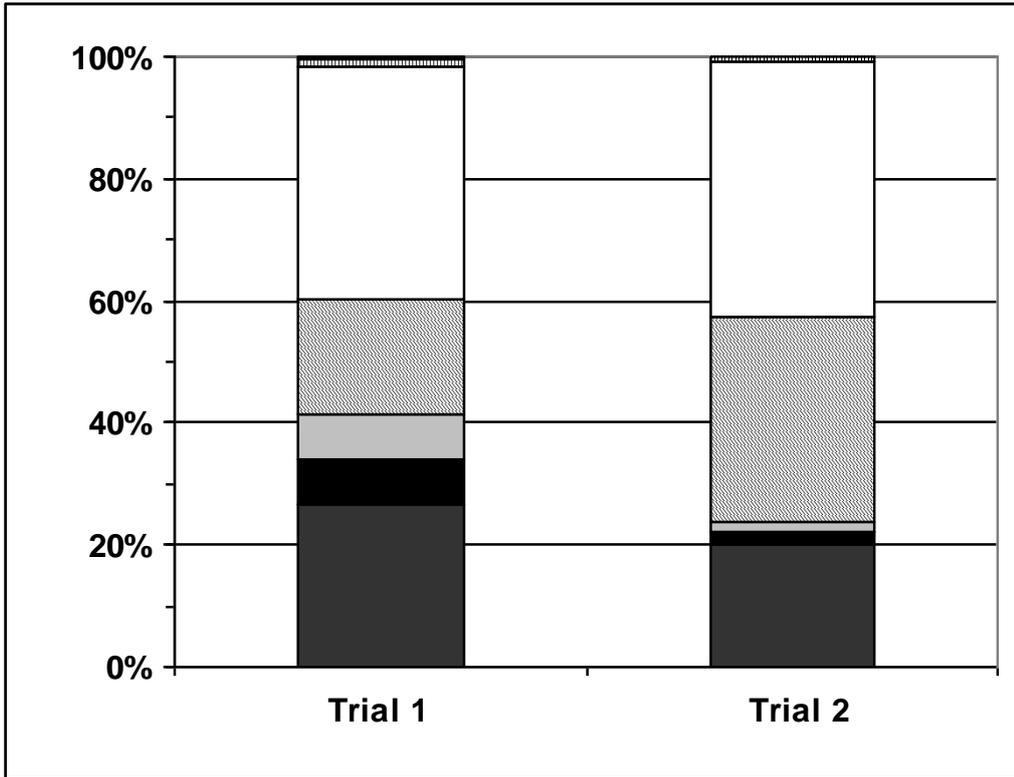
528 \* F = film type; FxZ= interaction between film and zone effects; MB-CF = zone close to biodegradable film; film; MB-  
 529 FF = zone far from biodegradable film; PE-CF = zone close to polyethylene film; PE-FF = zone far from polyethylene  
 530 film; Z = zone of sampling.

531

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
<i>Acremonium fusidioides</i>	X		
<i>Acremonium implicatum</i>	X		
<i>Acremonium strictum</i>	X		
<i>Acremonium zeae</i>	X		
<i>Alternaria alternata</i>	X		
<i>Aspergillus fumigatus</i>		X	X
<i>Cladosporium</i> spp.	X		
<i>Dipodascus australiensis</i>		X	X
<i>Epicoccum nigrum</i>	X		
<i>Fusarium proliferatum</i>	X		
<i>Fusarium verticillioides</i>	X		
<i>Galactomyces geotrichum</i>		X	X
<i>Mucor cyrcinelloides</i>		X	X
<i>Penicillium brevicompactum</i>	X		
<i>Penicillium roqueforti</i>		X	
<i>Penicillium expansum</i>		X	
<i>Penicillium funiculosum</i>		X	
<i>Penicillium resedanum</i>		X	
<i>Ramularia coccinea</i>	X		
Total fungal species	11	8	4

535  
 536

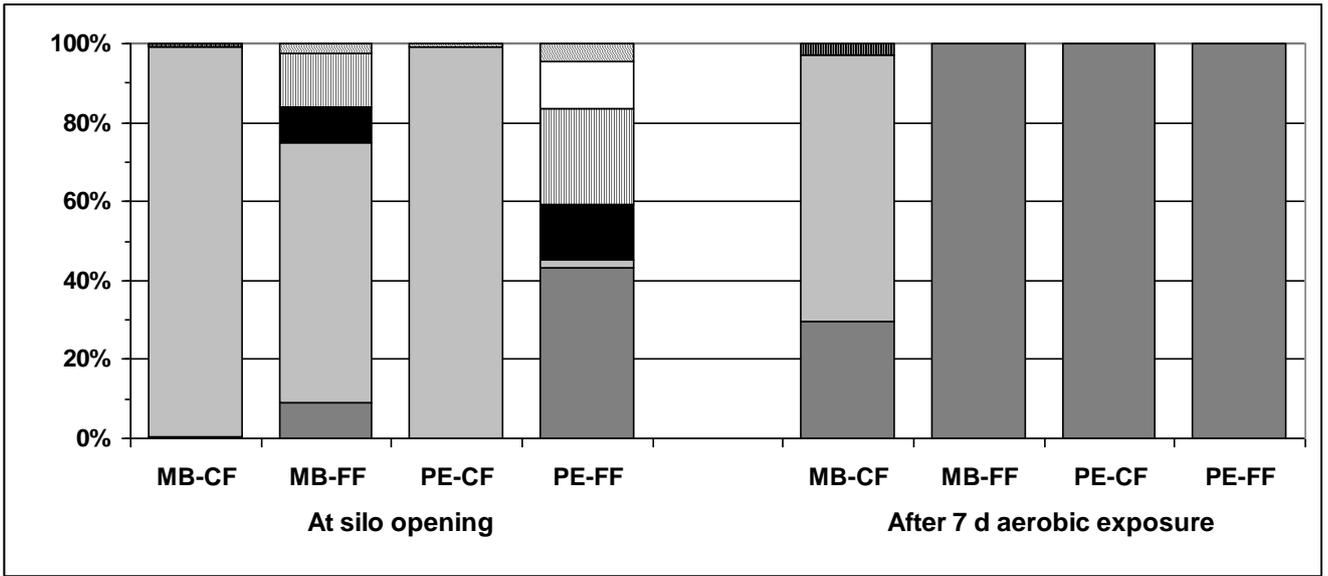


537

538

539 **Figure 1.**

540



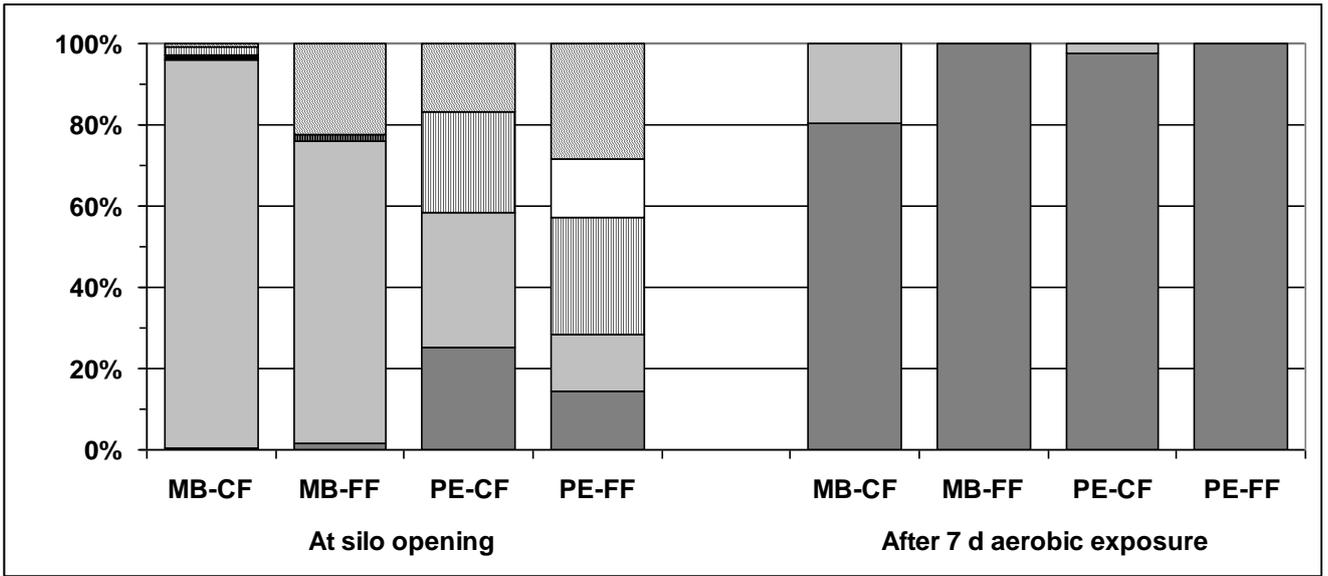
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544 **Figure 2.**

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546

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548

549 **Figure 3.**

550

551 **Supplementary material**

552

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

555

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

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