Effects of a prothioconazole- and tebuconazole-based fungicide on the yield, silage characteristics, and fungal mycobiota of corn harvested and conserved as whole-crop and high-moisture ear silages

Effects of a prothioconazole and tebuconazole-based fungicide on the yield, silage characteristics and fungal mycobiota of corn harvested and conserved as whole-crop and high-moisture ear silages

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Running Head: Mycobiome of corn silages

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ABSTRACT

Fungicides are commonly used to reduce the negative effects of fungal contamination on corn yield. This study analyzed the effect of prothioconazole and tebuconazole-based fungicide on the yield and silage characteristics of whole-crop (WCC) and high-moisture ear (HMC) silages and on the fungal community dynamics from the harvest to aerobic exposure. Two experiments were conducted on corn harvested as whole corn silage and or

as high-moisture corn. Half of the plots were untreated (NT) and the other half treated (T) with a prothioconazole and tebuconazole-based fungicide. Fresh herbage was ensiled and opened after 60 and 160 days of conservation. The silages were subjected to an aerobic stability test and sampled after 7 and 14 days of air exposure. The silages were analyzed for their chemical composition, fermentation profile, microbial counts and fungal mycobiota. The fungicide increased the yield per hectare in both WCC and HMC. However, the treatment did not affect the nutritional characteristics of WCC or HMC. The chemical, fermentative and microbial characteristics, DM losses and aerobic stability were affected by the conservation time, regardless of the treatment with fungicide in both WCC and HMC. *Fusarium*, *Alternaria*, *Aspergillus*, and *Penicillium* genera were identified as dominant before ensiling, but *Aspergillus* and *Penicillium* became dominant after silo opening and aerobic exposure. Yeast population during ensiling and aerobic deterioration resulted in a simplification, with *Pichia* and *Kazachstania* genera being dominant. The application of fungicide improved the DM, starch and NEL yield per ha but did not affect the silage quality.

IMPORTANCE

Fungi play a role as spoiling agents both before the harvesting of corn and during silage conservation and utilization. The adverse effects of fungal infestation include a reduction in the feed value and palatability, and a negative effect on human and animal health because of the risk of allergenic reactions and mycotoxin production. In order to avoid fungal contamination, fungicides are commonly used in agriculture during the cropping season. The aims of this study have been to analyze the effects of a prothioconazole and

tebuconazole-based fungicide on the yield and the chemical and microbial quality of whole-crop and high-moisture corn ear silages. Application of the fungicide improved the DM, starch and NEL yield per ha, but did not affect the nutritional composition of the plant. The mold counts and mold population were not affected by the fungicide application. A dominance of *Aspergillus* and *Penicillium* genera was observed during conservation and aerobic exposure.

KEYWORDS

Next Generation Sequencing, silage, mold, yeast, molecular identification.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most frequently cultivated crops and is usually harvested as a whole plant and conserved as silage, because it presents optimal characteristics for direct ensiling. The energy component of the feeding ration affects the feeding costs to a great extent, and thus the whole corn harvest has progressively been delayed to an advanced stage of maturity to maximize the starch production per hectare (1, 2). The late harvesting of corn leads to silages with a dry matter (DM) content of up to 40%, which in turn leads to a progressive worsening of the ensilability characteristics and a higher risk of aerobic deterioration during feed-out (3). High-moisture ear corn (HMC) silage has also become widespread in feeding rations as an alternative to feeding dry grain, due to its higher starch digestibility (4). However, the low moisture content and concentration of fermentable sugars in HMC limits the extent of the fermentation, thus resulting in silages prone to aerobic deterioration caused by yeast and mold (5).

The corn crop represents a habitat for many microorganisms, including fungi, which perform important ecological functions and can be both beneficial and harmful to their host plant (6, 7). Fungi play a spoiling agent role both before the corn harvest and during silage conservation and utilization. During crop growth, fungi attack plant cells, release toxins that kill the plant tissue to provide nutrients for their growth and, under stressful conditions, can produce mycotoxins (8, 9). Furthermore, unwanted fungal contamination can determine an increase in fiber and a decrease in digestibility of the corn plant (9).

The mold population in silage comes from the field, even though an additional fungal contamination can occur from the soil and ambient during harvesting and chopping (10, 11). Several fungi are able to grow on corn plants, but only a few can adapt to ensiling

conditions, and almost all of them are killed by anaerobiosis or greatly reduced in load (12, 13). The presence of molds in silage is linked to the infiltration of air into the mass during conservation. During storage, this is usually observed only in the peripheral layers of bunker silages in direct contact with the covers, but the whole silage can undergo aerobic deterioration and become moldy during the feed-out phase (14). The mold activity during aerobic deterioration follows the resurgence of yeast activity that had consumed some inhibiting factors such as organic acid, thereby increasing the pH of the silage (15). The adverse effects of the fungal infestation of silage include a reduction in feed value and palatability of the silage, and a negative effect on human and animal health because of the risk of allergenic reactions and mycotoxin production (16, 17).

Fungicides are commonly used in conventional agriculture to control fungal diseases (18), but also to improve growth efficiency and stress tolerance in order to obtain a crop yield enhancement (19). Furthermore, the use of foliar fungicides may be beneficial for the nutritional quality of a plant, as fungal contamination may decrease the *in vitro* neutral detergent fiber (NDF) digestibility when ensiled as corn silage (20, 21). Many fungicides with different biochemical targets and activity spectra are available for the control of corn diseases, and they are often combined in mixtures (22). One of the most widespread fungicides is the group of demethylation inhibitor fungicides (DMI), which are also known as azoles. Sterol biosynthesis inhibitors, and prothioconazole and tebuconazole in particular, inhibit fungal growth by acting on the biosynthesis of sterols, which are essential for the maintenance of cell membrane integrity (23, 24). Several works have reported that corn silages treated with fungicides had improved fermentation profiles during the ensiling period as well as improved feed efficiency (21). Nair et al. (25) reported that the application

of a foliar fungicide changed the chemical composition of barley, although it appeared to have little impact on the fungal community of barley silage.

The fungal population of corn is composed of a complex mixture of genera with colony-forming units ranging from 10^4 to 10^9 cfu/g (15, 13). The mold species that have regularly been isolated from silage at harvest belong to the *Penicillium, Fusarium, Aspergillus, Mucor, Geotrichum, Monascus,* and *Trichoderma* genera (13, 26). Once the ensiling process begins, the diversity of the community decreases because of oxygen exclusion and a low pH, with the main fungal genera being *Aspergillus* and *Penicillium* (13). Under these conditions, many fungi can enter into a viable but unculturable state that precludes their complete characterization through traditional methods (27). Because of the importance of the fungal populations associated with the ensiling process, many efforts have been made to characterize the microbiota using morphological, physiological, and biochemical analyses. The ability to extract microbial DNA from silages, and the technological advances in DNA sequencing and bioinformatics analysis have driven the recent advances in the knowledge of the bacterial and fungal communities present in silages (27).

Fungal contamination negatively affects corn yield and silage quality, and the aims of this study have thus been to i) analyze the effect of a prothioconazole and tebuconazole-based fungicide on the yield and silage characteristics of whole-crop (WCC) and high-moisture ear (HMC) silages and ii) analyze the fungal community dynamics from harvesting to aerobic exposure of WCC and HMC. We hypothesized that a fungicide treatment could reduce the fungal contamination in the field and thus improve the corn yield and silage quality.

MATERIALS AND METHODS

Crop and ensiling procedure

Two experiments were conducted in the 2016 growing season at the University of Turin experimental farm in the western Po plain, northern Italy (44°53'N, 7°41'E, altitude 232 m a.s.l.) on corn (*Zea mays* L.) cropped to be harvested for whole corn silage or high-moisture corn. A corn hybrid (P1547, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) was sown in April, at an intended planting density of 75,000 seeds/ha, in an experimental field of about 3 ha. The experimental field received 100 kg/ha K_2O (as potassium chloride) before sowing, 39 and 100 kg/ha of N and P_2O_5, respectively, (as diammonium phosphate) at sowing and 120 kg/ha N, as urea, at the 6-leaf stage. The crop was irrigated twice during the growing season. Weed control was performed by utilizing mesotrione (0.15 kg AI/ha), S-metolachlor (1.25 kg AI/ha) and terbuthylazine (0.75 kg AI/ha) (Lumax®, Syngenta Crop Protection S.p.A., Milan, Italy). The field was divided into six plots for each experiment and, at the beginning of flowering (61 BBCH scale), half of the plots were untreated (**NT**) and half were treated (**T**) with a commercial fungicide (Prosaro®, Bayer Crop Science: 12.7 g prothioconazole and 12.7 g tebuconazole per 100 g) applied at the dose of 1.0 L/ha. Two experiments were conducted: in Experiment 1, corn was harvested, as a chopped whole crop (WCC), using a precision forage harvester (Claas Jaguar 950, equipped with an 8-row Orbis head, Claas, Harsewinkel, Germany) to a theoretical cutting length of 12 mm; in Experiment 2, corn was harvested, as chopped high moisture corn (HMC, whole ear, cob, and grain), using a precision forage harvester (Claas Jaguar 950) to a theoretical cutting length of 12 mm. The treated and untreated plots were harvested separately for each experiment in order to obtain three field replicates. The fresh

148 herbage of each plot was divided into two 50-kg piles in order to obtain silos that were to
149 be opened in different conservation periods. The fresh forage was sampled prior to ensiling
150 and was then ensiled in 20 L plastic silos equipped with a lid that only enabled the release
151 of gas. The forage was packed by hand, and the average final packing density was 490 ±
152 33 kg fresh matter (FM)/m^3 and 698± 23 kg FM/m^3 for WCC and HMC, respectively. All
153 the laboratory silos were filled within two hours. The silos were weighed, stored at room
154 temperature in a controlled environment (20 ± 1°C) and then opened after 60 and 160 days
155 of storage. Each silo was weighed at opening, the first 50 mm layer was discarded, and the
156 remaining silage was mixed thoroughly and sub-sampled to determine the dry matter (DM)
157 content, chemical composition, fermentation profile, microbial counts, and for DNA
158 extraction. The DM losses due to fermentation were calculated as the difference between
159 the weight of the forage placed in each plastic silo at ensiling and the weight of the silage
160 at the end of conservation, corrected for the DM content of the forage and its respective
161 silage.
162 After sampling, the silages were subjected to an aerobic stability test, which involved
163 monitoring the temperature increase due to the microbial activity in the samples exposed
164 to air. An aliquot of about three kilograms from each silo was allowed to aerobically
165 deteriorate at a controlled temperature (20 ± 1°C) in 17 L polystyrene boxes (290 mm
166 diameter and 260 mm height). A single layer of aluminum foil was placed over each box
167 to prevent drying and dust contamination, but also to allow air penetration. The
168 temperatures of the environment and silage were measured hourly by means of a data
169 logger. Aerobic stability was defined as the number of hours the silage remained stable
170 before its temperature increased by 2°C above the ambient temperature, an increase that is

171 indicative of instability. The silage was sampled after 7 and 14 d of aerobic exposure to
172 quantify the fermentative and microbial changes in the silage during exposure to air. The
173 experimental design of the two experiments is reported in Table 1.
174
175 **Sample Preparation and Analyses**
176 The pre-ensiled material and silages were split into six sub-samples. One sub-sample was
177 analyzed immediately, for the DM content, by oven drying at 80°C for 24 h. Dry matter
178 was corrected, according to Porter and Murray (28), to consider the volatile compound
179 losses that occur at 80°C.
180 The second subsample was oven-dried at 65°C to a constant weight and was then air
181 equilibrated, weighed, and ground in a mill (Cyclotec Tecator, Herndon, VA, USA) to pass
182 a 1 mm screen. The dried samples were analyzed for total nitrogen (TN), according to the
183 Dumas method (method number 992.23, AOAC [29]), using a Primacs SN nitrogen
184 analyzer (Skalar, Breda, The Netherlands), for crude protein (CP) (total N x 6.25) and for
185 ash by ignition (method number 942.05, AOAC [29]). The starch concentration was
186 determined according to the AOAC methods (method number 996.11; AOAC [29]). The
187 neutral detergent fiber (NDF) was analyzed, using a Raw Fiber Extractor (FIWE, VELP
188 Scientifica, Usmate Velate, Italy), with the addition of heat-stable amylase (A3306, Sigma
189 Chemical Co., St. Louis, MO) and expressed on a DM basis, including residual ash, as
190 described by Van Soest et al. (30). The acid detergent fiber (ADF) was analyzed and
191 expressed on a DM basis, including residual ash.
192 A third fresh sub-sample was used to determine the water activity (a_w), pH, nitrate (NO_3),
193 and the buffering capacity. The water activity was measured at 25°C on a fresh sample

194 using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopts the
195 chilled-mirror dew point technique. The fresh forage was extracted for pH, nitrate, and
196 NH_3-N determination, using a Stomacher blender (Seward Ltd, Worthing, UK), for 4 min
197 in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The total nitrate
198 concentration was determined in the water extract, through semi-quantitative analysis,
199 using Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg
200 NO_3/kg DM). The buffering capacity was determined in the water extract, as described by
201 Plaine and McDonald (31).
202 A fourth sub-sample was extracted, using a Stomacher blender, for 4 min in H_2SO_4 0.05
203 mol/L at a 4:1 acid-to-sample material (fresh weight) ratio. An aliquot of 40 mL of silage
204 acid extract was filtered through a 0.20-µm syringe filter and used for quantification of the
205 fermentation products. The lactic and monocarboxylic acids (acetic, propionic, and butyric
206 acids) were determined, by means of high-performance liquid chromatography (HPLC), in
207 the acid extract. Ethanol and 1,2-propanediol were determined, by means of HPLC coupled
208 to a refractive index detector, on a Aminex HPX-87H column (Bio-Rad Laboratories,
209 Richmond, CA).
210 The fifth sub-sample was used for classical plate counts. An aliquot of 30 g was transferred
211 to a sterile homogenization bag, suspended 1:9 w/v in a peptone salt solution (1 g of
212 bacteriological peptone and 9 g of sodium chloride per liter) and homogenized for 4 min
213 in a laboratory Stomacher blender (Seward Ltd, London, UK). Serial dilutions were
214 prepared, and yeast and mold numbers were determined using the pour plate technique with
215 Yeast Extract Glucose Chloramphenicol Agar (YGC agar, DIFCO, West Molesey, Surrey,
216 UK) after incubation at 25°C for 3 and 5 d for yeast and mold, respectively. The yeast and

mold colony forming units (CFU/g) were enumerated separately, according to their macromorphological features. The Lactic Acid Bacteria (LAB) count was determined on De Man, Rogosa and Sharpe agar (MRS agar, DIFCO, West Molesey, Surrey, UK), plus natamycin (0.25 g/L) and incubated at 30°C for 3 d in anaerobic jars with a gas generating system (AnaeroGenTM, Thermo Fisher Scientific, Rodano (MI), Italy).

After micro and macro-morphological observations, representative fungal colonies belonging to the *Cladosporium*, *Alternaria*, *Fusarium*, *Penicillium* and *Aspergillus* genera were purified on Potato dextrose agar (PDA, Merck, Germany). DNA was extracted from the mycelium obtained from a 6-day-old culture on PDA using an Omega E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek Inc., Norcross, GA) according to the manufacturer's instructions. Partial amplification of the calmodulin gene (CmD) was obtained for species assignation of the isolates belonging to the *Aspergillus* genus, using a cmd5 and cmd6 primer pair (32), as reported in Prencipe et al. (33). Partial amplification of the β-tubulin gene (BenA) was obtained for the isolates belonging to *Penicillium*, using Bt2a and Bt2b primers (34), and the protocols reported in Prencipe et al. (35). Partial amplification of the internal transcribed spacers of the rDNA region (ITS) was obtained for the isolates belonging to *Cladosporium* and *Alternaria* genera, using ITS1 and ITS4 primers and the protocols reported in White et al. (36). Partial amplification of the translation elongation factor (TEF) was performed for the isolates belonging to the *Fusarium* genus with the EF1/EF2 primer pair (37) and protocols reported in Spadaro et al. (13). The PCR products were purified, using a QIAquick PCR purification Kit (Qiagen), and were sent to Macrogen Inc. (Amsterdam, the Netherlands) for sequencing. The consensus sequences were obtained using the DNA Baser program (HeracleBiosoft S.R.L., Arges, Romania) and

compared, using the BLAST program, with the sequences deposited on the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) to assign species identification.

DNA extraction, library preparation, and sequencing

The sixth sub-sample was used for DNA extraction. gDNA was extracted from 0.25 g of plant material that had been ground in liquid nitrogen, using a DNeasy Power Microbiome KIT (Qiagen, Milan, Italy), following the manufacturer's instructions. DNA was quantified using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and standardized at 5 ng/µL.

Mycobiota were evaluated by amplifying the D1 domain of 26S using primers and the conditions described by Mota-Gutierrez et al. (38). Library preparation was performed according to the Illumina metagenomic procedure. Sequencing was performed, by means of an MiSeq instrument (Illumina), with V3 chemistry, and 250-bp paired-end reads were generated, following the producer's instructions.

Bioinformatics analysis

After sequencing reads were assembled, quality filtered and processed by using QIIME2 software (39). Briefly, adapters were trimmed using Cutadapter and then quality filtered using the DADA2 algorithm (40), removing low-quality bases, while the chimeric sequences were removed using the dada2 denoise-paired plug-in of QIIME2. Amplicon Sequence Variants (ASVs), generated by DADA2, were used for taxonomic assignment, using the qiime feature-classifier plug-in against the manually built mycobiota database (38). The taxonomy assignment was double checked with BLAST suite tools. A QIIME2

diversity script was used to perform alpha and beta diversity analysis. The accession number sequences are available in the NCBI Sequence Read Archive under accession number PRJNA814208.

Statistical Analysis

The microbial counts were $\log_{10}$ transformed and presented on a wet weight basis. The values below the detection limit for yeast and mold (detection level: 10 cfu/g of silage) were assigned a value corresponding to half of the detection limit to calculate the average value. An unpaired *t*-test was used to analyze the effect of the treatment with Prosaro® on the agronomic, chemical, and microbial characteristics of WCC and HMC at harvest.

The data pertaining to the silage characteristics were analyzed for their statistical significance, via an analysis of variance, with their significance reported at a 0.05 probability level. Data were analyzed, with 3 replications, using the fungicide treatment and time of conservation as fixed factors. The used statistical model was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk},$$

where Y_{ijk} = observation, μ = overall mean, α_i = fungicide treatment effect, β_j = time of conservation effect, $\alpha\beta_{ij}$ = interaction effect, and ε_{ijk} = error. All the analyses were performed using the R software (R version 4.0.3 [41]). An ASV table was used to build a Principal Component Analysis and an ANOSIM statistical test was run through the vegan function of R to identify significant differences as a function of the time, type, or air exposure. A Wilcoxon matched pairs test was used for the difference in alpha diversity or ASV abundance as a function of the time, type or air exposure. Non-Normally distributed variables were presented as median values (interquartile range) and box plots were used to

represent the interquartile range between the first and the third quartiles, with error bars showing the lowest and the highest values. The P value was adjusted for multiple comparison by False Discover Rate.

RESULTS

Agronomic performances

The DM yield, nutritional characteristics, and starch and energy production per hectare are reported in Table 2 for the treated and untreated WCC and HMC. The fungicide treatment increased the yield per hectare in both WCC (P = 0.008) and HMC by 7% and 9% (P = 0.021), respectively. The main nutritional characteristics were not affected by the application of the fungicide, even though the higher yield in the treated crops allowed a higher NEL and starch yields per ha to be obtained than for the NT crops.

Silage characteristics

The chemical and microbial characteristics of the treated and untreated WCC and HMC before ensiling are reported in Table 3. The fungicide application did not lead to any difference in the chemical and microbial characteristics at harvest in either the WCC or HMC. The DM content and a_w were on average 46 and 65%, and 0.985 and 0.977, for WCC and HMC, respectively. The buffering capacity was 44 and 18 meq/kg DM for WCC and HMC, respectively. No nitrate content was detected, and the pH was typical of corn at harvesting. The yeast, mold, and LAB counts were around 6 log cfu/g.

The chemical, fermentative and microbial characteristics, DM losses, and aerobic stability of the treated and untreated WCC, after 60 and 160 days of conservation, are shown in

309 Table 4. All the WCC silages were well fermented with a pH value of around 3.8. No
310 differences were detected for the fermentative compounds in the T and NT WCC silages,
311 whereas lactic acid decreased, and acetic acid increased from 60 to 160 days of
312 conservation. The LAB count decreased as the days of conservation increased ($P < 0.001$).
313 The treated silages showed an increase in aerobic stability from 60 and 160 days of
314 conservation ($P = 0.024$). On the other hand, the yeast count increased in the NT silages,
315 and aerobic stability decreased during conservation.
316 The chemical, fermentative and microbial characteristics, DM losses and aerobic stability
317 of the treated and untreated HMC, after 60 and 160 days of conservation, are shown in
318 Table 5. All the HMC silages were well fermented, with a pH value of around 3.8 and a
319 higher lactic acid content than 20 g/kg DM. No differences were detected for the
320 fermentative compounds. The LAB, yeast, and mold counts decreased during conservation,
321 while no differences were observed between the T and NT silages. Aerobic stability
322 decreased in the T silages and increased in the NT HMC silages from 60 to 160 days of
323 conservation.
324 The yeast and mold counts of the treated and untreated WCC and HMC silages, after 7 and
325 14 days of air exposure, are shown in Figure 1. After 7 days of air exposure, the yeast count
326 of WCC was higher than 7 log cfu/g after both 60 and 160 days of conservation. The
327 increase in the mold count followed the same trend as that of yeast and reached higher
328 values than 7 log cfu/g after 14 days of air exposure. A similar result was observed for
329 HMC, although the value was lower.
330
331 **Molecular identification of *Fusarium*, *Aspergillus* and *Penicillium***

The isolates morphologically identified as *Cladosporium* spp. and *Alternaria* spp. during sampling were confirmed as such through the amplifications of the ITS region. The other representative fungal colonies isolated from plates were found to belong to the *Fusarium, Penicillium,* and *Aspergillus* genera and showed a dominance of *Fusarium* at harvesting in both WCC (35 to 51% of the total fungi) and HMC (36 to 81% of the total fungi). The main isolated species were *F. verticillioides*, *F. temperatum*, *F. proliferatum,* and *F. subglutinans*. *Penicillium* genus was mainly detected in HMC at harvesting and during the exposure of WCC and HMC to air, with the main species being identified as *P. roqueforti* and *P. paneum*. *Aspergillus* genus was detected at harvesting in both WCC and HMC. *Aspergillus* became the dominant genus during ensiling and aerobic exposure, with *A. fumigatus*, *A. flavus,* and *A. oryzae* var. *effusus* being the main species in WCC and *A. flavus* and *A. oryzae* var. *effusus* the main species in HMC.

Sequencing results and alpha diversity analysis

After sequencing and data analysis, a total of 3,162,755 reads were used with an average of 37,651 reads/sample and a median sequence length of 358 bp, and a satisfactory coverage was achieved of all the samples, that is, > 99% (Supplementary Table 1). By taking into account the rarefaction measure in the whole crop corn samples, we observed a significant reduction in the Shannon index as well as in the number of observations, when comparing the WCC_T0 and WCC_T60 samples with the other ones. On the other hand, we only observed a significant increase in the number of ASVs in the high-moisture corn samples from HMC_T60 to HMC_T60_7D, and in WCC_T60_14D. The fungicide treatment showed no statistical effect on the diversity indices.

355

356 **Mycobiota composition of WCC**

357 The yeast and fungal populations of WCC at harvest are reported in Figure 2, at silo
358 opening and after aerobic exposure. The ANOSIM analysis showed no significant
359 differences between the T and NT silages for each sampling time. The yeast population at
360 harvest showed a dominance of *Clavispora, Kurtzmaniella, Candida,* and *Pichia*. Even
361 though no statistical differences were detected, *Kurtzmaniella* and *Metschnikowia* were the
362 most abundant ASVs at ensiling, while *Clavispora* and *Hanseniaspora* were less abundant
363 in T than in NT. After 60 and 160 days of conservation, the yeast population had simplified
364 with a reduction of *Candida* and *Clavispora* and a dominance of *Pichia* and *Kazachstania*.
365 *Pichia* and *Kazachstania* were the main genera identified during aerobic exposure (more
366 than 90% on average). The fungal population at harvest was mainly represented by
367 *Cladosporium* (more than 80% on average). *Cladosporium, Alternaria,* and *Fusarium*
368 decreased during ensiling, whereas *Aspergillus* and *Penicillium* increased, as they were the
369 dominant genera during aerobic exposure.

370 By considering the whole mycobiota and the air exposure as discriminant factors, a
371 separation of the samples was observed, according to the air exposure variable
372 (Supplementary Figure 1, ANOSIM $P < 0.05$), and, as expected, most of the main ASVs
373 significantly decreased as the conservation time increased. However, after 7 days of air
374 exposure, *Kazachstania* was observed more, while *Aspergillus* and *Penicillium* were
375 mainly observed after 14 days of air exposure (data not shown, FDR <0.05). By comparing
376 the conservation factor, we observed a significant reduction in *Fusarium*, *Kazachstania,*

377 *Metschnikowia,* and *Penicillium* after 160 days, compared to after 60 days (data not shown,
378 FDR < 0.05).
379
380 **Mycobiota composition of HMC**
381 The yeast and fungal populations of HMC at harvest, at silo opening and after aerobic
382 exposure are reported in Figure 3. The ANOSIM analysis showed no significant differences
383 between T and NT silages for any sampling time. The yeast population at harvest showed
384 a dominance of *Candida, Clavispora, Kurtzmaniella,* and *Sporobolomyces.* Even though
385 no statistical differences were detected, *Sporobolomyces* was higher at ensiling (65% on
386 average) and *Candida* and *Kurtzmaniella* were lower in NT than in T. After 60 and 160
387 days of conservation, the yeast population diversity decreased, and a dominance of *Pichia*
388 and *Kazachstania,* which became dominant during aerobic exposure, was observed. The
389 fungal population at harvest was mainly represented by *Cladosporium* in the untreated
390 HMC and by *Cladosporium*, *Alternaria, Penicillium,* and *Fusarium* in the treated HMC.
391 *Cladosporium, Alternaria,* and *Fusarium* decreased during ensiling, whereas *Aspergillus*
392 and *Penicillium* increased as they were the dominant genera during aerobic exposure.
393 When using a Principal Component Analysis (PCA) on the ASV tables, and considering
394 the whole mycobiota, a separation of the samples was observed when air exposure was
395 taken into account (Supplementary Figure 2) and this result was confirmed by the
396 ANOSIM statistical test (P= 0.001, PC1=43.45; PC2=16.68). When taking into the account
397 the ASVs responsible for cluster separation, *Candida, Clavispora,* and *Hanseniaspora*
398 were significantly abundant in the 7D samples, and were drastically reduced after 14D,
399 while *Saccharomyces* was mainly associated with 14D (data not shown, FDR < 0.05). By

comparing the mycobiota of T60 and T160, an association between *Botrytis*, *Fusarium, Kazachstania, and Metschinikowia* T60 was observed, while *Penicillium* was associated with T160 (data not shown, FDR < 0.05).

DISCUSSION

In conventional agriculture, fungicides represent an important tool for the disease management of plants. Foliar pathogens decrease the photosynthetic area, which in turn leads to a yield reduction (9). The magnitude of the yield loss mainly depends on the severity of the disease and the plant growth stage at which the infection occurs (42). Demethylation inhibitor (DMI) fungicides, which are commonly referred to as azole fungicides, are effective against a wide range of fungal pathogens (43). Preventative fungicide applications during the cropping season can help to minimize yield losses due to diseases and reduce the risk of mycotoxin production (18). Despite concerns, foliar fungicide applications are often made to corn without pathogen pressure, due to the perceived or marketed yield benefits (44, 45). In the present experiment, the field application of fungicide improved the DM yield of both WCC and HMC. These results have confirmed the outcomes of several authors, who reported that the majority of such treatments resulted in a positive yield response, even in the absence of disease (9, 44). Furthermore, fungicides are often applied to corn plants to improve the nutritional quality of the plant material. Fungal contamination may cause an increase in lignification of the fiber in plants and, therefore, a decrease in NDF digestibility, which is one of the main factors that affects corn silage quality (21, 46). Blonde and Esker (47) reported a 1% decrease in the NDF concentration when comparing silages from corn plants treated with

423 a foliar fungicide with untreated ones. In the present experiment, the fungicide did not
424 affect the chemical composition of WCC or HMC, although the higher DM yield per ha
425 allowed higher NEL and starch yields per hectare to be reached.
426 We evaluated the quality of WCC harvested at a late stage of maturity and HMC to
427 maximize the energy component of the corn. The harvesting of WCC with a DM content
428 of up to 40% and the low moisture content and concentration of fermentable sugars in
429 HMC limits the extent of the fermentation, and results in silages prone to aerobic
430 deterioration (5). As expected, the high DM content limited fermentation, and in particular
431 that of acetic acid, which is known to strongly contrast yeast activity during air exposure
432 at feed-out. Consequently, after 7 days of air exposure, the silages showed higher yeast
433 counts than 7 and 4 log cfu/g for WCC and HMC, respectively. The fermentative profile
434 and the limited reduction of yeast during conservation of the silages are in agreement with
435 the results of Ferrero et al. (48) for WCC and da Silva et al. (5) for HMC. The application
436 of a fungicide showed no consistent difference in the effect on the yeast count or aerobic
437 stability between WCC and HMC, thus it could be hypothesized that other factors
438 influenced these parameters.
439 The total mold count at harvest did not differ between the treated and untreated WCC or
440 HMC. Similar results were found by Nair et al. (25) and Fountaine et al. (49). This can be
441 explained by the early plant growth stage at which the fungicide was applied. Fountaine et
442 al. (49) reported that a single application of the fungicide did not affect the fungal
443 population, although, when the number of fungicide applications was increased to three,
444 the fungal count decreased.

A large number of microbial species are able to live as saprophytes on the plant surface at the preharvest stage, including filamentous fungi, some of which may be pathogenic during plant development. The application of a foliar fungicide may change the chemical composition of the plant, even though it appears to have little or a moderate impact on the fungal community at harvest (18, 25). In our work, the fungal population of the fresh material at ensiling was not affected by the fungicide treatment and showed the presence of fungi that are commonly found on corn plants and ears (e.g., *Fusarium*, *Alternaria,* *Aspergillus,* and *Penicillium* genera). The fungal pathogens of corn include *Fusarium* species, which typically cause ear infections (ear rot or ear mold) and can contaminate grains with mycotoxins (i.e., trichothecenes, fumonisins, and zearalenone), which are toxic to mammals. Most of *Fusarium* spp., isolates were from plates belonging to the dominant *F. verticillioides*, *F. temperatum*, *F. proliferatum,* and *F. subglutinans* fungal species, as previously reported by Spadaro et al. (13).

An ensiling process generates a low pH and an anaerobic environment that inhibits the growth or even eliminates most fungi present at harvest (13). However, during conservation, small amounts of oxygen can enable fungi to survive in the anaerobic phase of ensiling and multiply during the feed-out phase, thus causing aerobic deterioration (15). Romero et al. (50) and Duniere et al. (51) reported that fungal diversity decreased during ensiling and aerobic exposure to a distinct and more stable community, even though their results were reported at a family level and not at a genus level. In the present experiments, the mold count after ensiling was reduced to below 3 log cfu/g. The *Aspergillus* genus was the one detected the most at silo opening, with the main species being identified as *A. fumigatus*, *A. flavus,* and *A. oryzae* var. *effusus*. During aerobic deterioration, and after

yeast activity, the mold counts increased, and the main detected genera were *Aspergillus* in WCC and *Penicillium* in the HMC silages. The dominance of *Aspergillus fumigatus* during aerobic deterioration has been identified on corn silage by several authors (13, 52, 53). Ferrero et al. (3) reported that *Aspergillus flavus* showed the ability to survive in anaerobic silage and revive when the inhibiting conditions were lost, while it was dominant during aerobic exposure and the AFB_1 concentration increased.

The metataxonomic approach displayed a dominance of *Fusarium, Aspergillus,* and *Penicillium*. A dominance of these genera was also reported by Alonso et al. (54) and Ogunade et al. (16).

The anaerobic and acidic environment of silages is unsuitable for yeasts, and their survival is affected by the degree of anaerobiosis and the concentrations of such organic acids as acetic and propionic acids (15). Several genera were identified at harvest, including *Candida*, *Clavispora*, *Kurtzmaniella*, and *Hanseniaspora*. The yeast counts decreased during conservation and the main genera detected at silo opening were *Nakaseomyces*, *Pichia*, *Candida,* and *Kazachstania*. Duniere et al. (51) reported that *Kazachstania* and *Pichia* represented almost 70% of the fungal core mycobiota of small grain cereal silage at opening. Keshri et al. (55) detected *Candida* as being dominant in WCC after 90 d of conservation. Santos et al. (56) studied the diversity of yeast populations in corn and in high-moisture corn silages. They reported that isolates obtained on malt agar plates showed that *Candida*, *Saccharomyces*, *Pichia*, and *Kazachstania* were the dominant genera. Drouin et al. (26) reported that the main yeast genera after fermentation were *Candida*, *Dipodascus*, *Hannaella*, *Hanseniaspora*, *Kazachstania*, and *Metschnikowia*.

In our experiment, a simplification of the yeast population was detected during aerobic deterioration with *Pichia* and *Kazachstania* genera being dominant, particularly in the first phase (7 days of air exposure). Lactate-assimilating yeasts, such as the *Candida*, *Pichia*, *Hansenula*, and *Endomycopsis* genera, are the primary initiators of aerobic spoilage. *Pichia* is a lactic acid assimilator, and it has been detected as distinctive bands in aerobically unstable silage at both silo opening and after exposure to air (15). Dunière et al. (51) observed that, during aerobic deterioration, most ASVs were assigned to *Kazhachstania* and *Pichia*. Drouin et al. (57) identified *Issatchenkia* and *Kazhachstania* as the dominant yeasts during aerobic deterioration. The authors explained that the high number of observed ASVs related to the *Issatchenkia* genus were due to the difficulties in the phenotypic classification of yeast species because of switching between the teleomorph and anamorphic states. This may have introduced classification errors in the databases, as already reported for *Candida krusei*, *Issatchenkia orientalis*, *Candida glycerinogenes*, and *Pichia kudriavzevii,* which have been shown to belong to the same species (58).

Although fungicides are necessary for crop protection, minimizing non-target effects and unintended consequences is critical to evaluate the sustainability of agricultural production systems (45). Azoles are highly active against many plant pathogens. However, a consistent non-target effect has been detected against phyllosphere yeasts. Fungicides have been shown to lower pathogen abundance in maize and soybean and to decrease the abundance of yeasts, thus indicating that they may impact non-target fungal and yeast populations indirectly (45). These findings can partially explain the different yeast populations found at harvest in our experiment.

The present work has reported that the application of a prothioconazole and tebuconazole-based fungicide improved the DM, starch and NEL yield per ha in corn harvested and conserved as whole-crop and high-moisture ear silages but did not affect the nutritional composition of the plant. The mold counts and mold populations were not affected by the fungicide application, since a dominance of *Cladosporium* and *Fusarium* was observed at harvest and *Aspergillus* and *Penicillium* genera during conservation and aerobic exposure. Hence, NGS methods can be used side by side with a traditional plate count method to identify and monitor the evolution of fungal populations during the ensiling process.

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705

Tables

Table 1. Experimental design of the two Experiments

	WCC		HMC	
	T	NT	T	NT
Harvest	WCC_T0_T	WCC_T0_NT	HMC_T0_T	HMC_T0_NT
Conservation	WCC_T60_T	WCC_T60_NT	HMC_T60_T	HMC_T60_NT
	WCC_T160_T	WCC_T160_NT	HMC_T160_T	HMC_T160_NT
Air exposure	WCC_T60_7D_T	WCC_T60_7D_NT	HMC_T60_7D_T	HMC_T60_7D_NT
	WCC_T60_14D_T	WCC_T60_14D_NT	HMC_T60_14D_T	HMC_T60_14D_NT
	WCC_T160_7D_T	WCC_T160_7D_NT	HMC_T160_7D_T	HMC_T160_7D_NT
	WCC_T160_14D_T	WCC_T160_14D_NT	HMC_T160_14D_T	HMC_T160_14D_NT

WCC = whole crop corn; HMC = high moisture ear corn, T = treated with prothioconazole and tebuconazole-based fungicide; NT = untreated; T0 = harvest; T60 = 60 days of conservation; T160 = 160 days of conservation; 7D = 7 days of air exposure; 14D = 14 days of air exposure.

711 Table 2. DM yield, nutritional characteristics, and starch and energy production per hectare

712 of the treated and untreated whole crop corn (WCC) and high-moisture ear corn (HMC)

	WCC				HMC			
	T	NT	SEM	*P*-value	T	NT	SEM	*P*-value
DM (%)	46.0	47.3	0.004	0.150	64.2	67.7	0.009	0.268
DM yield (t/ha)	22.58	21.10	0.346	0.008	16.00	14.63	0.334	0.021
Crude protein (g/kg DM)	74.1	74.4	0.912	0.892	91.9	89.4	0.702	0.053
NDFom (g/kg DM)	334.5	332.2	9.074	0.919	167.7	149.3	6.696	0.198
ADF (g/kg DM)	193.7	194.4	6.048	0.964	80.5	69.3	3.912	0.175
Lignin (g/kg DM)	29.4	30.3	0.690	0.595	-	-	-	-
Starch (g/kg DM)	373.1	381.2	10.72	0.765	582.0	609.3	8.492	0.109
NDF-D 30h (% NDFom)	49.51	47.91	0.565	0.175	51.30	48.80	1.190	0.349
NEL (MJ/kg DM)	6.46	6.42	0.041	0.667	8.28	8.33	0.018	0.184
NEL yield (GJ/ha)	145.9	135.5	2.722	0.028	132.4	121.9	2.577	0.032
Starch yield (t/ha)	8.43	8.04	0.266	0.554	9.31	8.91	0.103	0.024

713 DM = dry matter; ADF = Acid Detergent Fiber; NDFom = ash free Neutral Detergent Fiber; NDF-D 30h =

714 NDF degradability at 30 hours; NEL = Net Energy for Lactation; SEM = standard error of the mean;

715 NT = untreated; T = treated with prothioconazole and tebuconazole-based fungicide.

716 Table 3. Chemical, fermentative, and microbial characteristics of the treated or untreated

717 whole crop corn (WCC) and high-moisture ear corn (HMC) before ensiling

	WCC				HMC			
	T	NT	SEM	*P*-value	T	NT	SEM	*P*-value
Buffering capacity (meq/kg DM)	44				18			
pH	5.87	5.89	0.040	0.854	5.93	5.96	0.009	0.191
Nitrate (mg/kg DM)	<100				<100			
a_w	0.985	0.984	0.001	0.886	0.976	0.978	0.001	0.796
Yeast (log cfu/g)	6.42	6.74	0.080	0.030	6.58	6.57	0.008	0.505
Mold (log cfu/g)	6.23	6.35	0.068	0.454	6.05	6.21	0.059	0.232
Lactic acid bacteria (log cfu/g)	6.20	6.42	0.062	0.094	6.29	5.85	0.152	0.264

718 a_w = activity water; cfu = colony forming unit; DM = dry matter; SEM = standard error of the mean;

719 NT = untreated; T = treated with prothioconazole and tebuconazole-based fungicide.

720 Table 4. Chemical, fermentative and microbial characteristics, DM losses and aerobic
721 stability of the treated and untreated whole crop corn (WCC) after 60 and 160 days of
722 conservation

	60 d		160 d		*P*-value			
	T	NT	T	NT	SEM	TREAT	TIME	TxT
DM (%)	45.4	47.1	46.3	46.6	0.002	0.015	0.599	0.056
pH	3.79	3.82	3.79	3.81	0.004	0.003	0.928	0.258
Yeast (log cfu/g)	2.90	2.45	3.47	4.04	0.197	0.754	<0.001	0.027
Mold (log cfu/g)	2.28	2.50	2.17	1.53	0.186	0.566	0.162	0.262
Lactic acid bacteria (log cfu/g)	6.48	6.99	6.31	5.45	0.177	0.225	<0.001	<0.001
DM losses (%)	2.24	1.96	2.25	2.26	0.048	0.076	0.049	0.061
Aerobic stability (h)	94	150	102	84	8.9	0.105	0.024	0.008
Lactic acid (g/kg DM)	36.0	39.5	40.5	41.0	0.751	0.111	0.025	0.210
Acetic acid (g/kg DM)	7.6	7.6	9.2	8.8	0.269	0.677	0.005	0.673
1,2-propanediol (g/kg DM)	0.5	0.4	0.7	0.2	0.072	0.009	0.812	0.088
Ethanol (g/kg DM)	15.5	12.8	13.1	14.6	0.482	0.525	0.726	0.035

723 cfu = colony forming unit; DM = dry matter; SEM = standard error of the mean;
724 NT = untreated; T = treated with prothioconazole and tebuconazole-based fungicide.

Table 5. Chemical, fermentative, and microbial characteristics, DM losses and aerobic stability of the treated and untreated high moisture ear corn (HMC) after 60 and 160 days of conservation

	60 d		160 d		*P*-value			
	T	NT	T	NT	SEM	TREAT	TIME	TxT
DM (%)	64.1	66.4	62.7	65.8	0.005	0.006	0.211	0.617
pH	3.78	3.82	3.80	3.86	0.012	0.014	0.091	0.412
Yeast (log cfu/g)	3.30	2.74	1.68	2.10	0.218	0.815	0.003	0.105
Mold (log cfu/g)	2.83	2.55	1.08	1.00	0.276	0.489	<0.001	0.738
Lactic acid bacteria (log cfu/g)	7.02	7.08	6.62	6.66	0.074	0.568	0.002	0.871
DM losses (%)	1.29	1.37	1.56	1.39	0.041	0.469	0.090	0.075
Aerobic stability (h)	160	233	130	247	14.9	<0.001	0.188	0.004
Lactic acid (g/kg DM)	26.8	22.0	24.7	22.0	0.760	0.008	0.353	0.354
Acetic acid (g/kg DM)	3.8	3.8	4.2	4.4	0.111	0.642	0.026	0.603
1,2-propanediol (g/kg DM)	<0.1	<0.1	<0.1	<0.1	-	-	-	-
Ethanol (g/kg DM)	9.0	8.0	7.3	6.7	0.395	0.259	0.064	0.759

cfu = colony forming unit; DM = dry matter; SEM = standard error of the mean;

NT = untreated; T = treated with prothioconazole and tebuconazole-based fungicide.

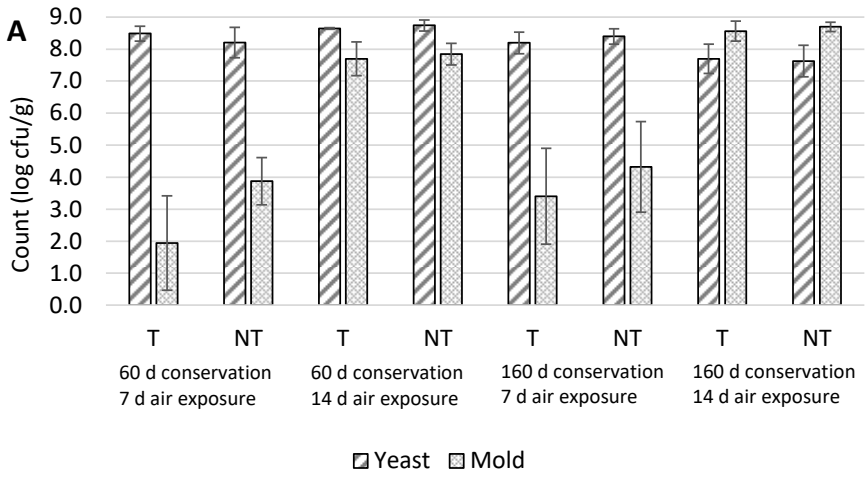
731 Supplementary Table 1. Diversity indices, observed ASVs, number of used reads and
732 coverages of the experiments

Sample	chao1	Shannon	Observed ASVs (n)	coverage (%)	Reads used (n)
WCC_T0_ND_NT	163.17	3.24	158	99.96	32998
WCC_T0_ND_T	195.09	4.15	189	99.96	33694
WCC_T60_ND_NT	182.92	4.36	179	99.97	46839
WCC_T60_ND_T	163.22	4.07	156	99.96	31229
WCC_T60_7D_NT	44.88	1.20	42	99.99	43065
WCC_T60_7D_T	59.04	1.44	53	99.98	46447
WCC_T60_14D_NT	34.81	1.99	33	99.99	37862
WCC_T60_14D_T	55.28	1.74	51	99.98	41524
WCC_T160_ND_NT	109.89	3.18	108	99.98	31480
WCC_T160_ND_T	84.45	2.54	82	99.98	32028
WCC_T160_7D_NT	57.79	1.73	50	99.98	43493
WCC_T160_7D_T	21.33	0.77	20	99.99	36316
WCC_T160_14D_NT	30.83	1.89	29	99.96	25058
WCC_T160_14D_T	27.80	2.17	25	99.99	41616
HMC_T0_ND_NT	65.23	1.45	61	99.93	22243
HMC_T0_ND_T	152.75	3.82	141	99.89	20790
HMC_T60_ND_NT	146.29	3.88	144	99.97	31635
HMC_T60_ND_T	133.64	3.33	131	99.98	30726
HMC_T60_7D_NT	93.19	2.07	89	99.97	43953
HMC_T60_7D_T	43.95	1.34	41	99.99	43926
HMC_T60_14D_NT	62.37	2.29	57	99.98	37447
HMC_T60_14D_T	33.48	1.66	32	99.99	37046
HMC_T160_ND_NT	113.24	3.15	108	99.98	46413
HMC_T160_ND_T	93.46	2.20	81	99.98	50550
HMC_T160_7D_NT	58.07	1.32	51	99.98	43966
HMC_T160_7D_T	105.53	1.89	90	99.96	40930
HMC_T160_14D_NT	30.50	1.75	28	99.99	45790
HMC_T160_14D_T	46.17	2.13	42	99.98	35188

733

734 **Figures**

735



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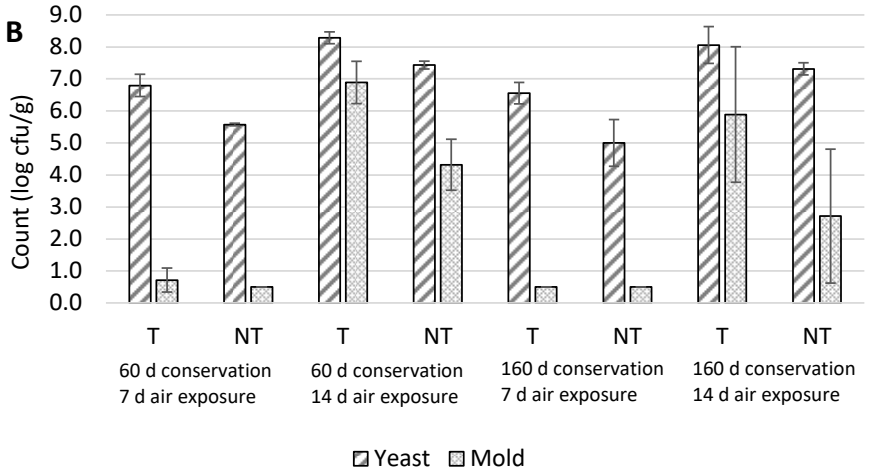
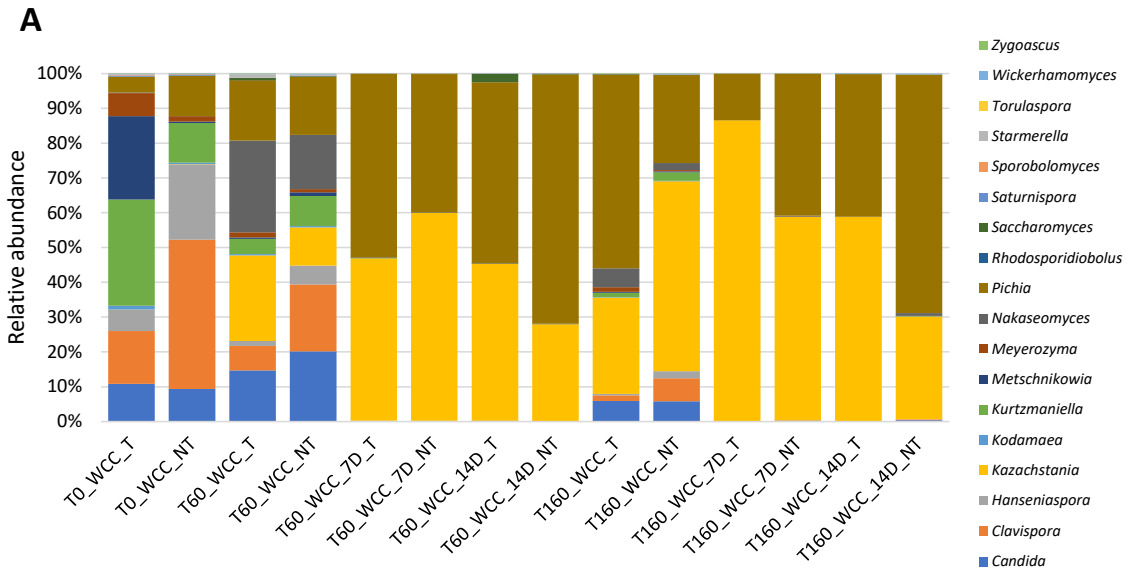


737

738 Figure 1. Yeast and mold counts of the treated (T) and untreated (NT) whole crop corn (A)
739 and high-moisture ear corn (B) after 7 and 14 days of air exposure (NT = untreated; T =
740 treated with a prothioconazole and tebuconazole-based fungicide).

741



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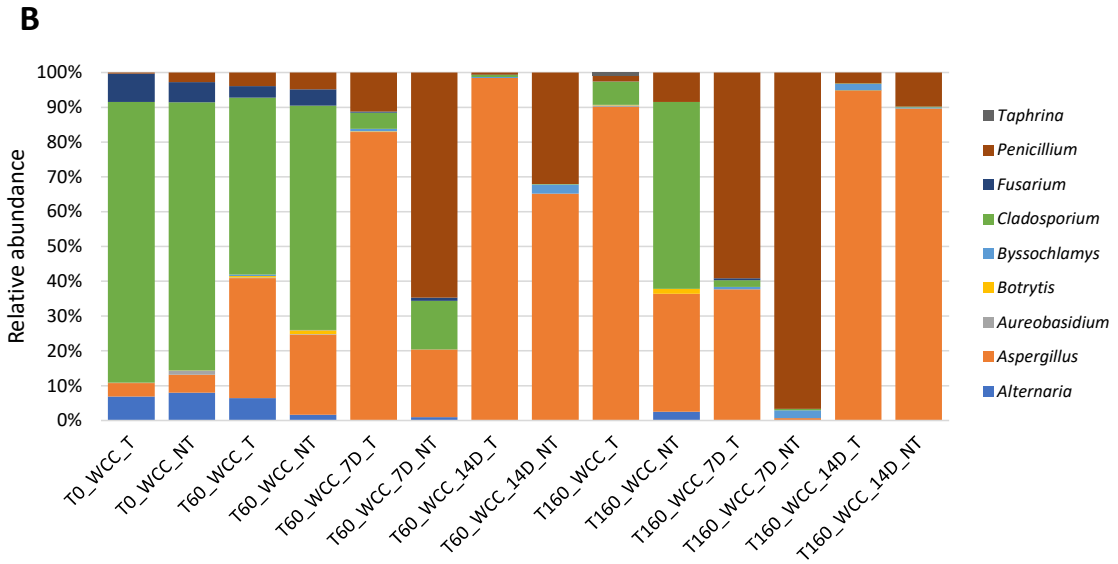


743

744 Figure 2. Yeast (A) and fungal (B) mycobiota of the whole crop corn treated (T) with a
745 prothioconazole and tebuconazole-based fungicide and the untreated (NT) whole crop corn
746 (WCC) at ensiling, at silo opening and during aerobic exposure (7D and 14D).

747

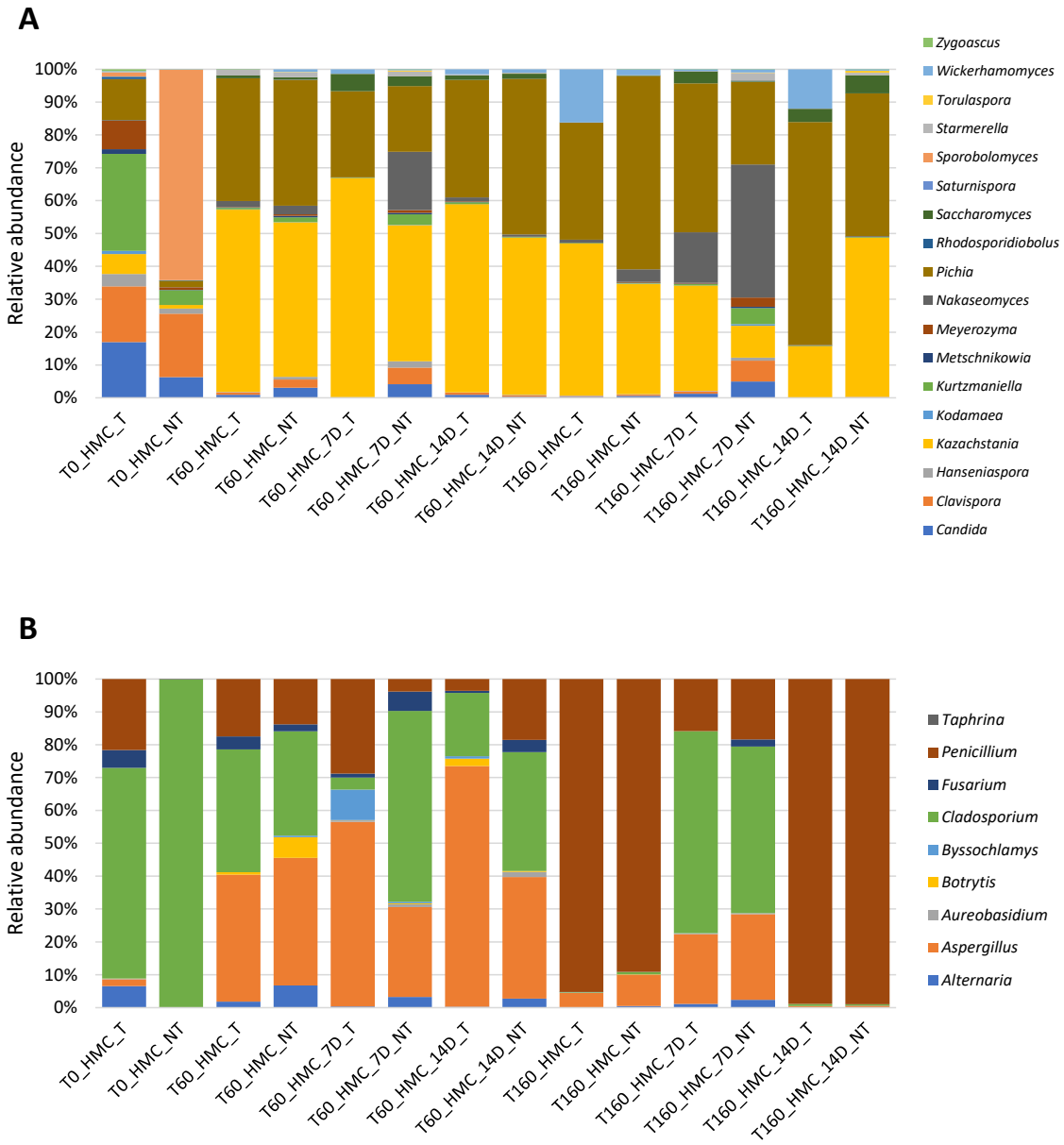
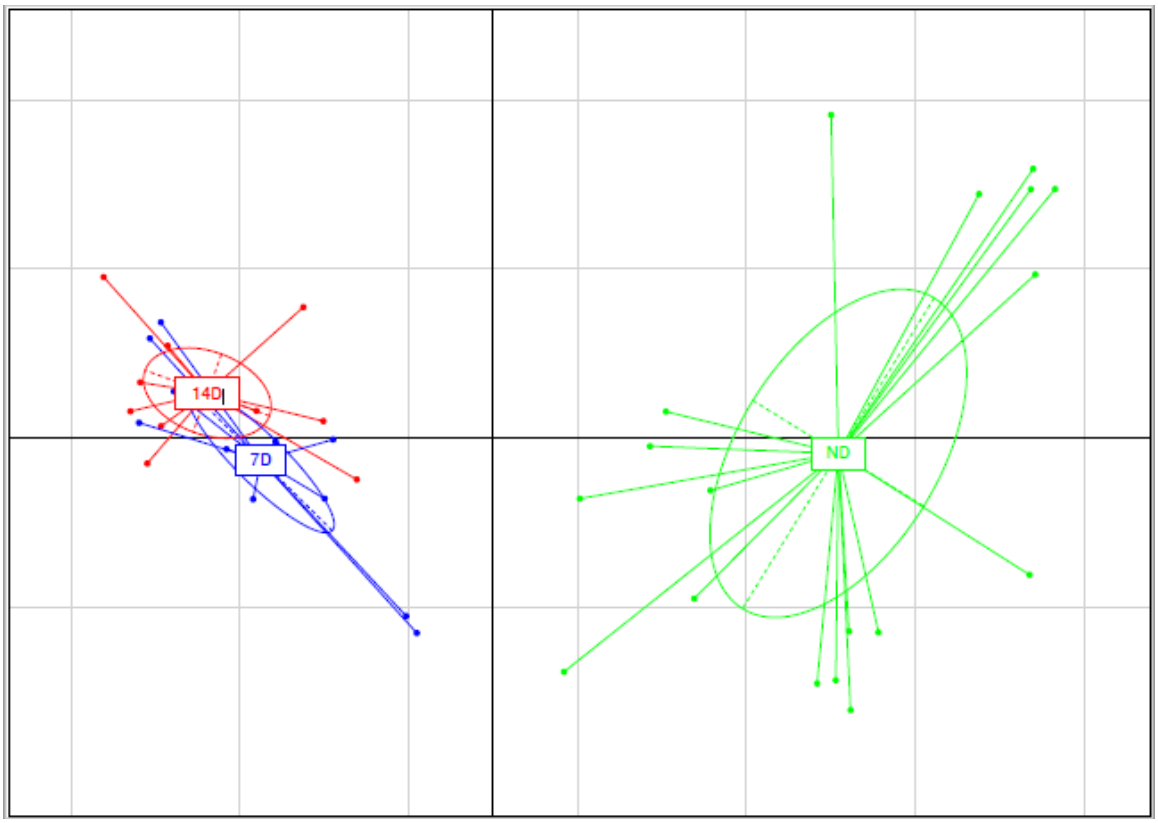
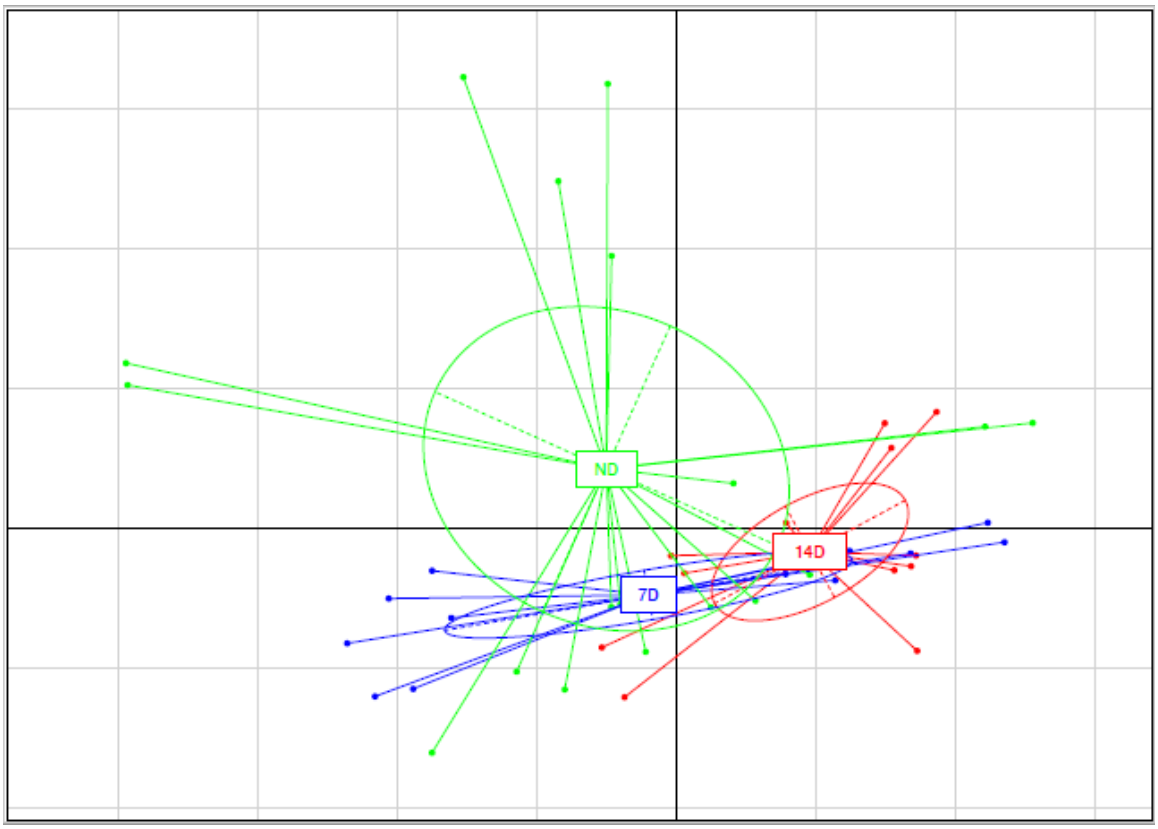


Figure 3. Yeast (A) and fungal (B) mycobiota of the high-moisture ear corn treated (T) with a prothioconazole and tebuconazole-based fungicide and the untreated (NT) high-moisture ear corn (HMC) at ensiling, at silo opening and during aerobic exposure (7D and 14D).



Supplementary Figure 1. Principal Component Analysis (PCA), based on the relative abundance of the ASVs of the whole crop corn silage at 7D (blue), 14D (red) and at silo opening (green). The first component (horizontal) accounts for 52.61% of the variance and the second component (vertical) accounts for 11.04%.



Supplementary Figure 2. Principal Component Analysis (PCA), based on the relative abundance of the ASVs of the high-moisture ear corn silage at 7D (blue), 14D (red) and at silo opening (green). The first component (horizontal) accounts for 40.20% of the variance and the second component (vertical) accounts for 14.88%.