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1 **TITLE**

2 Fungal Bioaerosols at Five Dairy Farms: A Novel Approach to Describe Workers' Exposure

3

4 **RUNNING TITLE**

5 Fungal Bioaerosols at Five Dairy Farms

6 **AUTHORS**

7 Hamza Mbareche[1,2], Marc Veillette[1], Guillaume J Bilodeau[3] and Caroline Duchaine[1,2]

8

9 **AUTHORS' AFFILIATION**

10 1. Centre de recherche de l'institut universitaire de cardiologie et de pneumologie de Québec,

11 Quebec City (Qc), Canada

12 2. Département de biochimie, de microbiologie et de bio-informatique, Faculté des sciences et de

13 génie, Université Laval, Quebec City (Qc), Canada

14 3.Pathogen Identification Research Lab, Canadian Food Inspection Agency (CFIA). Ottawa,

15 Canada

16

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20 **CORRESPONDING AUTHOR**

21 Mailing address: Caroline Duchaine, Ph.D., Centre de Recherche de l'Institut Universitaire de

22 Cardiologie et de Pneumologie de Québec, 2725 Chemin Ste-Foy, Québec, Canada, G1V 4G5.

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23 Phone: (418) 656-8711 ext. 5837. Fax: 418 656-4509. E-mail:
24 Caroline.Duchaine@bcm.ulaval.ca

25 Abstract

26 Occupational exposure to harmful bioaerosols in industrial environments is a real treat to the
27 workers. In particular, dairy-farm workers are exposed to high levels of fungal bioaerosols on a
28 daily basis. Associating bioaerosol exposure and health problems is challenging and adequate
29 exposure monitoring is a top priority for aerosol scientists. Using only culture-based tools do not
30 express the overall microbial diversity and underestimate the large spectrum of microbes in
31 bioaerosols and therefore the identification of new airborne etiological agents. The aim of this
32 study was to provide an in-depth characterization of fungal exposure at Eastern Canadian dairy
33 farms using qPCR and next-generation sequencing methods. Concentrations of
34 *Penicillium/Aspergillus* ranged from 4.6 x 10^6 to 9.4 x 10^6 gene copies/m^3 and from 1 x 10^4 gene
35 copies/m^3 to 4.8 x 10^5 gene copies/m^3 for *Aspergillus fumigatus*. Differences in the diversity
36 profiles of the five dairy farms support the idea that the novel approach identifies a large number
37 of fungal taxa. These variations may be explained by the presence of different and multiple
38 sources of fungal bioaerosols at dairy farms. The presence of a diverse portrait of fungi in air
39 may represent a health risk for workers who are exposed on a daily basis. In some cases, the
40 allergen/infective activity of the fungi may not be known and can increase the risks to workers.
41 The broad spectrum of fungi detected in this study includes many known pathogens and proves
42 that adequate monitoring of bioaerosol exposure is necessary to evaluate and minimize risks.

43 Importance

44 While bioaerosols are a major concern for public health, accurately assessing human exposure is

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45 challenging. Highly contaminated environments, such as agricultural facilities, contain a broad
46 diversity of aerosolized fungi that may impact human health. Effective bioaerosol monitoring is
47 increasingly recognized as a strategic approach for achieving occupational exposure description.
48 Workers exposure to diverse fungal communities is certain, as fungi are ubiquitous in the
49 environments and the presence of potential sources increase their presence in the air. Applying
50 new molecular approaches to describe occupational exposure is a necessary work around the
51 traditional culture approaches and the biases they introduce to such studies. The importance of
52 the newly developed approach can help to prevent worker's health problems.
53

54 Introduction

55 Exposure to airborne microbial flora or bioaerosols in the environment, whether from indoor or
56 outdoor sources, is an everyday phenomenon that may lead to a wide range of human diseases.
57 Compared to other well-described microbial habitats, such as water and soil, little is known
58 about the diversity of airborne microbes (1, 2, 3). Whether aerosolized from natural sources (e.g.,
59 wind) or human activities (e.g., industrial processes), the dispersal of bioaerosols can impact
60 public health due to the presence of highly diverse and dynamic microbial communities in urban
61 and rural environments. These impacts range from allergies to asthma and can lead to exposure
62 to pathogens (4, 5, 6, 7). Occupational exposure to harmful bioaerosols in industrial
63 environments can be worrisome depending on the types of raw materials present, and the
64 disturbance and the intensity of air movement and ventilation. For example, animal feeding
65 operations involve various sources of biological material potentially associated with respiratory
66 problems (8, 9, 10, 11, 12, 13).

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67 Fungal bioaerosols consist of spores, mycelium fragments and debris which are easily inhaled by
68 workers and cause myriad symptoms including allergies, irritation and opportunistic infections.
69 Long-term lung exposure to fungal bioaerosols can be associated with chronic diseases while the
70 effects of short-term exposure range from irritation of the eyes and nose to coughing and a sore
71 throat (14, 15). Dairy-farm workers are exposed to high levels of fungal bioaerosols on a daily
72 basis. In fact, fungal concentrations in the air at dairy farms were reported to be higher than
73 bacterial concentrations and may reach up to 10^{11} colony-forming units/m^3 (16). At dairy farms,
74 hay and straw are important sources of fungal bioaerosols, as fungi naturally colonize those
75 substrates, especially if there are high moisture levels (17, 18, 19). Building type and
76 management practices (e.g. free stall, use of various bedding materials, ventilation type) also
77 influence the fungal load in bioaerosols.

78 The inhalation of large concentrations of fungal bioaerosols can lead to a variety of respiratory
79 problems. The major allergy-related diseases caused by fungi are allergic asthma (20, 21, 22),
80 allergic rhinitis (23, 24), allergic sinusitis (25), bronchopulmonary mycoses (26, 27), and
81 hypersensitivity pneumonitis (28, 29, 30). The latter includes farmer's lung disease (allergic
82 alveolitis), a disease specific to dairy farm workers (31, 32). Furthermore, a component of the
83 fungal cell wall ((1–3)-β-D glucan), is believed to play a role in pulmonary inflammation,
84 increased sensitivity to endotoxins and pulmonary embolisms (33, 34, 35). Some respiratory
85 symptoms are also associated with fungal exposure including mucous membrane irritation
86 syndrome, nasal congestion, sore throat, and irritation of the nose and eyes (36, 37, 38, 39).

87 The link between exposure to fungi and occupational diseases is often difficult to prove due to
88 undocumented fungi in bioaerosols. This lack of information is primarily due to the methods
89 used to describe fungi present in the workplace. In diversity studies, culture methods are

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90 associated with well-known biases as only the viable/culturable portion of the samples is
91 represented. Using culture-independent molecular methods is a good solution for getting around
92 the non-viable/non-culturable limits of the commonly used culture-based methods. Molecular
93 methods are based on the detection of the genetic material of organisms present in a given
94 sample. Applying these methods to samples from composting and biomethanization
95 environments allowed the identification and quantification of fungal bioaerosols present and a
96 better understanding of human exposure (40, 41). In dairy farms, only culture-dependent
97 methods have been used to assess occupational exposure or ambient fungal aerosols (42, 43, 44,
98 45). All of the previous studies identified the same frequently encountered genera including
99 *Aspergillus*, *Penicillium, Cladosporium* and *Alternaria.* In Canada, the most recent study that
100 described the airborne fungal microflora in dairy farms is from 1999 (16).
101 Because of the dearth of information about fungal diversity and concentrations in bioaerosols at
102 dairy farms, the aim of this study was to provide an in-depth characterization of fungal exposure
103 at Eastern Canadian dairy farms using qPCR and next-generation sequencing methods.

104 Results

105 Concentrations of fungal bioaerosols using culture methods to capture the viable spores and
106 qPCR for DNA quantification of *Penicillium/Aspergillus* genera and *Aspergillus fumigatus*
107 species are shown in Fig.1. Using culture methods, the results ranged from 3.2 x 10^6 to 8.2 x 10^6
108 CFU/m^3 in samples from the five dairy farms (DF1 to DF5). A strong correlation was observed
109 between concentrations obtained by culture methods and those obtained by qPCR targeting
110 *Penicillium* and *Aspergillus* (*PenAsp*). Concentrations of *PenAsp* ranged from 4.6 x 10^6 to 9.4 x
111 10^6 gene copies/m^3 at the five dairy farms. Greater variance was observed in concentrations of
112 *Aspergillus fumigatus* which, ranged from 1 x 10^4 gene copies/m^3 at DF3 to 4.8 x 10^5 gene

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113 copies/m^3 at DF2. Concentrations of *Aspergillus fumigatus* at DF1, DF4 and DF5 were 3 x 10^4,
114 2.9 x 10^4 and 3.9 x 10^5 gene copies/m^3, respectively. The highest concentrations of *PenAsp*
115 coincided with the highest concentrations of *Aspergillus fumigatus* as observed at DF2 and DF5
116 (Fig.1). The gap between the two concentrations was more notable in results from DF1, DF3 and
117 DF4 where concentrations of *Aspergillus fumigatus* were lower.
118 Samples were separate by four categorical variables: *Type of milking*, *animal space, cattle feed,*
119 and *type of ventilation*. Concentrations of *PenAsp* between groups of samples within each of
120 those categories were compared. The same comparison was made for *Aspergillus fumigatus*
121 concentrations. No significant differences ($p \leq 0.05$) in concentrations were found between the
122 groups of samples for any of the four variables for either *PenAsp* or *Aspergillus fumigatus* (Table
123 5).
124 Fungal communities were described by Illumina Miseq sequencing of the ITS1 region of the
125 fungal ribosomal RNA encoding gene. After quality filtering, dereplication and chimera
126 checking, 307 304 sequences were clustered into 188 OTUs. In order to confirm that the
127 sequencing depth was adequate to describe the fungal diversity at each of the sampling sites,
128 rarefaction analyses were performed using the observed OTUs alpha diversity metric. The
129 lowest-depth sample parameter was used to determine the sequencing depth of the rarefaction
130 analyses which was approximately 40 000 sequences per sample. Samples with a sequencing
131 depth lower than 40 000 were excluded from analyses. The higher the sequencing depth, the
132 more likely it is that the true diversity of the fungi in aerosols is captured. All of the samples
133 from the five dairy farms met this criterion and were included in the analyses. The values shown
134 in Fig.2 were calculated following these steps: ten values from 10 to 40 000 sequences per
135 sample were randomly selected. For each of these values the corresponding number of OTUs

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136 observed was noted for all of the samples. The plateaus observed in the five curves shown in
137 Fig.2 indicate an efficient coverage of the fungal diversity, as no more OTUs were observed
138 even with much greater numbers of sequences per sample.
139 Many next-generation sequencing surveys of microbial communities aim to compare the
140 composition of different groups of samples (beta diversity). This multivariate approach can be
141 used to assess the effects of several environmental factors on the microbial content of the
142 samples. The environmental factors, or "variables", are used to separate the samples into
143 different groups. In this case, the same four variables used to categorize qPCR concentrations
144 were also used for the next generation sequencing multivariate analysis and included: *Animal*
145 *space*, *Cattle feed, Type of milking* and *Type of ventilation*. One of the techniques commonly
146 used by microbial ecologists relies on the creation of a dissimilarity matrix like the Bray-Curtis
147 index. This index was used to evaluate the distance, taken pairwise between samples
148 (representing how closely related samples are). The index uses numbers between 0 and 1, where
149 0 means the two samples have the same composition and 1 means that they do not share any
150 species. Because the Bray-Curtis dissimilarity matrix uses the absolute abundance of OTUs, it is
151 necessary to use a rarefied OTU table as the input for the dissimilarity calculation. One function
152 of multivariate analyses is to represent inter-sample distances in a 2-dimensional space using
153 ordination (55). To evaluate ordination patterns, one of the most common methods used is the
154 Principal coordinate analyses (PCoA). In this case, the input used for ordination calculation and
155 clustering was the dissimilarity matrix calculated above. The matrix was transformed to
156 coordinates and then plotted using the principal coordinates script in QIIME. Table 6 shows a
157 summary of the results from the PCoA analyses (the PCoA figure is presented as a
158 supplementary file 1). The three principal coordinate axes captured more than 90% of the

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159 variation in the DF samples. Samples were coloured according to the four variables to visualize
160 and identify sample clustering. Samples closer to one another are more similar than those that are
161 further away from each other. No obvious sample clustering was observed for any of the four
162 variables. Though they were not clearly clustered, calculations based on *Animal space* and *Cattle*
163 *feed* were close together than the others. The samples from confined spaces were grouped far
164 from those from the semi-confined space. The forage samples were more closely grouped
165 compared to the samples with concentrates and forage & concentrates combinations. No patterns
166 were observed when samples were coloured according to the *Type of milking* or *Type of*
167 *ventilation*.

168 To determine the statistical significance of the variance observed in the PCoA analyses, a
169 PERMANOVA test was performed on the Bray-Curtis dissimilarity matrix. This non-parametric
170 test allows for the analysis of the strength that each variable have in explaining the variations
171 observed between samples (sample clustering). It is based on the ANOVA experimental design
172 but analyzes the variance and determines the significance using permutations, as it is a non-
173 parametric test (56). Whereas ANOVA/MANOVA assumes normal distributions and a
174 Euclidean distance, PERMANOVA can be used with any distance measure as long as it is
175 appropriate to the dataset. The same variables used for color clustering in the PCoA analyses
176 were used with the PERMANOVA test for statistical significance of sample clustering. The
177 QIIME compare categories script was used to generate the statistical results. Results from the
178 PERMANOVA are consistent with the color clustering observations made based on the PCoA
179 analyses. Using a significance of 0.05, the only variables that exhibited significant differences
180 among sample groupings were *Animal space* (p-value = 0.04) and *Cattle feed* (p-value = 0.05).

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181 The two other variables tested did not exhibit significant differences (*Type of milking* p-value =
182 0.61 and *Ventilation* p-value = 0.90).
183 The taxonomy of the microbes in the air samples collected from the dairy farms was determined
184 by comparing Illumina sequences to the UNITE database. Of the 12 fungal classes detected in
185 samples from the dairy farms (Fig.4) six classes seem to be dominant: *Eurotiomycetes,*
186 *Dothideomycetes, Wallemiomycetes, Agaricomycetes, Sordariomycetes* and *Tremellomycetes*.
187 However, there is variability in this dominance between the diversity profiles from the five dairy
188 farms. At DF3 and DF5 the class *Eurotiomycetes* have much greater relative abundance than the
189 other classes. In DF2 samples, *Dothideomycetes* and *Wallemiomycetes* are more abundant than
190 the other classes. The *Sordariomycetes* class is particularly more abundant at DF1 compared to
191 the other farms. Fungi from the class *Tremellomycetes* have greater relative abundance at DF4
192 than any of the other farms. In fact, DF4 has the most diverse profile, in contrast to samples from
193 DF3 where the class *Eurotiomycetes* represents 70% of the relative abundance.
194 *Ustilaginomycotina* were detected only in samples from DF4.
195 Relative abundance of taxa was analyzed more thoroughly by examining the 20 most abundant
196 genera at each dairy farm (Fig.4). From this list, only six fungi were present at all five of the
197 dairy farms: *Aspergillus*, *Penicillium*, *Wallemia, Aureobasidium, Pleosporales* and
198 *Tremellomycetes*. OTUs that were not identifiable to the genus level were identified to the
199 highest taxonomic level (e.g class *Tremellomycetes* and order *Pleosporales*). Similar to
200 observations made based on fungal class, diversity profiles of the genera present were quite
201 variable between the five farms. The least diverse profile was observed in samples from DF3
202 where *Penicillium* occupied 67% of the abundance. The most diverse profiles were from DF1,
203 DF4 and DF5 as they exhibited the greatest variety of fungal genera. In DF2 samples, 52% of the

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204 abundance was made up of *Wallemia* (31 %) and *Bipolaris* (21 %). The diversity profiles from
205 the five dairy farms are larger than what is shown in Fig.4. Due to graphical limitations, only the
206 most abundant fungi are represented. *Piptoporus* and *Microascus* were identified only at DF1.
207 *Exobasidiomycetes*, *Microdochium*, *Dissoconium* and *Parastagonospora* were present at DF2
208 exclusively. *Tubilicrinis* was detected only at DF3. *Mycoacia, Phlebia, Ustilago* and *Ganoderma*
209 were identified solely at DF4. Finally, *Whickerhamomyces* was specific only to samples from
210 DF5.

211 The diversity of fungi identified using the culture method was compared with the fungal
212 diversity obtained using next generation sequencing (NGS). Using NGS, fungal genera
213 representing greater than 1% of the total abundance of the five dairy farms combined are
214 presented in Fig.5. For species identified using the culture approach, the fungi identified at more
215 than one dairy farm were grouped together. The fungi that were detected only once by culture
216 were *Trichoderma, Microdochium, Phoma, Apiospora, Botrytis, Conyothirium, Millerozyma,*
217 *Neosetophoma, Irpex,* and *Debaryomyces*. Those species detected at more than one farm and
218 their relative abundances are presented in Fig.6. The relative abundance of fungi identified by
219 culture was calculated as follows: for each fungus, the number of times that it was isolated from
220 the five dairy farms was calculated. Based on this sum, a percentage of relative abundance was
221 calculated for each fungus and appears in the list in Fig.5. Only four fungi were detected by both
222 approaches: *Penicillium*, *Aspergillus*, *Bipolaris* and *Sarocladium*. Of the 16 fungi isolated using
223 culture techniques, three (*Hyphopichia, Gibellulopsis et Myceliophtora)* were not detected by
224 NGS. The remaining 13, though they do not appear on the list, were detected with a total
225 abundance of less than 1%. Many fungi genera were present but with a total relative abundance
226 of less than 1% making the diversity profile more exhaustive than what is shown in the figures.

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227 ## Discussion

228 Using molecular tools has enabled us to provide an in-depth description of the complex
229 biodiversity of bioaerosols in various occupational environments (11, 57, 58, 40, 41). At dairy
230 farms, molecular approaches targeting bacteria allow for a better understanding of the causes of
231 occupational respiratory diseases (59, 60). Since hay and straw are important sources of fungal
232 aerosols and are often ubiquitous at dairy farms, characterizing the fungal diversity of
233 bioaerosols is essential to better understand their role in occupational exposure. As mentioned
234 previously, most studies concerning dairy farm exposure use culture-based methods to study
235 aerosolized fungi. To address the major bias associated with culture methodology, which
236 represents only the viable portion of bioaerosols, this study also used a molecular approach
237 combining qPCR and next generation sequencing to describe the bioaerosol fungal exposure at
238 five dairy farms.

239 The *PenAsp* qPCR assay is a good indicator of the total quantities of *Aspergillus*, *Penicillium* and
240 *Paecilomyces* conidia in air samples (61). Results of this study showed a strong correlation
241 between concentrations of culturable fungi and *PenAsp.* This correlation supports the idea of
242 using the qPCR *PenAsp* assay as an indicator of total fungal concentration in exposure studies.
243 No significant differences were observed in fungal concentrations obtained from the five dairy
244 farms using qPCR and culture. These concentrations are comparable to concentrations obtained
245 using culture methods almost two decades ago from Eastern Canadian dairy farms (16, 59). This
246 suggests that dairy-farm workers are still at risk for developing diseases linked to fungal
247 exposure. Furthermore, *Aspergillus fumigatus* was specifically quantified in aerosols from areas
248 at dairy farms where humans work because it is a known pathogen that causes aspergillosis,
249 allergic bronchopulmonary aspergillosis and is involved in other pulmonary diseases (62, 63). In

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250 some cases, the gap between the concentrations of *PenAsp* and *Aspergillus fumigatus* can be
251 used as an indicator of the diversity of *Aspergillus* and *Penicillium* genera in air samples.
252 The qPCR analysis allowed the quantification of potentially hazardous fungal spores in
253 bioaerosols. No particular correlation was found between the types of ventilation, animal
254 confinement, cattle feed and milking methods, and concentrations of *PenAsp* and *Aspergillus*
255 *fumigatus* in aerosols from dairy farms. These results prove that no matter how different the
256 building attributes, animal confinement and types of milking activities are, exposure to fungal
257 bioaerosols should be considered regardless of the modernity of the method used.
258 The MiSeq Illumina sequencing depth used in this study was adequate for covering the true
259 diversity of fungi in the samples. Targeting the ITS1 genomic region provided an in-depth
260 analysis of the fungal composition of bioaerosols at the five dairy farms. The methodology
261 applied also revealed the variations in fungal communities present in the air (40, 41). Differences
262 in the diversity profiles support the idea that this approach identifies a large number, if not all of
263 the taxa that are responsible for the fungal community changes. These variations in diversity
264 profiles may be explained by the presence of different and multiple sources of fungal bioaerosols
265 at dairy farms. Four variables were chosen to examine these differences more closely. The use of
266 multivariate analyses, PCoA, coupled with a PERMANOVA test, offers a robust statistical
267 significance of sample clustering using distance matrices. Both analyses (PCoA and
268 PERMANOVA) resulted in the same conclusions in regards of sample clustering confirming
269 their usefulness as tools to visualize and measure sample clustering. The main source of the
270 variation in diversity is associated with cattle feed type. Dairy cattle are fed a wide range of
271 feedstuffs, from forage (grasses, legumes, hay, straw, grass silage and corn silage) to
272 concentrates (barley and maize). The presence of *Ustilaginomycotina* and *Exobasidiomycetes*

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273 could be explained by the presence of wheat and other grasses. These classes of fungi include the
274 plant pathogen *Tilletia* known to affect various grasses. Biochemical changes in these products,
275 like pH and water content, can affect their fungal composition (64, 65). Animal confinement also
276 affected the fungal composition of bioaerosols. The semi-confined environment consists of an
277 enclosure where dairy cattle have freedom to move around inside the enclosed space. The
278 confined spaces allow no freedom of movement and each cow has its own space. These
279 differences in the density of cows seem to have an impact on the fungal bioaerosols. The type of
280 milking whether automated or manual, and the type of ventilation, either automatic or manual
281 does not seem to have an effect on the fungal content of the bioaerosols collected. However, a
282 limited number of dairy farms were visited during this study and multivariate analyses and
283 sample clustering methods are known to perform better with a large number of samples. A larger
284 number of air samples collected from different dairy farms would be useful to support the
285 findings that milking method and or types of ventilation influences fungal bioaerosol variability.
286 Other factors like building attributes, handling of feed, seed and silage, and method of spreading
287 the bedding can affect the fungal content of the bioaerosols at dairy farms (66, 67, 68, 69). While
288 PCoA gives a cursory assessment of the variables that affect sample clustering, these variables
289 can often be harder to define. A set of chosen explanatory environmental factors does not
290 guarantee that they have true explanatory power. There is always the possibility that an
291 unexplored covariate is the real causal influence on the microbial ecology of the samples (70).
292 Further research including larger sample sizes and additional variables should be conducted.
293 *Agaricomycetes* are a group of fungi known for their role in wood-decaying activities and in
294 ectomycorrhizal symbiosis (71, 72). The presence of agricultural planting material/products may
295 explain the larger proportions of *Agaricomycetes* identified at DF1 and DF2 compared to the

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296 three other farms. Conversely, *Eurotiomycetes* are a class of fungi linked to processes like
297 fermentation used in food processing. Many genera of this class are natural decomposers and are
298 involved in food spoilage (73,74). The presence of natural or processed foods at DF3, DF4 and
299 DF5 might explain the greater abundance of *Eurotiomycetes* detected in the air at those farms.
300 Additionally, the prevalence of *Eurotiomycetes* might also be explained by the presence of silage
301 which is a fermented, high-moisture stored fodder used to feed cattle (75). Members of
302 *Dothideomycetes* and *Tremellomycetes* include several important plant pathogens that grow on
303 wood debris or decaying leaves (76, 77). *Wallemiomycetes* were detected at all five dairy farms.
304 They were most prevalent at DF1 and DF2, representing 20% and 32% of genera detected,
305 respectively. This class includes one order (*Wallemiales*), containing one family (*Wallemiaceae*),
306 which in turn contains one genus (*Wallemia*) (78). These fungi can grow over a wide range of
307 water activity from 0.69 a_w to 0.997 a_w (79). Water activity is the vapour pressure of water in the
308 product divided by vapour pressure of pure water at the same temperature. High a_w support more
309 microbial growth. *Wallemia* have been isolated in air samples from dairy farms in previous
310 studies (80). Airborne *Wallemia* are suspected of playing a role in human allergies like bronchial
311 asthma (81). A study conducted in France identified *Wallemia* as a causative agent of farmer's
312 lung disease (82). Other prevalent fungal genera commonly found at dairy farms were identified
313 in this study: *Aspergillus*, *Penicillium*, *Cladosporium*, *Alternaria, Nigrospora* and *Periconia*.
314 For relative abundance, differences observed in the diversity profiles obtained by next generation
315 sequencing (NGS) and culture methods may be explained by the hypothesis that the culture
316 approach may be biased toward fungi from the rare biosphere. These results are consistent with
317 the conclusions made by Shade and his collaborators (83) regarding the complementarity of
318 culture-dependent and culture-independent approaches to studying bacterial diversity. The

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319 premise of their study is that culture-dependent methods reveal bacteria from the rare biosphere
320 and provide supplemental information to that obtained using a NGS approach. In the current
321 case, this complementarity is true only for abundance. As mentioned previously, only three fungi
322 were detected exclusively by culture, while more than a hundred fungi were identified by NGS
323 and not by culture. This is consistent with the concept that culture methods may reveal less
324 abundant taxa in an environment while NGS provides a more exhaustive diversity profile. To the
325 best of our knowledge this is the first research to compare both approaches for examining
326 aerosols at dairy farms.

327 The application of the NGS approach revealed a large fungal diversity profile in bioaerosols
328 released from five dairy farms. The presence of a diverse portrait of fungi in air may represent a
329 health risk for workers who are exposed on a daily basis. In some cases, the allergen/infective
330 activity of the fungi may not be known and can increase the risks to workers. More specifically,
331 the following fungi detected are known allergens and/or are opportunistic pathogens:
332 *Aspergillus, Malassezia, Wallemia, Emericella, Fusarium, Alternaria* and *Candida. Malassezia*
333 causes skin disorders and can lead to invasive infections in immunocompetent individuals (84).
334 *Emericella* is a taxon of teleomorphs related to *Aspergillus.* Species of this group are known
335 agents of chronic granulomatous disease (CGD; 85). *Acremonium* causes fungemia in
336 immunosuppressed patients (86). *Fusarium* species are responsible for a broad range of health
337 problems, from local and systemic infections to allergy-related diseases such as sinusitis, in
338 immunodepressed individuals (87). *Alternaria* is an important allergen related to asthma (88).

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339 # Methodology

340 ## Environmental Field Samples

341 Air samples were collected from five dairy farms in Eastern Canada during summer 2016. At
342 each farm, a sampling site was designated based on where activities that generate the most
343 bioaerosols took place. The buildings at each farm exhibited differences in building type and
344 characteristics (age, volume, ventilation), number of animals present (cows), methods of milking
345 (automatic or manual) and types of animal feed animal were given. Table 1 presents a
346 description of the sampling sites at each dairy farm. At each sampling site, three air samples
347 were collected during the morning milking activity, when workers are exposed to the most
348 bioaerosols, for a total of 15 samples.

349 ## Air Sampling

350 A liquid cyclonic impactor Coriolis µ® (Bertin Technologies, Montigny-le-Bretonneux, France)
351 was used for collecting air samples. The sampler was set at 200 L/min for 10 minutes (2m^3 of air
352 per sample) and placed within 1-2 meters of the source. The air flow in the sampler creates a
353 vortex through which air particles enter the Coriolis cone and are impacted in the liquid. Fifteen
354 millilitres of a phosphate buffer saline (PBS) solution with a concentration of 50 mM and a pH
355 of 7.4 were used to fill the sampling cone.

356 ## Culture-Based Approach to Study Fungal Diversity

357 One millilitre of the 15ml Coriolis sampling liquid was used to perform a serial dilution from 10^0
358 to 10^{-4} concentration/ml. The dilutions were made using 0.9% saline and 0.1% Tween20 solution
359 and were performed in triplicate. Tween20 is a detergent that makes spores less hydrophobic and
360 easier to collect. One hundred microlitres of each triplicate were plated on Rose Bengal Agar

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361 with chloramphenicol at a concentration of 50 μg/ml. Half of the petri dishes were incubated at
362 25°C for mesophilic mould growth and the other half at 50°C for thermophilic mould growth,
363 specifically *Aspergillus fumigatus*. After 5 days of incubation, moulds were identified and counts
364 were translated into CFU/m^3.

365 *Identification of Isolates*

366 Spores from cultured fungi were recovered in one millilitre of a 0.9 % saline and 0.1% Tween20
367 solution and stored in an Eppendorf tube. Two hundred microlitres of the collection liquid were
368 placed in an FTA card (sample collection card; Qiagen, Mississauga, Ontario, Canada). Five
369 punches from the inoculated zone of the FTA card were placed in a microtube and washed three
370 times with the FTA purification agent. The washing step is mandatory as it allows the removal of
371 the chemical substrates in the FTA card that may alter the subsequent amplification step. Forty-
372 eight microlitres of the master mix solution described in table 2 were placed in each microtube
373 followed by amplification and sequencing of the ITS genomic region. The protocol described by
374 White and his collaborators (46) was performed at the CHU (*Centre hospitalier de l'Université*
375 *Laval*). The following oligonucleotides were used for the ITS region amplification:
376 ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'
377 ITS4: 5'-TCCTCCGCTTATTGATATGC-3'
378 The identification of the isolates was made by comparing the sequences obtained with sequences
379 in the UNITE database.

380 Fungal Spore Concentration by Filtration

381 The following methods are described in detail by Mbareche and his coauthors 2018 (47). Briefly,
382 the 45ml Coriolis suspension was filtered through a 2.5cm polycarbonate membrane (0.2-mm
383 pore size; Millipore) using a vacuum filtration unit. The filters were placed in a 1.5ml Eppendorf

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384 tube with 750µl of extraction buffer (bead solution) from a MoBio PowerLyser® Powersoil®
385 Isolation DNA kit (Carlsbad, CA, U.S.A) and a 0.3cm tungsten bead. The filters were flash-
386 frozen by placing the Eppendorf tube in a 99% ethanol solution and dry ice. The frozen filters
387 were then pulverized using the tungsten steel bead in the Eppendorf tube in a bead-beating
388 machine (a Mixer Mill MM301, Retsch, Düsseldorf, Germany) set at a frequency of 20
389 movements per second for 20 minutes. The liquid containing the pulverized filter particles was
390 used as aliquot for the first step of the DNA extraction procedure.

391 ## DNA Extraction

392 Using the same bead-beating machine, a second bead-beating step using glass beads at a
393 frequency of 20 movements per second for 10 minutes was performed to ensure that all of the
394 cells were ruptured. Next, a MoBio PowerLyser® Powersoil® Isolation DNA kit (Carlsbad, CA,
395 U.S.A) was used to extract the total genomic DNA from the samples following the
396 manufacturer's instructions. Next the DNA was eluted in a 100µl buffer and stored at -20°C until
397 subsequent analyses.

398 ## Real-Time PCR Quantification

399 PCR was performed with a Bio-Rad CFX 96 thermocycler (Bio-Rad Laboratories, Mississauga,
400 CANADA). The PCR mixture contained 2µl of DNA template, 0.150 µM per primer, 0.150 µM
401 probe, and 7.5µl of 2× QuantiTect Probe PCR master mix (QuantiTect Probe PCR kit; Qiagen,
402 Mississauga, Ontario, Canada) in a 15-µl reaction mixture. The results were analyzed using Bio-
403 Rad CFX Manager software version 3.0.1224.1015 (Bio-Rad Laboratories). *Aspergillus*
404 *fumigatus* was used as positive control and for the standard curves of both qPCR analyses
405 (*Penicillium/Aspergillus* and *Aspergillus fumigatus*). Table 3 presents the primers, probes and
406 PCR protocol used in this study.

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407 ## Next-Generation Sequencing

408 The rRNA fungal gene ITS1 was used for the next-generation sequencing analyses.
409 Amplification of the amplicons, equimolar pooling and sequencing were performed at the
410 Plateforme d'analyses génomiques (IBIS, Université Laval, Quebec City, Canada). Briefly,
411 amplification of the ITS1 regions was performed using the sequence specific regions described
412 by Tedersoo et al. (2015) (48) and references therein, using a two-step dual-indexed PCR
413 approach specifically designed for Illumina instruments. First, the gene-specific sequence was
414 fused to the Illumina TruSeq sequencing primers and PCR was carried out on a total volume of
415 25 µL of liquid made up of 1X Q5 buffer (NEB), 0.25 µM of each primer, 200 µM of each of the
416 dNTPs, 1 U of Q5 High-Fidelity DNA polymerase (NEB) and 1 µL of template cDNA. The PCR
417 started with an initial denaturation at 98°C for 30 s followed by 35 cycles of denaturation at 98°C
418 for 10 s, annealing at 55°C for 10 s, extension at 72°C for 30s and a final extension step at 72°C
419 for 2 min. The PCR reaction was purified using an Axygen PCR cleanup kit (Axygen). Quality
420 of the purified PCR products was verified with electrophoresis (1% agarose gel). Fifty to 100-
421 fold dilution of this purified product was used as a template for a second round of PCR with the
422 goal of adding barcodes (dual-indexed) and missing sequence required for Illumina sequencing.
423 Cycling conditions for the second PCR were identical to the first PCR but with 12 cycles. The
424 PCR reactions were purified as above, checked for quality on a DNA7500 Bioanlayzer chip
425 (Agilent) and then quantified spectrophotometrically with a Nanodrop 1000 (Thermo Fisher
426 Scientific). Barcoded Amplicons were pooled in equimolar concentration (85 ng/µl) for
427 sequencing on the illumina Miseq.

428 The oligonucleotide sequences used for amplification are presented in Table 4.

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429 Please note that primers used in this work contain Illumina specific sequences protected by
430 intellectual property (Oligonucleotide sequences © 2007-2013 Illumina, Inc. All rights reserved.
431 Derivative works created by Illumina customers are authorized for use with Illumina instruments
432 and products only. All other uses are strictly prohibited.).

433 Bioinformatics Workflow

434 The bioinformatics workflow used in this study was developed during a compost study by
435 Mbareche et al. (40). Briefly, after demultiplexing the raw FASTQ files, the reads generated
436 from the paired end sequencing using Mothur v 1.35.1 were combined (49*)*. Quality filtering was
437 performed using Mothur by discarding reads with ambiguous sequences. Reads shorter than 100
438 bp and longer than 450 bp were also discarded. Similar sequences were combined to reduce the
439 computational burden, and the number of copies of the same sequence was displayed. This
440 dereplication step was performed using USEARCH version 7.0.1090 (50). The selected region of
441 fungal origin was then extracted from the sequences with ITSx which uses HMMER3 (51) to
442 compare input sequences against a set of models built from a number of different ITS region
443 sequences found in various organisms. Only the sequences belonging to *fungi* were kept for
444 further analyses. Operational taxonomic units (OTUs) with a 97% similarity cut-off were
445 clustered using UPARSE 7.1 (52). UCHIME was used to identify and remove chimeric
446 sequences (53). QIIME version 1.9.1 (54) was used to assign taxonomy to OTUs based on the
447 UNITE fungal ITS reference training data set for taxonomic assignment and to generate an OTU
448 table. The fungal diversity analysis was achieved by using different QIIME scripts. The alpha
449 and beta diversity scripts used are listed in the following link: http://qiime.org/scripts/.

450 Statistical Analyses

451 Concentrations of *PenAsp* were compared with the Kruskal-Wallis one-way analysis of variance.

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452 The test was performed using the software R version 3.3.2 with RStudio Version 0.99.486. The
453 same analysis was performed comparing concentrations of *Aspergillus fumigatus.*
454 To determine the statistical significance of the variation observed with the multivariate analyses
455 (PCoA), a PERMANOVA test was performed on the Bray-Curtis dissimilarity matrix. The
456 *compare categories* QIIME script was used to generate the statistical results. Because
457 PERMANOVA is a non-parametric test, significance is determined through permutations. The
458 number of permutations used is 999. P-value ≤ 0.05 was considered statistically significant.
459 Detailed information about the performance of the test are presented in the multivariate section
460 of the results.

461 Conclusion

462 Bioaerosols from Eastern Canadian dairy farms contain high concentrations of highly diverse
463 fungi. This study demonstrated that fungal bioaerosols have large diversity profiles. It also adds
464 another piece to the puzzle regarding the complexity of bioaerosols relative to the sources
465 present. This study also highlights the importance of using a high-throughput sequencing method
466 combined with real-time PCR assay for quantification and an in-depth characterization of fungal
467 diversity in bioaerosols to better assess occupational exposure. As air samples were collected
468 during activities where workers are present, this study shows that human exposure to harmful
469 fungi may be higher during milking activities (automatic or manual), as well as during handling
470 of feed and silage and when spreading bedding. Based on the results of this investigation, the
471 authors strongly recommend taking action to reduce workers' exposure to bioaerosols. Such
472 measures include increased air-exchange rates, better confinement and source ventilation. If
473 these measures cannot be applied, we recommend skin and respiratory protection for workers
474 who are exposed on a daily basis as a means to reduce continuous exposure to harmful fungi

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475 present in bioaerosols. The broad spectrum of fungi detected in this study includes many know
476 pathogens and proves that adequate monitoring of bioaerosol exposure is necessary to evaluate
477 and minimize risks.

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724 **Table 1:** Description of the sampling sites and the parameters affecting the sampling

725 environments

	Type of milking	*Animal space*	*Cattle feed*	*Ventilation*	*Temperature*	*Time of sampling*
DF1	manual	confined	forage	natural	22°C	6 am – 9 am
DF2	automatic	confined	forage	mechanical	21°C	7 am – 10 am
DF3	manual	confined	concentrates	natural	19°C	7 am – 10 am
DF4	automatic	confined	concentrates & forage	mechanical	20°C	6 am – 9 am
DF5	manual	semi-confined	forage	mechanical	23°C	11 am – 2 pm

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728 **Table 2**: Master mix of the ITS amplification reaction

Substance	Concentration	Volume
PCR-mix (IDT, Coralville, États-Unis)	5x	10µl
$MgCl_2$ (IDT, Coralville, États-Unis)	25 nM	6µl
Taq DNA polymerase (IDT, Coralville, États-Unis)	5 µ/µl	0.5µl
Primer ITS1 (Promega, Madison, États-Unis)	100 µM/769 µl IDTE Buffer pH8	0.125µl
Primer ITS4 (Promega, Madison, États-Unis)	100 µM/1399 µl IDTE Buffer pH8	0.125µl
dNTP (IDT, Coralville, États-Unis)	10 nM	0.5µl
ADN	-	2µl of ADN (5 punch of the FTA-card)
H_2O	-	30.75µl

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730 **Table 3**: Primers, probes and protocol used for qPCR quantification of selected microorganisms

Microorganisms and references	Primers and probes	PCR protocol
Penicillium, Aspergillus and *Paecilomyces variotii* http://www.epa.gov/microbes/moldtech.html	**PenAsp1mgb** (Taqman) PenAspR1: 5'-GCCCGCCGAAGCAAC-3' PenAspF1: 5'-CGGAAGGATCATTACTGAGTG-3' PenAspP1mgb: 5'-FAM-CCAACCTCCCACCCGTG-TAMRA-3'	Activation: 94°C-3min Denaturation: 94°C-15sec Annealing/extension: 60°C-60sec Cycles: 40
Aspergillus fumigatus and Neosartoya fischeri http://www.epa.gov/microbes/moldtech.html	**Afumi** (Taqman) AfumiR1: 5'-CCGTTGTTGAAAGTTTTAACTGATTAC-3' AfumiF1: 5'-GCCCGCCGTTTCGAC-3' AfumiP1: 5'-CCCGCCGAAGACCCCAACATG-3'	Activation: 94°C-3min Denaturation: 94°C-15sec Annealing/extension: 60°C-60sec Cycles: 40

731

732 **Table 4**: Primers used for Illumina amplification

First-PCR primer	**ITS1Fngs**: 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTCATTTAGAGGAAGTAA-3' **ITS2**: 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTGCGTTCTTCATCGATGC-3'
Second-PCR primer	**Generic forward**: 5'-AATGATACGGCGACCACCGAGATCTACAC[index1]ACACTCTTTCCCTACACGAC-3' **Generic reverse**: 5'-CAAGCAGAAGACGGCATACGAGAT[index2]GTGACTGGAGTTCAGACGTGT-3'

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734

735 **Table 5:** Comparison of p-value of the concentrations obtained by qPCR between groups of

736 samples within four environmental factors using Kruskal-Wallis one-way analysis of variance.

Environmental factors	*PenAsp* (p-value)	*Aspergillus fumigatus* (p-value)
Animal Space (confined vs semi-confined)	0.09	0.06
Cattle feed (forage vs concentrates vs concentrates & forage)	1	0.07
Type of milking (automatic vs manual)	0.74	0.07
Type of Ventilation (mechanical vs natural)	0.8	0.08

737

738

739 **Table 6: Summary of the parameters and results of the principal coordinates analysis of air**

740 **samples collected from five dairy farms including the statistical significance of the sample**

741 **clustering. The PCoA was calculated using the Bray-Curtis dissimilarity based on ITS1**

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742 **sequences.** The three principal coordinate axes captured over 90% of the variation in the input
743 DF samples**.** The statistical significance of the variation observed with the PCoA analyses was
744 determined using a PERMANOVA statistical test. Four environmental variables were used for
745 sample clustering, only two were statistically significant (*Animal space* and *Cattle feed*).

Environmental factors	Sample clustering	Permanova (p-value)
Animal Space (confined vs semi-confined)	√	0.04
Cattle feed (forage vs concentrates vs concentrates & forage)	√	0.05
Type of milking (automatic vs manual)	X	0.61
Type of Ventilation (mechanical vs natural)	X	0.90

746
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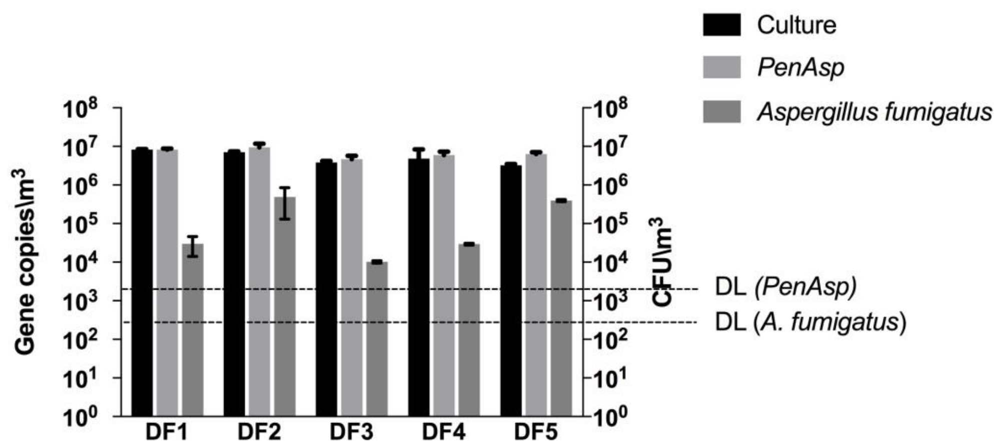

748

749 **Figure 1: Concentrations of viable spores of mesophilic fungi (from culture),**
750 ***Penicillium/Aspergillus* (*PenAsp* from qPCR) and *Aspergillus fumigatus* (from qPCR) in air**
751 **samples collected from five different dairy farms**. The detection limit of the qPCR run was 2 x
752 10^3 Gene copies/m^3 for *PenAsp* and 5 x 10^2 Gene copies/m^3 for *Aspergillus fumigatus*.

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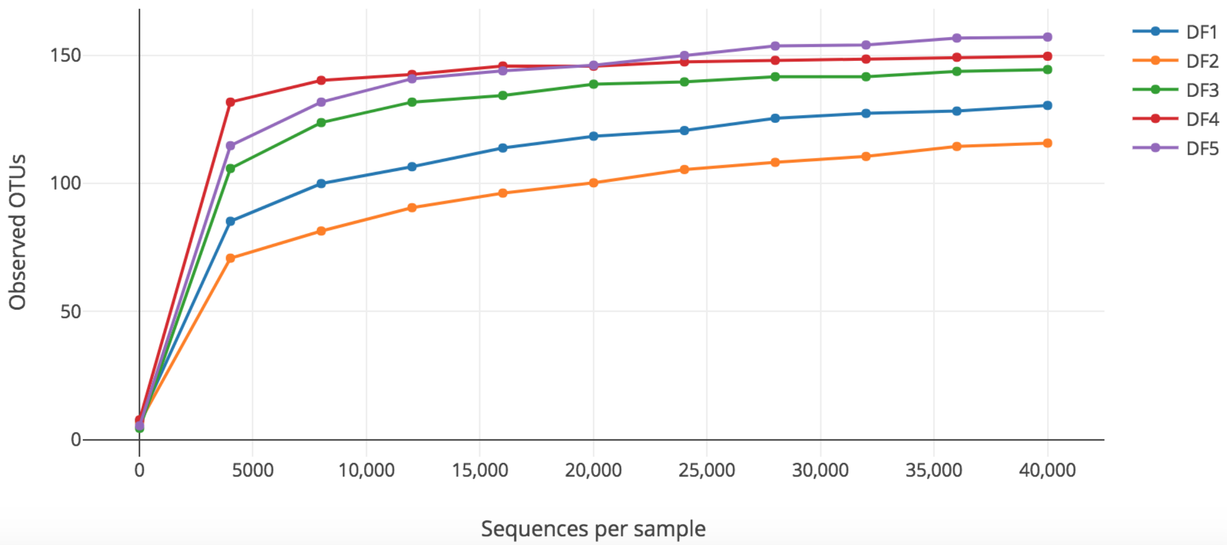

753

754 **Figure 2: Rarefaction curves obtained from the number of observed OTUs and the**
755 **sequences per sample for air samples from the five dairy farms visited.** The plateau of the
756 curves started at around 5000 sequences.

757

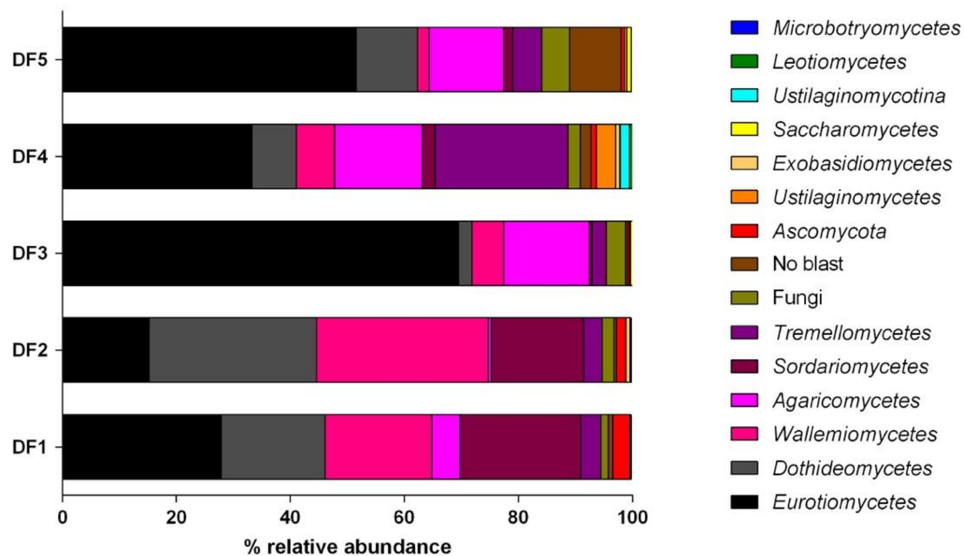

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Figure 3: Relative abundance of fungi classes detected in air samples from five dairy farms using next-generation sequencing. While 12 classes were detected, six dominate the diversity profiles. Those six classes have different distributions among the five dairy farms visited.

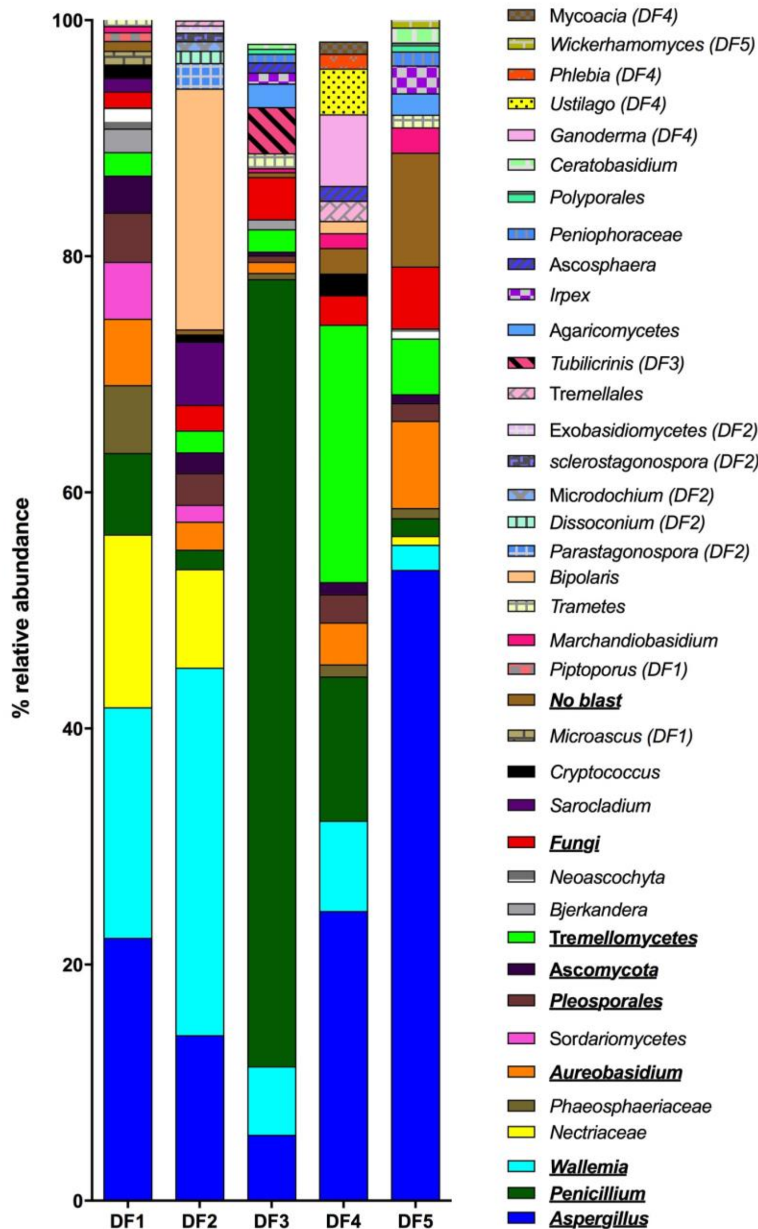

Figure 4: Relative abundance of fungal genera detected in air samples from five dairy farms. The 20 most abundant genera from each dairy farm were included in the analyses. The

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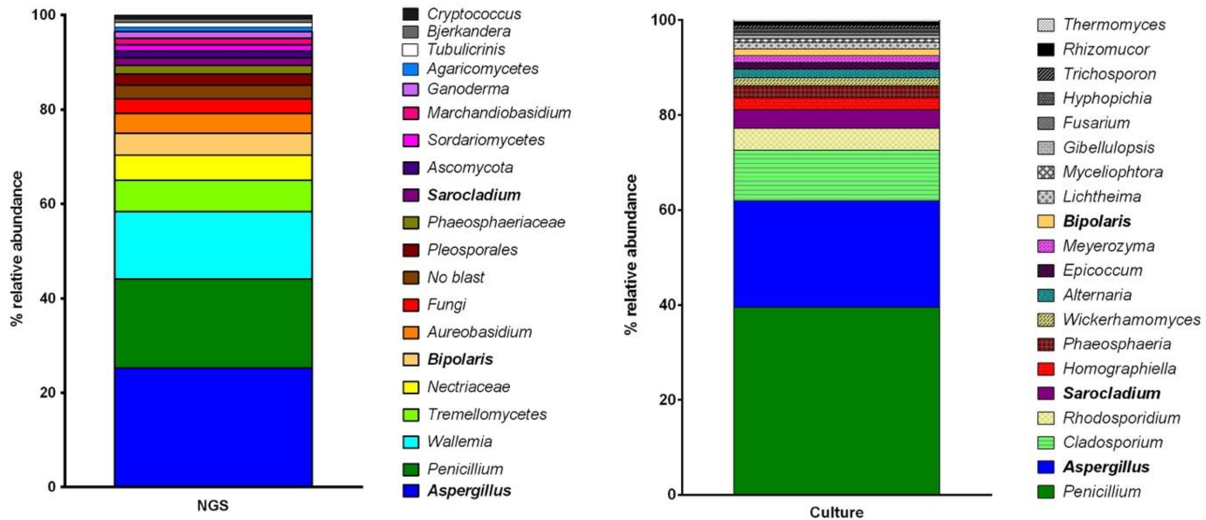
766 underlined bold fungi were common in all the dairy farms. The fungi that were detected only in
767 one dairy farm have the DF identification after their name.
768



769

770 **Figure 5: Relative abundance of fungal genera identified by next generation sequencing**
771 **(NGS) and culture in air samples collected from five dairy farms.** Fungi in bold character are
772 common to both approaches.
773