Mycobiome analyses of critically ill COVID-19 patients suggests antifungal prophylaxis may be protective against *A. fumigatus*-associated mortality

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- Processing of specimens and generation of data: DW, SG, MB, JP, JPG, FR and HG.
- Analysis and interpretation of data: DW, SG, MH, MJB and PB
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Abstract

Rationale: Covid-associated pulmonary aspergillosis (CAPA) is a life-threatening complication in patients with severe COVID-19. Previously, acute respiratory distress syndrome in patients with COVID-19 has been associated with lung fungal dysbiosis, evidenced by reduced microbial diversity and *Candida* colonisation. Increased fungal burden in the lungs of critically ill COVID-19 patients is linked to prolonged mechanical ventilation and increased mortality. However, specific mycobiome signatures associated with severe COVID-19 in the context of survival and antifungal drug prophylaxis have not yet been determined and such knowledge could have an important impact on treatment.

Objectives: To understand the composition of the respiratory mycobiome in critically ill COVID-19 patients with and without CAPA, the impact of antifungal use and its role in patient outcome.

Methods: We performed a multi-national study of 39 COVID-19 patients in intensive care units (ICU) with and without CAPA. Respiratory mycobiome was profiled using ITS1 sequencing and *Aspergillus fumigatus* burden was further validated using qPCR. Fungal communities were investigated using alpha diversity, beta diversity, taxa prevalence and taxa abundances.

Measurements and Main Results: Respiratory mycobiomes were dominated by *Candida* and *Aspergillus.* There was no significant association with corticosteroid use or CAPA diagnosis and respiratory fungal communities. Increased *A. fumigatus* burden was associated with mortality. The use of antifungals at ICU admission was associated with an absence of *A. fumigatus.*

Conclusions: Our findings suggest that systemic antifungal treatment at ICU admission may be protective against *A. fumigatus*-associated mortality in CAPA.

58

59 Introduction

60 COVID-19 is a pulmonary disease caused by severe acute respiratory syndrome coronavirus 2.
61 There have been over 500 million confirmed cases of COVID-19 since December 2019 with
62 mortality > 6 million[1]. Around 5% of patients with COVID-19 require admission into the
63 intensive care unit (ICU)[2,3] and, 50% of those patients need mechanical ventilation[4], thus
64 increasing the risk of hospital-acquired pneumonia[5]. The role of the pulmonary microbiome
65 and its associations with disease outcomes in patients with COVID-19 has been explored since
66 the beginning of the pandemic[6–8]. However, our knowledge on the role of fungi in the
67 pathophysiology of COVID-19 is limited. Specifically, the association between respiratory
68 mycobiome composition and patient outcome, and the interplay of antifungal use, is yet to be
69 investigated.

70 Mycobiome sequencing of the upper respiratory tract (nasopharyngeal swabs) suggests
71 COVID-19 infection significantly reduces fungal diversity, with a higher abundance of
72 *Alternaria* and *Cladosporium* spp., and a lower abundance of other taxa such as *Candida* and
73 *Aspergillus* in those infected[9]. In the lower respiratory tract (tracheal aspirates), bacterial and
74 fungal microbiome analyses of patients with severe COVID-19 have shown changes over time
75 that might be linked to antimicrobial pressure[10]. A variety of respiratory mycobiome clusters
76 were identified, including those dominated by *Candida* and *Cladosporium.* Using 18S qPCR in
77 bronchoalveolar lavage (BAL) samples it has been reported that critically ill COVID-19 patients
78 with high fungal burdens are less likely to be liberated from mechanical ventilation[11].
79 However, the taxa responsible for this outcome remains unclear. Lastly, mycobiome
80 sequencing of BAL found COVID-19 patients with acute respiratory distress syndrome (ARDS)

81 to be associated with reduced fungal diversity and an increase in *Candida* colonisation[12]. In
82 patients without *Candida* colonisation, an increased abundance of an unclassified *Ascomycota*
83 species was identified. However, little is known of the fungal species present in the airways of
84 patients with fungal disease, in particular *Aspergillus*-related pulmonary infections, with the
85 exception of fungal allergic diseases[13].

86 Covid-associated pulmonary aspergillosis (CAPA) is an important complication of COVID-19.
87 CAPA has mainly been described in critically ill patients. Multicentre cohort studies of CAPA
88 conducted in the ICU setting report incidence rates varying between 10-15% per centre[14–16].
89 Nevertheless, mortality rates in patients with CAPA were double that observed in critically ill
90 COVID-19 patients without CAPA[17,18]. Airway epithelial cell damage due to viral replication and
91 COVID-19 associated downregulation of interferon γ signalling pathway, aberrant immune
92 responses due to ARDS, corticosteroids, azithromycin, or the use of immunomodulators have
93 been linked with susceptibility to CAPA[19–22]. Here we performed a multi-national mycobiome
94 analysis of 39 respiratory samples from critically ill COVID-19 patients with and without CAPA.

95 Methods

96 **Study design, participants, and sample collection**

97 This study was based on a multinational retrospective study on the prevalence of invasive
98 pulmonary aspergillosis in critically ill COVID-19 patients in intensive care units (ICUs) during
99 2020[15]. Inclusion criteria consisted of: PCR confirmed COVID-19 infection, bronchoscopy or
100 tracheal aspiration performed during routine clinical investigations, and chest imaging
101 available seven days before or after respiratory samples were collected. Patients less than 18
102 years of age were excluded. Respiratory samples not passing quality control (described in PCR
103 & Sequencing section) were also excluded. Respiratory specimens obtained at ICU admission

104 or during ICU stay were collected, aliquoted (at least 1ml) and stored at -80 °C. Criteria for
105 defining aspergillosis were according to previous guidelines[23] with the following
106 modifications: COVID-19 requiring ICU admission was included as an additional host factor,
107 tracheal aspirates were equated to bronchoalveolar lavage (BAL) fluid for microbiological
108 tests, and serum and BAL GM was added as entry criterion. For a summary of full patient
109 demographics, see Table E1.

110 IRB approval was obtained at each participating center: Medical University of Graz EC #32-296
111 ex 19/20. University of Genoa Liguria Region Ethics Committee registry number 163/2020.

112 **Sample processing**

113 BAL DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method[24]. Full
114 details on sample processing are provided in the online supplement. Briefly, for mycobiome
115 analysis, the ITS1 region was amplified using Nextera XT compatible versions of ITS1[25] and
116 ITS2degen (a degenerate version of ITS2 primer) (see Table 1). The presence of *A. fumigatus*
117 in respiratory samples was validated using a TaqMan probe assay targeting the ITS1 region[26].

118 **Data analysis**

119 Paired end reads were subject to quality trimming at Q30 and a minimum length filter of 75
120 nucleotides using bbduk[27] (BBMap v38.22). Primer sequences were removed using Cutadapt[28]
121 (v1.18). Reads were mapped to UNITE database using bowtie2[29] (v2.3.5.1). Count data was
122 further processed in R (v4.1.3) using the following packages: phyloseq[30] v1.38.0, vegan[31] v2.5-
123 7, DESeq2[32] v1.34.0, stringr[33] v1.4.0, ggplot2[34] v3.3.5 and tidyr[35] v1.2.0.

124 To minimise contamination in the mycobiome data, samples were subject to a multi-staged
125 quality control process using negative extraction control (NEC) samples, along with qPCR and

126 sequencing data. Firstly, samples from a DNA extraction batch with *A. fumigatus* detected (Ct
127 <40) in the corresponding NEC by qPCR were excluded. Secondly, samples were excluded if
128 they produced less than 500 fungal reads (read count filter) and/or a lower number of fungal
129 reads than the corresponding NEC sample (NEC count filter). Of 99 initial samples, 22 were
130 removed due to contamination of an extraction batch, 35 samples did not pass the read count
131 filter and 3 did not pass the NEC count filter.

132 Abundances were standardised to the median sequencing depth. Extremely low abundance
133 taxa were removed by only retaining those occurring > 0.2% in any sample. DESeq2 was used
134 to identify significantly differentially abundant taxa (adjusted *p* value < 0.05 and basemean >
135 500). Differences in diversity (Shannon, Chao1 and observed OTUs) were assessed using
136 pairwise Wilcoxon rank sum tests. PERMANOVA test was used to assess differences in Bray-
137 Curtis ordination.

138

139 Results

140 **Patient cohort**

141 The respiratory mycobiome of 91 critically ill COVID-19 patients in the intensive care unit (ICU)
142 was analysed using internal transcribed spacer 1 (ITS1) amplicon sequencing of BAL. Samples
143 from 39 patients harboured significant fungal communities which passed quality control (See
144 Data analysis section in methods). Table 1 describes demographic and clinical characteristics
145 of the 39 patients maintained in the mycobiome analysis, stratified by CAPA diagnosis. Six
146 patients had CAPA (15%). Patients were from Genoa, Graz and Rennes (64%, 26% and 10%,
147 respectively). Mean age of those with and without CAPA was 61 and 64, respectively. Most
148 patients received systemic corticosteroids (74% overall, 83% of those with CAPA and 73% of

149 those without CAPA). No patients diagnosed with CAPA received systemic antifungals at the
150 time of ICU admission. Mortality at end of follow-up in patients with CAPA was 33% (2/6) while
151 mortality in patients without CAPA was also 33% (11/33).

152 **Table 1.** Clinical and demographic characteristics of patients with and without probable
153 CAPA diagnosis.

Variable	**Patients with CAPA (N=6)**	**Patients without CAPA (N=33)**
Age		
Mean (SD)	61 (3.4)	64 (7.9)
valid (missing)	6 (0)	32 (1)
Sex		
male	100% (6)	79% (26)
female	0% (0)	18% (6)
missing	0% (0)	3% (1)
Ethnicity		
Caucasian	100% (6)	91% (30)
other		6.1% (2)
missing	0% (0)	3% (1)
BMI > 30		
yes	50% (3)	18% (6)
no	50% (3)	79% (26)
missing	0% (0)	3% (1)
Smoking		
yes	33% (2)	12% (4)
no	67% (4)	85% (28)
missing	0% (0)	3% (1)
Institution		
Graz	33% (2)	24% (8)
Genoa	17% (1)	73% (24)
Rennes	50% (3)	3% (1)
Hematology oncology		
yes	17% (1)	9.1% (3)
no	83% (5)	88% (29)
missing	0% (0)	3% (1)
Solid organ transplant		
yes	17% (1)	3% (1)
no	83% (5)	94% (31)
missing	0% (0)	3% (1)
Cardiovascular disease		
yes	67% (4)	45% (15)

no	33% (2)	52% (17)
missing	0% (0)	3% (1)
Pulmonary disease		
yes	33% (2)	24% (8)
no	67% (4)	73% (24)
missing	0% (0)	3% (1)
Diabetes Mellitus		
yes	17% (1)	9.1% (3)
no	83% (5)	88% (29)
missing	0% (0)	3% (1)
Corticosteroids		
yes	83% (5)	73% (24)
no	17% (1)	24% (8)
missing	0% (0)	3% (1)
Tocilizumab		
yes	17% (1)	6.1% (2)
no	83% (5)	91% (30)
missing	0% (0)	3% (1)
Azithromycin		
yes	33% (2)	33% (11)
no	67% (4)	61% (20)
missing	0% (0)	6.1% (2)
Systemic antifungals at ICU admission		
yes	0% (0)	15% (5)
no	100% (6)	6.1% (2)
missing	0% (0)	79% (26)
Life Support		
mechanical	100% (6)	70% (23)
ECMO		3% (1)
noninvasive		6.1% (2)
mechanical & noninvasive		12% (4)
none		6.1% (2)
missing	0% (0)	3% (1)
Duration ICU (days)		
Mean (SD)	27 (11)	32 (28)
valid (missing)	6 (0)	32 (1)
Palliative (Day 28 or 32)		
yes	67% (4)	48% (16)
no	33% (2)	48% (16)
missing	0% (0)	3% (1)
Survival (at end of follow up)		
yes	33% (2)	33% (11)
no	67% (4)	64% (21)
missing	0% (0)	3% (1)

Days from ICU admission to CAPA		
Mean (SD)	6.2 (3.5)	-
BAL GM (ODI > 1)		
positive	83% (5)	9.1% (3)
negative	17% (1)	82% (27)
missing	0% (0)	9.1% (3)
BAL PCR		
positive	50% (3)	0% (0)
negative	0% (0)	58% (19)
missing	50% (3)	42% (14)
BAL culture		
positive	50% (3)	0% (0)
negative	50% (3)	97% (32)
missing	0% (0)	3% (1)
BAL LFD		
positive	17% (1)	6.1% (2)
negative	0% (0)	9.1% (3)
missing	83% (5)	85% (28)
Tracheal Aspirate GM		
positive	0% (0)	3% (1)
negative	0% (0)	3% (1)
missing	100% (6)	94% (31)
Tracheal Aspirate PCR		
positive	33% (2)	0% (0)
negative	17% (1)	21% (7)
missing	50% (3)	79% (26)
Tracheal Aspirate Culture		
positive	17% (1)	0% (0)
negative	33% (2)	3% (1)
missing	50% (3)	97% (32)
Serum GM (> 0. 5)		
positive	33% (2)	0% (0)
negative	50% (3)	42% (14)
missing	17% (1)	58% (19)
Bronchial Aspirate culture		
positive	17% (1)	0% (0)
negative	33% (2)	21% (7)
missing	50% (3)	79% (26)

154 CAPA; COVID-19 associated pulmonary aspergillosis. BMI; body mass index. ICU; intensive care unit. ECMO;
155 extracorporeal membrane oxygenation. BAL; bronchoalveolar lavage. GM; galactomannan. LFD; lateral flow
156 device.

157 ***Candida* and *Aspergillus spp.* dominate respiratory mycobiome communities in critically ill**

158 **COVID-19 patients**

159 Median read counts per sample for the 39 samples that passed our quality metric was 57,913

160 (range 6,970 – 342,640). Total taxa identified was 58, with a median of 7 taxa per sample

161 (range 2 – 28 taxa). At the genus level, there were 36 taxa in total and a median of 5 taxa per

162 sample (range 1 – 22). There was no significant clustering between samples batches (Fig. E1),

163 suggesting that sample processing had no impact on mycobiome communities. Mycobiomes

164 were predominantly consisted of *Candida* and *Aspergillus* species (Fig. 1A). *Candida albicans*,

165 *Aspergillus fumigatus* and *Candida parapsilosis* were the most abundant species (Fig. E2A-B).

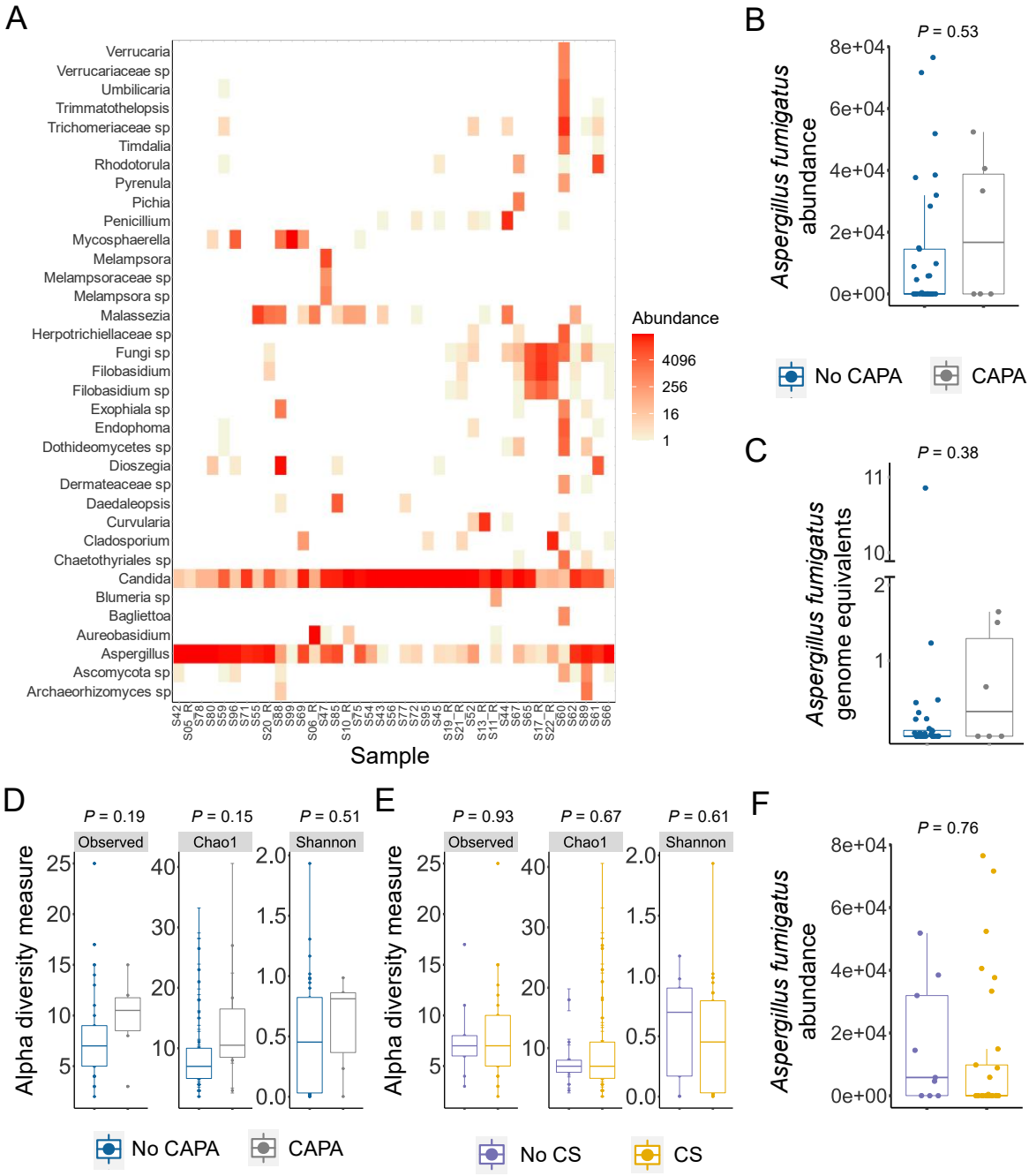

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Fig. 1. *Aspergillus* and *Candida spp.* dominate the respiratory mycobiome in critically ill COVID-19 patients. (A) *Aspergillus* and *Candida* were the main genus observed in the lungs from COVID-19 patients included in the study (n = 39). Median *A. fumigatus* abundance was higher in patients with coronavirus associated pulmonary aspergillosis (CAPA) compared to those without CAPA, measured as (B) Raw sequence reads generated by ITS1 sequencing (Median ~16,700 vs. 35) and (C) *A. fumigatus* specific qPCR (Median 0.33 vs. 0 genome equivalents) As one outlier (Grubbs test, $P < 0.0001$) had a genome equivalents value > 10, a Y axis break is included for ease of visualising majority of data points. (D) Alpha diversity measures (Observed OTUs, Chao1 and Shannon) trended towards higher diversity in CAPA patients (E) Corticosteroid treatment caused no apparent effect on alpha diversity as measured by Observed OTUs and Chao1. Shannon diversity trended towards lower diversity in patients receiving corticosteroids. (F) Median *A. fumigatus* abundance (sequence reads) was lower in patients receiving corticosteroids (Median 8 reads compared to 5,800 in those without corticosteroids). Hypothesis testing was applied using Wilcoxon Rank Sum tests. CS: Corticosteroids. Boxplot data represent median and interquartile range.

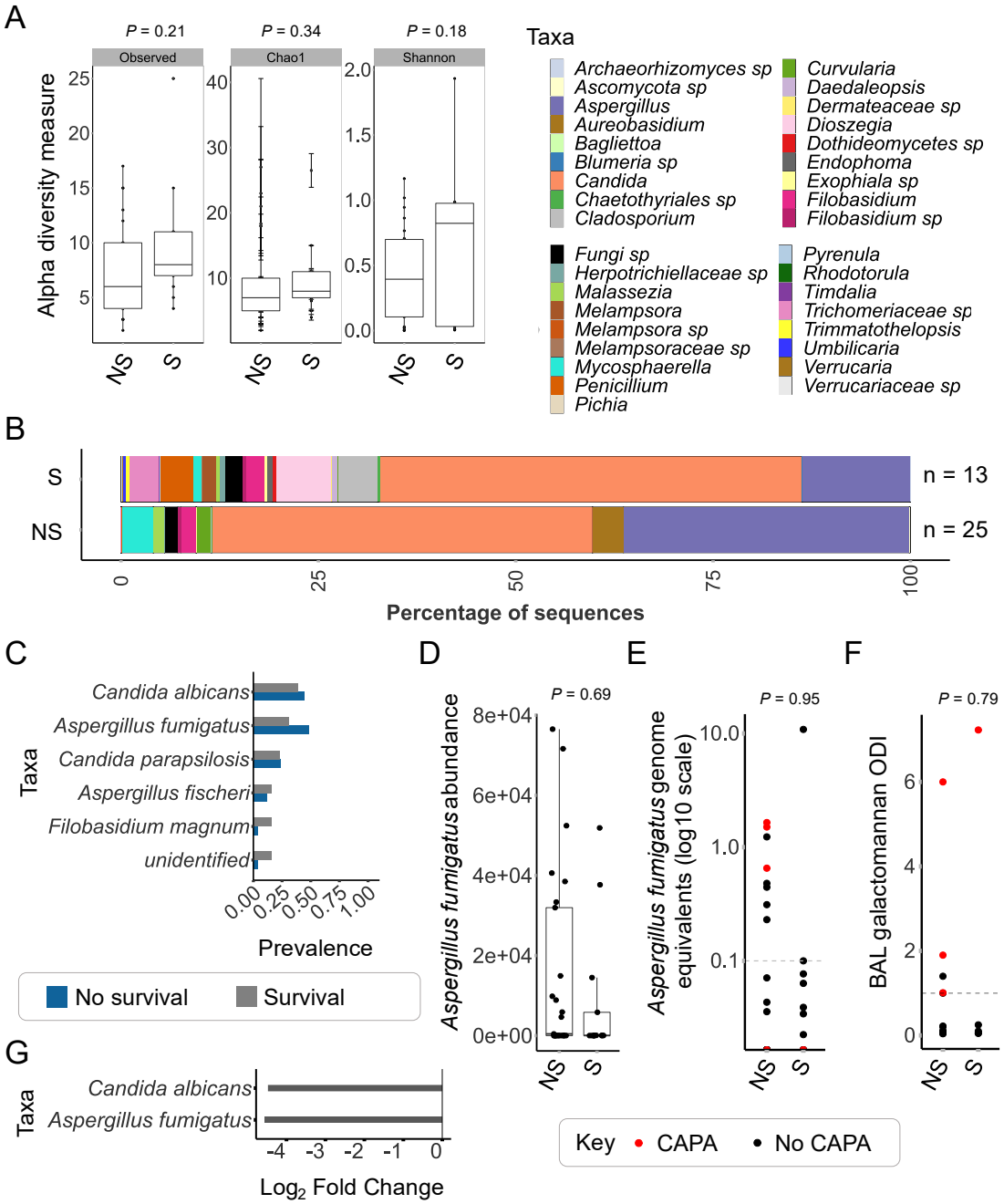
167 **CAPA patients have higher median *A. fumigatus* abundance and trend towards lower**
168 **mycobiome diversity whereas corticosteroid use has a less apparent effect on the**
169 **mycobiome**

170 In our study, higher median *A. fumigatus* levels were observed in CAPA when assessed by read
171 count (Median ~16,700 vs. 35) and (C) *A. fumigatus* specific qPCR (Median 0.3 vs. 0 genome
172 equivalents) (Fig. 1B-C), but there was overlap between the patient groups and, possibly due
173 to low sample size for CAPA patients, statistical significance was not reached. *A. fumigatus*
174 burden in patients without CAPA was varied, as some patients had little to no *A. fumigatus*
175 and others contained particularly high burden. Furthermore, species differential abundance
176 analysis did not find significant differences between *A. fumigatus* levels based on CAPA status.
177 Instead, *Cladosporium delicatulum, Mycosphaerella tassiana* and *Filobasidium magnum* were
178 found to be at higher abundance in CAPA patients (Fig. E4). In addition, probable CAPA
179 patients had higher median α diversity (Observed OTUs, Chao1 and Shannon), however, this
180 difference was not significant, and no significant effect on β diversity (Bray-Curtis ordination)
181 was observed (Fig. 1D, Fig. E3).

182 Corticosteroid use resulted in lower median α diversity (Shannon only), however, this
183 difference was not significant (Fig. 1E). Median levels of *A. fumigatus* were lower in the
184 corticosteroid treated group as assessed by sequencing (8 vs. ~5,800 reads). and qPCR (0 vs.
185 0.1 genome equivalents) (Fig.1F, Fig. E3). However, patients on corticosteroids were highly
186 heterogenous in terms of *A. fumigatus* abundance, some harboured little to no *A. fumigatus*
187 and others displayed considerably high amounts. Species differential abundance analysis
188 found no significant differences between corticosteroid usage. In addition, corticosteroid use
189 had no significant impact on β diversity (Bray-Curtis ordination) (Fig. E3).

190 **Increased *A. fumigatus* burden is associated with mortality**

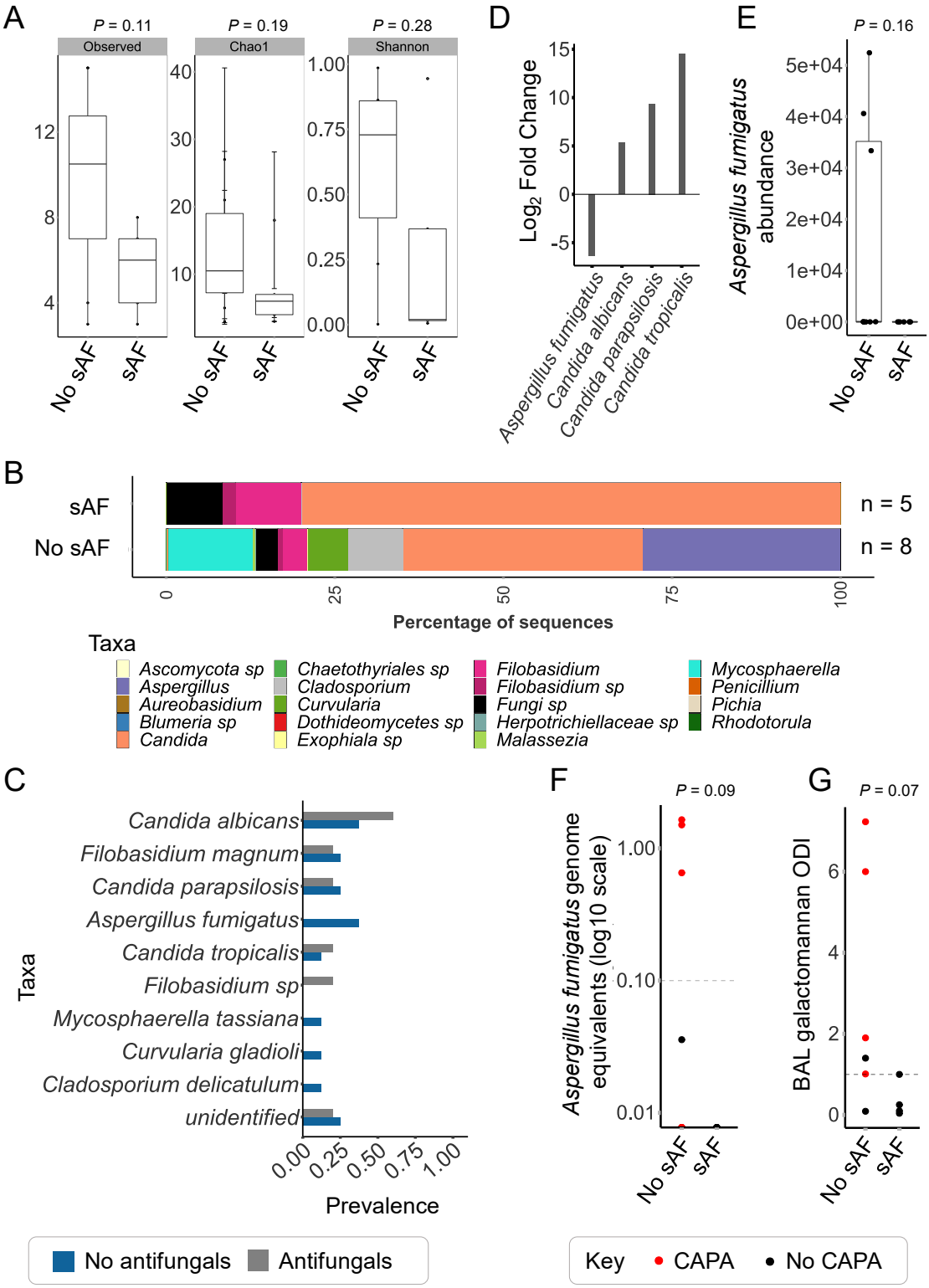
191 The mycobiome of individuals surviving CAPA (at the end of follow-up) showed a trend
192 towards higher median α diversity (Fig. 2A). Alongside an increased number of OTUs, grouped
193 mean abundances indicated a lower level of *Aspergillus* was present upon survival (Fig. 2B).
194 Differences in species prevalence suggested this difference was due to a reduction in *A.*
195 *fumigatus* in particular (Fig. 2C). Furthermore, mycobiome differential abundance analysis
196 using DESeq2 found *A. fumigatus* and *Candida albicans* to be significantly less abundant upon
197 survival, with log fold change values of -4.3 and -4.6, respectively (*padj* < 0.05, baseMean >
198 500) (Fig. 2D). Although *A. fumigatus* counts and genome equivalents (as determined by qPCR)
199 trended towards lower levels upon survival, these changes were not significant by Wilcoxon
200 Rank sum tests (Fig. 2E-F). Notably, *A. fumigatus* qPCR burden in those that did not survive
201 appeared to be bimodal; 8 samples had a burden above a threshold of 0.1 haploid genome
202 equivalents (HGE), 3 of which were patients with probable CAPA. Most patients which
203 survived had HGE =< 0.1, excluding one outlier which had extremely high burden (> 10, higher
204 than any other sample). In addition, BAL galactomannan index values were not significantly
205 different between the patient groups, with again an outlier in the survival group having a very
206 high BAL GM index (Fig. 2G).



207 **Fig. 2. A higher *A. fumigatus* burden is associated with mortality in critically ill COVID-19 patients.** (A) Alpha diversity measures (Observed OTUs, Chao1 and Shannon) trended towards higher diversity in critically ill COVID-19 patients which survived. (B) At the genus level, BAL ITS1 mycobiomes from patients which survived (n =13) indicated a lower proportion of *Aspergillus*, and an apparent increase in the number of observed taxa overall. (C) Increased *A. fumigatus* burdens in lungs of COVID-19 patients which did not survive (blue, 0.5 prevalence) compared to those that did survive (grey, 0.3 prevalence). Taxa were counted if present at over 5% in a sample, and only taxa found in at least 10% of samples of either group are shown. (D) Analysis using DESeq2 identified *A. fumigatus* and *C. albicans* to be at significantly lower abundance in patients which survived. S: Survival; NS: No survival. Data represent median and interquartile range. (E) Median *A. fumigatus* abundance (raw sequence reads) was not significantly different between the patients. (F) *A. fumigatus* burden measured by qPCR in patients which did not survive appeared biomodal, with some patients exhibiting a high burden (>0.1 genome equivalents). (G) BAL galactomannan levels between patient groups were not significantly different. Hypothesis testing in A and D-F was applied using Wilcoxon Rank Sum tests.

208 **Systemic antifungal use at intensive care unit admission reduces *A. fumigatus* burden in**
209 **critically ill COVID-19 patients and is associated with COVID-19 survival**

210 Use of systemic antifungals at ICU admission in critically ill COVID-19 patients resulted in
211 significantly reduced α diversity when analysing raw mycobiomes (prior to filtering for very
212 rare taxa). Upon removal of very rare taxa, there was a trend towards reduced median α
213 diversity upon systemic antifungal use at ICU admission (Fig. 3A). At the genus level, there
214 appeared to be a strong reduction in *Aspergillus* counts that correlated with treatment (Fig.
215 3B). At the species level, the most prevalent taxa in patients without systemic antifungal
216 treatment was *A. fumigatus*, whereas this species was not detectable in patients receiving
217 treatment (Fig. 3C). Furthermore, differential abundance analysis of mycobiome data found a
218 significant reduction of *A. fumigatus* in patients which received systemic antifungal treatment
219 (LFC -6.3, *padj* 0.04) (Fig. 3D). This analysis also found *Candida albicans, Candida parapsilosis*
220 and *Candida tropicalis* had significantly higher abundance in COVID-19 patients receiving
221 antifungal treatment at ICU admission (LFC 5.3, 9.4 and 14.5, respectively). Although no
222 significant difference was found between *A. fumigatus* counts or qPCR burden in groups with
223 or without systemic antifungals, three patients not receiving antifungals did have high *A.*
224 *fumigatus* levels and were also positive for CAPA (Fig. 3E-F). BAL galactomannan index was
225 not statistically different between patients with or without systemic antifungal use, however,
226 all individuals receiving treatment were GM negative (ODI < 1) whereas only half of the
227 patients without treatment were GM negative (Fig.3G).



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229 **Fig. 3. *A. fumigatus* is associated with the absence of systemic antifungals** at **intensive care unit**

230 **admission in critically ill COVID-19 patients.** (A) Alpha diversity measures (Observed OTUs, Chao1 and

231 Shannon) trended higher diversity in COVID-19 patients without systemic antifungals at ICU admission

232 (sAF). (B) At the genus level, BAL ITS1 mycobiomes from COVID-19 patients which received sAF (n =8)

233 display an absence of *Aspergillus.* (C) Species prevalence in each group indicated a complete lack of *A.*
234 *fumigatus* in patients received sAF (grey) compared to those that did not (blue). Taxa were counted if
235 present at over 5% in a sample, and only taxa found in at least 10% of samples of either group are
236 shown. (D) Analysis using DESeq2 identified *A. fumigatus* to be at significantly lower abundance in the
237 mycobiome of patients who received antifungals, compared to patients who did not. In contrast, three
238 *Candida* species were found to be significantly more abundant in the patients receiving antifungals. (E)
239 Median *A. fumigatus* abundance (raw sequence reads) was not significantly different between the
240 patients. (F) *A. fumigatus* burden measured by qPCR in COVID-19 patients which did not receive sAF
241 appeared bimodal, with three patients exhibiting a high burden (~1 genome equivalents). Notably
242 these patients were all diagnosed with COVID19-associated pulmonary aspergillosis (CAPA)(red). (G)
243 All COVID-19 patients receiving antifungals where BAL galactomannan negative (1 or lower). Half of
244 patients not receiving antifungals were galactomannan positive. Hypothesis testing in A and E-G was
245 applied using Wilcoxon Rank Sum tests. sAF: Systemic antifungals.

246 Our findings suggest an association between *A. fumigatus* abundance and mortality in
247 critically ill COVID-19 patients, and that systemic antifungals at ICU significantly reduces *A.*
248 *fumigatus* levels. Therefore, we combined these factors to assess the association between *A.*
249 *fumigatus* and survival outcomes depending on the presence or absence of systemic
250 antifungal treatment. Upon combining these factors, it was apparent that a higher level of
251 *Aspergillus* was associated with mortality only in COVID-19 patients who had not received
252 systemic antifungal treatment (Fig. 4A). Presence of *A. fumigatus* in only those patients that
253 did not survive or receive systemic antifungal treatment was confirmed by qPCR data (Fig. 4B).
254 Therefore, these findings suggest systemic antifungal treatment at ICU admission may have
255 been protective against *A. fumigatus*-associated mortality in patients with severe COVID-19.

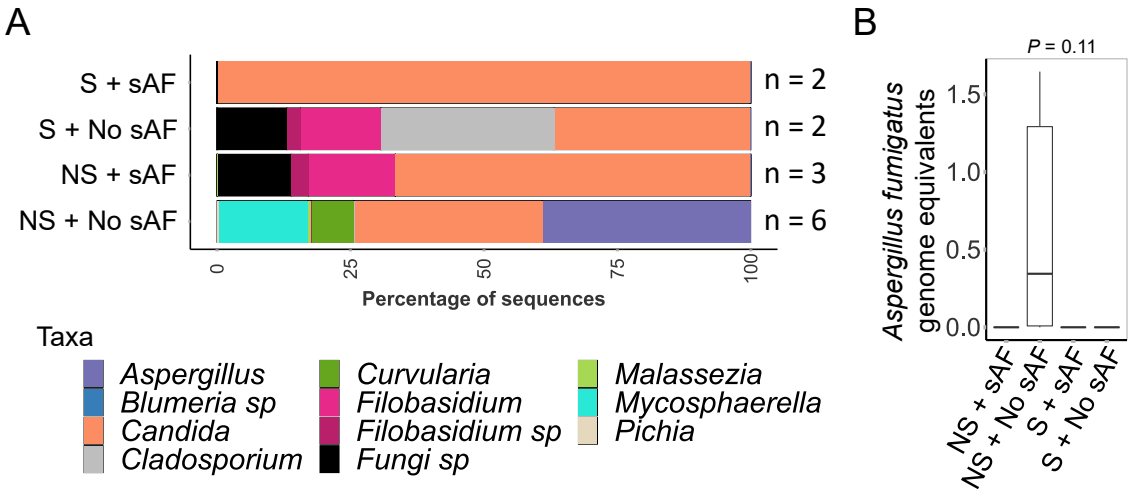

Fig. 4. *A. fumigatus* is associated with mortality in patients with COVID-19 who have not received systemic antifungals at intensive care unit admission. (A) When combining the use of systemic antifungals at ICU and survival outcomes, *Aspergillus* was only found in the BAL ITS1 mycobiomes from patients which did not receive sAF or survive (n=6). (B) *A. fumigatus* levels (measured by qPCR) did not differ significantly between groups (Wilcoxon Rank Sum test); however, *A. fumigatus* burden was observed only in the patient group which did not survive or receive systemic antifungals at ICU admission. sAF: Systemic antifungals. S: Survival. NS: No survival.

Discussion

This multinational study found that higher *Aspergillus fumigatus* burdens in critically ill COVID-19 patients are associated with increased mortality. In addition, the association of *A. fumigatus* with mortality was found only in patients who did not receive antifungal treatment at ICU admission, suggesting prophylactic use of antifungals in severe COVID-19 patients may reduce *A. fumigatus*-associated mortality in this cohort.

The respiratory mycobiomes of critically ill COVID-19 patients described here were dominated by *Candida* and *Aspergillus.* Previous studies using similar patient groups have also reported fungal communities in the lung to be dominated by *Candida*[10,12]. Furthermore, one study identified a significant increase in unidentified Ascomycota spp. in patients without *Candida* colonisation[12]. Due to the reported incidence of CAPA in severe COVID-19, the authors hypothesised that *Aspergillus* could be present in these patients and, although

271 mycobiome samples were mostly negative for *Aspergillus*, the presence of *Aspergillus* was
272 confirmed by PCR in follow up BAL samples in over 20% of patients.

273 A recent report suggests higher fungal burden in the lung microbiota of patients with
274 proven/probable CAPA[11]. However, a considerable overlap in fungal burdens between those
275 with and without CAPA was noted. Our study found CAPA patients had higher median levels
276 of *A. fumigatus* and trended towards higher fungal diversity. However, these findings did not
277 meet statistical significance, which may have been driven by the low sample size (n = 6 in
278 CAPA group). Some patients within the non-CAPA group harboured considerable levels of *A.*
279 *fumigatus.* It is known that high *Aspergillus* burdens can be found in the lungs of healthy
280 individuals[36]. These observations suggest that as long as a sufficient level of Aspergillus is
281 present in the lung, other factors such as disease susceptibility or strain virulence in the
282 context of CAPA may be more important than burden in the outcome of infection. This study
283 was limited to one sample time point, and it would be interesting to assess how *Aspergillus*
284 burden changes during the course of CAPA or COVID-19 infection.

285 It has been suggested that corticosteroid treatment increases lung fungal burden
286 (particularly increased abundance of *A. fumigatus*)[13] and lowers Shannon diversity[37] in
287 asthmatic patients. In contrast, no significant differences were found between mycobiome
288 diversity or taxa abundance in respiratory fungal communities of COPD patients with or
289 without inhaled corticosteroid treatment[38]. Another recent study found that alterations in
290 the airway mycobiome in COPD were not significantly affected by oral or inhaled
291 corticosteroid use[39]. Large cohort studies suggest systemic corticosteroids are a risk factor
292 for CAPA[16,19], however, there are no specific reports of the influence corticosteroid use has
293 on lung fungal communities in COVID-19. In our study, patients receiving corticosteroids

294 displayed lower median levels of *A. fumigatus* and lower fungal diversity (by Shannon metric
295 only) compared to those not receiving corticosteroids. However, these differences did not
296 meet statistical significance. As most (~74%) patients received corticosteroids in this cohort,
297 this may have contributed to low power in these statistical comparisons, warranting further
298 data on this topic.

299 A high fungal burden has previously been associated with a lower likelihood of release from
300 mechanical ventilation and increased mortality risk in severe COVID-19 patients[11]. However,
301 as this study utilised pan-fungal qPCR to identify burden, there was no indication of the
302 specific fungal taxa responsible for this association. Our findings suggest that higher levels of
303 *A. fumigatus* in particular are associated with increased mortality in severe COVID-19. There
304 are no previous reports on the impact of antifungal use on the respiratory mycobiome in
305 COVID-19 patients. In this study, the use of antifungals at ICU admission appeared protective
306 against *A. fumigatus*-associated mortality.

307 Our study has investigated the composition of respiratory fungal communities in critically ill
308 COVID-19 patients with and without CAPA. *Candida* and *Aspergillus* were predominant in the
309 respiratory communities. CAPA diagnosis was associated with higher median *A. fumigatus*
310 level and fungal diversity, and a higher prevalence of *A. fumigatus* was associated with
311 mortality and a lack of systemic antifungals at ICU admission. Our data provides evidence
312 suggesting the use of prophylactic antifungals in seriously ill COVID-19 patients may prevent
313 *A. fumigatus*-associated mortality. A limitation of this study is the small number of patients
314 included, particularly the low number of CAPA cases. In addition, incomplete clinical data
315 with respect to antifungal use reduced the sample sizes for this comparison, which may have
316 resulted in low power for these statistical tests. Therefore, a further study of a larger cohort

317 is warranted to further understand the association between prophylactic antifungal use, the
318 presence of *A. fumigatus* in the respiratory mycobiome and patient outcome in COVID-19
319 critical care.

320

321 Conflicts of Interest

322

323 MH received research funding from Gilead, Astellas, MSD, Euroimmune, IMMY, Scynexis,
324 Pulmocide, F2G and Pfizer, outside the submitted work.

325 MJB is a former employee and has previously received research funding from F2G outside
326 the submitted work.

327 In the past 5 years SG has received speaker fees from Gilead Sciences and research grant
328 support from Pfizer outside of the submitted work.

329 Outside the submitted work, DRG reports investigator-initiated grants from Pfizer, Shionogi,
330 and Gilead Italia and speaker and/or advisor fees from Pfizer and Tillotts Pharma.

331 Outside the submitted work, MB reports research grants and/or personal fees for
332 advisor/consultant and/or speaker/chairman from Bayer, BioMérieux, Cidara, Cipla, Gilead,
333 Menarini, MSD, Pfizer, and Shionogi. In the past 5 years JPG has received speaker fees from
334 Gilead Sciences, MundiPharma, Pfizer, and Shionogi outside of the submitted work. In the
335 past 5 years HG has received speaker fees from Gilead Sciences. JP has received speakers'
336 fees from Gilead Sciences, Pfizer, Swedish Orphan Biovitrum, Associated of Cape Cod outside
337 of the submitted work, served at advisor boards Gilead Sciences and Pfizer, and holds stocks
338 of NovoNordisk and AbbVie Inc. All remaining authors declare no competing interests.

339

340

341

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346 the NIHR of the Department of Health and Social Care.

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440

441

442 Supplementary data

443 Raw sequence data has been deposited at the NCBI sequence read archive (SRA) under accession
444 number PRJNA905224. Code used for analysis is available at
445 https://github.com/Danweaver1/COVID_respiratory_mycobiome.

446

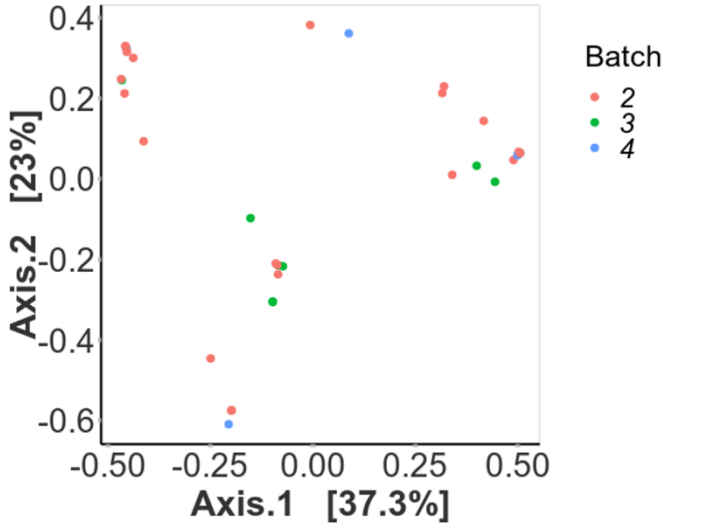

Fig. E1. Mycobiome Bray-Curtis ordination by sample batch

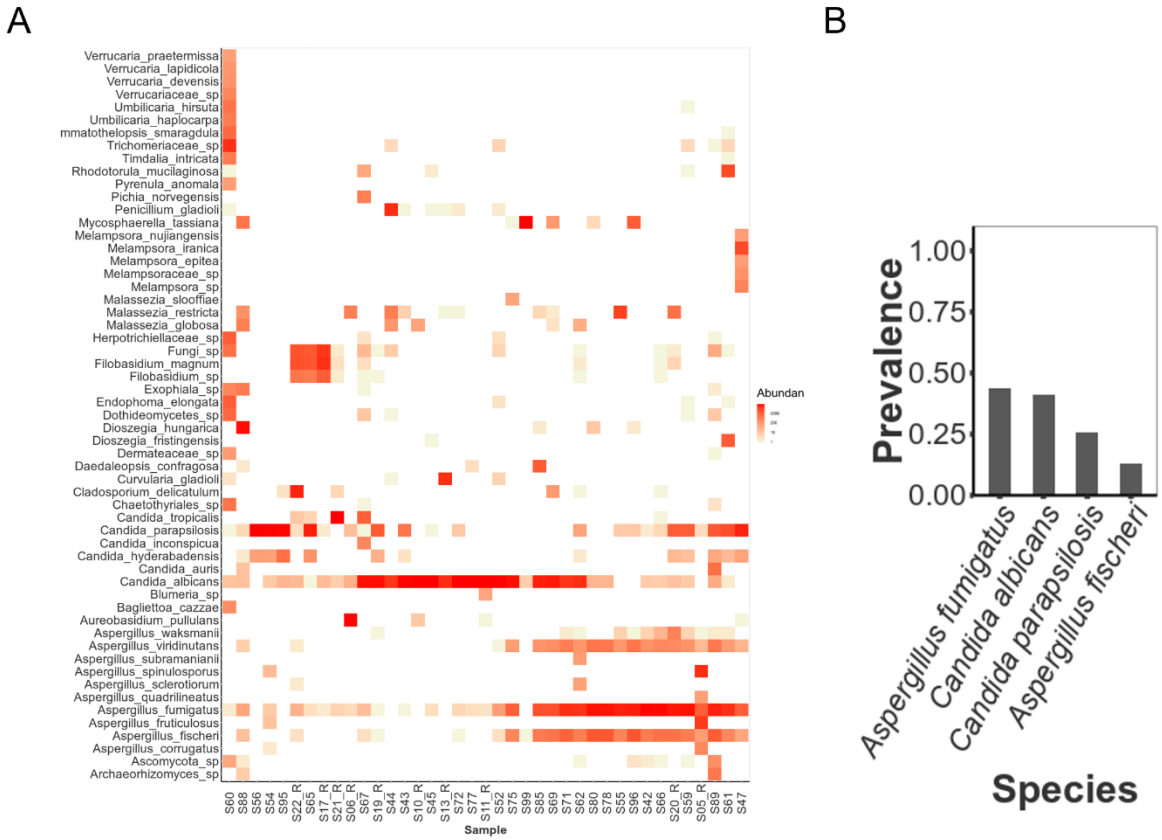

Fig. E2. ITS1 respiratory mycobiomes at the species level. (A) Heatmap of fungal species identified in all samples. To remove extremely rare taxa, only those present at > 0.2% in one sample were retained. (B) *A. fumigatus* and *C. albicans* are the most prevalent species. Taxa were counted if present at over 5% in a sample, and only taxa found in at least 10% of samples of either group are shown.

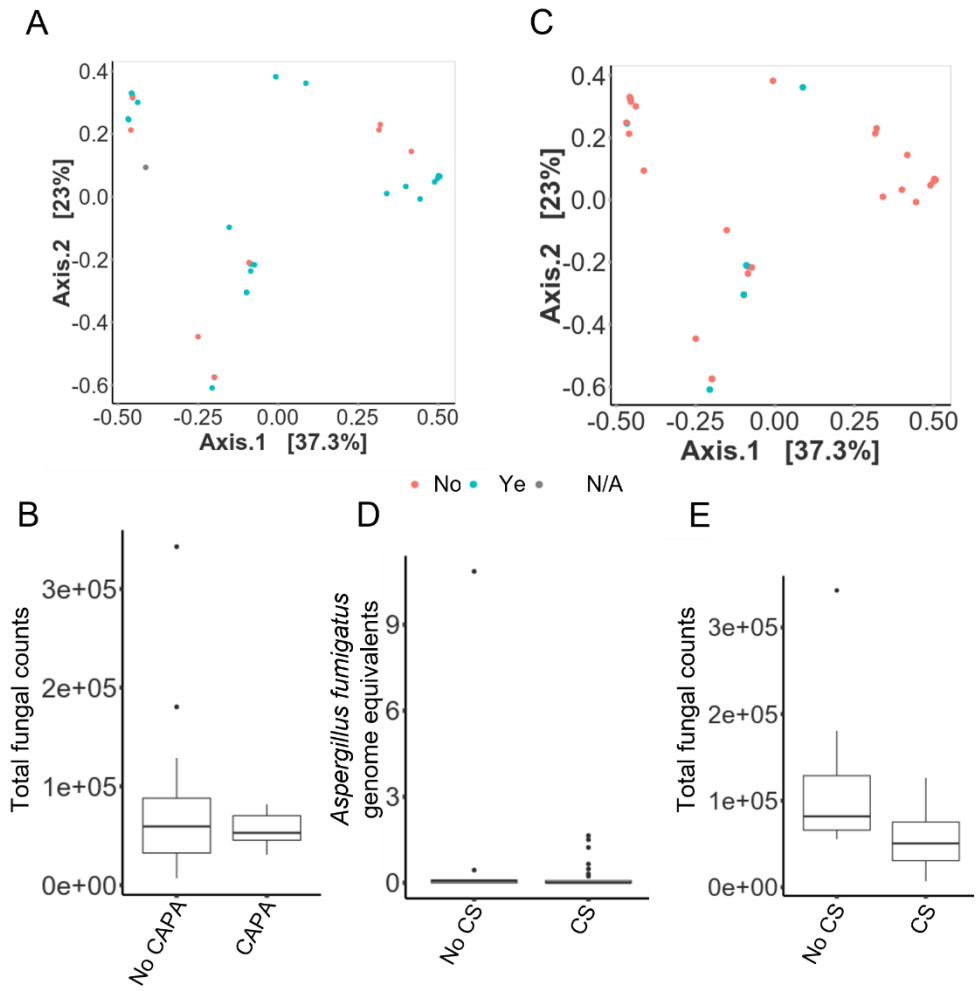

Fig. E3. CAPA diagnosis and corticosteroid use had no significant impact on fungal burden, *Aspergillus fumigatus* levels or beta diversity.

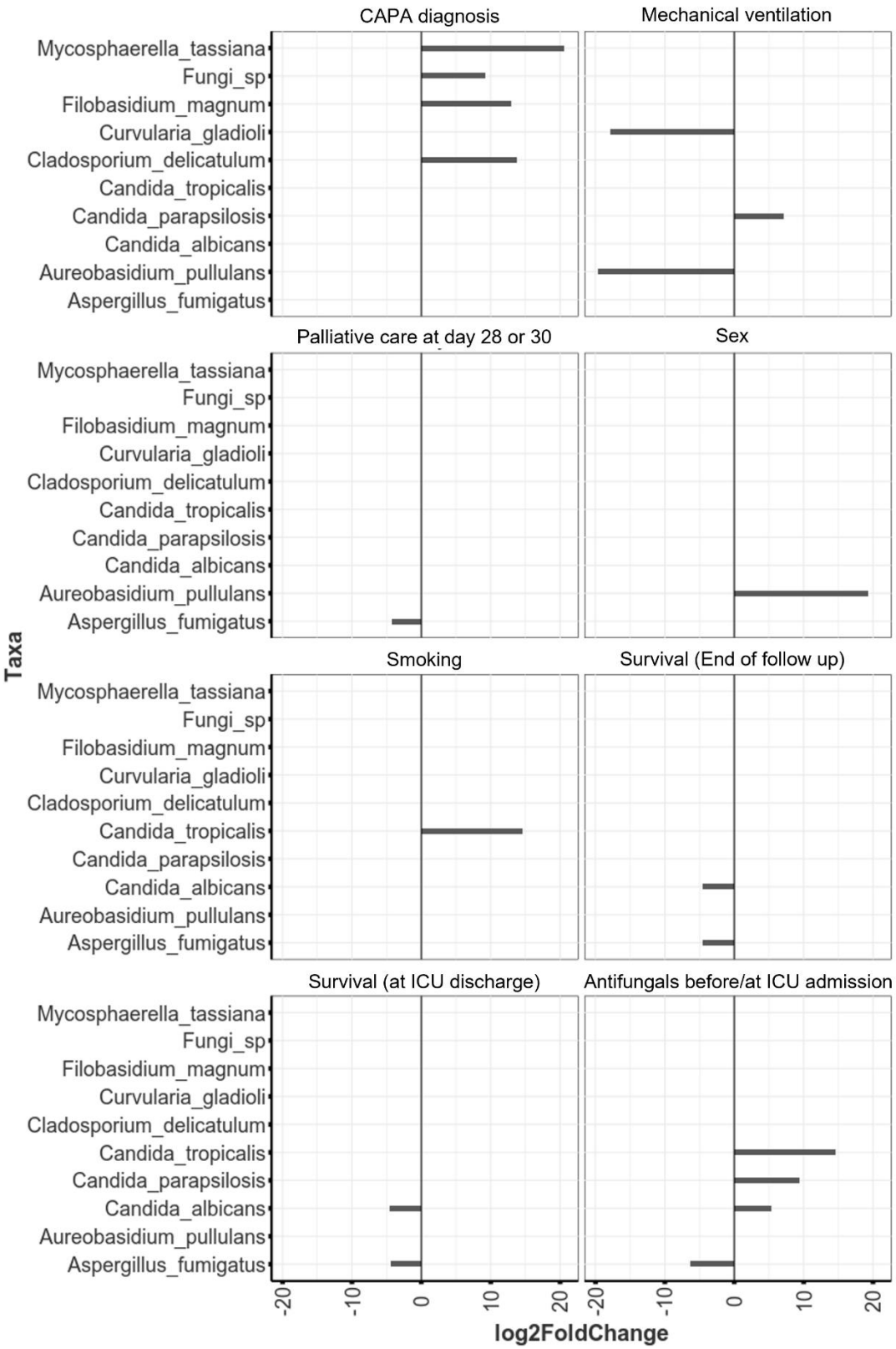

Fig. E4. Significantly differentially abundant taxa identified by DESeq2 analysis.

Table E1. Full clinical patient demographics

Characteristics	N=39
Age	
Mean (SD)	64 (7.4)
valid (missing)	38 (1)
Sex	
male	82% (32)
female	15% (6)
missing	2.6% (1)
Ethnicity	
Caucasian	92% (36)
other	5.1% (2)
missing	2.6% (1)
BMI > 30	
yes	23% (9)
no	74% (29)
missing	2.6% (1)
Smoking	
yes	15% (6)
no	82% (32)
missing	2.6% (1)
Institution	
Graz	26% (10)
Genoa	62% (24)
Rennes	13% (5)
Hematology oncology	
yes	10% (4)
no	87% (34)

missing	2.6% (1)
Solid organ transplant	
yes	5.1% (2)
no	92% (36)
missing	2.6% (1)
Cardiovascular disease	
yes	49% (19)
no	49% (19)
missing	2.6% (1)
Pulmonary disease	
yes	26% (10)
no	72% (28)
missing	2.6% (1)
Diabetes Mellitus	
yes	10% (4)
no	87% (34)
missing	2.6% (1)
Corticosteroids	
yes	74% (29)
no	23% (9)
missing	2.6% (1)
Tocilizumab	
yes	7.7% (3)
no	90% (35)
missing	2.6% (1)
Azithromycin	
yes	33% (13)
no	62% (24)
missing	5.1% (2)

Systemic antifungals at ICU admission	
yes	13% (5)
no	21% (8)
missing	67% (26)
Life Support	
mechanical	74% (29)
ECMO	2.6% (1)
noninvasive	5.1% (2)
mechanical & noninvasive	10% (4)
none	5.1% (2)
missing	2.6% (1)
Duration ICU (days)	
Mean (SD)	31 (26)
valid (missing)	38 (1)
Palliative (Day 28 or 32)	
yes	51% (20)
no	46% (18)
missing	2.6% (1)
Survival (at end of follow up)	
yes	33% (13)
no	64% (25)
missing	2.6% (1)
Antifungal Treatment Outcome	
yes	5.1% (2)
no	5.1% (2)
partial	0% (0)
missing	90% (35)
Primary Treatment	
yes	5.1% (2)

no	0% (0)
missing	95% (37)
Antifungals initiated for CAPA	
yes	18% (7)
no	0% (0)
missing	82% (32)
Antifungal initiated	
Posa Isa	5.2% (2)
Isav	0% (0)
IsavCasp	2.6% (1)
LAmb	0% (0)
Voriconazole	10% (4)
missing	82% (32)
BAL GM (ODI > 1)	
positive	21% (8)
negative	72% (28)
missing	7.7% (3)
BAL LFD	
positive	7.7% (3)
negative	7.7% (3)
missing	85% (33)
BAL PCR	
positive	7.7% (3)
negative	49% (19)
missing	44% (17)
BAL culture	
positive	7.7% (3)
negative	90% (35)
missing	2.6% (1)

Bronchial Aspirate culture	
positive	2.6% (1)
negative	23% (9)
missing	74% (29)
Tracheal Aspirate culture	
Positive	2.6% (1)
negative	7.7% (3)
missing	90% (35)
Tracheal Aspirate GM	
positive	2.6% (1)
negative	2.6% (1)
missing	95% (37)
Tracheal Aspirate PCR	
positive	5.1% (2)
negative	21% (8)
missing	74% (29)
Serum GM (> 0.5)	
positive	5.1% (2)
negative	44% (17)
missing	51% (20)

Table E2. Full results for significantly differentially abundant taxa determined by DESeq2 analysis when assessing CAPA diagnosis, survival, and systemic antifungal use at ICU admission.

Variable	Taxa	base-Mean	log2Fold-Change	lfcSE	stat	p	Adjusted p
CAPA diagnosis	Cladosporium delicatulum	4389.2	13.71	4.00	3.42	0.000619	0.003719
	Filobasidium magnum	1165.8	12.93	3.13	4.13	3.59E-05	0.000368
	Fungi sp	1017.7	9.18	2.62	3.51	0.000456	0.003719
	Mycosphaerella tassiana	312608.4	20.62	4.01	5.15	2.65E-07	3.62E-06
Survival (at end of follow up)	Aspergillus fumigatus	8266.3	-4.55	1.30	-3.50	0.000462	0.017097
	Candida albicans	83190.1	-4.48	1.42	-3.15	0.001611	0.029809
Systemic anti-fungals at ICU admission	Aspergillus fumigatus	943.1	-6.33	2.43	-2.6036	0.009225	0.042435
	Candida albicans	66157.1	5.32	2.13	2.499698	0.01243	0.047648
	Candida parapsilosis	8112.9	9.35	2.68	3.486701	0.000489	0.003749
	Candida tropicalis	51931.1	14.52	2.99	4.85916	1.18E-06	1.36E-05

Supplementary methods

DNA extraction

BAL DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method[24]. Negative extraction controls (NECs) consisting of molecular grade Tris-EDTA (TE) buffer (Promega) were included with each sample batch. First, 500 µl samples were incubated with 0.1% dithiothreitol (Thermo Scientific) at 37 °C for 45 minutes. To lyse fungal cells, CTAB DNA Extraction Buffer (Generon Ltd) was added and a short mechanical disruption (2 x 20 seconds FastPrep-24) using glass beads (425-600µM, Sigma) was performed, followed by 3 cycles of alternated heating to 65 °C and gentle vortexing for 10 mins each. Cell lysates were treated with 4 µl RNase A (100 mg/ml, Sigma) for 30 mins prior to DNA isolation using 1:1 ratio of Phenol-Chloroform (25:24:1, Merck) and 1:1 Chloroform:isoamyl alcohol (24:1, VWR). DNA

478 was precipitated at -20 °C overnight using 2 volumes of ice-cold 100% ethanol and 0.1 volumes
479 of 3M sodium acetate pH 4.8. Pelleted DNA was washed in 100% ethanol and twice in 70%
480 ethanol. Final DNA was suspended in 50 µl TE buffer.

481 **qPCR**

482 The presence of *A. fumigatus* in respiratory samples was validated using a TaqMan probe
483 assay targeting the ITS1 region[26]. Assays were performed on an Applied Biosystems 7500 fast
484 system using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). Standard curves
485 were a nine sample 10-fold dilution series beginning at 100 ng *A. fumigatus* genomes per
486 reaction.

487 **PCR & Sequencing**

488 The ITS1 region was amplified using Nextera XT compatible versions of ITS1[25] and ITS2degen
489 (a degenerate version of ITS2 primer) (see Table 1). PCR setup used Phusion Green Hot Start
490 II HF PCR master mix to create 25 µl reactions with 300 nM forward primer, 1.2 µM reverse
491 primer and 2 µl DNA. Parameters included annealing temperature of 55 °C for 40 cycles. PCRs
492 included positive (*Aspergillus niger* genomic DNA), negative (molecular grade water) and
493 negative extraction controls (NECs) from DNA extractions. Controls were processed fully and
494 sequenced alongside the BAL samples. For sequencing library preparation, samples were
495 processed as per Illumina fungal metagenomic demonstrated protocol (Starting at 'Clean up'
496 section) using Nextera XT index kit v2 (Illumina). However, this protocol was modified to
497 include sample pooling prior to the second clean up. Libraries were sequenced 2x150 on an
498 Illumina iSeq100, with a final library loading concentration of 50 pM.

499 **Data analysis**

500 Paired end reads were subject to quality trimming at Q30 and a minimum length filter of 75
501 nucleotides using bbduk[27] (BBMap v38.22). Primer sequences were removed using Cutadapt[28]
502 (v1.18). Reads were mapped to UNITE database using bowtie2[29] (v2.3.5.1). Count data was
503 further processed in R (v4.1.3) using the following packages: phyloseq[30] v1.38.0, vegan[31] v2.5-
504 7, DESeq2[32] v1.34.0, stringr[40] v1.4.0, ggplot2[41] v3.3.5 and tidyr[35] v1.2.0.

505 To minimise contamination in the mycobiome data, samples were subject to a multi-staged
506 quality control process using NEC samples, along with qPCR and sequencing data. Firstly,
507 samples from a DNA extraction batch with *A. fumigatus* detected (Ct <40) in the
508 corresponding NEC by qPCR were excluded. Secondly, samples were excluded if they
509 produced less than 500 fungal reads (read count filter) and/or a lower number of fungal
510 reads than the corresponding NEC sample (NEC count filter). Of 99 initial samples, 22 were
511 removed due to contamination of an extraction batch, 35 samples did not pass the read
512 count filter and 3 did not pass the NEC count filter.

513 Abundances were standardised to the median sequencing depth. Extremely low abundance
514 taxa were removed by only retaining those occurring > 0.2% in any sample. DESeq2 was used
515 to identify significantly differentially abundant taxa (adjusted p value < 0.05 and basemean >
516 500). Differences in diversity (Shannon, Chao1 and observed OTUs) were assessed using
517 pairwise Wilcoxon rank sum tests. PERMANOVA test was used to assess differences in Bray-
518 Curtis ordination.

519

520