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Mycobiome profiling of nasopharyngeal region of SARS-CoV-2 infected individuals

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1 **Mycobiome profiling of nasopharyngeal region of SARS-CoV-2 infected individuals**

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24 **Abstract**

25 The present cross-sectional study aims to explore the fungal community composition of the

26 nasopharyngeal region of SARS-CoV-2 infected individuals and how the infection influences the

27 mycobiome therein. The infection significantly ($p<0.05$) influenced the alpha diversity.

28 Interestingly, a higher abundance of *Cladosporium* and *Alternaria* was noted in the infected

29 individuals and inter-individual variation in mycobiome composition was well supported by beta

30 dispersion analysis ($p < 0.05$). Moreover, decrease in *Aspergillus* abundance was observed in

31 infected patients across the four age groups. This study provides insight into the alteration in

32 mycobiome during the viral disease progression and demands continuous investigation to monitor

33 fungal infections.

34 **Keywords:** Mycobiome, SARS-CoV-2, Nasopharyngeal, COVID-19, Pandemic

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1. Introduction

49 Coronavirus disease caused by the novel severe acute respiratory syndrome coronavirus (SARS-

50 CoV-2) which predominantly affects the respiratory system has been widely studied from a

51 panoramic perspective owing to its pandemic nature that has overwhelmed the global healthcare

52 system since the end of 2019 [1]. Of the notable features of SARS-CoV-2 such as high

53 transmissibility and rapid mutational capacity; the wide spectrum of clinical manifestations

54 patients' exhibit ranging from mild to severe and requiring brief to prolonged hospitalization has

55 further challenged disease prognosis and treatment [2]. Recently, several studies have tried to

56 answer the question as to why certain infected individuals exhibit a mixed set of symptoms with a

57 different magnitude of severity while a majority remain asymptomatic [3]. Although the

58 heterogenous immune status among the individuals and their response to infections remain at the

59 center of this argument at large, plausible interaction between the host, microbiome, and disease

60 severity/progression has added a layer to this understanding [1]. Despite the fact that fungi have a

61 significant contribution in human respiratory and chronic infections; this group of organisms has

62 received shallow attention in human microbiome studies. [1,4-7]. In the light of COVID- 19

63 pandemic, most of the microbiome studies have focused on understanding the role of bacteria in

64 SARS-CoV-2, neglecting the importance of fungi [8-10]. Considering the fact that COVID-19

65 involve a dysregulated immune response with cytokine storm and impaired T cell response during

66 severe illness [11,12] and the role of fungi to shape immunological responses and T cell action has

67 been previously reported [13]. Hence, it is important to perform fungal profiling of SARS-CoV-2

68 infected individuals because very few studies have been performed to understand the alteration of

69 fungal populations during COVID-19 [1,7,14-17]. These studies have reported an increase in the

70 abundance of *Candida* sp. along with the decrease in species diversity and richness in COVID-19

71 patients. It has been observed that dominating fungal species are highly variable among patients

72 even within the groups [16]. There are reports where several fungal taxa have been depleted in

73 critically ill patients [15-17] and acute respiratory distress syndrome in COVID-19 was

74 characterized by lung dysbiosis and decreased fungal diversity [7]. Since nasal cavity is one of the

75 main entry points for the SARS-CoV-2 infection, it would be interesting to have a better

76 understanding of SARS-CoV-2 infection on autochthonous mycobiome composition in

77 nasopharynx of COVID-19 patients. The recent spike in the COVID-19-associated mucormycosis

78 (an invasive fungal infection) cases in India provides an opportunity to consider the importance of

79 mycobiome in future viral pandemics [18]. The current study is designed to assess the effect of

80 SARS-CoV-2 infection on the composition of nasopharyngeal mycobiome in COVID-19 patients

81 and to further understand the association of these changes with host conditions. This work is in

82 continuation to our previous study where we assessed the prevalence of opportunistic bacterial

83 pathogens in SARS-CoV-2 infected individuals [19].

84 **2. Materials and Methods**

85 A total of 89 nasopharyngeal swabs previously collected from patients of SARS-CoV-2 infection

86 were used for the mycobiome analysis [19]. Details of the recruited subjects, clinical

87 characteristics, and real-time PCR testing for COVID-19 as per the ICMR guidelines were

88 described in [19]. Sample collection was performed as per the standard Indian Council of Medical

89 Research (ICMR), Government of India, guidelines. Swab samples were immediately put in Viral

90 Transport Medium (VTM) and was transported in cold chain conditions and triple packaging to

91 the laboratory of B J Government Medical College, Pune for COVID-19 real-time Polymerase

92 Chain Reaction (RT-PCR). Out of the 89 nasopharyngeal samples, DNA from 80 samples yielded

93 amplification of ITS1 region using primer set (ITS1F and ITS2R) [20]. These 80 samples were

94 used for further downstream processing and demographic characteristics are presented in Table

95 S1. The resultant amplicons were processed for library preparation, the barcoded libraries were

96 pooled in equimolar concentration and sequenced on the Illumina MiSeq platform using 2 X 250

97 bp v2 chemistry. The PCR negative control was also sequenced to remove contaminants from the

98 main datasets. The obtained raw reads were quality checked using FastQC [21]. The reads were

99 pre-processed and analyzed using DADA2 package v1.6.0 [22]. in R 3.6.0. Non-chimeric, error

100 free reads were used for taxonomic assignment using UNITE database [23]. Decontam package

101 was used to remove contaminants from the datasets using prevalence-based method [24]. Phyloseq

102 v3.4.2 R package [25]. was used to generate alpha and beta diversity matrices. Pairwise Wilcoxon

103 test was used to compare the changes in the alpha diversity parameters in the infected and non-

104 infected individuals. Principal Co-ordinate Analysis (PCoA) was performed with Bray-Curtis

105 matrix using phyloseq package. Permutational ANOVA (PERMANOVA) was performed between

106 the study groups using Bray-Curtis dissimilarity matrix to assess the difference in beta diversity.

107 A permutation-based test of multivariate homogeneity of group dispersions (PERMDISP) was

108 conducted using betadisper function of vegan package. Linear discriminant analysis Effect Size

109 (LEfSe) was performed to find out the differentially enriched taxa between groups. The raw ITS1

110 gene amplicon sequencing data generated in this study was submitted to NCBI SRA database and

111 it is available under the BioProject ID: PRJNA707350.

112 **3. Results**

113 Using ITS1 region, fungal community composition of the nasopharyngeal region of the SARS-

114 CoV-2 infected individuals showed significant decrease ($p < 0.05$) in the number and richness of

115 fungal taxa than the non-infected individuals (Fig 1a). Out of the total detected ASVs, only 309

116 ASVs were found to be shared between the two cohorts (Fig 1b). The ratio of *Basidiomycota* to

117 *Ascomycota* was not significantly differed between these two groups (p > 0.05) as depicted in

118 Figure 1c. Increased average relative abundance of *Alternaria* and *Cladosporium* together with

119 decreased count of *Aspergillus*, *Candida*, *Olpidium*, *Saitozyma*, *Mortierella*, and *Wallemia* was

120 observed in the infected individuals (Fig 1d). However, an inter-individual mycobiome variation

121 was observed in the infected individuals with dominance of a few fungal taxa such as *Albifimbria*,

122 *Cutaneotrichosporon*, *Sarocladium*, *Hannaella*, *Chaetomium*, and *Kluyveromyces* (Fig S1).

123 LefSe-based analysis found 10 differentially abundant fungal ASVs affiliated to *Cladosporium*,

124 *Aspergillus*, *Wallemi*a, *Candida*, and *Olpidium* between the infected and non-infected individuals

125 at FDR-adjusted p < 0.1. Furthermore, PCoA was performed to assess the overall difference in the

126 mycobiome community composition between infected and non-infected individuals.

127 PERMANOVA analysis displayed difference (p<0.007) in the overall mycobiome community

128 structure between infected and non-infected individuals (Fig 1e). However, beta-dispersion

129 analysis described the higher inter-individual variation in infected subjects than non-infected ones

130 (PERMDISP, p <0.0008).

131 We further investigated the association of mycobiome with host age as SARS-CoV-2 was found

132 to have more pronounced effect on older age group. We segregated our subjects into four distinct

133 age groups (age group 1: 0-15 years; age group 2: 16-30 years; age group 3: 31-45 and age group

134 4: 46 and above) and found that alpha diversity decreased significantly (p <0.05) in infected

135 individuals across all the age groups (Fig S2). Abundance of *Aspergillus* and *Saitozyma* was found

136 to be decreased in all the age groups of infected individuals as compared to non-infected ones (Fig

137 2a). Interestingly, the relative abundance of *Candida* was found to be decreased in infected

138 individuals within age group 1 and 2 and vice-versa for age group 3 and 4 (Fig 2a). *Cladosporium*

139 read count was enhanced in all the age groups of infected individuals. Similar trend in the

140 abundance pattern of *Alternaria* was also observed, except for age group 2 (Fig 2a). Notably, few

141 taxa were enhanced in specific age groups such as *Papiliotrema* in age group 1, *Kluyveromyces* in

142 age group 2, and *Wallemia* in age group 1 (Fig 2a). No significant differences were observed

143 between the categorical age groups using PCoA (Fig 2b) and Pairwise PERMANOVA ($p > 0.05$,

144 FDR corrected) (Table S2).

145 We further asked to understand the relationship between fungal composition and asymptomatic

146 and/or symptomatic conditions of infected individuals. No significant difference was observed in

147 the alpha diversity parameters between asymptomatic and symptomatic infected individuals.

148 Additionally, beta diversity was not affected significantly in these two conditions (PERMANOVA,

149 $p>0.05$) (Fig 2c). We did not find very significant changes in the relative abundance of the taxa,

150 however, few genera such as *Albifimbria, Wallemia, Sarocladium, Kluyveromyces,* etc. were found

151 to be abundant in the asymptomatic individuals (Fig 2d). However, inter-individual variation in

152 fungal composition was clearly observed across the asymptomatic and symptomatic infected

153 subjects (Fig S3). For example, *Cladosporium* and *Papiliotrema* constituted the major proportions

154 of the fungal constituents in few of the symptomatic subjects.

155 **4. Discussion**

156 The upper respiratory system is consistently exposed to air and forms a unique microbiota and

157 mycobiota [26]. Even though the abundance or biomass of latter is found to be very low in

158 comparison to its bacterial counterpart, the shift in its composition is well observed in

159 immunocompromised patients with respiratory or chronic diseases [4,27,28]. The present study is

160 aimed to understand the impact of SARS-CoV-2 infection on nasopharyngeal mycobiome of the

161 infected individuals. Our results showed the disruption and diminution in the fungal species

162 richness in the nasopharyngeal region. Similar observation was reported by Lv et al. [15] in gut

163 mycobiome of COVID-19 and healthy controls. Furthermore, reduction in fungal diversity in

164 Bronchoalveolar lavage (BAL) samples from patients with COVID-19 with *Candida* spp.

165 colonization in comparison to uncolonized ones was reported [7]. On contrary, Soffritti et al. [14]

166 reported an increase in species richness in oral mycobiome of COVID-19 patients. Such changes

167 clearly indicate that SARS-CoV-2 infection has pronounced effect on the mycobiome composition

168 and is site-specific.

169 Interestingly, even though we have not found significant changes in the major taxa, increased

170 abundance of two known opportunistic pathogens and decreased in *Aspergillus, Wallemia,*

171 *Candida*, etc. in our study highlighted the influence of SARS-CoV-2 infection on fungal

172 composition [Fig 1]. In the recent years, *Cladosporium* is becoming increasingly important

173 opportunistic pathogen, and known to cause superficial and invasive infections in human [4].

174 Similarly, *Alternaria* spp. were detected in asthmatic patients and also been reported from allergic

175 bronchopulmonary mycosis, hypersensitivity pneumonitis, and allergic sinusitis and rhinitis

176 [29,30]. Increment in such taxa in COVID-19 patients is of great concern, hence it is imperative

177 to investigate the underlying pathogenesis in SARS-CoV-2 like infections. On contrary to previous

178 reports, our data describe the decrease in *Candida* populations (which form the major portion of

179 the human mycobiome and have been associated with various respiratory diseases) in the infected

180 individuals [7,12]. However, it has been reported that *Candida* spp. colonization was significantly

181 higher in BAL samples from COVID-19 patients, while patients which were not colonized by

182 *Candida* showed the distinct mycobiome profile with higher abundance of unclassified fungi from

183 the *Ascomycota* phylum [7]. In line with this, decrease in *Candida* members in our study has

184 promoted the preponderance of opportunistic pathogens (*Cladopsorium* and *Alternaria*) in

185   COVID-19 patients. Recently, Lv et al. [15] has shown the association between various metabolic

186   markers and fungal groups in COVID-19 and H1N1 infected patients, which might be responsible

187   for increased viral load, hypersensitivity, and secondary infections. Our study further tried to

188   identify the unique fungal taxonomic markers associated with a particular age group. As a result,

189   we have found the association of few fungal taxa which were either decreased or increased in

190   particular age groups. These changes might be the results of COVID-19 or impaired host

191   mechanisms. For example, *Aspergillus* populations was found to be decreased in all the age groups,

192   while abundance of *Candida* was found to be more prominent in patients with older age group,

193   this might be due to their higher susceptibility to *Candida* infection or impaired host defense

194   mechanisms. Conversely, our study did not find significant variation in the fungal mycobiome

195   profiling of the infected asymptomatic versus symptomatic patients. Inter-individual variations

196   were well evident between these two conditions; hence we can hypothesize that inter-individual

197   variation might be one of the factors responsible for symptomatic and asymptomatic nature of the

198   disease. To further understand the inter-kingdom association between fungus and bacterial

199   populations in infected patients as compared to non-infected individuals, we compared the fungus

200   taxonomic profile with our pervious study on these recruited samples [19]. We have reported the

201   increment of *Pseudomonas* in the nasopharynx of COVID-19 infected individuals; antagonistic

202   association between *Pseudomonas aeruginosa* and *Candida albicans* has been reported by [26].

203   Overall decrease in abundance of *Candida* in the infected patients might be due to the negative

204   effect of *Pseudomonas* on its growth [26]. It has been documented that symbiotic gut fungi can

205   promote local and systemic immunity by providing complementary microbial stimulation and

206   decrease host susceptibility to colitis and H1N1 virus infection [31]. Therefore, in the present

207   study, depletion of commensal fungi in COVID-19 patients might lead to the loss of their beneficial

208 functions. The main limitation of our study is the low number of the recruited individuals which

209 did not enable us to ascertain the fungal composition with robust statistical analysis, especially in

210 developing effective prevention strategies based on mycobiome profile. Therefore, longitudinal

211 studies with higher number of subjects along with detailed immunological profiling would

212 certainly define the biomarkers and open unique therapeutic opportunities to prevent the

213 development of severe symptoms and combat SARS and other viral infections.

214 **Ethical clearance**

215 The study was approved by the Institutional Ethical Committees of both National Centre for Cell

216 Science, Pune, India and BJ Medical College, Pune, India.

221 **Declaration of competing interest**

222 Authors declare no competing interest.

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Figure Legends

Figure 1. Compositional differences in nasopharyngeal mycobiome between patients infected with SARS-CoV-2 and non-infected subjects. Alpha diversity measures between infected and non-infected individuals (a). Venn diagram-based identification of core and distinct ASVs between the cohorts (b). Relative abundance of major taxa at phylum (c) and genus level (d). PCoA based analysis to assess the difference in fungal community composition between the infected and non-infected individuals (e).

Figure 2. Association between mycobiome and host types (age and conditions). Mycobiome profile of major genera in SARS-CoV-2 infected and non-infected individuals across different age groups (a). PCoA based analysis to assess the difference in fungal community composition across different age groups (b). PCoA based analysis to assess the difference in fungal community composition between asymptomatic and symptomatic SARS-CoV-2 infected individuals (c). PERMANOVA analysis did not yield significant difference ($p > 0.05$). Relative abundance of major genera between asymptomatic and symptomatic SARS-CoV-2 infected individuals (d). Number of individuals belonged to each age category: [Infected ones: Age group 1: 8; Age group 2: 16; Age group 3: 12; Age group 4: 20] and [Non-Infected ones: Age group 1: 9; Age group 2: 7; Age group 3: 5; Age group 4: 3].

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