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A HIGH-RISK AIRWAY MYCOBIOME IS ASSOCIATED WITH FREQUENT EXACERBATION AND MORTALITY IN COPD

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Take home message: The airway mycobiome in COPD is important and associates with exacerbations, survival, and systemic immune responses.

Abstract

Introduction: The COPD bacteriome associates with disease severity, exacerbations, and mortality. While COPD patients are susceptible to fungal sensitization, the role of the fungal mycobiome remains uncertain. **Methods:** We report the largest multicenter evaluation of the COPD airway mycobiome to date including participants from Asia (Singapore and Malaysia) and the United Kingdom (Scotland) when stable (n=337) and during exacerbations (n=66) as well as non-diseased controls (n=47). Longitudinal mycobiome analyses performed during and following COPD exacerbations (n=34) were examined in terms of exacerbation frequency, two-year mortality, and the occurrence of serum specific-IgE against selected fungi. **Results:** A distinct mycobiome profile is observed in COPD compared to controls evidenced by increased alpha diversity (Shannon-index) ($p<0.001$). Significant airway mycobiome differences including greater inter-fungal interaction (by co-occurrence) characterise very frequent COPD exacerbators ($\geq$ 3 exacerbations per year) (PERMANOVA, adjusted $p<0.001$). Longitudinal analyses during exacerbations and following treatment with antibiotics and corticosteroids did not reveal any significant change in airway mycobiome profile. Unsupervised clustering resulted in two clinically distinct COPD groups, (1) with increased symptoms (CAT score) and *Saccharomyces* dominance and (2) with very frequent exacerbations and higher mortality characterized by *Aspergillus*, *Penicillium* and *Curvularia* with a concomitant increase in serum specific IgE levels against the same fungi. During acute exacerbations of COPD, lower fungal diversity associates with higher two-year mortality. **Conclusion:** The airway mycobiome in COPD is characterized by specific fungal genera associated with exacerbations and increased mortality.

Keywords: COPD, Mycobiome, Fungi, Mortality, Survival

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Centre; First Resources Ltd; Genting Plantation, and Olam International outside the submitted work. All other authors have no conflicts of interest to declare.

Introduction

The role of fungi and, in particular *Aspergillus* species in chronic respiratory disease states including asthma, bronchiectasis and cystic fibrosis (CF) is increasingly being recognized and researched [1-4]. A major challenge in clinical settings is the varied presentation of fungal-associated disease that ranges from simple nodules to allergic sensitization and includes chronic non-specific illness and life-threatening invasive disease [5, 6]. Patients with asthma, bronchiectasis, CF and, more recently COPD have been shown to be susceptible to fungal sensitization and allergic bronchopulmonary mycoses (ABPM) with poorer clinical outcomes [2, 3, 5, 7].

Despite prior work illustrating the importance of fungal sensitization, the true significance of fungal infection remains poorly defined in COPD [4, 7, 8]. Prior work suggests associations with disease severity and worse clinical outcomes [4, 9, 10]. For example *Aspergillus* sensitization in COPD is associated with increased symptoms, lung function decline, exacerbations and the presence of bronchiectasis [4, 7, 8, 11]. Long-term inhaled corticosteroid use and frequent oral steroid bursts during exacerbations impair host immunity and may predispose patients to fungal-associated disease [12, 13]. While isolation of fungi from the airway can be associated with clinical disease, the significance of detecting fungi without overt invasive fungal disease in stable COPD remains unknown [10]. Positive fungal culture is reported in up to 43% of COPD patients and has been associated with inhaled corticosteroid dose, activation of the host response and airway neutrophilia [4].

Prior studies on the COPD bacterial microbiome has shown associations with disease severity, exacerbations and mortality, however, the role of fungal mycobiome has lacked dedicated study [14-19]. Although present in lower absolute abundance compared to bacteria, a healthy airway

mycobiome does exist demonstrating a predominance of C*andida, Saccharomyces and Grammothele,* and fungal dysbiosis is linked to disease severity in asthma, bronchiectasis and CF [2, 20, 21]. In COPD however, only a single study has assessed the role of the mycobiome in a specific patient sub-group with HIV and found that *Pneumocystic jirovecii* was the predominant airway taxa [22]. Therefore, no study to date has evaluated the role of the airway mycobiome in COPD, and, related it to treatment and clinical outcomes.

Here, we describe a large multicentre study characterising the airway mycobiome in COPD participants from Singapore, Malaysia, and the United Kingdom. In addition, we provide a longitudinal analysis of the mycobiome during and following COPD exacerbations, and, relate it to clinical outcomes and the systemic immune response in COPD.

Methods

Study Recruitment: *Non-diseased (healthy)*: Non-COPD subjects were recruited from community volunteers at Nanyang Technological University, Singapore with $FEV_1/FVC>0.7$ with normal FEV_1 (≥80% predicted); *Chronic obstructive pulmonary disease (COPD) cohorts*: Individuals with COPD aged ≥40 years were prospectively recruited in three countries (Singapore, Malaysia and the United Kingdom) across five hospital sites into three separate independent cohorts as follows: Stable COPD (n=337); acute exacerbation of COPD (n=66) and a longitudinal COPD cohort (n=34). COPD was diagnosed based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [23]. Participants with any prior history of asthma (defined by variable symptoms and expiratory airflow limitation according to GINA guidelines), those receiving long term oral steroids (>5mg/day) and/or other immunosuppressive therapy, malignancy on active therapy and/or active mycobacterial disease were excluded. Hospital sites included Singapore General Hospital, Changi General Hospital and Tan Tock Seng Hospital (all Singapore), University Malaya Medical Centre (Kuala Lumpur, Malaysia) and Ninewells Hospital (Dundee, Scotland, UK). Recruitment for the stable and longitudinal COPD cohorts occurred between January 2014 and June 2019, and acute exacerbations of COPD were recruited between January 2014 and January 2018. COPD participants recruited into the stable or

acute exacerbation arms were respectively defined as follows: stable COPD was defined as the absence of an exacerbation in the four week period immediately preceding study recruitment and an acute exacerbation defined as an acute worsening of respiratory symptoms (increase dyspnea, cough, sputum purulence and volume or wheeze) necessitating treatment with antibiotics and/or corticosteroids based on GOLD guidelines [23]. Participants in the acute exacerbation cohort (n=66) were followed prospectively over a two-year period following sampling during an acute exacerbation and their mortality outcome recorded (as documented on the death certification). Only respiratory causes of death were considered and defined as death secondary to pneumonia, chronic obstructive pulmonary disease and/or respiratory failure. A separate cohort of COPD participants were recruited into a longitudinal study arm (n=34) with airway specimens samples collected at baseline (during stability), within 24 hours of a documented exacerbation and then at two weeks post-exacerbation and upon completion of treatment. All participants in the longitudinal arm received standard treatment with an oral antibiotic (200mg doxycycline on day one followed by 100mg for 6 days or 625mg twice daily co-amoxiclav) for one week and a five-day course of prednisolone at 30mg or 40mg/day based on the patients weight. At recruitment, a representative airway (sputum) sample and blood draw (for serum) was performed for each patient, and, complete demographic and clinical data (including GOLD group, stage and treatment received) for each respective cohort was collected and are detailed in Table 1. We define frequent COPD exacerbators (FE) as ≥2 exacerbations per year, very frequent exacerbators (vFE) as ≥3 exacerbations per year and non-frequent exacerbators (non-FE) as <2 exacerbations per year based on the year preceding recruitment [24]. Use of inhaled corticosteroids was defined as daily maintenance doses of at least 100µg fluticasone propionate or an equivalent inhaled corticosteroid. This study was approved by the Institutional Review Boards (IRBs) of all participating hospitals and institutions.

Full details on ethical approvals, specimen collection and processing, DNA extraction and mycobiome sequencing, specific-IgE assays and statistical analysis are provided in the supplementary material.

Results

The airway mycobiome in stable COPD is diverse and illustrates geographic variation. The airway mycobiome exhibits significantly greater diversity in stable COPD (n=337) compared to non-diseased (healthy) individuals (p=0.0006) (Figure 1a). Characterized by distinct fungal genera including *Alternaria, Aspergillus, Cladosporium, Cryptococcus, Mycosphaerella, Penicillium, Trametes* and *Wickerhamomyces*, the COPD mycobiome also contains other fungi, shared with a healthy mycobiome but at a higher proportion (Figure 1b and 1c). Geographic variation between mycobiome profiles were observed from participants recruited in Singapore and Kuala Lumpur (SG/KL) (n=175) compared to Dundee (DD) (n=162) where *Saccharomyces, Curvularia, Aspergillus, Schizophyllum, Penicillium, Grammothele* predominate in SG/KL and *Cladosporium, Debaryomyces, Hanseniaspora, Trametes* and *Wickerhamomyces* in DD cohorts respectively by linear discriminant analysis (LDA) (LDA score >3.5, $p<0.05$) (Figure 1d-1e and E6a). Importantly, mycobiome composition remains unaltered by GOLD ABCD group or GOLD lung function grade in stable COPD (Figures E1, E2).

Frequent COPD exacerbators illustrate altered airway mycobiomes demonstrating increased fungal interaction and unaffected by acute exacerbation, antibiotics and/or corticosteroids. Very frequent COPD exacerbators (vFE; n=92) demonstrate airway mycobiomes discriminated by *Wickerhamomyces* (LDA>3.5) (Figure 2a, E6b). These mycobiomes also illustrate contrasting β-diversity compared to non-exacerbators (non-FE; n=245) (median distance to centroid: 0.61; IQR: 0.49-0.67 versus 0.53; IQR: 0.39-0.69; $p<0.05$) (Figure 2b and 2c). Differences in mycobiome profiles between vFE and non-FE remain significant following permutational multivariate ANOVA

(PERMANOVA) adjusted for age, gender, smoking pack year exposure, body mass index, geographic origin, and inhaled corticosteroid use (p=0.0022). When fungal burden was assessed semi-quantitatively by PCR, derived amplicon concentrations between non-FE and vFE COPD groups were comparable (Figure E7). Having detected significant differences in the airway mycobiome of vFE, we next evaluated fungal interactions within the mycobiome using network analyses. Employing the top fifteen fungal genera (all with >1% abundance and present in at least >5% of the participants), we identified an increased number of fungal interactions in the vFE group compared to non-FEs (Figure 2d, Table E2). In vFEs, the key taxa responsible for maintaining network integrity include *Alternaria, Aspergillus, Cryptococcus, Curvularia, Lodderomyces, Malassezia, Penicillium* and *Saccharomyces* which demonstrate the highest number of interactions (edge counts) and their critical (indicated by stress centrality) and influential roles (measured by betweenness centrality) in maintaining mycobiome network integrity (Table E2). Prospective and longitudinal analysis of the mycobiome in n=34 participants with COPD was then assessed at baseline (pre-exacerbation), within 24 hours of an acute exacerbation and again two weeks post exacerbation following treatment with one week of oral antibiotics (either doxycycline or co-amoxiclav) and five days of oral corticosteroids (prednisolone). No significant changes were observed to airway mycobiome profiles or to their α- (Shannon diversity: p=0.9393 and Simpson index: p=0.9144) or β-diversity (PERMANOVA: p=0.6963) over the course and treatment of an acute exacerbation (Figure 2e – 2h, E6c). In addition, when mycobiome profiles were compared between stable COPD participants receiving long-term treatment with inhaled corticosteroids, no differences were detectable suggesting that the altered mycobiome observed in vFE is not strongly influenced by exacerbations, antibiotics, oral or inhaled corticosteroids (Figure E3).

Unsupervised hierarchical clustering of the COPD mycobiome reveals two distinct patient clusters with variable clinical outcomes. We next sought to determine if specific mycobiome signatures or individual fungal genera relate to COPD outcomes. Unsupervised hierarchical clustering (n=337) (excluding *Candida* as this taxa represents >50% relative abundance) revealed two distinct

COPD patient clusters (Figure 3a). Cluster 1 (n=178) is characterised by *Saccharomyces* (Figure 3b) while cluster two (n=159) associates with significant levels of *Aspergillus*, *Curvularia* and *Penicillium* (Figure 3c-3e). Multivariate logistic regression with adjustment for age, gender, BMI, smoking pack year history and lung function (FEV_1% predicted), and where cluster 2 was the dependent variable, demonstrates the clinical relevance of the two identified clusters in that cluster 1 demonstrates significant symptomatology (CAT score >10) (odds ratio (OR): 0.45, 95% CI 0.24-0.85, p=0.0135) and cluster 2 a higher number of exacerbations (OR 1.12, 95% CI 1.01-1.24, p=0.0393) and higher mortality (OR: 2.43, 95% CI 1.04-5.69, p=0.0408: 31 deaths (9.2%) from 337 participants) (Figure 3f). As additional confirmation of the increased symptoms (CAT score >10) in cluster 1, logistic regression was repeated with CAT score >10 as the 'dependent variable' and adjusted for age, gender, BMI, lung function, mortality and exacerbations. The odds ratio for CAT score >10 in cluster 1 was 2.41 (95% CI: 1.25-4.66; p=0.01) confirming the increased symptomatology in this cluster. As the mycobiome in vFE COPD exacerbators is altered (Figure 2a-2d), we next assessed the occurrence of vFE between our two identified clusters and found no significant difference in the number of vFEs between clusters (n=41 (23.0%) in cluster 1 versus n=48 (30.2%) in cluster 2; p=0.173). Overall, cluster 2 is characterised by *Aspergillus, Curvularia* and *Penicillium*, and demonstrates poorest clinical outcome with increased exacerbations (median: 2, interquartile range (IQR) 0-3 versus cluster 1 with median 1, IQR 0-2; p=0.0016) and higher mortality (n=21 (13.2%) versus cluster 1 with n=10 (5.6%), p=0.0266) (Figure 3f).

A 'high risk' COPD mycobiome characterised by *Aspergillus, Curvularia* and *Penicillium* associates with systemic immune responses to these fungi. We next evaluated if a systemic and specific immune response was detectable to these fungi in participants belonging to this "high-risk" cluster employing a subset of stable COPD participants recruited from Singapore and Kuala Lumpur into our original cohort (n=42). Systemic specific-IgE (sIgE) binding using immuno-dot blot assays were screened against a panel of fungi including *Aspergillus, Curvularia* and *Penicillium* (all characteristic of cluster 2) and *Cladosporium, Fusarium, Schizophyllum* and *Trametes* (as controls, no

specific association with either cluster). We detected a significantly elevated sIgE response to *Aspergillus*, *Curvularia* and *Penicillium* (all $p<0.05$) in participants from cluster 2, corresponding with the increased relative abundance of the same respective taxa from their airway (Figure 4a-4c). Importantly, no significant differences in systemic sIgE response were observed between the clusters using the control fungi that illustrate no cluster association (Figure E4).

COPD mycobiomes illustrating lower fungal diversity during acute exacerbations associate with worse two-year survival. While no significant change was observed to airway mycobiomes including α- or β-diversity over longitudinal assessment during acute COPD exacerbations (AECOPD), we next assessed if mycobiome profiles examined during an AECOPD in an independent subset of n=66 participants (recruited from Singapore and Dundee) and followed over two-years related to survival outcomes (Table E3). Mycobiome composition differed between COPD survivors (alive at two years; n=51) and non-survivors (from a respiratory cause of death at two years; n=15) and, in the latter illustrated a significantly lower α-diversity ($p<0.01$) with inverse hazard ratio (HR) (lower α-diversity associated with high mortality) after adjustment for age, gender, BMI, smoking pack year history, lung function (FEV_1) and comorbidities including cardiovascular disease disease, diabetes mellitus, osteoporosis, malignancy, and anxiety disorder (Shannon index HR: 0.25; 95% CI: 0.07-0.085; $p=0.03$ and Simpson index HR 0.07; 95% CI: 0.005-0.88 ; $p=0.04$) (Figure 5a-5d). Of the fungal genera characterizing the 'high risk' mycobiome cluster 2 described above, only *Penicillium* was exclusively detected in non-survivors, while *Aspergillus* and *Curvularia* were identified in varying proportions between survival groups (Figure 5a). Interestingly, in addition to *Penicillium*; *Cladosporium*, *Trametes* and *Lodderomyces* were identified at higher proportions in (but not exclusive to) non-survivors (Figure 5a). Non-survivors further illustrate contrasting β-diversity compared to survivors with no differences observed between their country of origin (adjusted PERMANOVA $p<0.05$) (Figure 5e). Collectively these data suggest that targeted analyses of mycobiome profiles during AECOPD may have prognostic implications and identify patients who are at higher risk of death.

Discussion

Here, we report the largest multicenter evaluation of the airway mycobiome in COPD including participants recruited in Asia (Singapore and Malaysia) and the United Kingdom (Dundee) and additionally evaluate longitudinally mycobiome profiles during and following exacerbations.

The COPD mycobiome is diverse and demonstrates geographic variation. Very frequent COPD exacerbators are characterized by altered mycobiomes and increased inter-fungal interaction, however, mycobiome composition and diversity remains unaffected by the exacerbation event in itself, antibiotics and/or corticosteroid use suggesting that perturbation of the COPD mycobiome results from factors other than treatment. Two important patient clusters were identified based on mycobiome signatures, one characterised by significant symptoms and *Saccharomyces* and the other by exacerbations, higher mortality, and *Aspergillus, Penicillium* and *Curvularia*. The latter group demonstrate systemic immune responses to these fungi. Lower mycobiome diversity during exacerbations represents an important risk for two-year mortality.

In line with work in bronchiectasis, we observe differing mycobiome profiles between participants recruited in different countries [2]. The detected geographic variability may be attributed to contrasting climates, humidity, and air quality, all important for fungal growth and survival. Host factors including genetics, lifestyle and dietary differences may contribute further but remain beyond the scope of this work. Importantly, the respiratory tract represents an organ system in continuous communication with the external environment, a rich fungal source, allowing temporary passage and potentially retention of environmental fungi under appropriate conditions. The importance of geographic location and the specific environmental influence on host mycobiome profiles in relation to COPD has also been the subject of prior work [7]. Using metagenomic sequencing of outdoor and indoor air, a measurable sensitisation response to environmental fungi relates to COPD outcomes. Taken together with the findings presented in this study, geographic location and local environment remain important determinants of the host mycobiome.

Prior studies in COPD demonstrate alterations in the bacterial microbiome particularly in frequent exacerbators [25, 26]. This current study identifies significant changes to the airway mycobiome in very frequent COPD exacerbators (vFEs, ≥3 exacerbations per year) who interestingly, illustrate a more complex mycobiome characterised by increased antagonistic inter-fungal interaction. COPD exacerbations may promote initial bacterial dysbiosis and disruption to fungal communities may only occur as exacerbation frequency increases. Beside an alteration in fungal community membership with increased exacerbation frequency, dynamic variation also occurs. The increased negative (co-exclusive) interactions observed in very frequent COPD exacerbators may result in fungal overgrowth, particularly of specific fungal taxa such as *Wickerhamomyces* which in turn drives the observed clinical phenotype. These fungi are widespread in natural habitats including soil, trees and plants, however, has previously been a reported cause of clinical fungaemia [27]. Although very frequent COPD exacerbators generally receive significantly more treatments including antibiotics and/or systemic steroids compared to non-exacerbators, longitudinal mycobiome analysis reveals its relative stability despite such treatment and even during exacerbations, consistent with work performed in cystic fibrosis (CF), suggesting alternate mechanisms to explain the observed fungal dysbiosis in very frequent exacerbators [28, 29]. As bacteria and viruses (rather than fungi) are commonly identified triggers of COPD exacerbations, it is not unexpected that the airway mycobiome remains stable during AECOPD in our longitudinal analysis. However, with increased exacerbation frequency and, its associated immune-inflammatory change, a 'threshold' in very frequent COPD exacerbators may be reached where alterations to the airway mycobiome begin to appear. Such change, occurring over time, likely involves a complex interaction between microbial kingdoms, the surrounding environment, and the underlying host immune-inflammatory response rather than an acute alteration during a single COPD exacerbation where bacterial and/or viral change may be more apparent [16]. Therefore, the airway mycobiome in COPD is likely most useful in identifying patients at greatest risk of longer-term adverse outcomes including a risk of frequent exacerbations and mortality [30, 31]. Interestingly, inhaled corticosteroid use demonstrates no impact on mycobiome profiles. This contrasts with bacteriome studies in COPD, where microbiome change is observed over the course and treatment of exacerbations, which are influenced further by COPD therapy [16].

Differences in study outcomes between bacterial and fungal microbiomes suggest that bacteria and fungi play different roles during exacerbations and are differently impacted by COPD treatments.

While our current understanding of the mycobiome's contribution to respiratory disease pathogenesis remains indeterminate, available data does suggest that specific mycobiome profiles do exist in chronic respiratory disease states with an increased abundance of *Candida* and *Aspergillus* in CF, *Psathyrella, Malassezia, Termitomyces* and *Grifola* in asthma, and *Aspergillus, Cryptococcus* and *Clavispora* in bronchiectasis [2, 20, 32-34]. In the context of COPD, prior mycobiome studies have focused purely on the HIV-infected COPD patient group where the key change to airway mycobiomes observed was an increased abundance of *Pneumocystic jirovecii* [22]. Importantly, our work did not detect any *Pneumocystic* fungi in our COPD cohorts, however, we did identify specific fungal taxa associated to COPD including *Alternaria, Aspergillus, Cladosporium, Cryptococcus, Mycosphaerella, Wickerhamomyces, Trametes* and *Penicillium*. Differences in the chosen study populations and methodology used likely accounted for at least some of these differences.

Unsupervised clustering analysis reveals two clinically important COPD patient groups: one with increased symptoms (CAT score) and *Saccharomyces* dominance and a second with increased exacerbations and higher mortality characterized by *Aspergillus, Penicillium* and *Curvularia*. In the latter group, systemic specific-IgE responses to *Aspergillus, Penicillium* and *Curvularia* were detectable. The first group, characterized by increased COPD symptoms and *Saccharomyces* is particularly interesting because existing data in COPD and more recently bronchiectasis suggests that the presence of symptoms can indicate a risk of future exacerbations and a response to treatment [35-39]. Whether these concepts hold true regarding airway fungi in COPD remains to be established. The second patient group characterized by *Aspergillus, Penicillium* and *Curvularia* should be considered 'high risk', warrant early identification and close clinical follow up. *Aspergillus, Penicillium* and *Curvularia* are thermophilic fungi with survival capabilities in the human airway and pathogenic potential. Importantly, these fungi commonly associate with allergic bronchopulmonary mycoses (ABPM) and airway isolation of *Aspergillus* and *Penicillium* have been associated with sensitization

and reduced lung function in asthma [40]. The importance of fungal sensitization in COPD has recently been described by our group and contributes to COPD exacerbations in line with earlier studies in asthma, where fungal sensitization demonstrates key roles in disease severity and exacerbations [3, 7]. Therefore, it is plausible that increases in specific airway fungi in COPD activates host immunity with resulting sensitization and poorer clinical outcomes [7]. Importantly despite striking geographical, environmental, and ethnic differences both clusters were consistently identified in participants from Asia and the UK.

Evaluating the COPD mycobiome during acute exacerbations reveals that a loss of diversity is a signal for increased two-year mortality. This is consistent with prior bacteriome studies in COPD where survival, assessed at one year following exacerbation demonstrates a similar lack of diversity [17]. Fungal genera characterizing poorer survival includes *Penicillium, Cladosporium, Trametes* and *Lodderomyces*. Of interest, *Penicillium* also characterized the "high risk" cluster in the stable COPD state suggesting that its specific role in COPD pathogenesis warrants further study. The fungi identified in the non-survivors have been associated with invasive fungal infection and/or sensitization in humans, however their specific roles in COPD remains to be fully expounded. The COPD mycobiome clearly holds important information with potential prognostic implications and may be used to identify 'high risk' patients with worse two-year survival following an acute exacerbation.

Our study is the largest and, to our best of our knowledge, the first multicenter COPD study to evaluate the airway mycobiome including longitudinal sampling. Despite its clear strengths, our work is limited by a small healthy cohort for comparison, all recruited from a single site (Singapore) and therefore may not be generalizable to the wider healthy population. In addition, our cohort of acute exacerbations of COPD was relatively small (n=66) and, therefore the survival analysis limited, however it does represent the largest study of the airway mycobiome in COPD to date, complete with two-year survival data. Further investigations building on this work in larger cohorts will help to explicate the clinical significance and survival-related aspects of our findings. While we report unique

mycobiome signatures in COPD, we did not specifically exclude patients with bronchiectasis-COPD overlap (BCO) (24%; n=81 out of 337) although importantly, no differences between mycobiome profiles were detectable between patients with COPD (n=256) and patients with BCO (n=81). In addition, we did not assess the COPD mycobiome's potential interaction with other microbiomes (bacterial and viral). Future studies integrating the various microbiomes accounting for the host response will be important in understanding the specific impact of the mycobiome in COPD pathogenesis. Despite optimizing our ITS protocol as previously published, inherent limitations persist in the field including weak fungal references databases that preclude confident resolution to the species level [41]. In addition, targeted amplicon sequencing lacks functional annotation when compared to metagenomic sequencing approaches, and therefore our findings presented here can only describe associations and cannot imply causation or demonstrate a pathogenic role for any of the detected fungal taxa. Future work focused on mechanistic confirmation should be pursued. Unlike bacterial microbiome studies in COPD [19, 42], we did not detect associations between disease severity and the COPD mycobiome, however, it does appear that beyond a certain 'threshold' of COPD exacerbation frequency that mycobiome composition is influenced, exemplified by our "very frequent exacerbator" group. The COPD mycobiome may therefore represent a 'marker' for specific disease traits in COPD that requires further study. Finally, our systemic specific IgE assays were performed only in a subset of participants and with selected fungi and is an avenue for further exploration. A validation of our study findings with a more comprehensive fungal allergen panel and inclusion of other ethnic populations should be pursued in future work. In summary, we show that the COPD mycobiome provides important information in terms of clinical outcomes and prognosis and should be further evaluated.

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Table 1

Characteristics	Non-diseased (Healthy)	Stable COPD (Overall)	Stable COPD (SG/KL)	Stable COPD (Dundee (DD))	AECOPD (Overall)	AECOPD (SG/KL)	AECOPD (Dundee (DD))	Longitudinal (pre-during-post COPD exacerbation)
N	47	337	175	162	66	29	37	34
Age (years), Median (IQR)	39 (28-63)	72(67-77)	72 (67-77)	72 (66-77)	69 (64-76)	68 (63-72)	70 (65-77)	70 (65-77)
BMI (Kg/m^2), Median (IQR)	22.5 (19.6-25.7)	24.7 (21.9-29.0)	22.5 (20.2-26.3)	27.0 (24.0-31.0)	25.0 (21.0-30.6)	21.7 (17.5-27.9)	28.0 (24.3-31.8)	25.0 (23.0-29.9)
Gender (Male), n (%)	17 (36.2)	273 (81.0)	167 (95.4)	106 (65.4)	53 (80.3)	29 (100)	24 (64.9)	26 (76.5)
Current smoker, n (%)	0 (0.0)	186 (55.2)	61 (34.9)	125 (77.2)	44 (66.7)	11 (38.0)	33 (89.2)	27 (79.4)
Ex-smoker, n (%)	0 (0.0)	151 (44.8)	114 (65.1)	37 (22.8)	22 (33.3)	18 (62.0)	4 (10.8)	7 (20.6)
Smoking pack years, Median (IQR)	NA	50.0 (33.1-64.5)	50.0 (40.0-80.0)	40.0 (30.0-54.5)	41.5 (37.8-67.3)	49.0 (40.0-75.0)	40.0 (30.0-60.0)	40.0 (26.3-69.5)
CAT Score, Median (IQR)	NA	20.0 (12.0-25.0)	20.0 (11.3-25.0)	19.0 (13.0-25.0)	23.0 (18.0-27.0)	22.0 (17.5-27.0)	23.0 (18.8-27.3)	23.0 (17.0-27.3)
FEV_1 (% predicted), Median (IQR)	96.5 (85.8-107.3)	59.0 (42.0- 74.5)	52.0 (37.0-66.0)	67.5 (51.8-79.3)	51.7 (41.0-73.0)	49.0 (41.0- 59.0)	61.8 (43.4-79.9)	61.8 (50.7-76.9)
FEV_1/FVC (% predicted), Median (IQR)	86.6 (81.5-93.1)	53.0 (44.0-63.0)	55.0 (44.0-65.0)	52.0 (43.1-60.9)	50.7 (42.3-59.8)	49.0 (43.0-59.0)	51.0 (42.0-60.0)	49.0 (41.0-56.0)
No. of Exacerbations/year, Median (IQR)	NA	1 (0-3)	0 (0-2)	2 (1-4)	3 (2-4)	2 (1-3)	3 (2-4)	3 (2-4)
GOLD Group, n (%)								
A	NA	81 (24.0)	45 (25.7)	36 (22.2)	3 (4.5)	0 (0.0)	3 (8.1)	1 (2.9)
B		104 (30.9)	76 (43.4)	28 (17.3)	1 (1.5)	7 (24.1)	1 (2.7)	5 (14.7)
C		41 (12.2)	8 (4.6)	33 (20.4)	10 (15.2)	0 (0.0)	10 (27.0)	0 (0.0)
D		111 (32.9)	46 (26.3)	65 (40.1)	52 (78.8)	22 (75.9)	23 (62.2)	28 (82.4)
GOLD Grade								
1	NA	57 (16.9)	20 (11.4)	37 (22.8)	12 (18.2)	3 (10.4)	9 (24.3)	8 (23.5)
2		163 (48.4)	71 (40.6)	92 (56.8)	29 (43.9)	11 (37.9)	18 (48.7)	19 (55.9)
3		92 (27.3)	66 (37.7)	26 (16.1)	20 (30.3)	13 (44.8)	7 (18.9)	4 (11.8)
4		25 (7.4)	18 (10.3)	7 (4.3)	5 (7.6)	2 (6.9)	3 (8.1)	3 (8.8)
LABA monotherapy, n (%)	NA	8 (2.4)	3 (1.7)	5 (3.1)	1 (1.5)	0 (0.0)	1 (2.7)	1 (2.9)
LAMA monotherapy, n (%)	NA	34 (10.1)	20 (11.4)	14 (8.6)	5 (7.6)	4(13.8)	1(2.7)	2 (5.9)
LABA/LAMA, n (%)	NA	112 (33.2)	70 (40.0)	42(25.9)	13(19.7)	6 (20.7)	7 (18.9)	6 (17.6)
LABA/ICS, n (%)	NA	41 (12.1)	14 (8.0)	27 (16.7)	5 (7.6)	1 (3.4)	4 (10.8)	4 (11.8)
LABA/LAMA/ICS, n (%)	NA	130(38.6)	63 (36.0)	67 (41.4)	38 (57.5)	17 (58.7)	21 (56.8)	19 (55.9)
SAMA/SABA, n (%)	NA	12 (3.6)	5 (2.9)	7 (4.3)	4 (6.1)	1 (3.4)	3 (8.1)	2 (5.9)
Macrolide, n (%)	NA	34 (10.1)	10 (5.7)	24 (14.8)	8 (12.1)	1 (3.4)	7 (18.9)	5 (14.7)

Table 1: Demographics of the non-diseased (healthy) and COPD cohorts. Data is presented as the number of participants (n) (with percentage; %) or median (and interquartile range; IQR) as appropriate. BMI: body mass index, CAT: COPD assessment test, FEV_1: forced expiratory volume in the 1st second, FVC: forced vital capacity, IQR: interquartile range, NA: not applicable, GOLD: global initiative for COPD, SAMA: short acting muscarinic antagonist, SABA: short-acting beta agonist, LAMA: long-acting muscarinic antagonist, LABA: long-acting beta agonist, ICS: inhaled corticosteroid.

Figure 1: The airway mycobiome in COPD is diverse and demonstrates geographic variation (a) Boxplots illustrating α-diversity (Shannon index) between non-diseased (healthy) (n=47), and participants with stable COPD (n=337) (b) Stacked bar charts illustrating the relative abundance and (c) prevalence (present at >1% relative abundance) of various fungal genera between non-diseased (healthy) individuals (n=47) and participants with COPD (n=337) (d) Airway mycobiome composition between participants with COPD from Singapore and Kuala Lumpur (SG/KL) and Dundee (DD) respectively with the top 25 fungal genera illustrated by colour coded relative abundance (e) Linear Discriminant Analysis (LDA) effect size (LEfSe) illustrating the most discriminant fungal genera between respective groups. Respective colouration denotes group membership: SG/KL (dark turquoise) and DD (purple). COPD: Chronic obstructive pulmonary disease. ***$p<0.001$.

Figure 2: Very frequent COPD exacerbators (vFE) illustrate an altered airway mycobiome characterized by increased fungal interactions which is unaffected by exacerbation, antibiotics or corticosteroids. (a) Stacked bar charts illustrating the relative abundance of the top 25 fungal genera between vFE (n=92) and non-frequent (Non-FE) COPD exacerbators (n=245) (b) Principle Coordinate Analysis (PCoA) illustrating β-diversity between the vFE (pink) and non-FE (light blue) exacerbation groups using the Bray-Curtis dissimilarity index (c) Box plot of the average distance to

centroid between groups illustrating significant differences based on the preceding PCoA; *p<0.05 (d) Co-occurrence analysis between the COPD exacerbation groups revealing a more complex and increased fungal interaction present within the airway mycobiome in vFE compared to non-FE COPD exacerbators. Fungal genera demonstrating >1% relative abundance in >5% of the study population are illustrated. Colouration denotes if an interaction is positive (co-occurrence) (blue) or negative (co-exclusion) (red). (e) Stacked bar charts illustrating the relative abundance of the top 25 fungal genera in the airway mycobiome following longitudinal analysis in n=34 participants with COPD at baseline (B) (pre-exacerbation), during exacerbation (E) and following treatment (PE) (post-exacerbation) which illustrates no significant change by (f) Shannon index (g) Simpson index and (h) Principle Coordinate Analysis (PCoA) plot. Dotted lines connect each individual patient at the respective timepoints. ns: non-significant.

Figure 3: Unsupervised hierarchical clustering of fungal genera from the airway mycobiome in COPD reveals two distinct clusters with variable clinical outcomes (a) Heatmap illustrating the relative abundance of the various fungal genera within the mycobiome in their two distinct clusters differentiated based on abundance of (b) *Saccharomyces* (c) *Aspergillus* (d) *Curvularia* and (e) *Penicillium.* Colouration denotes cluster membership: Cluster 1 (purple) and Cluster 2 (light green) (f) Forest plot with adjusted odds ratio (OR) and 95% confidence interval (CI) illustrating clinical differences between the two clusters. Cluster 1 has significantly greater symptoms (CAT score>10) (p<0.05) while Cluster 2 demonstrates significantly more exacerbations (p<0.05) and higher mortality (p<0.05). Circles represent ORs, circles colouration indicate significant level: p<0.05 (red), not significant (grey), and error bars 95% confidence interval. *p<0.05.

Figure 4: A 'high risk' COPD mycobiome (Cluster 2) characterised by *Aspergillus, Curvularia* and *Penicillium* demonstrates a measurable systemic immune response to these fungi. Systemic specific-IgE (sIgE) binding (expressed as $\log_{10}$ OD intensity) to (a) *Aspergillus* (b) *Curvularia* and (c) *Penicillium* was measured in a subset of stable COPD participants from Singapore and Kuala Lumpur (SG/KL) (n=42) and illustrated as bar plots for comparison between clusters. *p<0.05.

Figure 5: COPD mycobiomes with lower fungal diversity at exacerbation associate with higher two-year mortality (a) Bar plots illustrating the prevalence rates of various fungal genera (with relative abundance >1%) from COPD mycobiomes obtained at acute exacerbation (n=66) between survivors (alive) (n=51) and non-survivors (dead) (n=15) at two-year follow up. The text and bar coloration represents the predominant taxa between the survival groups: non-survivors (dead) (blue), survivors (alive) (grey). Previously identified cluster-associated taxa are highlighted: cluster 1 (purple), cluster 2 (light green). A significantly decreased α-diversity of the mycobiome is observed in COPD non-survivors (dead) compared to survivors (alive) measured by the (b) Shannon and (c) Simpson indexes between groups (d) Forest plot with adjusted hazard ratio (HR) and 95% confidence interval (CI) illustrating inverse association with increase α-diversity indexes in survivors (alive) at two-year follow up. Circles represent HRs (p<0.05) and error bars 95% confidence interval. HRs are adjusted for age, gender, smoking pack year history and body mass index (e) Principle Coordinate Analysis (PCoA) illustrating β-diversity between survivors (alive; grey) and non-survivors (dead; blue) at two-year follow up using the Bray-Curtis dissimilarity index. Patient origin are indicated by circles (SG-KL) and triangles (DD) respectively SG/KL: Singapore and Kuala Lumpur; DD: Dundee. *p<0.05.

Figure 1

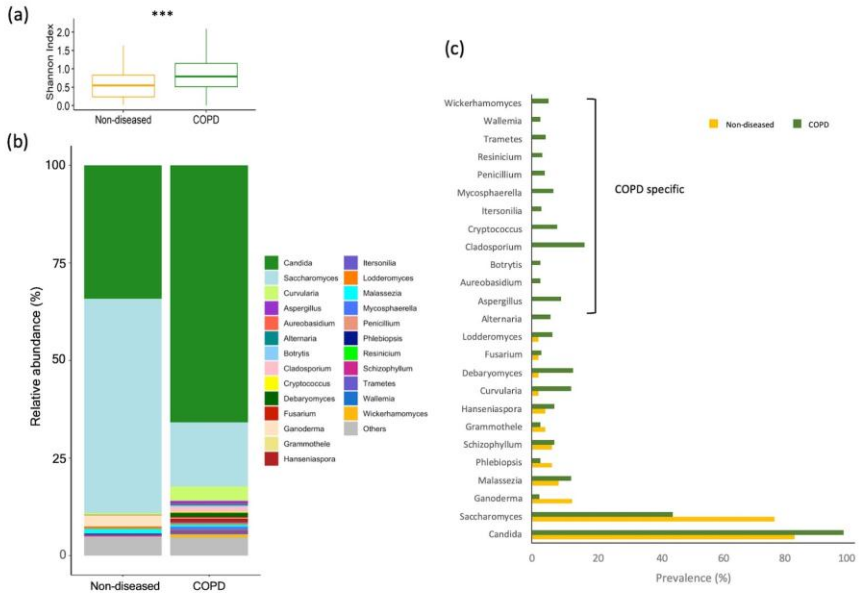


Figure 1

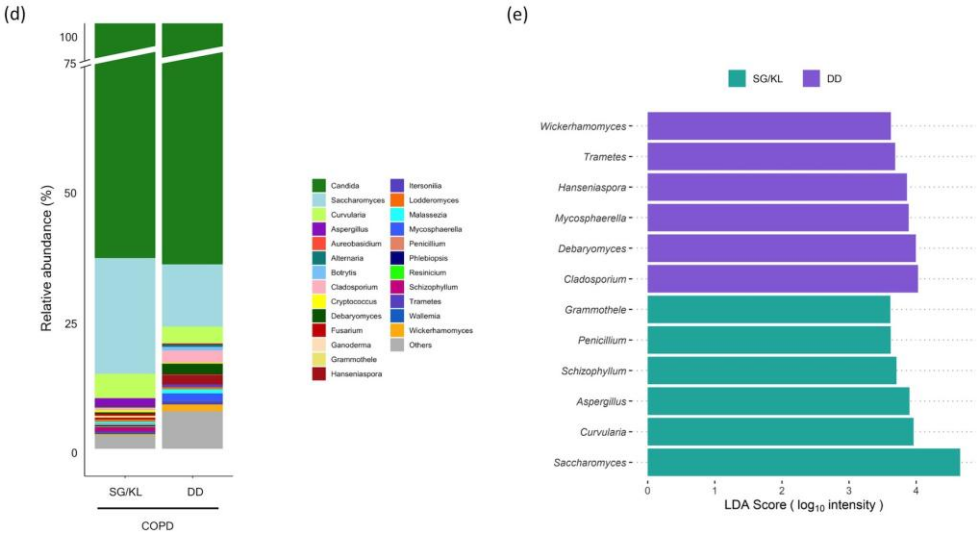
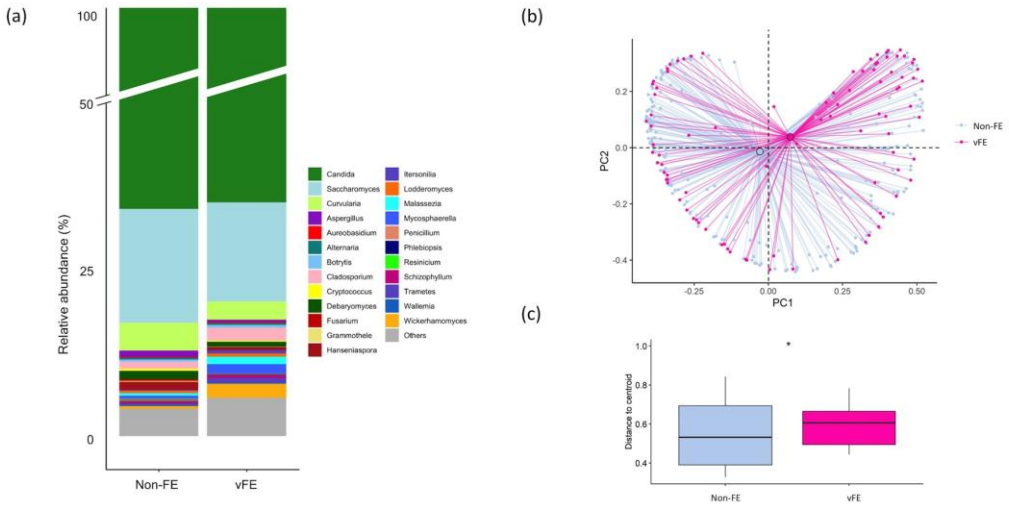


Figure 2



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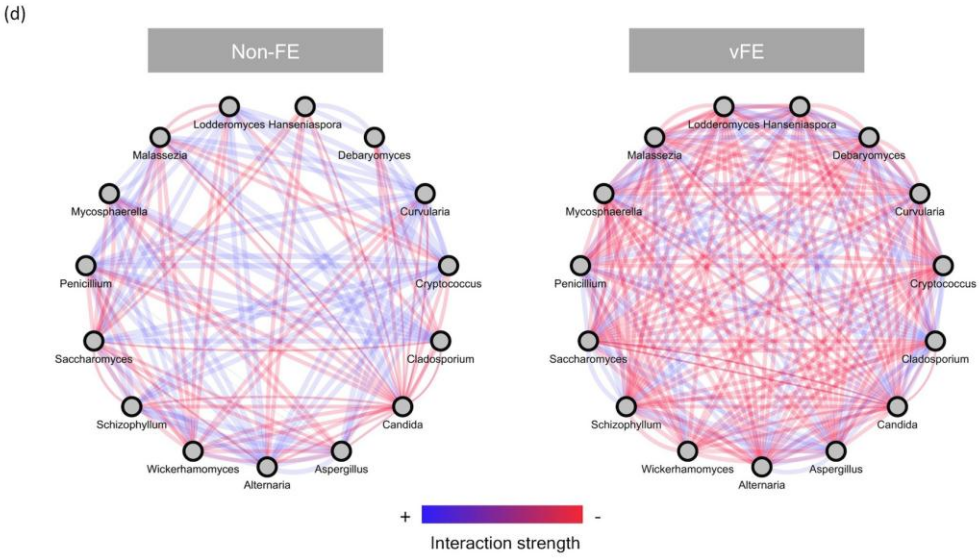

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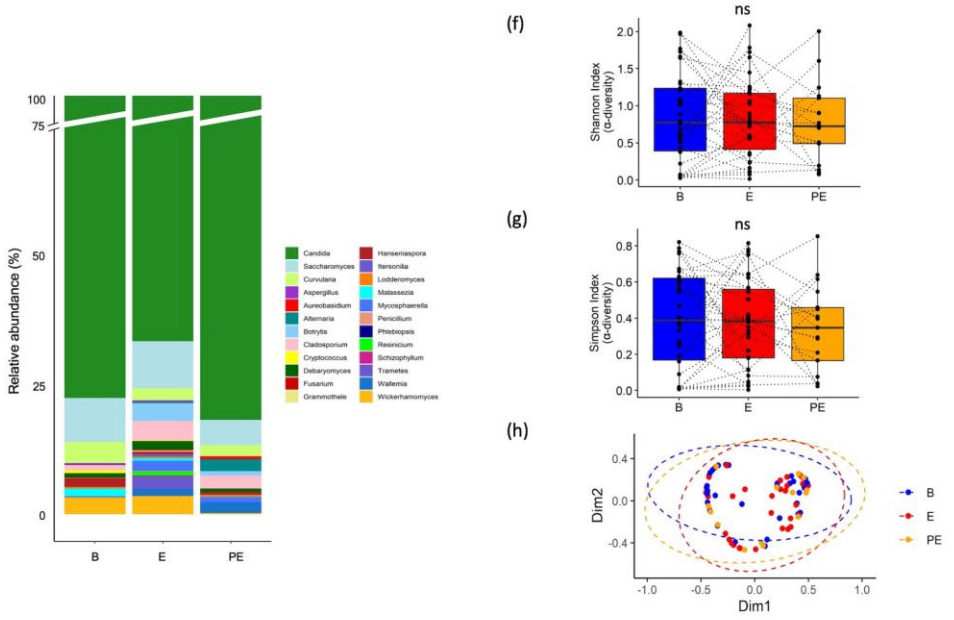

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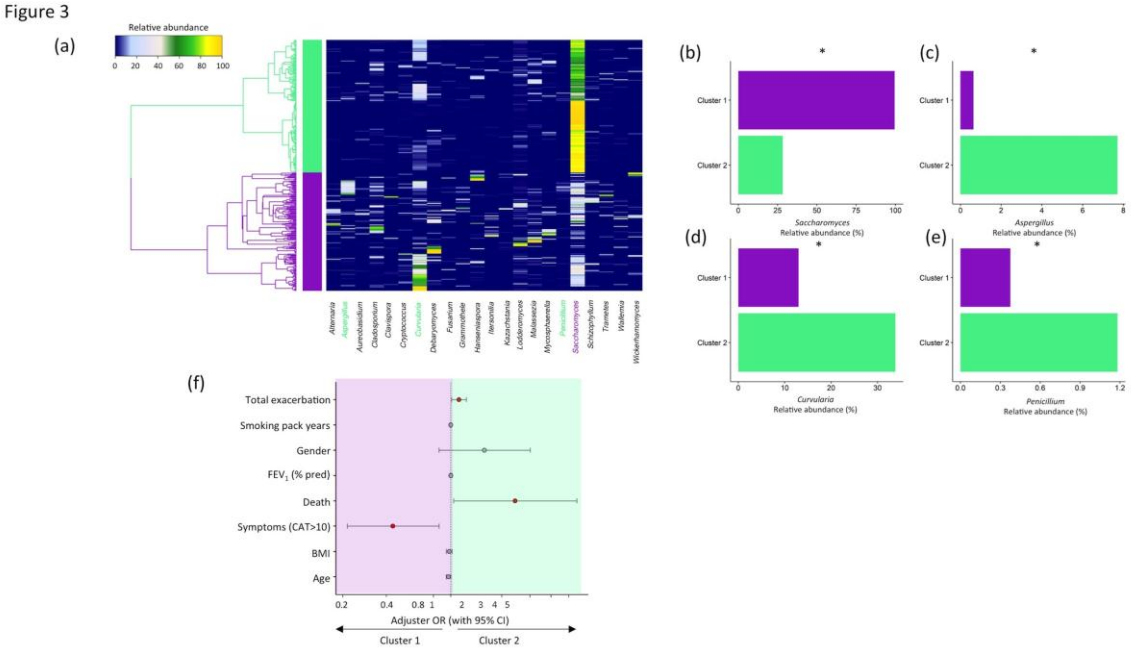

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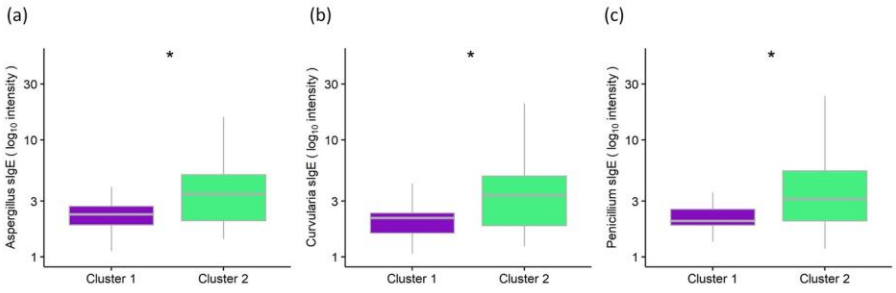
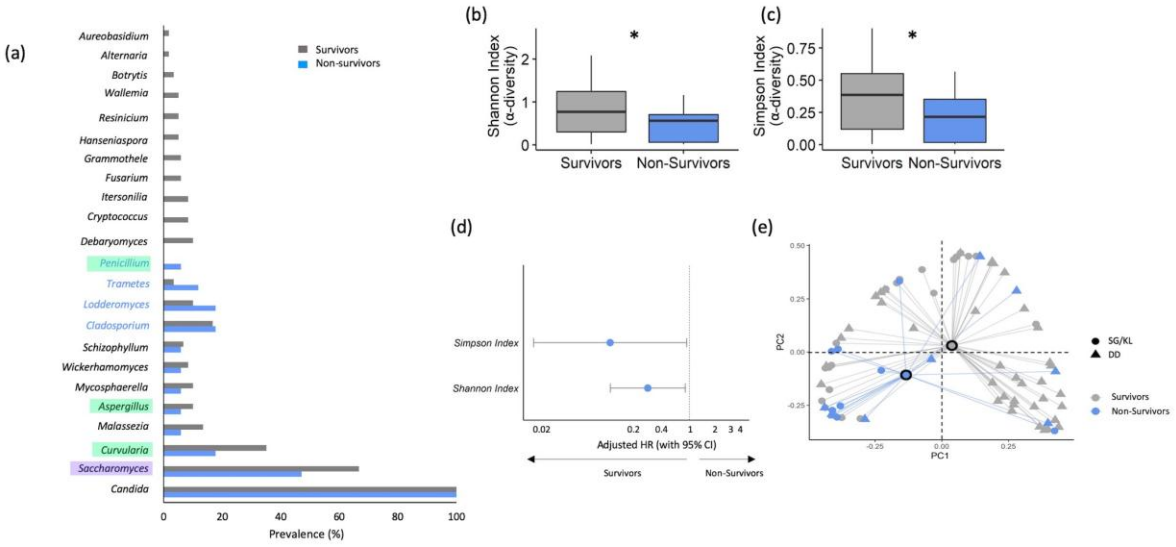


Figure 5



Online supplement

A HIGH-RISK AIRWAY MYCOBIOME IS ASSOCIATED WITH FREQUENT EXACERBATION AND MORTALITY IN COPD

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SUPPLEMENTARY MATERIALS AND METHODS

Ethics Statement: This study was approved by **t**he Institutional Review Boards (IRBs) of all participating hospitals and institutions. Written informed consent was obtained from all participants. Reference numbers pertaining to ethical approvals at each site were as follows: CIRB 2016/2549, CIRB: 2016/2715, CIRB 2017/2933, CIRB 2017/2109 (all mutually recognized by DSRB, Singapore), UMMC 2018725-6524 (Malaysia). Non-diseased (healthy) control recruitment was approved by Nanyang Technological University (NTU) under IRB-2017-12-010 (Singapore). Patients in the UK were recruited under approval granted by the East of Scotland Research Ethics Committee (13/ES/0030).

COPD specimen collection and processing (sputum and serum): *Sputum:* Spontaneously expectorated or induced 'representative' sputum was obtained from a deep cough with the assistance of a chest physiotherapist (where appropriate) and collected in sterile containers and transported (on ice) for evaluation [1]. An equal volume of Sputasol (Oxoid Limited, UK) was added to each sample and shaken for 15 minutes at 37℃. Sputasol-homogenised samples mixed with two volumes of RNAlater (Life Technologies, New Zealand) were stored at -80˚C for subsequent DNA extraction and mycobiome analysis as described [2]. *Serum and Plasma:* Where appropriate, venous blood draws were performed and specimens collected in serum or EDTA vacutainer tubes (BD biosciences), centrifuged at 1300g for 10 minutes at 18℃ to separate serum, where appropriate, which was used for subsequent immunological studies. All specimens from clinical sites were transported promptly, appropriately and processed centrally in Singapore to ensure consistency and standardization of all assessments. All sputum was extracted using the same DNA extraction kit (Zymo reagents) and stored at -80ºC. To ensure quality control of materials transported from sites outside Singapore, specimens were temperature controlled and their integrity checked on arrival to Singapore before experimental use. All experiments were performed at a single site: Nanyang Technological University, Singapore.

Non-diseased (healthy) sputum collection: Spontaneously expectorated sputum was obtained through directed coughing using the Huff cough manoeuvre by direct supervision and with clear instruction from trained medical personnel [3]. Specimens were collected in sterile containers and transported (on ice) to the laboratory for further processing and evaluation.

Sputum DNA extraction: Sputum DNA was extracted using the Zymo DNA isolation kit with an appropriate blank control (Zymo Research, Irvine, CA, USA) as per manufacturer's instructions. Briefly, sputum was centrifuged at 13,000g for 10 minutes and resultant pellets resuspended with elution buffer. Next, the homogenised samples were incubated at 55℃ for 10 minutes with storage buffer and proteinase K. Subsequently, samples containing Genomic binding buffer was transferred to the Zymo-spin column and centrifuged for 1 minute at 12,000g. The columns were washed and final DNA eluted into 50uL of elution buffer. All DNA was quantified using a Qubit. fluorometer 2.0 double-stranded DNA (dsDNA) assay (Invitrogen, Carlsbad, CA, USA).

PCR amplification, library preparation, mycobiome sequencing and data processing: The Fungal Internal Transcribed Spacer (ITS)-2 region was selected for amplification using the following primers: Fseq: ATGCCTGTTTGAGCGTC; Rseq: CCTACCTGATTTGAGGTC [4]. Primers were selected based on published work illustrating that these were most optimal for mycobiome sequencing approaches in respiratory specimens [4]. Forward and reverse adaptors were added to the ITS-2 primer pairs (Integrated DNA technologies, IDT, Coralville, Iowa, USA) as per Illumina fungal metagenomics protocols. PCR conditions and cycles were optimised as follows: 10 ul KAPA HiFi HotStart ReadyMix polymerase (Kapa Biosystems, Salt River, Cape Town, South Africa) was added to 4 uL (2uM) of primers (2 ul each forward and reverse) (IDT, Coralville, Iowa, USA) and 6uL of sputum DNA (total volume: 20 uL). Initial denaturation was performed at 95℃ for 3 minutes followed by 35 cycles at 95℃ for 15 seconds, 50℃ for 15 seconds and 72℃ for 30 seconds with a final extension step at 72℃ for 7 minutes using a Verti Thermal cycler (Invitrogen, Carlsbad, CA, USA). After amplification, the PCR products were visualized using a 1.5% agarose gel to ensure

successful amplification and an appropriately sized band. PCR products were then purified using Ampure XP beads (Beckman Coulter Genomics, part #A63881) with a modified two-step clean up: first with an 0.6 ratio volume of Ampure XP beads to PCR product, followed by an 0.8 ratio where supernatant was collected in-between and a final elution performed in 20 uL elution buffer. Second stage index PCR and clean up were performed to include the index sequences and flow-cell attachment regions. Agarose gel electrophoresis (1.5%) was performed to ensure successful purification and the absence of primer dimers prior to pooling of the library. Final libraries were denatured and quantified using the Kappa library quantification kit (Kapa Biosystems, Salt River, Cape Town, South Africa) and pooled libraries of 8.5pM with 20% phiX loaded onto the Illumina MiSeq platform (2x 101bp reads) (Illumina, San Diego, CA, USA) in parallel to extraction blanks and respective negative controls for the various PCR steps. Mycobiome sequencing was then performed on an Illumina MiSeq platform (2x 101bp reads) (Illumina, San Diego, CA, USA). ITS analysis was performed using Illumina Miseq platforms as previously described [4]. Briefly sequences were mapped to the UNITE database (v7.2) described by Köljalg *et al.* using the Illumina ITS Metagenomics pipeline (version 1.0.1; Basespace labs, San Diego, CA, USA) with high-performance implementation of the Ribosomal Database project (RDP) classifier [5, 6]. All sequence data has been uploaded to the National Center for Biotechnology Information (NCBI) Sequence read archives (SRA) under project accession PRJNA609892. https://dataview.ncbi.nlm.nih.gov/object/PRJNA609892?reviewer=fgrp8fvt98lcf28lk7icfmqu8m

Negative controls and extraction blanks: A total of fourteen negative control samples (i.e. PBS) and extraction blanks (i.e. Zymo reagents) were sequenced and included with each respective sequencing run. The average DNA concentration (in nanograms) and number of assigned reads (at genus level) compared between the sputum samples and blank controls are illustrated in Figure E5. Average sequence read count per ul for extraction blanks, sequencing blanks and the sputum samples were 342, 9 and 42,556 respectively (Figure E5). Contamination was identified using the R "Decontam" package with a prevalence threshold of 0.5 [7]. All fungal genera identified as contaminants

(including those present in the sequencing blanks, as illustrated in Table E1) were removed from downstream analysis.

Immuno-dot-blot assays to measure specific-IgE responses against fungi: Using plasma from a subset of n=42 COPD patients as previously described, specific IgE (sIgE) immuno-dot blot assays against the following fungi were performed: *Aspergillus, Curvularia, Penicillium, Cladosporium, Schizophyllum, Fusarium* and *Trametes* using methodology as previously described [8]. Briefly, fungi were isolated from Singapore outdoor air using the Spin-Air agar impactor (IUL S.A., Barcelona, Spain). The air was impacted onto various nutrient media, single colonies selected and re-streaked onto fresh plates. This process was repeated for three generations until single fungal isolates were obtained. Final fungal identity of isolated fungi was confirmed by Sanger sequencing. Immuno-dot blots were then performed as follows: crude fungal proteins (from isolated fungi) were blotted in duplicate onto nitrocellulose membranes with Bovine Serum Albumin (BSA) as protein control and PBS as negative control. The membranes were blocked with 1X PBS 0.1% Tween-20 for 40 minutes followed by exposure to patient serum overnight (16 hours). Washing steps were then performed in triplicate using 1X PBS 0.05% Tween-20. The washed membrane was then incubated with anti-human IgE antibodies (1:1000) conjugated to alkaline phosphatase (Sigma Aldrich, USA) for 2 hours. Nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3'-indolyphosphate (BCIP) solution (Thermo Fisher Scientific) was next added to detect alkaline phosphatase activity for 10 minutes and data analyzed using Syngene imaging software [8]. Inter- and intra-assay reproducibility was 90% and 95% respectively and specific Ig-E binding calculated and subsequently presented as optical density (OD) intensity.

Statistical analysis: Data was analyzed using R (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria.) and python (version 3.8). Shapiro-Wilk tests were to assess data for normality distribution.

Statistical analysis: Data was analyzed using R (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria.) and python (version 3.8). Shapiro-Wilk tests were to assess data for

normality distribution. Data are presented as medians with interquartile range (non-normally distributed data) or mean with standard deviation (normally distributed data). For between group comparisons, Wilcoxon's, Mann-Whitney-U and/or Kruskal-Wallis tests with Benjamin-Hochberg correction for false discovery was performed as appropriate. Categorical data was analyzed using Chi-squared or Fisher-exact tests as appropriate. Contamination was identified with R "Decontam" package with a prevalence threshold of 0.5 [7]. The fungal genera identified as contaminants were removed from further downstream analysis (Table E1). Shannon and Simpson diversity indexes were calculated with the "*diversity*" function of the R package "vegan". Bray-Curtis dissimilarity index was generated with the "*vegdist"* function of the R package "vegan". Beta diversity was visualized using principal coordinate analysis (PCoA) plots with Bray-Curtis dissimilarity. The distance to centroids were calculated using the "*betadisper"* function of the R package "vegan". Between group differences were obtained with permutation-based testing of multivariate homogeneity of group dispersion (PERMDISP2). For multivariate analysis adjusted for age, gender, body mass index, smoking status (as pack year exposure), post bronchodilator FEV_1, inhaled corticosteroid use and country of origin, permutational multivariate analysis of variance using distance matrices (PERMANOVA) was computed with 10,000 permutations using the "*adonis"* function of the R package "vegan". Linear discriminant analysis effect size (LEfSe) was performed using the webtool: http://huttenhower.sph.harvard.edu/galaxy/ and a cut-off Linear Discriminant Analysis (LDA) score of >3.5. Graphs were generated using the R package "ggplot2" and significance level defined as $p<0.05$.

Cluster analysis: As *Candida* was the predominant fungal genera within the COPD mycobiome (≥65% relative abundance), it was excluded from clustering analyses to allow for the identification, if relevant, of other differentiating fungi in relation to COPD outcomes. Unsupervised hierarchical clustering with Ward's minimum variance was performed with the "*hclust*" function of the R" Cluster*"* package and visualized with *heatmap.2* using the R "gplot" package. Optimal clusters were assessed with the R "Nbclust" package. Cluster stability was assessed using Jaccard similarities index with bootstrapping over 100 iterations. The mean Jaccard similarities index for clusters 1 and 2

respectively were 0.91 and 0.92 suggesting that the identified clusters were highly stable (Jaccard index ≥0.75 suggests valid and stable clusters [9]).

Co-occurrence network analysis: Co-occurrence network analyses was generated using methodologies described by *Fraust et al,* with slight modification [10]. Pearson's correlation, Spearman's correlation, Bray-Curtis index, Generalized Boosted Linear Models (GBLMs) and mutual information were all ensembled with a weighted Sime's test and visualized using Cytoscape (version 3.7.2) [11]. Fungal genera present in the airway mycobiome in at least 5% of the total population at an abundance of >1% were included in co-occurrence analyses.

Table E1

Genus	Probability
Dichomitus	0.028
Trichosporon	0.057
Dekkera	0.085
Hortaea	0.127
Puccinia	0.127
Polyporus	0.141
Pichia	0.258
Tinctoporellus	0.276
Wickerhamia	0.327
Hyphodontia	0.456

Table E2

Non- FE			
Microbes	No. of interactions (Edges)	Critical (Stress Centrality)	Influential (Betweenness Centrality)
Candida	***28***	***79***	***0.131846***
Saccharomyces	***24***	***51***	***0.068446***
Alternaria	22	36	0.048775
Penicillium	22	29	0.03046
Cryptococcus	20	28	0.044562
Malassezia	18	19	0.019183
Curvularia	18	14	0.011748
Aspergillus	18	14	0.013139
Lodderomyces	17	10	0.008272
Cladosporium	13	12	0.019885
Mycosphaerella	14	10	0.010178
Debaryomyces	8	6	0.008425
Hanseniaspora	8	4	0.00641
Wickerhamomyces	18	14	0.011517
Schizophyllum	14	2	0.001221

Top taxa

vFE			
Microbes	No. of interactions (Edges)	Critical (Stress Centrality)	Influential (Betweenness Centrality)
Alternaria	**28**	**11**	**0.006207**
Aspergillus	**28**	**11**	**0.006207**
Cryptococcus	**28**	**11**	**0.006207**
Curvularia	**28**	**11**	**0.006207**
Lodderomyces	**28**	**11**	**0.006207**
Malassezia	**28**	**11**	**0.006207**
Penicillium	**28**	**11**	**0.006207**
Saccharomyces	**28**	**11**	**0.006207**
Debaryomyces	26	8	0.00464
Cladosporium	25	4	0.002198
Candida	26	2	0.000881
Hanseniaspora	26	2	0.000881
Mycosphaerella	26	2	0.000881
Schizophyllum	26	2	0.000881
Wickerhamomyces	19	1	0.000423

Top taxa

Table E3

Patient Characteristics	Survivor	Non-survivor	p-value
N	51	15	
Age (years), Median (IQR)	67 (63-73)	72 (69-78)	0.02
BMI (Kg/m^2), Median (IQR)	26.0 (22.0-30.8)	22.3 (17.8-26.0)	0.03
Gender (Male), n (%)	42 (82.4)	11 (73.3)	ns
Current smoker, n (%)	36 (70.6)	8 (53.3)	ns
Ex-smoker, n (%)	15 (29.4)	7 (46.7)	
Smoking pack years, Median (IQR)	40.0 (30.0-67.0)	50.0 (40.0-65.0)	ns
CAT Score, Median (IQR)	22.5 (18.0-27.0)	24.0 (19.0-27.0)	ns
FEV_1 (% predicted), Median (IQR)	51.9 (41.5-72.6)	50.0 (38.6-69.9)	ns
FEV_1/FVC (% predicted), Median (IQR)	51.0 (42.5-59.5)	50.0 (41.5-59.0)	ns
No. of Exacerbations/year, Median (IQR)	3 (2-4)	2 (2-4)	ns
GOLD Group, n (%) A B C D	 2 (3.9) 7 (13.7) 9 (17.7) 33 (64.7)	 1 (6.7) 1 (6.7) 1 (6.7) 12 (80.0)	ns
GOLD Grade, n (%) 1 2 3 4	 10 (19.6) 23 (45.1) 15 (29.4) 3 (5.9)	 2 (13.3) 6 (40.0) 5 (33.4) 2 (13.3)	ns
LABA monotherapy, n (%)	1 (2.0)	0 (0.0)	ns
LAMA monotherapy, n (%)	3 (5.9)	2 (13.3)	ns
LABA/LAMA, n (%)	9 (17.7)	4 (26.7)	ns
LABA/ICS, n (%)	4 (7.8)	1 (6.7)	ns
LABA/LAMA/ICS, n (%)	32 (62.7)	6 (40.0)	ns
SAMA/SABA, n (%)	2 (3.9)	2 (13.3)	ns
Macrolide, n (%)	6 (11.8)	2 (13.3)	ns

Table E1: Summary of fungal genera identified as contaminants using the "Decontam" R package. P: true probability of a genera being present in an examined sample. Cut off <0.5 in prevalence was identified as a contaminant.

Table E2: Summary of network analyses based on fungal genus comparing very frequent (vFE) with non-frequent (non-FE) COPD exacerbators. The number of interactions (edge counts), critical (stress centrality) and influential (betweenness centrality) measures are illustrated for between group comparisons. The top taxa with the highest number of interactions, which are most influential and critical within the network are highlighted.

Table E3: Demographics of survivors (n=51) and non-survivors (n=15) within the acute exacerbation of COPD (AECOPD) cohort. Data is presented as the number of patients (n) (with percentage; %) or median (and interquartile range; IQR) as appropriate. BMI: body mass index, CAT: COPD assessment test, FEV_1: forced expiratory volume in the 1^{st} second, FVC: forced vital capacity, IQR: interquartile range, NA: not applicable, GOLD: global initiative for COPD, SAMA: short acting muscarinic antagonist, SABA: short-acting beta agonist, LAMA: long-acting muscarinic antagonist, LABA: long-acting beta agonist, ICS: inhaled corticosteroid.

Figure E1: Airway mycobiome profiles are unaffected by GOLD ABCD group in patients with stable COPD (a) Stacked bar charts illustrating the relative abundance of the top 25 fungal genera between patients in GOLD A (n=81), B (n=104) ,C (n=41) and D (n=111) group, which illustrates no significant change in α-diversity measured by the (b) Shannon and (c) Simpson index and (d) Principle Coordinate Analysis (PCoA) plot between the groups. GOLD: global initiative for COPD. ns: non-significant.

Figure E2: Airway mycobiome profiles are unaffected by GOLD lung function (FEV_1) grade in patients with stable COPD (a) Stacked bar charts illustrating the relative abundance of the top 25 fungal genera between patients in GOLD 1 (n=57), 2 (n=163) , 3 (n=92) and 4 (n=25) grouping, which illustrates no significant change in α-diversity measured by the (b) Shannon and (c) Simpson index and (d) Principle Coordinate Analysis (PCoA) plot between the groups. GOLD: global initiative for COPD. ns: non-significant.

Figure E3: Airway mycobiome profiles are unaffected by treatment with inhaled corticosteroids in patients with stable COPD (a) Stacked bar charts illustrating the relative abundance of the top 25 fungal genera between patients receiving long-term treatment with an inhaled corticosteroid (ICS) (n=166) and those not receiving ICS (n=171) which illustrates no significant change in α-diversity measured by the (b) Shannon and (c) Simpson index and (d) Principle Coordinate Analysis (PCoA) plot between the groups. ns: non-significant.

Figure E4: No significant different in the systemic immune response to control fungi allergens between clusters. Systemic specific-IgE (sIgE) binding (expressed as $\log_{10}$ OD intensity) to (a) *Cladosporium* (b) *Fusarium* (c) *Schizophyllum* and (d) *Trametes* illustrated as bar plots for comparison between clusters. ns: non-significant.

Figure E5: Box and whisker plots illustrating (a) DNA concentration (in nanograms, ng), (b) the number of assigned reads (at genus level), (c) volume of sample added to sequencing pool (in microlitre, µl) and (d) the number of assigned reads per microliter (µl), compared between sputum samples and negative controls: DNA extraction blanks (n=4) and sequencing blanks (n=10). Error bars represent the standard deviation (SD). (e) Stacked bar charts illustrating the taxonomy assignment for negative control samples: DNA extraction blanks (E1-E4) and sequencing blanks (S1-10).

Figure E6: Violin plots illustrating the (log10) relative abundance of individual fungal genera between (a) COPD participants from Singapore and Kuala Lumpur (SG/KL) (n=175) and Dundee (DD) (n=162), (b) Non-frequent (non-FE) (n=245) and very frequent (vFE) (n=92) COPD exacerbators and (c) longitudinal analysis in n=34 COPD participants at baseline (B) (pre-exacerbation), during exacerbation (E) and following treatment (post-exacerbation) (PE).

Figure E7: Scattered boxplot illustrating no significant differences in the Internal Transcribed Spacer (ITS) burden between non-frequent (non-FE) and very frequent COPD exacerbators (vFE). ns: non-significant.

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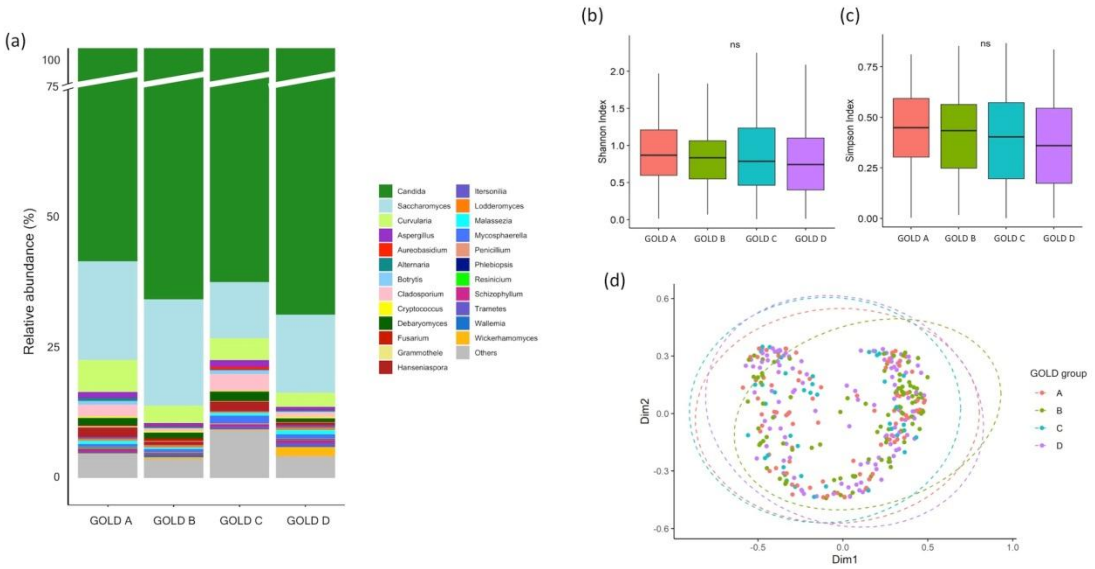
Figure E1



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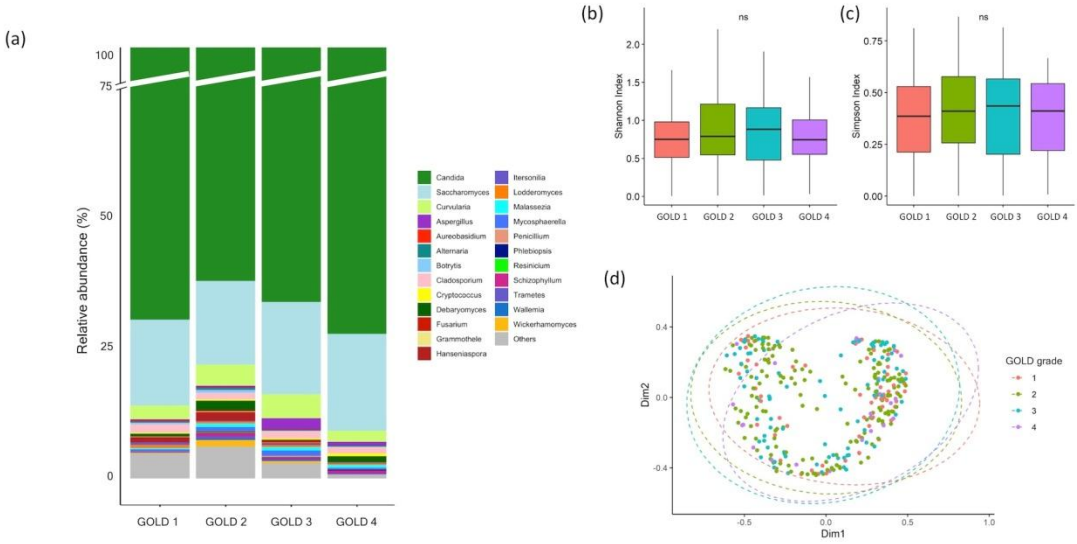

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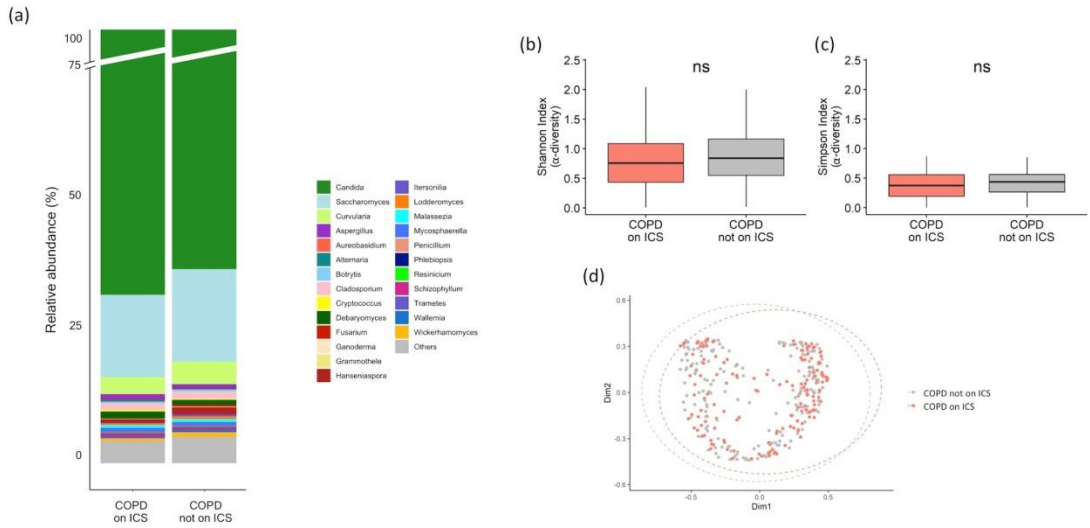

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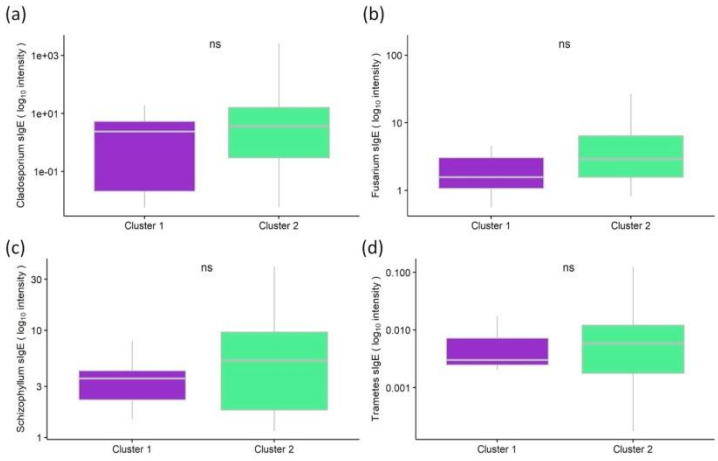

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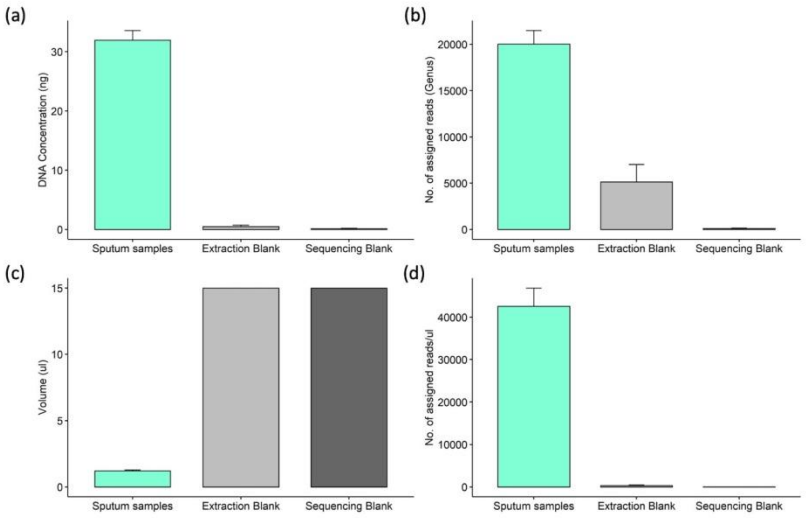

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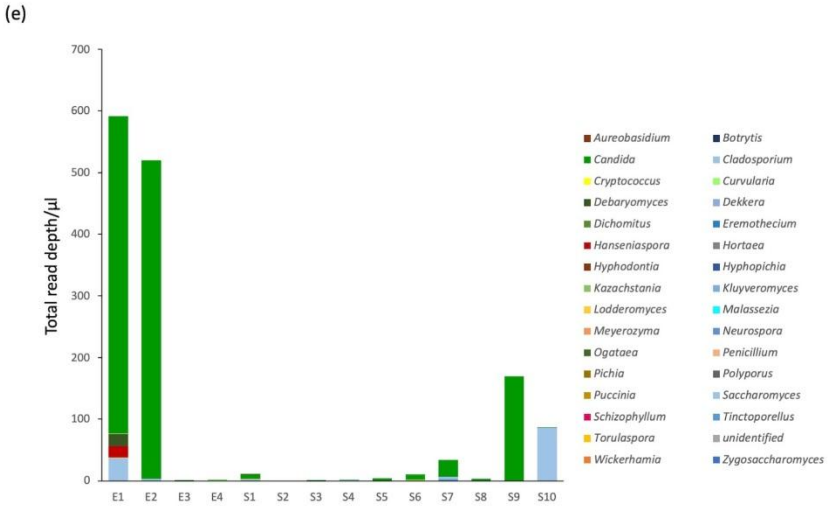

Figure E6

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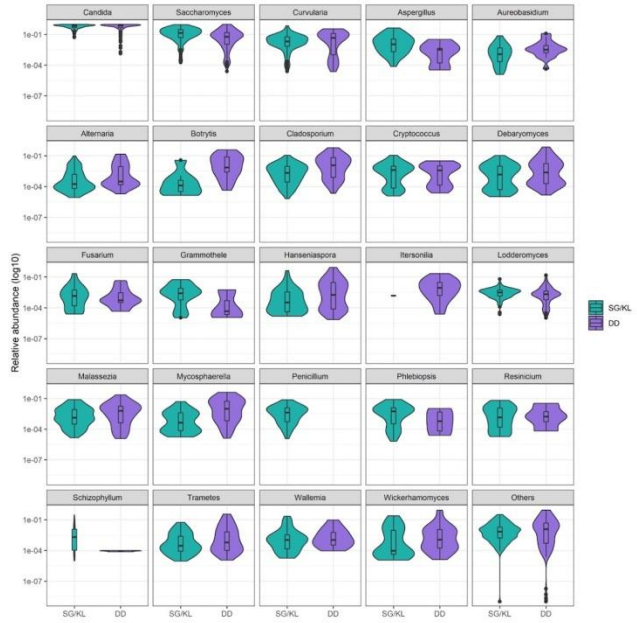

Figure E6

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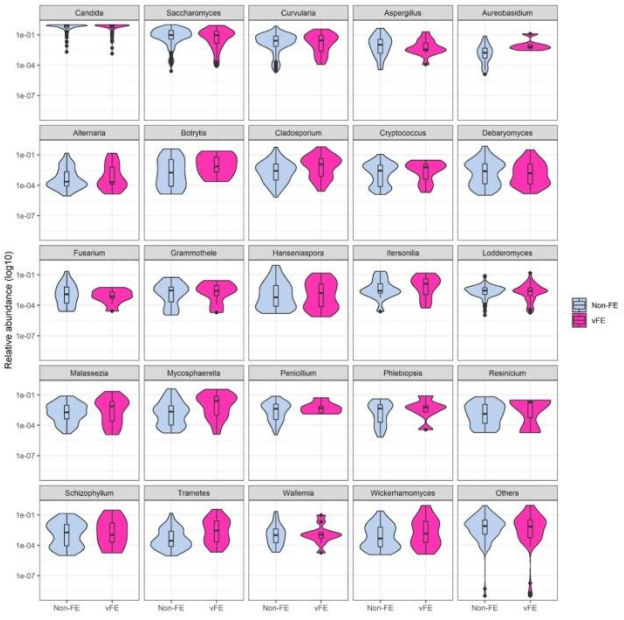

Figure E6

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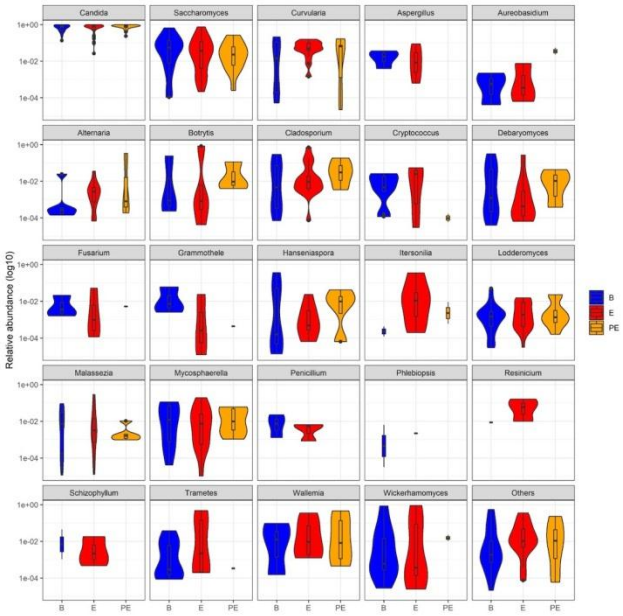

Figure E7