The sinonasal mycobiota in chronic rhinosinusitis and control patients*

Michael Hoggard[1], Melissa Zoing[2], Kristi Biswas[2], Michael W. Taylor[1,3], Richard G. Douglas[2]

[1] School of Biological Sciences, The University of Auckland, Auckland, New Zealand

[2] School of Medicine, The University of Auckland, Auckland, New Zealand

[3] Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Auckland, New Zealand

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Background: While bacterial associations with chronic rhinosinusitis (CRS) are increasingly well described, fewer studies have examined the fungal component of the sinonasal microbiota. Here we present a study of the sinonasal mycobiota in a cohort of 144 patients (106 patients with CRS and 38 controls).

Methodology: Fungal communities were characterised by analysis of mucosal swab samples of the left and right middle meatuses via ITS2 marker amplicon sequencing on the Illumina MiSeq platform. Fungal associations with previously published bacterial community and inflammatory cytokine and cell data for this cohort (collected at the same intra-operative time point) were also investigated.

Results: *Malassezia* spp. were ubiquitous and often highly predominant. Season of sampling explained more of the variability in the data than any of the clinical parameters. The predominant *Malassezia* sp. was distinct in patients with cystic fibrosis compared to those without. However, distinctions in the mycobiota were not evident between any other patient groupings assessed, and few fungal-bacterial or fungal-inflammatory associations were observed.

Conclusions: This study confirms the prominent place of *Malassezia* spp. within the upper respiratory tract. Overall, few distinctions between patient groups were evident, and these data lend further support to the hypothesis that fungal community types may have no direct causative association with idiopathic CRS. Additional studies incorporating a broader array of inflammatory markers are required to assess whether these ubiquitous fungi nonetheless play an exacerbating role in some sensitive individuals.

Key words: chronic rhinosinusitis, fungi, *Malassezia*, microbiome, next-generation sequencing

Introduction

The upper respiratory chronic inflammatory condition chronic rhinosinusitis (CRS) affects approximately 5% of the population[1,2], causing considerable burden on patient quality of life[1,2], and on healthcare systems globally[3]. Immune[4,5], genetic[6], environmental exposure[7], and microbial[8–15] causes have been investigated. However, the aetiology of CRS remains poorly understood.

Fungi have been implicated in a wide array of conditions, including lung infections, chronic obstructive pulmonary disease, chronic pulmonary aspergillosis, asthma linked to fungal allergy, pneumonia, and chronic subcutaneous infections[16]. In CRS, fungal-specific subtypes include allergic fungal rhinosinusitis and the presence of fungal balls[17]. However, fungi are not currently thought to play a direct causative role in idiopathic CRS[18]. Fungi are ubiquitous in the upper respiratory tract, with no clear specific association with CRS[2,19–25]. Furthermore, there is no evidence for the efficacy of antifungals in idiopathic CRS[18,26]. Nonetheless, fungi can influence both the host immune system and the other members of the resident microbiota in a myriad of ways[7,27–31], and it remains unclear whether fungi play modifying or exacerbating roles in CRS.

The bacterial component of the human microbiota has been increasingly well described. We are beginning to better under-

stand patterns that underlie the difference between health and CRS, including bacterial community dysbiosis in CRS (involving altered community structure and diversity)[9,11,12,32,33], and associations with inflammatory signalling[9,34]. Comparably fewer studies have examined the fungal component of the sinonasal microbiota (the mycobiota)[24,25,35,36], and fewer still have investigated bacterial-fungal associations.

In this study, we present the first comprehensive parallel assessment of fungal, bacterial, and immunological associations in CRS. The sinonasal mycobiota in patients with CRS (n = 106) and control subjects (n = 38) were characterised via ITS2 marker-based amplicon sequencing. Furthermore, previously published bacterial community data (generated from the same sample genomic DNA)[11,14] and local inflammatory cell and signalling data[14,34] were analysed to investigate associations with the mycobiota.

Material and Methods

Subjects and sample collection

In total, 144 subjects were included in this study, including 50 CRS without nasal polyps (CRSsNP), 49 CRS with nasal polyps (CRSwNP), seven CRS with cystic fibrosis (CRSwCF) patients, and 38 control subjects undergoing a similar surgical procedure for indications other than CRS (including dacrocystorhinostomy and pituitary adenoma surgery). The prospective recruitment pool included all patients electing to undergo endoscopic sinonasal surgery to be undertaken by a single ENT surgeon (RGD) at two tertiary care centres in Auckland, New Zealand. Patients consenting to participate in the study were recruited and categorised as CRS (n = 106) or non-CRS control patients (n = 38). CRS subjects were defined as per the European Position Paper (EPOS2012) guidelines[1]. Exclusion criteria included sinonasal vasculitis, immunodeficiency, and patients under 18 years of age. Clinical severity scores (Lund-Mackay score; symptom severity score based on ratings of five nasal symptoms) and patient demographics data were collected at the time of surgery. Mucosal swab samples from the left and right middle meatuses were collected intra-operatively under endoscopic guidance, and sample DNA extracted using AllPrep DNA/RNA Isolation kits (Qiagen, Hilden, Germany), as previously described[11,14] (previously extracted genomic DNA were obtained directly for this study).

Institutional ethical approval was obtained for this study from the New Zealand Heath and Disability ethics committee (NZX/08/12/126), and written informed consent was obtained from all participants.

PCR and preparation for sequencing

The fungal ITS2 marker was PCR amplified using primers ITS3-ITS4[37] incorporating Nextera adapters, under the following conditions: initial enzyme activation and denaturing for 15 min at 95°C, 35 cycles of 95°C for 30 s, 52°C for 30 s, and 70°C for 60 s, and a final extension step of 70°C for 7 min. PCR reaction mixes were as previously described[36], incorporating HotStar DNA polymerase (Qiagen, Hilden, Germany), together with ~70 ng template gDNA per reaction. Triplicate PCR products for each sample were pooled and purified using Agencourt AMPure beads (Beckman-Coulter, Brea, CA, USA) as per the manufacturer's instructions, normalised to ~1 ng/µl, and submitted to the sequencing provider (Auckland Genomics Ltd., Auckland, New Zealand) for library preparation and sequencing on the Illumina MiSeq platform (2 x 250 bp paired-end reads). Raw sequences have been uploaded onto the SRA-NCBI database (BioProject accession: PRJNA498816).

Bioinformatics

Sequences were processed in usearch (v10)[38–43] via a bioinformatics pipeline specifically developed for ITS amplicon sequences as previously described[36]. Samples were subsampled to an even depth of 1000 reads per sample. For nine subjects, fungal community data were only successfully obtained for samples from one side (either the left or right middle meatus). For further detail, the full bioinformatics processing pipeline is available online: https://github.com/mhog025/Microbiota-amplicon-bioinformatics.

Previously published inflammatory and bacterial data

Local (mucosal) inflammatory data were available for 113 subjects, including $CD3^+$ T, $CD20^+$ B, $CD68^+$ macrophage, plasma, eosinophil, and neutrophil cell counts, and local concentrations of interleukin- (IL-)2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17A, interferon- (IFN-)γ, and tumour necrosis factor (TNF) (from sample collections at the same intra-operative time point)[14,34]. Ninety nine of these subjects had also previously been categorised into 8 distinct subject clusters on the basis of local inflammatory profile and key clinical factors including asthma and polyposis[34]. These subject clusters were used as an additional stratification for between-group comparisons for this subset of patients.

Previously published bacterial community data (amplified from the same sample template DNA as fungal amplicons) were available for 140 subjects[11,14]. Sequences were re-processed with an updated version of the bioinformatics pipeline used previously for these data, with zero-radius operational taxonomic units (ZOTUs) (analogous to amplicon sequence variants (ASVs)) generated in lieu of 97% similarity OTUs, the Silva Living Tree Project database (v123)[44] used for taxonomic assignments, and additional filtering for cross-talk included. Samples were subsampled to even depth of 400 reads/sample. All samples from 11 subjects had insufficient reads to meet this subsampling threshold and these patients were excluded from the bacterial data set. Full details of the processing pipeline are available online: https://github.com/mhog025/Microbiota-amplicon-bioinformatics.

Table 1. Summary of participants' data.

Variables[a]	Subject groups				Unadjusted test p-value[c]
	Controls (n=38)[b]	CRSsNP (n=50)[b]	CRSwNP (n=49)[b]	CRSwCF (n=7)[b]	
Demographics and clinical scores					
Age	46 (19 to 84)	47 (19 to 68)	50 (18 to 71)	31 (22 to 50)	0.098
Gender	23/38 (61%)	24/50 (48%)	19/48 (40%)	6/7 (86%)	0.076
European	24/37 (65%)	42/50 (84%)	39/48 (81%)	6/7 (86%)	0.227
Asthma	4/38 (11%)	18/50 (36%)	35/49 (71%)	1/7 (14%)	**0.000**
Aspirin sensitivity	1/38 (3%)	2/50 (4%)	15/49 (31%)	0/7 (0%)	**0.000**
Antibiotics[d]	1/38 (3%)	6/50 (12%)	8/49 (16%)	5/7 (71%)	**0.000**
Steroids[d]	0/38 (0%)	6/50 (12%)	5/49 (10%)	3/7 (43%)	**0.006**
Revision Surgery[e]	-	14/50 (28%)	22/48 (46%)	6/6 (100%)	**0.000**
Symptom_score	-	13 (2 to 25)	15.5 (5 to 25)	17.5 (7 to 21)	0.151
Lund-Mackay	-	13 (5 to 21)	17 (6 to 24)	16 (12 to 24)	**0.000**
Fungal communities[f]					
ZOTU1_*Malassezia*	470 (20 to 966)	386 (12 to 980)	446 (12 to 933)	47 (8 to 274)	**0.004**
ZOTU2_*Malassezia*	102 (1 to 836)	91 (1 to 679)	79 (1 to 690)	28 (3 to 918)	0.735
ZOTU3_*Davidiella*	1 (0 to 266)	1 (0 to 455)	4 (0 to 385)	3 (0 to 208)	0.972
ZOTU6_*Malassezia*	0 (0 to 330)	0 (0 to 310)	0 (0 to 323)	51 (0 to 339)	**0.021**
ZOTU7_*Malassezia*	1 (0 to 173)	4 (0 to 294)	4 (0 to 358)	0 (0 to 7)	0.112
ZOTU11_*Malassezia*	1 (0 to 127)	3.5 (0 to 264)	3 (0 to 168)	0 (0 to 14)	0.335
ZOTU13_*Leptosphaerulina*	0 (0 to 336)	0 (0 to 307)	0 (0 to 899)	0 (0 to 2)	0.621
ZOTU10_*Malassezia*	0 (0 to 229)	0 (0 to 229)	0 (0 to 95)	38 (0 to 903)	0.060
ZOTU8_*Malassezia*	5 (0 to 207)	0 (0 to 362)	1 (0 to 272)	0 (0 to 23)	0.074
ZOTU4_*Pleosporales*_unclassified	0 (0 to 324)	0 (0 to 441)	0 (0 to 468)	0 (0 to 242)	0.203
ZOTU14_*Aspergillus*	0.5 (0 to 288)	0 (0 to 161)	0 (0 to 89)	2 (0 to 121)	0.201
ZOTU9_*Malassezia*	0 (0 to 109)	1 (0 to 587)	1 (0 to 85)	1 (0 to 7)	0.815
ZOTU5_*Malassezia*	0 (0 to 97)	0 (0 to 716)	0 (0 to 569)	0 (0 to 1)	0.718
ZOTU12_*Cladosporium*	0 (0 to 245)	0 (0 to 179)	0 (0 to 117)	0 (0 to 29)	0.518
ZOTU17_*Malassezia*	0 (0 to 184)	0 (0 to 270)	1 (0 to 83)	0 (0 to 12)	0.583
ZOTU18_*Rhodotorula*	0 (0 to 317)	0 (0 to 123)	0 (0 to 79)	0 (0 to 33)	0.641
ZOTU26_*Agaricomycetes*_unclassified	0 (0 to 98)	0 (0 to 155)	0 (0 to 498)	0 (0 to 0)	0.457
ZOTU20_*Cryptococcus*	0 (0 to 312)	0 (0 to 215)	0 (0 to 32)	0 (0 to 1)	0.980
ZOTU24_*Galactomyces*	0 (0 to 11)	0 (0 to 243)	0 (0 to 809)	0 (0 to 0)	0.935
ZOTU16_*Malassezia*	0 (0 to 549)	0 (0 to 1)	0 (0 to 412)	0 (0 to 1)	0.428
Richness	12 (3 to 26)	11 (3 to 28)	13 (3 to 26)	12 (5 to 22)	0.855
Shannon diversity	1.87 (0.28 to 3.32)	1.775 (0.14 to 3.33)	1.95 (0.56 to 3.56)	2.25 (0.54 to 2.62)	0.990
Simpson diversity	0.37 (0.13 to 0.94)	0.414 (0.13 to 0.97)	0.385 (0.1 to 0.86)	0.318 (0.22 to 0.84)	0.988
Intra-patient dissimilarity[g]	0.319 (0.02 to 0.83)	0.413 (0.01 to 0.99)	0.391 (0.03 to 1.00)	0.268 (0.10 to 0.98)	0.851

[a] Categorical variables are summarised as proportion yes/total (%), except for gender, which is given as proportion female. Continuous variables are summarised as median (range). [b] Total cohort numbers for each group are given. The differences in total numbers for each variable reflect missing data for some subjects. [c] Difference between groups tested using Fisher's exact test for categorical variables and the Kruskal-Wallis test for continuous variables. Significant p-values are expressed in bold ($\alpha = 0.05$). [d] Antibiotics and/or steroids in the 4 weeks prior to surgery. [e] Whether patients had previously undergone surgery for CRS prior to the current appointment. [f] Fungal community data, including the 20 most abundant fungal ZOTUs, community diversity, and intra-patient fungal community dissimilarity. [g] How different the fungal communities were between the left and right middle meatus for each patient, based on weighted Bray-Curtis dissimilarity. CRS, chronic rhinosinusitis; CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; CRSwCF, CRS with cystic fibrosis.

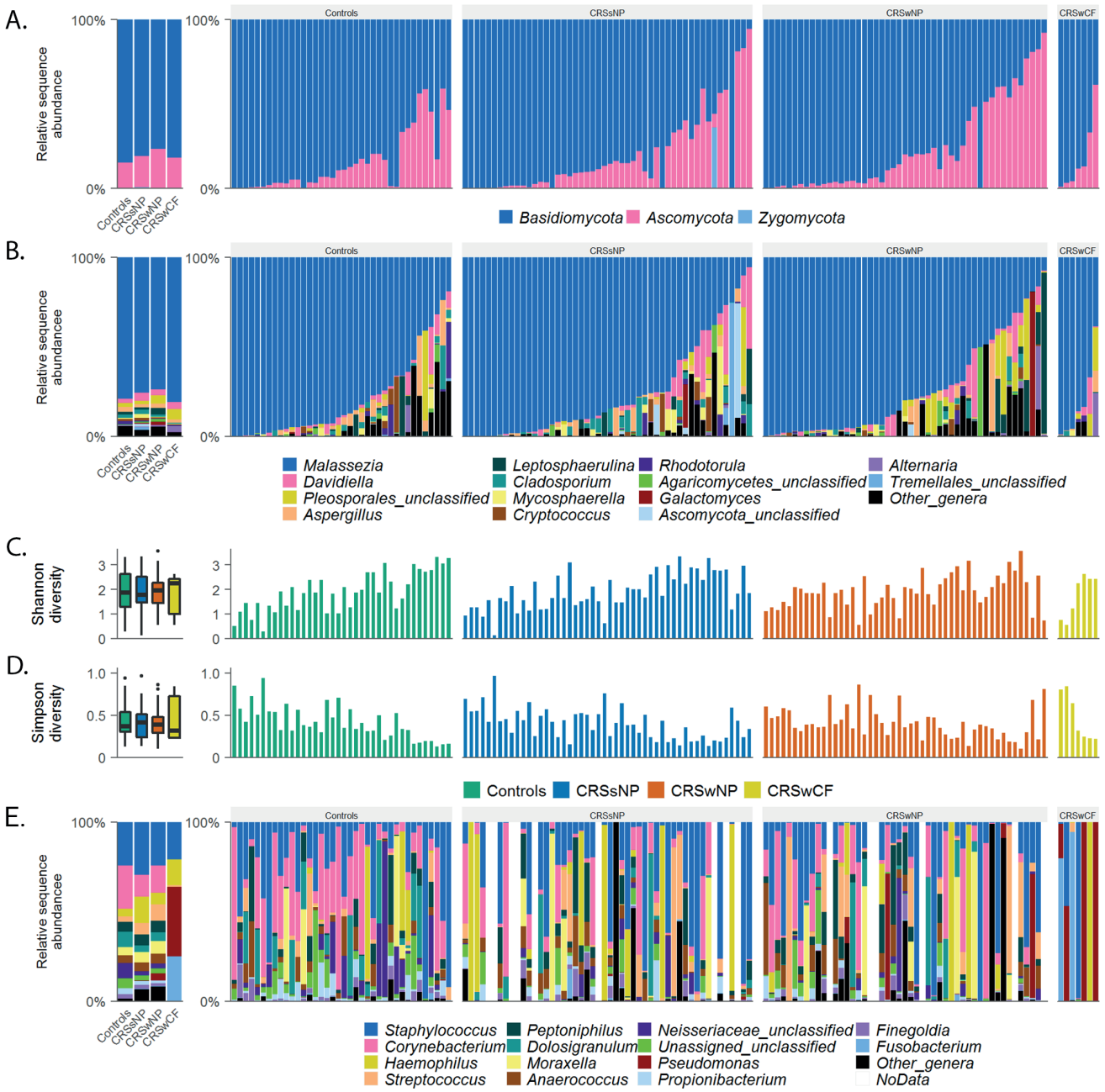

Figure 1. Fungal and bacterial community data summaries. A. Phylum level and B. genus level taxonomic summary plots of fungal community assemblages. Box and bar plots of fungal community alpha diversity for Shannon (base 2) (C.) and (D.) Simpson indices. E. Genus level bacterial community plots for each patient. Samples are grouped by standard clinical phenotypes (controls, CRS without nasal polyps (CRSsNP), CRS with nasal polyps (CRSwNP), CRS with cystic fibrosis (CRSwCF)). Group summaries are presented to the left, and data for each individual subject is presented to the right, with samples within each group ordered by relative abundance of the genus *Malassezia*.

Data analyses and statistics

Alpha diversity (richness, Shannon [base 2], and Simpson) and beta diversity (weighted Bray-Curtis dissimilarity) metrics were calculated in usearch. Statistical analyses and the generation of plots were conducted in R (v3.4.1)[45].

Spearman correlations incorporated the 20 most abundant fungal ZOTUs, the 20 most abundant bacterial ZOTUs, fungal and bacterial alpha diversity metrics, and the available inflammatory data. Between-group pairwise testing of variables, ordination (non-metric multidimensional scaling (nMDS)), beta-dispersion and adonis analyses, and intra-patient dissimilarity comparisons were conducted for each of the following: standard clinical phenotypes (based on polyposis); subtypes based on asthma; inflammatory subject clusters[34]; seasons; and antibiotics usage

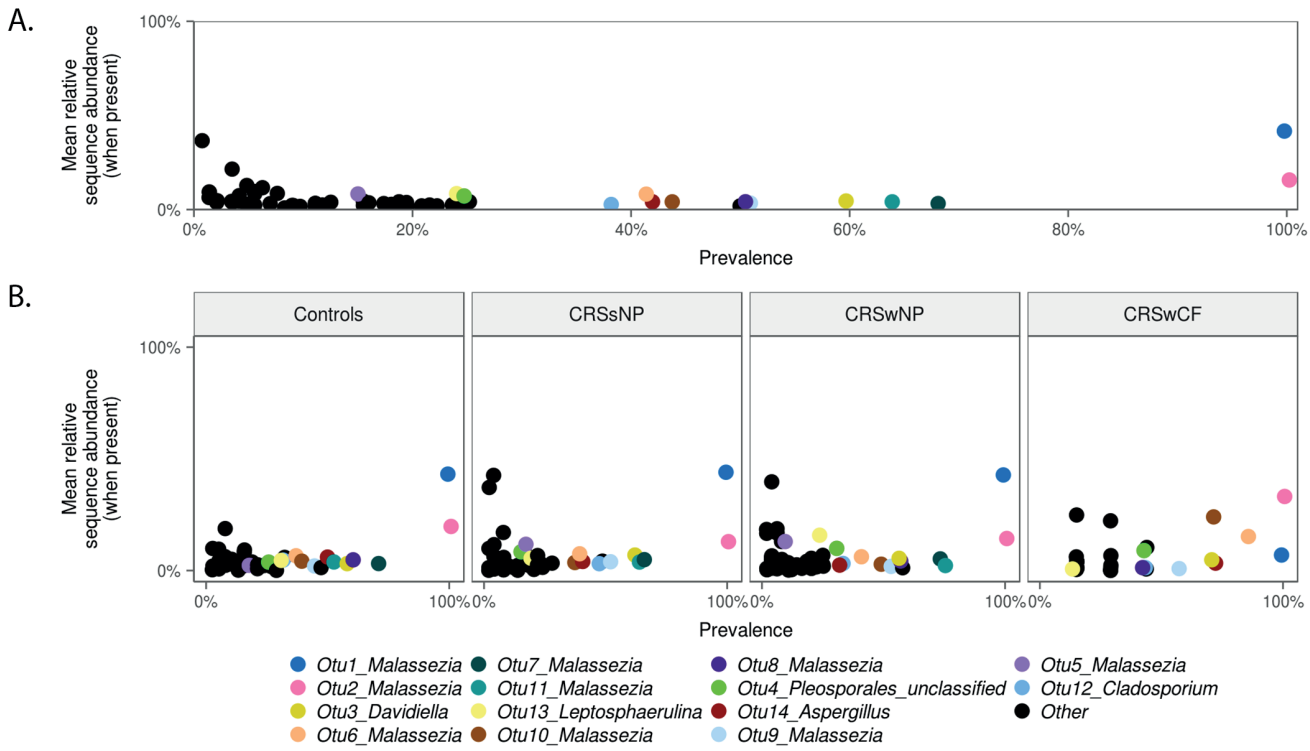

Figure 2. Prevalence and relative dominance of fungal ZOTUs. Dot plots of the prevalence of each fungal ZOTU against their dominance within the fungal communities when present (based on mean relative sequence abundance when present in any given community). A. ZOTU prevalence against dominance for all samples in this study. B. Samples grouped by standard clinical CRS phenotypes (controls, CRS without nasal polyps (CRSsNP), CRS with nasal polyps (CRSwNP), CRS with cystic fibrosis (CRSwCF)). The 14 most abundant ZOTUs are colour-coded, all other ZOTUs are coloured black.

(Y/N) or corticosteroids usage (Y/N) in the four weeks prior to surgery. Pairwise testing was conducted using Fisher's exact, Kruskal-Wallis, Wilcoxon Rank Sum, and Dunn's tests as previously described[34], with Bonferroni adjustment for multiple comparisons. Intra-patient dissimilarity comparisons (comparing between the left and right middle meatus within each patient) were conducted based on weighted Bray-Curtis dissimilarity data for each patient's pair of samples. Intra-patient community dissimilarity analyses incorporated all data (analysing left and right middle meatus samples for each patient separately). For all remaining analyses, data from the left and right side of each patient were merged to accommodate intra-subject variability and provide a patient-level picture of the microbial communities.

Results

Demographics (comparing between standard clinical phenotypes based on polyposis)

CRSwNP patients significantly more often had concomitant asthma and/or aspirin sensitivity than control subjects and CRSsNP patients, and significantly more often had asthma than CRSwCF patients (p-values < 0.05) (Table 1).

Fungal communities

With the exception of a single representative of *Zygomycota* (*Phycomyces*) that was only present in one subject, all identified fungi were from the phyla *Basidiomycota* or *Ascomycota* (Figure 1A). Members of the genera *Malassezia* were commonly highly dominant in the community sequence data (median [IQR] = 86% [66 to 96%]) (Figure 1B). The 20 most abundant ZOTUs were predominantly represented by members of *Malassezia* (11 ZOTUs; median range = 0% to 41% each), together with ZOTUs of *Davidiella*, *Leptosphaerulina*, unclassified *Pleosporales*, *Aspergillus*, *Cladosporium*, *Rhodotorula*, unclassified *Agaricomycetes*, *Cryptococcus*, and *Galactomyces* (Figure 2A).

Fungal, bacterial, and inflammatory associations

ZOTU1_Malassezia was associated with several fungal and bacterial variables (Figure 3), including positive correlations with bacterial ZOTUs of *Haemophilus*, *Corynebacterium*, *Finegoldia*, and *Anaerococcus*, as well as fungal community evenness (Simpson). ZOTU1_*Malassezia* also negatively correlated with fungal ZOTUs of *Aspergillus*, *Cladosporium*, *Davidiella*, *Leptosphaerulina*, an unclassified *Pleosporales*, and fungal community diversity (richness and Shannon). In general, however, there were few other significant correlations between bacterial and fungal variables.

Intra-patient (fungal community) dissimilarity (comparing between the left and right middle meatuses within each patient)

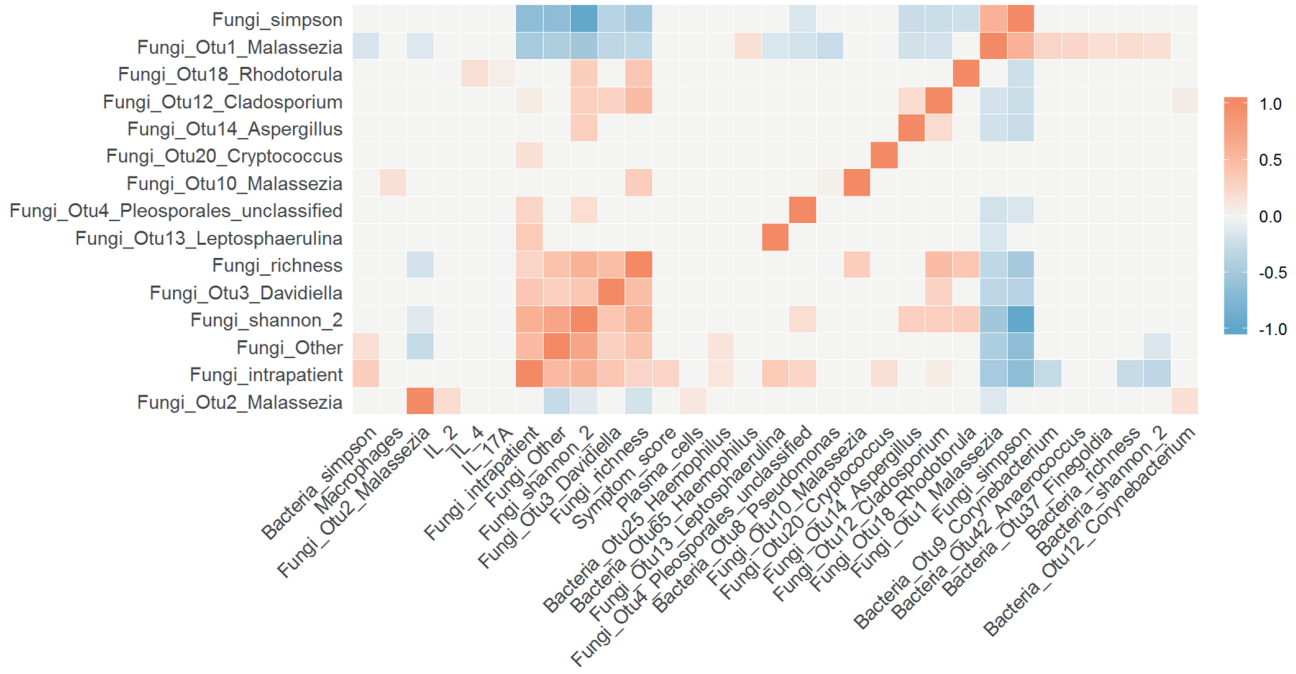

Figure 3. Fungal, bacterial, and inflammatory associations. Heat map of Spearman correlations between fungal, bacterial, and inflammatory variables. Colour-coding represents significant positive (red; scale 0 to 1) and negative (blue; scale 0 to -1) correlations. Significance testing was based on upper tail probabilities, using the R function cor.test (α = 0.05). Non-significant pairwise associations are uncoloured. The 20 most abundant fungal and bacterial ZOTUs, fungal and bacterial diversity indices, fungal intra-patient dissimilarity, and all available inflammatory marker data were included in the analysis, with variables ordered by hierarchical clustering of correlation coefficients. A subset of variables of interest is presented here.

correlated with several variables. These included positive associations with patient symptom score and ZOTUs of *Davidiella*, *Leptosphaerulina*, *Pleosporales*_unclassified, and *Cryptococcus*, and a negative association with ZOTU1-_*Malassezia* (Figure 3). With respect to inflammatory associations, ZOTU10_*Malassezia* significantly positively correlated with macrophage cell counts, ZOTU18_*Rhodotorula* positively correlated with IL-4 and IL-17A, and ZOTU2_*Malassezia* positively correlated with IL-2 and plasma cells (Figure 3). However, overall, there were few correlations between the inflammatory variables and fungal variables.

Comparisons between patient groups

There were few significant differences in fungal communities between groups based on standard clinical phenotypes (controls, CRSsNP, CRSwNP, CRSwCF). In pairwise testing, relative abundances of two ZOTUs of Malassezia (ZOTU1 and ZOTU6) were significantly different in CRSwCF compared to Controls, CRSsNP, and CRSwNP (Figure 2B): ZOTU1 was significantly lower in CRSwCF (median [IQR] = 4.7% [3% to 62%]) compared to the other groups (39% < medians < 47%), and ZOTU6 significantly higher in CRSwCF (5% [3% to 21%]) than all other groups (all medians = 0%) (p-values < 0.02)) (Table 1). These differences were similarly reflected when patients were instead partitioned on the basis of concomitant asthma (controls, CRS without asthma [CRSsAsthma], CRS with asthma [CRSwAsthma], CRSwCF) (p-values < 0.025). Several of the 20 ZOTUs with the highest relative sequence abundance had median values of zero for all four phenotypic groups (Controls, CRSsNP, CRSwNP, CRSwCF), suggesting that many of the dominant ZOTUs were nonetheless not consistently observed across most patients. Exceptions included several ZOTUs of *Malassezia* and one of *Davidiella*, which were more consistently observed over the whole cohort.

When a subset of subjects was partitioned on the basis of inflammatory subject clusters (as previously defined for 99 of the patients in this study[34]) there were no noteworthy significant differences for any fungal ZOTUs in pairwise comparisons.

When grouped by season of sampling, the relative abundance of ZOTU1_*Malassezia* was significantly higher in summer (62% [30% to 74%]) compared to autumn (30% [10% to 58%]) (p = 0.049). ZOTU13_*Leptosphaerulina* and ZOTU4_*Pleosporales*_unclassified were both significantly higher in summer and autumn than in winter or spring months (p-values < 0.04).

In alpha diversity (Figure 1C-D) and nMDS and beta-dispersion analyses, there were no significant differences in pairwise comparisons whether subjects were partitioned on the basis of polyposis, asthma, seasons, inflammatory clusters, or whether

subjects had taken antibiotics or corticosteroids in the four weeks prior to surgery. Adonis analysis identified that partitioning subjects on the basis of polyposis significantly explained 5.0% of the variation in the fungal community data ($p = 0.001$), and partitioning on subtypes based on asthma significantly explained 5.2% ($p = 0.001$). Of the subject groupings assessed, seasons over the full two years of sampling (i.e. eight consecutive seasons) explained the highest amount of variation in the data (7.7%; $p = 0.026$).

Finally, there were no significant differences in intra-patient fungal community dissimilarity based on any of the assessed subject groups (p-values > 0.25).

Discussion

The advent of culture-independent, molecular-based tools for characterizing complex microbial communities has dramatically changed our understanding of microbes living in association with the human body. While the bacterial portion of the sinonasal microbiota is increasingly well understood[8–11,14,15,33], fungi have received comparably less attention in the next-generation sequencing era.

This study characterised the sinonasal fungal communities (mycobiota) in 106 patients with CRS patients and 38 controls using the fungal ITS2 target marker. Fungal associations with previously published bacterial community and inflammatory profile data for this cohort (collected at the same intra-operative time point)[11,14,34] were also investigated.

The sinonasal mycobiota

Previous studies describing sinonasal fungal communities have been conflicting. In particular, the presence or dominance of *Malassezia* spp. has been markedly different between studies. *Malassezia* spp. were initially largely overlooked due to special requirements for culture[31]. *Malassezia* spp. were later identified in some studies as highly prevalent and dominant[25,35,36], while others highlighted *Aspergillus* spp.[22,23] or *Cryptococcus neoformans*[24] as the dominant taxa. A diverse range of other taxa has also been identified in lower relative abundance in both culture-dependent and -independent studies[19,22–25,35,36,46].

A recent methodological comparison indicated that disparities between studies are likely largely shaped by biases introduced by primer selection[36]. These include a strong bias against *Malassezia* spp. by several methods, while ITS2 marker data most closely represented fungal communities typical of the human mycobiota[36]. The data presented here represent the first ITS2-based large cohort study of the sinonasal mycobiota in CRS. These data further confirm the overwhelming prevalence and dominance of *Malassezia* spp. in the sinonasal mycobiota, together with numerous other taxa in lower abundance, including *Davidiella*, *Aspergillus*, *Cladosporium*, *Cryptococcus*, *Curvularia*, *Eurotium*, *Candida*, *Saccharomyces*, and unclassified *Pleosporales* and *Tremellales* spp.

With the exception of CRSwCF patients, there were few differences in fungal community types whether subjects were partitioned on the basis of polyposis, asthma, or inflammatory clusters. Season of sampling explained more of the variation within the data than dividing patients on the basis of these clinical groupings. Notably, the most abundant representative of *Malassezia* (ZOTU1) had significantly higher relative abundance in summer than autumn. In community data based on relative sequence abundances, seasonal changes in highly predominant taxa will strongly influence the relative representation (and subsequent associations observed) of all other taxa in the community. As such, seasonality should always be considered in future such studies.

Additional studies using a standardised methodology are required to assess whether these patterns hold for different geographic regions. Ultimately, this will also allow multi-centre meta-analyses to investigate subtle associations that may be present, but are difficult to detect without very large (and well-described and stratified) patient groups.

Malassezia spp.: the predominant taxa in the sinonasal mycobiota

Malassezia spp. were ubiquitous (detected in 100% of subjects) and often predominant. *Malassezia* spp. tend to be underrepresented in ITS marker sequence data[36]. Thus, the marked dominance of *Malassezia* in the sinonasal tract is likely to be even stronger than that represented here.

Malassezia have been thought to be involved in an array of conditions, including seborrheic dermatitis, atopic eczema, atopic dermatitis, folliculitis, dandruff, psoriasis, onychomycosis, pityriasis versicolor, and sepsis in immunocompromised neonates[28,47,48]. However, a definitive involvement is yet to be established for the majority of these conditions[28,47,48]. *Malassezia* spp. are now known to be ubiquitously associated with humans[28,49], and the data here further suggest that *Malassezia* spp. are present in the upper respiratory tract at comparable relative abundance in both CRS and control subjects. Thus, these taxa are unlikely to play a direct pathogenic etiological role in CRS. Whether an aberrant immune response to these ubiquitous fungi or defective and/or damaged mucosal epithelial integrity subsequently enables *Malassezia* spp. (or other fungi) to exacerbate or influence the course of CRS, requires further study.

Significant differences in CRSwCF patients

Few between-group differences in fungal community types were observed, with the exception of CRSwCF patients. In particular, the *Malassezia* spp. present in CRSwCF compared with non-CF patients may differ at the species or strain level. Antibacterials have previously been shown to alter fungal communities due to the flow-on effects of altering the bacte-

rial community[27]. A long history of antibacterial usage in CF patients may subsequently influence the fungal communities present. Alternatively, this may simply reflect the effects of differences in environmental exposure. In this cohort, CF patients were often already hospitalised prior to surgery for CRS. Many taxa identified in studies of the respiratory tract are also commonly present in the environment, including *Davidiella, Epicoccum, Leptosphaerulina, Alternaria, Xenobotryosphaeria, Cryptococcus, Aureobasidium, Sporobolomyces*, and *Debaryomyces* spp. [50]. Differential exposure of the patients to indoor versus outdoor airborne fungal spores may influence the structure of the community detected by amplicon sequencing. Further research detailing the spatial and temporal differences between fungi in different indoor (such as the home, workplace, and hospital and clinic spaces) and outdoor environments are required to better understand the role this might play in shaping the specific fungi present in the respiratory tract.

Fungal, bacterial, and inflammatory associations

Multi-directional interactions between fungi, bacteria, and the mucosa can influence the overall structure and function of the microbial community as a whole[27], as well as both suppress or promote immune responses[28–31]. We hypothesised that patterns of bacterial community disturbance or instability previously identified in CRS[8,11,12,33], as well as associations with underlying inflammation[9,10,34], might similarly be reflected in the fungal portion of the microbiota.

Few specific fungal-bacterial associations were identified, suggesting that there may be little inter-kingdom linkage within the sinonasal microbiota. Notably, intra-patient fungal community dissimilarity (comparing the left and right middle meatus) was positively correlated with patient symptom score. This reflects a relation between fungal community dynamics and severity of CRS which was also apparent for the bacterial communities of this cohort[11]. Whether this fungal pattern influences the specific nature and degree of local inflammation is unclear from these data, however. Differentiating patients on the basis of putative inflammatory endotypes identified no associations with fungal community patterns, and, overall, few specific fungal-inflammatory associations were apparent. Increased responsiveness of inflammatory cells to fungal stimuli, including *Alternaria* and *Cladosporium*, has been observed in CRS patients previously[46]. While *Alternaria*, *Cladosporium*, and *Davidiella* (a *Cladosporium* teleomorph) were present in this cohort, no differences were observed for these taxa between CRS and controls (or any of the patient groupings assessed).

It remains unclear whether fungal community types have any relation to the various inflammatory processes associated with CRS. However, only a small selection of markers was studied, and fungi may yet influence other aspects of inflammatory signalling not covered here.

Functional resident community or transient inhaled spores?

A key limitation of amplicon sequencing-based mycobiota studies is the inability to determine whether or not the identified fungi represent a functional community that is actually established at the sampling site. This is particularly problematic in the respiratory tract. Observations may reflect inhaled fungal material transiently passing through the respiratory tract, but which do not actually establish and live in association with the mucosa.

To the extent that the observed fungal taxa simply reflect inhaled environmental fungal material, this signal may overwhelm the identification of genuinely resident fungal taxa that do indeed have a relationship with the bacterial taxa present and/or underlying inflammatory disease processes. The hypothesis that the observed mycobiota might largely represent inhaled fungal material is supported by the finding that season of sampling explained more of the variability in the data than any clinical variables. If differences observed for CRSwCF patients are due to differential environmental (indoor/hospital vs. outdoor) exposure, this also provides a further line of evidence.

Fungi may still play a role in disease even if they do not establish as a local functional community. Whether they are established and propagating fungi or merely fungal spores (that in health are naturally transient due to effective mucociliary clearance) will influence the nature of their possible involvement. This includes as active pathogens (such as via the production of toxins), indirect/secondary roles (such as via influencing the bacterial microbiota which in turn may influence the inflammatory process), or simply as an antigen load that promotes or exacerbates the inflammatory process.

Summary and concluding remarks

This study represents the largest sequencing-based study on the CRS-associated mycobiota to date, and confirms the prominent place of *Malassezia* spp. within the upper respiratory tract. In addition to *Malassezia* spp., members of the genera *Davidiella, Aspergillus, Cladosporium, Cryptococcus*, Unclassified *Pleosporales* and *Tremellales, Curvularia, Eurotium, Candida*, and *Saccharomyces* were also common. With the exception of CRSwCF patients, distinctions in fungal communities were not evident between any of the patient groupings assessed, and few associations were observed between fungi and patterns in bacterial communities or local concentrations of inflammatory markers. These data lend further support to the hypothesis that fungal community types may have no direct causative involvement in idiopathic CRS, and further support the current recommendation against the use of antifungals in the treatment of these patients.

Season of sampling explained more of the variability in the data than any of the clinical parameters, suggesting that communities observed here might be strongly influenced by and/or reflect inhaled environmental fungal material rather than an

established functional community. Further research is warranted to differentiate between actual local functional communities and inhaled environmental fungal material. Additionally, studies assessing a broader array of inflammatory markers, together with well-designed in vitro studies, will ultimately further address whether ubiquitous fungi might still play an exacerbating role in immunologically compromised or aberrantly sensitive individuals.

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Authorship contribution

MH contributed to study design, sample processing, data analyses, and writing of the manuscript, under the supervision of MWT, KB, and RGD. MZ and RGD contributed to patient recruitment and sample collection. MWT provided laboratory space and materials. All authors contributed to critical review and editing of the manuscript.

Conflict of interest

The authors declare that no conflict of interest exists.

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Assoc. Prof. Michael W. Taylor
School of Biological Sciences
The University of Auckland
Auckland
New Zealand

Tel: +64 9 923 2280
Fax: +64 9 373 7416
E-mail: mw.taylor@auckland.ac.nz