Journal of Dental Research
1–9

DOI: 10.1177/0022034520915879
journals.sagepub.com/home/jdr

The Salivary Mycobiome Contains 2 Ecologically Distinct Mycotypes

B.Y. Hong[1,2*], A. Hoare[1,3*], A. Cardenas[1], A.K. Dupuy[4], L. Choquette[1,2], A.L. Salner[5], P.K. Schauer[5], U. Hegde[6], D.E. Peterson[1], A. Dongari-Bagtzoglou[1], L.D. Strausbaugh[2], and P.I. Diaz[1]

Abstract

A broad range of fungi has been detected in molecular surveys of the oral mycobiome. However, knowledge is still lacking on interindividual variability of these communities and the ecologic and clinical significance of oral fungal commensals. In this cross-sectional study, we use internal transcribed spacer 1 amplicon sequencing to evaluate the salivary mycobiome in 59 subjects, 36 of whom were scheduled to receive cancer chemotherapy. Analysis of the broad population structure of fungal communities in the whole cohort identified 2 well-demarcated genus-level community types (mycotypes), with *Candida* and *Malassezia* as the main taxa driving cluster partitioning. The *Candida* mycotype had lower diversity than the *Malassezia* mycotype and was positively correlated with cancer and steroid use in these subjects, smoking, caries, utilizing a removable prosthesis, and plaque index. Mycotypes were also associated with metabolically distinct bacteria indicative of divergent oral environments, with aciduric species enriched in the *Candida* mycotype and inflammophilic bacteria increased in the *Malassezia* mycotype. Similar to their fungal counterparts, coexisting bacterial communities associated with the *Candida* mycotype showed lower diversity than those associated with the *Malassezia* mycotype, suggesting that common environmental pressures affected bacteria and fungi. Mycotypes were also seen in an independent cohort of 24 subjects, in which cultivation revealed *Malassezia* as viable oral mycobiome members, although the low-abundance *Malassezia sympodialis* was the only *Malassezia* species recovered. There was a high degree of concordance between the molecular detection and cultivability of *Candida*, while cultivation showed low sensitivity for detection of the *Malassezia* mycotype. Overall, our work provides insights into the oral mycobiome landscape, revealing 2 community classes with apparently distinct ecologic constraints and specific associations with coexisting bacteria and clinical parameters. The utility of mycotypes as biomarkers for oral diseases warrants further study.

Keywords: oral mycobiome, microbiome community classes, fungal-bacterial interactions, microbial ecology, saliva, salivary diagnostics

Introduction

Fungi have been documented as oral inhabitants for many decades, with cultivation reports showing high prevalence of *Candida, Rhodotorula, Cryptococcus, Penicillium, Aspergillus*, and *Cladosporium* (Young et al. 1951; Monteiro-da-Silva et al. 2014). Recent molecular surveys expanded the range of oral fungi, with individual samples sometimes showing hundreds of taxa (Ghannoum et al. 2010; Abusleme et al. 2018). Fungi are ubiquitous in food and the environment; therefore, it is unclear which of these taxa constitute functional oral mycobiome components. *Malassezia*, a cultivable fungus, is detected in high proportions in sequenced oral samples (Dupuy et al. 2014; Abusleme et al. 2018), but its role in the oral ecosystem is unknown. Most research has focused on the role that species from a single genus, *Candida*, play in disease states such as oral thrush (candidiasis) or caries (Falsetta et al. 2014; Abusleme et al. 2018; Xiao et al. 2018; Bertolini et al. 2019), but a broader view of oral mycobiome communities in states of symbiosis and dysbiosis is lacking. The ecologic factors that shape the mycobiome and the role that commensal fungi, other than *Candida*, play in oral ecosystem perturbations are still unknown.

Enhanced understanding of the oral mycobiome could have clinical implications. A common approach to evaluate microbiome populations involves unsupervised classification methods, such as clustering, to find groups of individuals who share communities of similar composition. Different bacteriome

[1]Department of Oral Health and Diagnostic Sciences, School of Dental Medicine, UConn Health, Farmington, CT, USA
[2]The Jackson Laboratory for Genomic Medicine, Farmington, CT, USA
[3]Laboratorio de Microbiología Oral, Facultad de Odontología, Universidad de Chile, Santiago, Chile
[4]Department of Molecular and Cell Biology, University of Connecticut, Storrs, CT, USA
[5]Hartford Healthcare Cancer Institute at Hartford Hospital, Hartford, CT, USA
[6]Department of Medicine, UConn Health, Farmington, CT, USA
*Authors contributing equally to this article.

A supplemental appendix to this article is available online.

Corresponding Author:
P.I. Diaz, Department of Oral Health and Diagnostic Sciences, School of Dental Medicine, UConn Health. 263 Farmington Ave, Farmington, CT 06030-1710, USA.
Email: pdiaz@uchc.edu

community classes associated with certain host characteristics have been reported in fecal, vaginal, and skin samples (Arumugam et al. 2011; Wu et al. 2011; Ding and Schloss 2014; Zhou et al. 2014). Oral commensal bacterial populations appear highly homogeneous (Zhou et al. 2014), but population distributions of oral fungi have not been evaluated.

Accordingly, the current study evaluated the population structure of salivary mycobiome communities in 59 subjects, 36 of whom were undergoing cancer chemotherapy. Using 3 unsupervised classification methods, we analyzed the community-wide distribution of salivary fungi to explore stratification patterns of ecologic or clinical significance. We uncovered 2 fungal community types and evaluated their relationships with coexisting bacteria and host medical and oral health characteristics. Community types were confirmed in an independent cohort of 24 subjects in which we pursued the cultivability of fungi driving mycobiome partitioning.

Methods

Subject Recruitment and Collection of Demographic and Clinical Information

Cohort 1 was part of a larger study (Hong et al. 2019) and consisted of subjects diagnosed with nonoral solid tumors who were scheduled to receive chemotherapy and noncancer controls. The current study included 59 of these subjects (36 cancer and 23 noncancer), who were selected per the availability of complete demographic, clinical, internal transcribed spacer 1 (ITS-1), and 16S rRNA gene amplicon data from a baseline visit (Hong et al. 2019).

Subjects were enrolled according to a protocol approved by the Institutional Review Board at UConn Health (IE-11-037J-2). All participants provided written informed consent, and the study complied with STROBE guidelines. Inclusion and exclusion criteria were described by Hong et al. (2019) and appear in the Appendix Methods. Medical information was collected from questionnaires and medical charts. Subjects received an oral evaluation, including assessment of periodontal status via the Community Periodontal Index of Treatment Needs (Ainamo et al. 1982), presence and type of prosthetic restorations, presence of visible cavitated caries lesions according to the World Health Organization (WHO) criteria, and oral hygiene status per the plaque index (Silness and Loe 1964). An additional cohort (cohort 2; Institutional Review Board protocol 14-162-2), including 12 subjects receiving chemotherapy and 12 noncancer controls, was enrolled to evaluate the cultivability of *Candida* and *Malassezia*.

Salivary Microbiome Evaluation

Saliva was collected and DNA extracted and amplified as previously described (Hong et al. 2019). Samples from cohort 1 were evaluated by sequencing of the bacterial 16S rRNA gene V1–V2 hypervariable region and the fungal ITS-1 region. Only ITS-1 sequencing was performed for cohort 2. Sequences are available in the National Center for Biotechnology Information's Sequence Read Archive (PRJNA399163 and PRJNA593057).

Cultivation of Candida *and* Malassezia

Supplemented CHROMagar Malassezia was used for cultivation of *Candida* and *Malassezia*. Isolates were identified after Sanger ITS-1 sequencing.

Statistical Analyses

Three approaches were employed to evaluate community types: Dirichlet multinomial mixture (DMM) models, partitioning around medoids (PAM), and unsupervised hierarchical clustering. Linear discriminant analysis effect size (LEfSe; Segata et al. 2011) was used to evaluate differentially abundant taxa. Correlations were evaluated via Spearman rank tests and stepwise logistic regression. Benjamini-Hochberg false discovery rate was applied for multiple comparison adjustments. Additional details appear in the Appendix Methods.

Results

Identification of 2 Discrete Fungal Community Types (Mycotypes) in Saliva

Cohort 1 characteristics are summarized in the Table, and fungal taxa detected are shown in Appendix Table 1. The existence of different fungal community classes in the whole cohort was explored through unsupervised methods. An analysis with DMM models suggested that the data set contained 2 genus-level clusters (Appendix Fig. 1). Visualization of these clusters via nonmetric multidimensional scaling ordination showed 1 highly cohesive cluster and 1 with greater sample spread (Fig. 1A). Unsupervised hierarchical clustering revealed 2 clusters, one more homogeneous than the other, and complete agreement with DMM in assignment of samples to clusters (Fig. 1B). Evaluation of clusters with PAM indicated 2 main clusters, although a small number of samples showed potential for forming separate groups (Appendix Fig. 2). The silhouette width, a measure of cluster cohesiveness and separation, was 0.57 for 1 PAM cluster and 0.64 for the second cluster, with an average silhouette width of 0.60, which is considered a moderately strong value (Wu et al. 2011; Koren et al. 2013). Overall, these results indicated the existence in saliva of 2 distinct fungal community types, which we refer to as mycotypes.

The composition of mycotypes was then examined. As shown in Figure 1B, 1 mycotype was characterized by high *Malassezia* proportions (1.5% to 98%), while *Candida* was the dominant taxon in the second mycotype (59% to 99%), suggesting that these genera are the main drivers of mycotype partitioning. The *Malassezia*-enriched mycotype was more diverse than *Candida*-enriched communities (Fig. 1C). Consistent with these findings, LEfSe evaluation of taxa that differed between mycotypes showed that *Malassezia* and

Table. Demographic and Clinical Characteristics of Study Participants Included in Cohort 1.

Variable	Cancer ($n = 36$)	Noncancer ($n = 23$)	*P* Value
Age	57.64 ± 12.05	48.65 ± 14.42	**0.0171**[a]
Male	50.0	26.1	0.103[b]
White	91.7	100.0	0.274[b]
Current smoker[c]	16.7	10.5	0.700[b]
Proton pump inhibitor use	25.0	8.7	0.174[b]
Inhaler steroid use	8.3	0.0	0.274[b]
Steroid premedication	16.7	0.0	0.072[b]
Inhaler steroid or premedication	25	0.0	**0.009**[b]
No. of teeth	26 (0 to 32) [21 to 28]	28 (0 to 32) [27 to 28]	**0.007**[d]
Prosthetic teeth or oral appliance	80.6	69.6	0.363[b]
No. of teeth replaced by prostheses	2 (0 to 32) [1 to 5]	2 (0 to 28) [0 to 7]	0.671[d]
Removable prosthesis	11.1	8.7	>0.999[b]
Visible cavitated caries lesions	33.3	21.7	0.391[b]
No. of teeth with visible caries lesions	0 (0 to 11) [0 to 1]	0 (0 to 2) [0 to 0]	0.202[d]
Plaque index	1.05 (0.0 to 2.5) [0.6 to 1.5]	0.60 (0.1 to 1.3) [0.5 to 1.0]	**0.007**[d]
At least 1 periodontal pocket >5.5 mm	5.6	4.5	>0.999[b]
Salivary flow rate, mL/min	0.38 (0.05 to 1.46) [0.29 to 0.54]	0.45 (0.07 to 0.95) [0.23 to 0.59]	0.810[d]
Peripheral absolute neutrophil count, ×1,000/mm^3 (blood)	6.29 (2.23 to 18.41) [3.56 to 11.99]	3.25 (1.87 to 5.35) [2.88 to 4.02]	<**0.001**[d]
Cancer diagnosis		NA	NA
Squamous cell carcinoma	44.4		
Breast cancer	30.6		
Adenocarcinoma	19.4		
Other	5.6		

Values are presented as follows: for normally distributed continuous variables, mean ± SD; for nonnormally distributed continuous variables, median (range) [interquartile range]. Statistical tests for continuous data were applied according to data distribution. For nominal variables, data are presented as percentage of subjects positive. Bold indicates $P < 0.05$.
NA, not applicable.
[a]Independent sample *t* test.
[b]Fisher's exact test.
[c]Smoking descriptive statistics for the noncancer group are based on $n = 19$, as there were 3 cases with missing smoking data.
[d]Mann-Whitney *U* test.

several other taxa were enriched in 1 cluster, while only *Candida* was enriched in the second, less diverse mycotype (Fig. 1D).

Distributions of *Malassezia* and *Candida* species were then evaluated. Figure 1E shows that the most abundant *Malassezia* species and the most discriminative between mycotypes were *Malassezia restricta* and *Malassezia globosa*, while *Candida albicans* was the most abundant and differentially enriched species in the *Candida* mycotype (Fig. 1F). Mycotype clustering was due to differences in relative abundances of *Candida* and *Malassezia* rather than in presence/absence. As seen in Figure 1E and F, some *Candida* mycotype subjects had low levels of *Malassezia* and vice versa.

Associations between Mycotypes and Host Characteristics

Correlations of mycotypes with demographic, medical, and oral health characteristics of subjects were then evaluated. Although both mycotypes occurred in subjects with cancer and controls, the *Candida* mycotype was more frequent in the cancer group ($P = 0.015$; Fig. 2A). Variables that differed between groups (Table) and could explain this difference were then examined. Age and recent history of chemotherapy were not correlated with mycotypes. However, as seen in Figure 2B, receiving steroid premedication (administered to certain subjects with cancer 1 to 5 d prior to sampling) and having higher peripheral neutrophil counts as a consequence of steroid intake (Mishler and Emerson 1977) were positively correlated with the *Candida* mycotype. Other variables that possibly explained the higher *Candida* mycotype frequency in subjects with cancer were inhaler steroid use and plaque index, which showed higher values in subjects with cancer (Table), and were positively correlated with the *Candida* mycotype (Fig. 2B). In a partial correlation analysis, the association of cancer and mycotypes became nonsignificant ($P = 0.098$) after controlling for steroid intake (via any route) but remained significant when adjusting for the plaque index ($P = 0.049$), suggesting that steroid use was the main factor explaining the higher prevalence of the *Candida* mycotype in cancer subjects. Variables that did not differ between cancer and noncancer groups but were associated with mycotypes included smoking, utilizing a removable prosthesis, and number of teeth with visible caries lesions, which showed a positive correlation with the *Candida*

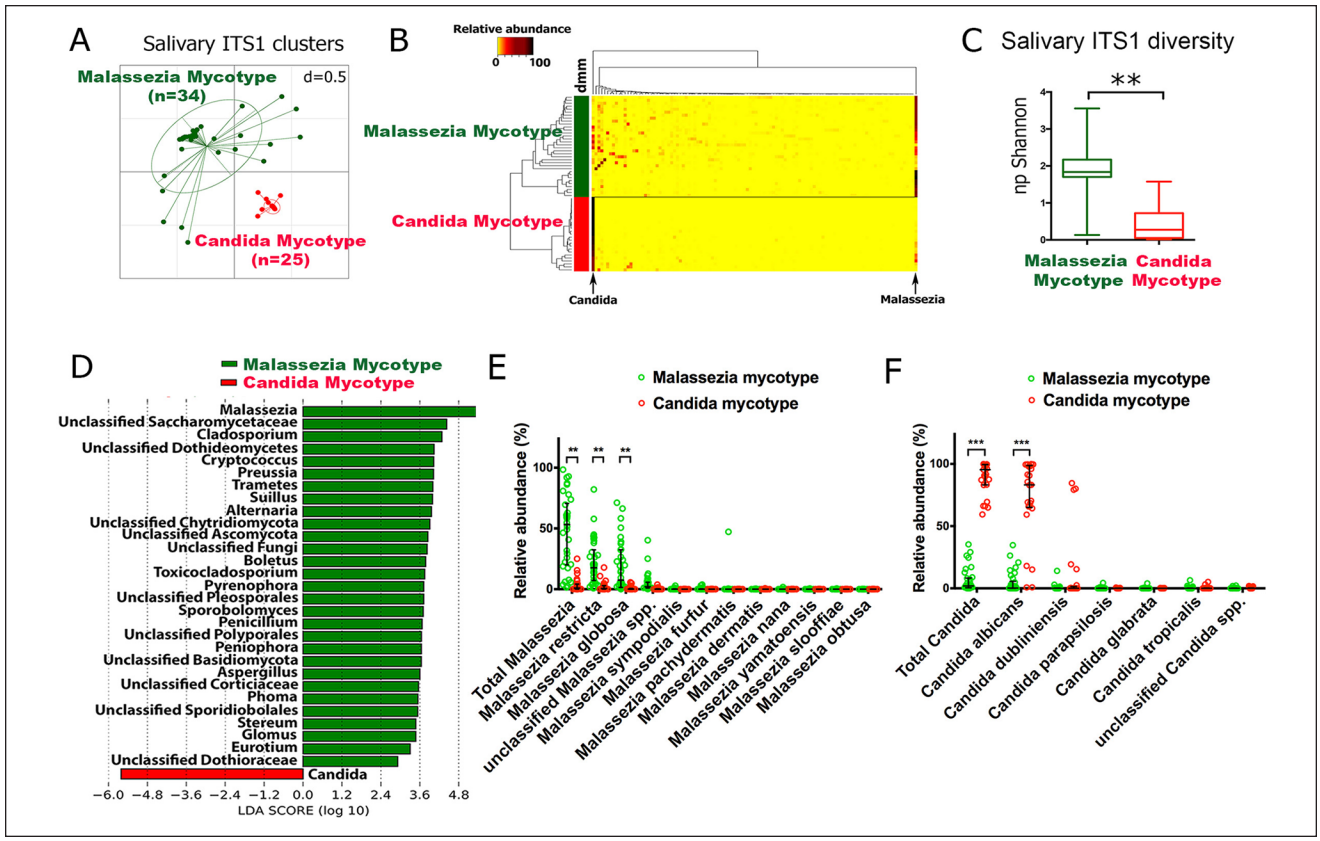

Figure 1. Characterization of salivary mycotypes in 59 subjects (cohort 1). (**A**) Nonmetric multidimensional scaling plot based on theta$_{YC}$ distances. Samples were colored according to Dirichlet multinomial mixture models analysis, which indicated that the data contained 2 clusters. (**B**) Unsupervised hierarchical clustering analysis also revealed 2 main clusters, as indicated in the dendrogram on the left side. The cluster to which each sample was assigned after Dirichlet multinomial mixture models analysis is indicated by the column bar. Genus-level relative abundances are shown in the heat map. (**C**) Differences in alpha diversity of the *Candida* and *Malassezia* mycotypes. Values are presented as median, interquartile range, and range. $^{**}P < 0.001$ as determined by a Mann-Whitney rank test. (**D**) Linear discriminant analysis effect size evaluation of differences in relative abundances of fungal genera between mycotypes. Relative abundances of species of (**E**) *Malassezia* and (**F**) *Candida* according to mycotypes. Individual data points, median, and interquartile ranges are shown. $^{**}P < 0.001$ and $^{***}P < 0.0001$ as determined by Mann-Whitney rank tests.

mycotype, while number of teeth was negatively correlated (Fig. 2B). A stepwise logistic regression model was then constructed incorporating cancer, steroids (via any route), number of teeth, plaque index, removable prosthesis, caries, and smoking as predictors and mycotype as outcome. Only steroid and plaque index were retained in the model, with steroid showing an odds ratio of 10.23 (95% CI, 0.98 to 106.28) and borderline significance ($P = 0.05$) and with plaque index showing an odds ratio of 6.23 (95% CI, 1.15 to 33.65) and $P = 0.033$ (Appendix Fig. 3). The prediction accuracy of this model for the *Candida* mycotype was 78.9%.

Correlations of Salivary Candida *and* Malassezia *Proportions with Host Characteristics*

We next evaluated correlations of *Malassezia* and *Candida* individual proportions and clinical characteristics. *Candida* was higher in subjects with cancer (Appendix Fig. 4) and positively correlated with steroids, smoking, removable prosthesis, presence of caries, number of teeth with caries lesions, and plaque index (Fig. 2C). At a species level, *C. albicans* positively correlated with steroids, smoking, removable prosthesis, and proton pump inhibitor use. Confirming their exclusive relationship, *Malassezia*, including *M. restricta* and *M. globosa* individual proportions, negatively correlated with the same variables. *Malassezia* proportions were also positively correlated with the presence of at least 1 periodontal pocket >5.5 mm, although this finding should be interpreted with caution as only 3 individuals had this clinical characteristic.

Bacterial Communities Coexisting with Mycotypes

DMM analysis indicated that salivary bacterial communities contained 2 weakly separated clusters (Appendix Fig. 1, Fig. 3A). PAM analysis showed a low silhouette width (0.13 for $k = 2$), confirming the homogeneity of salivary bacterial communities (Appendix Fig. 5). However, if bacterial communities associated with each mycotype were contrasted, communities associated with the *Malassezia* mycotype were more diverse than those associated with the *Candida* mycotype (Fig. 3B). These results paralleled the fungal diversity differences seen

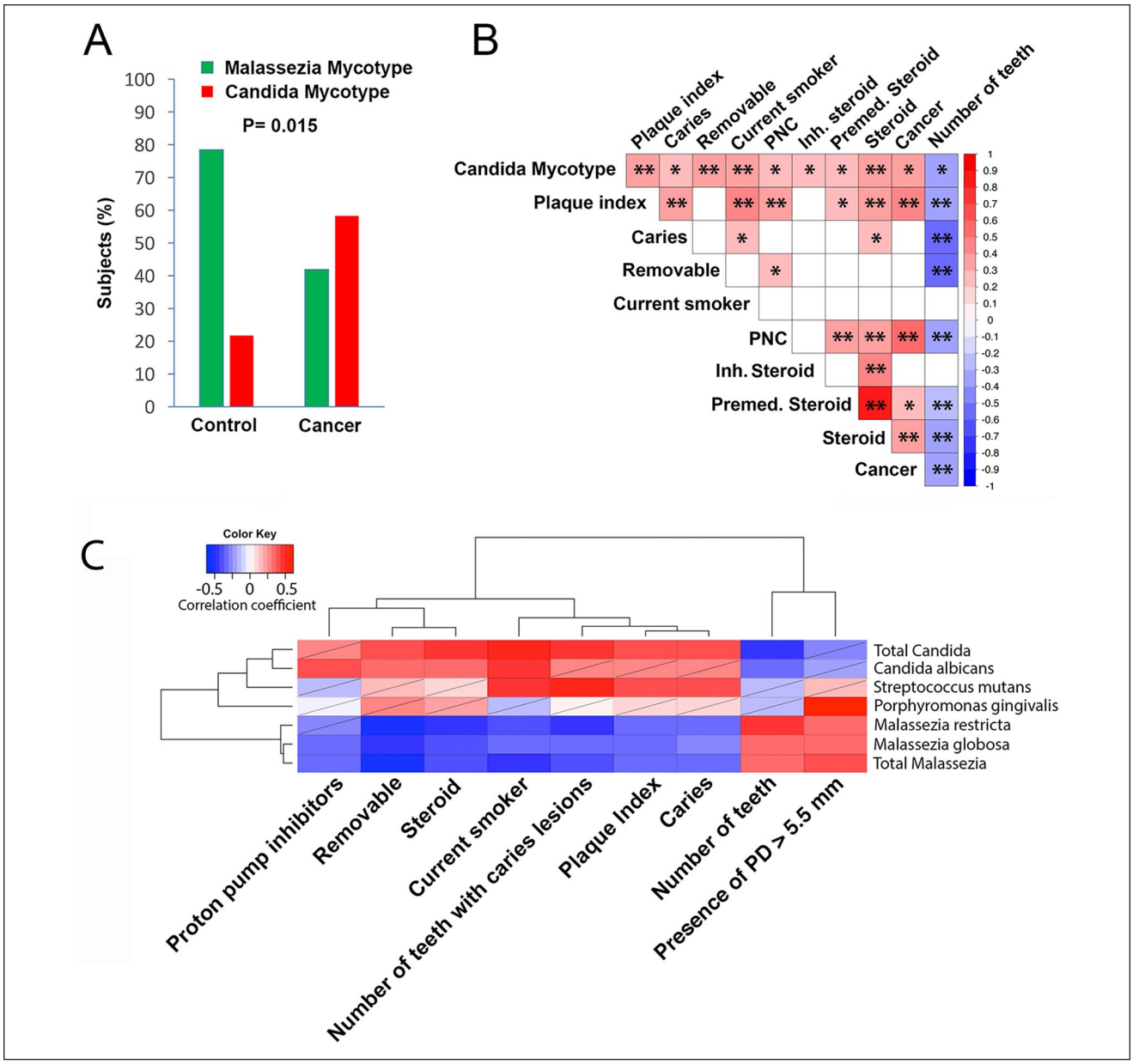

Figure 2. Association of mycotypes with host characteristics. (**A**) Prevalence of mycotypes according to cancer status. Bars show percentage of subjects in which the specific mycotype was detected. Differences evaluated via chi-square. (**B**) Correlogram depicts correlations (Spearman) between mycotypes (0, *Malassezia*; 1, *Candida*) and host parameters. Only those host variables that showed a significant correlation with mycotypes after multiple-test adjustment are shown. Colors indicate correlation coefficients. *$P < 0.01$ and **$P < 0.001$. Inh. Steroid, use of corticosteroids as an inhaler or nasal spray; PNC, peripheral neutrophil counts; Premed. Steroid, patients who received intravenous corticosteroids days prior to sampling. (**C**) Correlations (Spearman) between relative abundances of *Candida* and *Malassezia* (genus-level totals and individual species abundances) and host characteristics. Only taxa that showed significant correlations are depicted. Colors indicate correlation coefficients, and diagonal crossed lines indicate nonsignificant correlations (after multiple-comparison adjustment). As a reference, correlations of the salivary abundances of the caries-associated bacterium *Streptococcus mutans* and the periodontitis-associated bacterium *Porphyromonas gingivalis* are included. PD, periodontal probing depth.

between mycotypes (Fig. 1C), suggesting that a common environmental pressure affected bacteria and fungi. LEfSe showed that, indeed, metabolically distinct bacteria were differentially enriched in mycotypes, with aciduric bacterial species, such as *Lactobacillus salivarius, Lactobacillus ultunensis*, and *Propionibacterium acidifaciens*, enriched in subjects harboring the *Candida* mycotype, while for the *Malassezia* mycotype, subjects had higher proportions of anaerobic species of *Fusobacterium, Porphyromonas, Prevotella, Treponema*, and *Leptotrichia*, among others. These results were confirmed in a correlation analysis (Fig. 3D), which showed that *Candida* proportions positively correlated with bacteria known to have high acid tolerance, such as lactobacilli and *Veillonella* spp., while *Malassezia* proportions positively correlated with bacterial anaerobes typically associated with gingival inflammation, that rely on amino acid catabolism and prefer more basic environmental pH

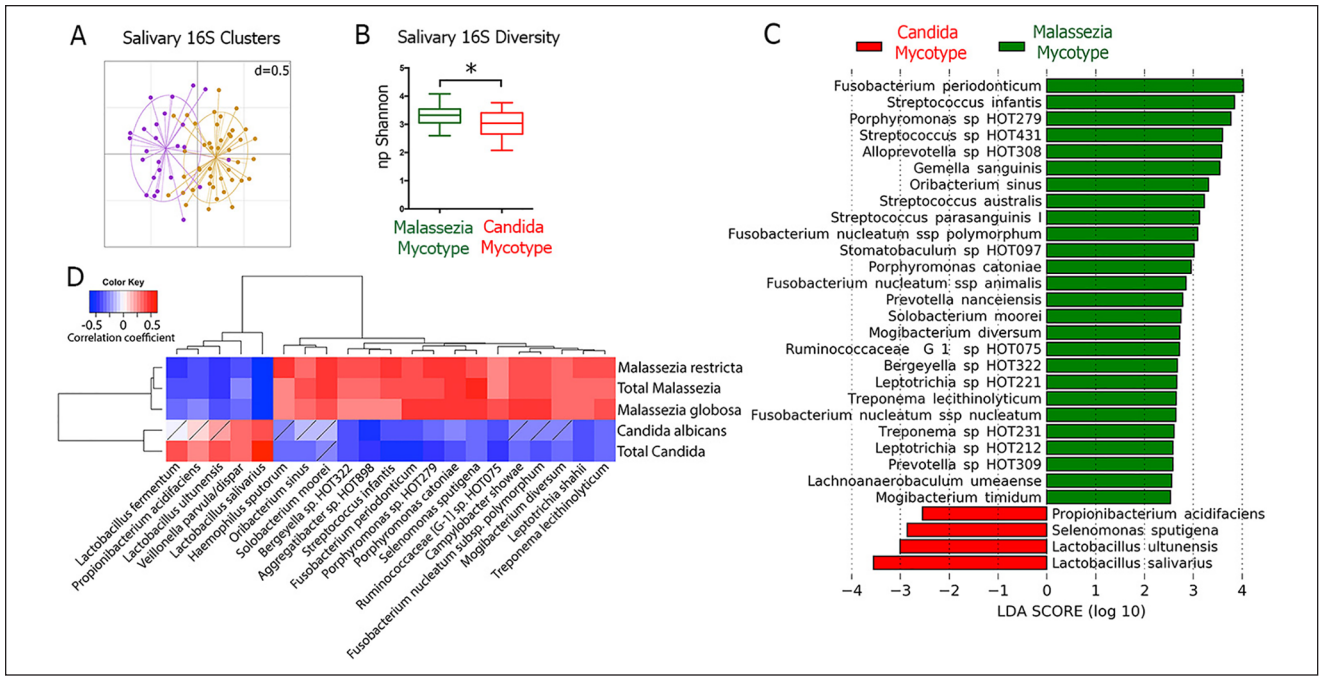

Figure 3. Bacterial communities associated with mycotypes. (**A**) Clusters contained in the species-level bacterial community data. Graph is a nonmetric multidimensional scaling ordination plot based on $theta_{YC}$ distances with samples colored according to Dirichlet multinomial mixture models analysis. (**B**) Differences in diversity of bacterial communities associated with mycotypes. Values are presented as median, interquartile range, and range. *$P < 0.01$ as determined by Mann-Whitney rank test. (**C**) Linear discriminant analysis effect size evaluation of differences in relative abundances of bacterial species coexisting with each mycotype. (**D**) Correlations (Spearman) between relative abundances of *Candida* and *Malassezia* (genus-level totals and individual species abundances) and bacterial relative abundances. Only taxa that showed significant correlations with *Candida* and *Malassezia* are depicted. Colors indicate correlation coefficients, and diagonal crossed lines indicate nonsignificant correlations (after multiple-comparison adjustment).

(e.g., *Porphyromonas* spp., *Fusobacterium* spp., *Treponema lecithinolyticum, Selenomonas sputigena*, and *Leptotrichia shahii*, among others). Altogether, our data show that although salivary bacterial communities do not contain discrete clusters, specific bacterial species with distinct metabolic requirements are enriched in each mycotype.

Cultivability of Mycotypes

The observed mycotypes and their cultivability were evaluated in a second cohort (cohort 2; Appendix Table 2). All but 1 subject in cohort 2 yielded ITS-1 amplicons. Mycobiome analysis showed 2 clusters with distinct levels of *Candida* and *Malassezia* (Fig. 4A). Similar to cohort 1, *C. albicans, M. restricta*, and *M. globosa* were the most abundant species (Appendix Table 3). Molecular mycotypes did not differ in frequency between control and cancer groups (chi-square, $P = 0.795$; Fig. 4A). However, as observed in cohort 1, the *Candida* mycotype positively correlated with number of teeth with visible caries lesions ($r = 0.556$, $P = 0.006$).

Although *Malassezia* have been observed in oral samples by molecular methods (Dupuy et al. 2014; Abusleme et al. 2018), to our knowledge, oral *Malassezia* have not been cultivated. Therefore, the cultivability of *Malassezia*, as compared with that of *Candida*, was evaluated with a microbiological medium that allows growth of both genera. Figure 4B shows *Malassezia* and *Candida* load in subjects harboring each mycotype. *Candida* was principally cultivated from the *Candida* mycotype group, while 3 individuals—2 classified in the *Malassezia* mycotype group and 1 undetermined due to a negative polymerase chain reaction result—yielded *Malassezia* colonies. Figure 4C demonstrates concordance between the molecular *Candida* mycotype classification and cultivability of *Candida* (sensitivity, 1; specificity, 0.82). However, cultivation did not allow detection of subjects harboring the *Malassezia* mycotype (sensitivity, 0.14; specificity, 1).

All fungi recovered by cultivation are shown in Figure 4D and Appendix Table 4. Only 1 species of *Malassezia, M. sympodialis*, was recovered on agar. Colonies of *Pichia, Exophiala, Rhodotorula, Cryptococcus*, and *Clavispora* were also observed. Colony morphologies of these fungi on CHROMagar Malassezia are shown in Appendix Figure 6.

Confirming the association of *Candida* and caries, *Candida* cultivable load showed a strong positive correlation with number of teeth with caries ($r = 0.616$, $P = 0.001$; Fig. 4E) and caries presence ($r = 0.458$, $P = 0.024$). At the species level, *C. albicans* and *Candida parapsilosis* cultivable load positively correlated with number of teeth with caries ($r = 0.446$, $P = 0.029$; $r = 0.431$, $P = 0.035$).

Discussion

Despite considerable intersubject variability in the human microbiome composition, different community types have been

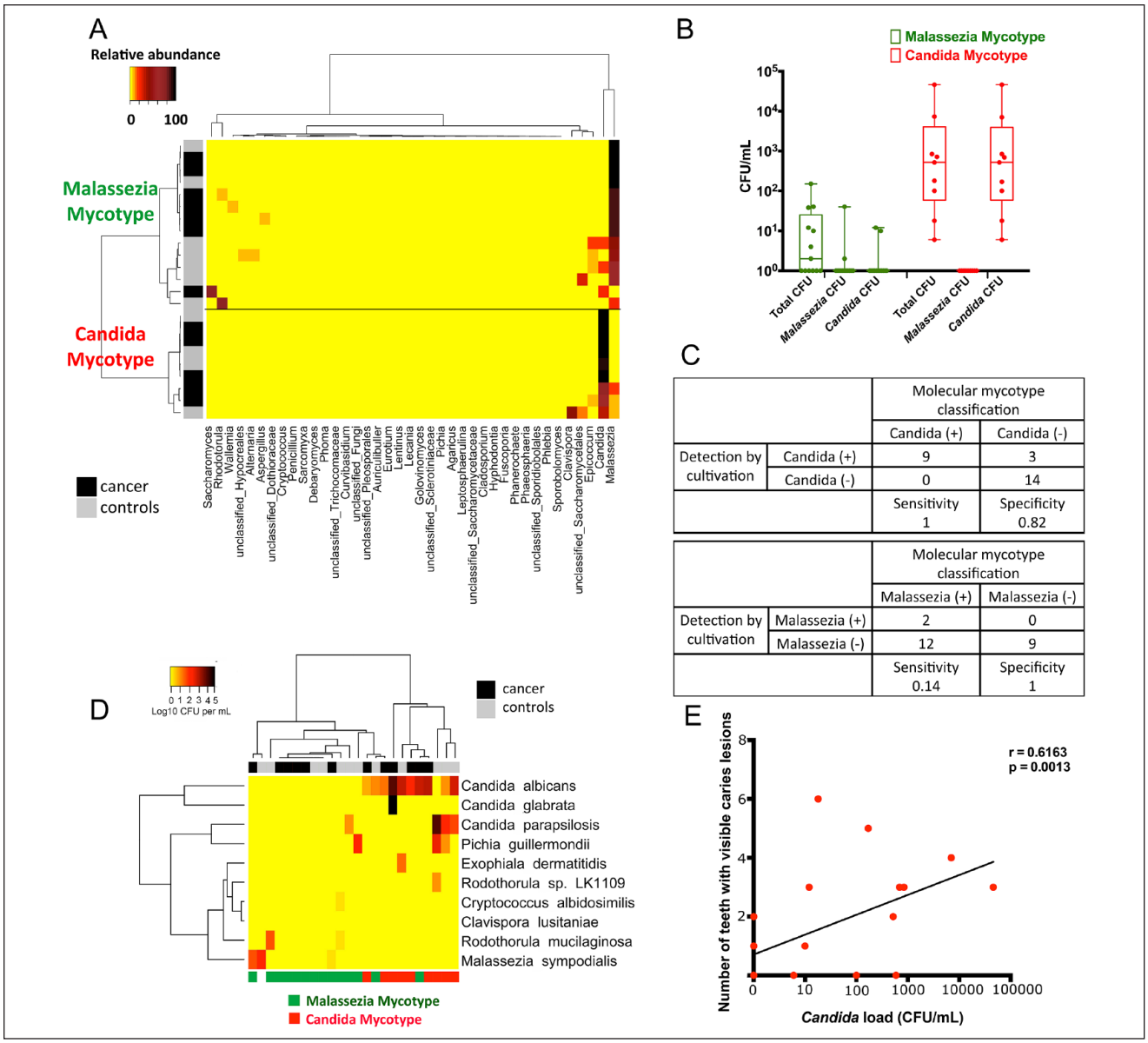

Figure 4. Cultivability of *Candida* and *Malassezia* in relation to mycotypes. A second cohort of 24 subjects was enrolled, and their salivary mycobiomes were characterized by ITS-1 (internal transcribed spacer 1) sequencing and cultivation. (**A**) Unsupervised hierarchical clusters and heat map show genus-level relative abundances of salivary fungi. Notice 2 clusters with *Malassezia* and *Candida* as the main distinctive taxa. (**B**) Total fungal, *Candida*, and *Malassezia* salivary cultivable load according to molecular mycotype classification. (**C**) Ability of cultivation to detect molecular mycotypes. (**D**) Heat map shows loads of fungi detected via cultivation in relation to cancer and mycotype groupings. (**E**) Correlation (Spearman) between number of teeth with visible cavitated caries lesions and salivary cultivable load of *Candida*.

described (Arumugam et al. 2011; Gajer et al. 2012; Zhou et al. 2014). Compositional analyses of fecal communities have revealed 3 community types, known as enterotypes (Arumugam et al. 2011), although others have questioned the status of enterotypes as discrete clusters (Koren et al. 2013; Knights et al. 2014). The oral mycotypes found in this study represent clusters with a degree of separation much higher than that of enterotypes. By comparison, the silhouette width for enterotypes has been reported at ~0.2 (Zhou et al. 2014; Costea et al. 2018), while that for salivary mycotypes was 0.6. Moreover, different partitioning methods agreed in the optimal number of clusters and sample assignment, and an independently enrolled cohort sequenced with a slightly different approach (different primers and sequencing platform) showed similar fungal distribution patterns. Our data strongly suggest the existence of 2 discrete salivary fungal communities. Due to the high dominance of *Candida*, the *Candida* mycotype was more cohesive and less diverse than the *Malassezia* mycotype. When the LEfSe alpha value (Fig. 1D) was relaxed to 0.1, only 1 other taxon, *Saccharomyces*, appeared enriched in the *Candida* mycotype. In contrast, the *Malassezia* mycotype was more diverse, showing *Malassezia* as the dominant community member but

accompanied by other co-occurring fungi (Fig. 1B, D). Analysis of a larger and more diverse population of subjects could reveal whether this binary partitioning and the specific community structures are maintained. The temporal stability of mycotypes within subjects also warrants further investigation.

Mycotype associations with host characteristics were explored to better understand ecologic and host constraints determining mycotypes. Our cohorts included subjects undergoing cancer chemotherapy, since we have an ongoing interest in predisposing factors leading to oral complications of cancer treatment (Diaz et al. 2019; Hong et al. 2019). In cohort 1, the *Candida* mycotype was more prevalent in subjects with cancer and correlated with their intake of corticosteroids, which have been associated with oral *Candida* colonization (Pereira Tdos et al. 2014). Several lines of evidence also pointed to the oral environment as a selective constrain for mycotypes. The relationship of plaque index, the only variable retained after logistic regression, and mycotypes may result from coadhesive interactions between *Candida* and dental plaque bacteria, which may facilitate the retention of the fungus, while *Candida* promotes greater bacterial biomass accumulation (Gregoire et al. 2011; Xu et al. 2017). It should be noted, however, that the number of covariates included in the logistic regression model was large given the sample size and some of the variables included showed collinearity. Therefore, a larger study is needed to validate the independent relationships between oral characteristics and mycotypes. Apart from dental plaque levels, caries also showed a relationship with mycotypes. *Candida* prefers glucose or lactate as carbon sources and has been shown to thrive in carbohydrate-rich polymicrobial communities (Chiew 1989; Ene et al. 2012; Koopman et al. 2015). Moreover, in agreement with our findings, levels of *Candida* and saccharolytic acidogenic bacteria have been shown to correlate (Kraneveld et al. 2012). Therefore, it appears that the *Candida* mycotype is associated with aciduric oral conditions. In contrast, *Malassezia* spp. are incapable of carbohydrate fermentation and depend on lipids (Senczek et al. 1999; Wu et al. 2015). Saliva, gingival crevicular fluid, and the host diet could represent lipid sources for *Malassezia*, which possess a diverse array of secretory lipases (Larsson et al. 1996; Wu et al. 2015). Moreover, the *Malassezia* mycotype was associated with bacteria that rely on amino acid fermentation and prefer slightly basic conditions for growth, again suggesting oral pH as a mycotype determinant.

The described mycotypes may have diagnostic utility. We recently reported a multivariate model able to predict oral candidiasis during chemotherapy (Diaz et al. 2019). Since *Candida* and *Malassezia* salivary proportions were significant predictors in the reported model, mycotypes could represent another discriminatory parameter. Mycotypes may be useful as screening tools for tooth-associated diseases, but further research is needed. Due to the site specificity of caries, the use of saliva as a diagnostic tool has been questioned (Mira 2018). However, our data show a correlation between salivary proportions of *S. mutans* and caries. The salivary *Candida* mycotype, *Candida* proportions, and cultivable *Candida* load positively correlated with caries. Additional research is required to assess the utility of mycotypes as caries biomarkers, in particular with more sensitive tools to document initial disease stages. The relationship of *Malassezia* and periodontitis needs further study in a larger cohort. It would also be important to discern whether *Candida* and *Malassezia* serve only as diagnostic indicators or if they participate in dysbiotic events at diseased sites.

We investigated whether *Malassezia* are viable oral mycobiome members. Using a lipid-containing medium, we cultivated *M. sympodialis*, a species present at <1% abundance according to ITS-1 sequencing in subjects that yielded colonies. The abundant *M. restricta* and *M. globosa* were not recovered, although skin derived–type strains of these species grew under the cultivation conditions used. This suggests that *Malassezia* are viable oral mycobiome components, but the cultivation requirements of oral *M. restricta* and *M. globosa* need additional study. Since the most abundant *Malassezia* species present were not recovered, it was not possible to estimate the total fungal load. Total fungi present in mycotypes may differ, but more work is needed to develop appropriate methods to measure load. We decided against employing universal ITS primers and quantitative polymerase chain reaction to estimate load, since the high inter- and intraspecies variability in the copy number of the rRNA gene cluster region among fungi is likely to bias load estimates (Rustchenko et al. 1993; Diaz et al. 2017; Lofgren et al. 2019). Such copy number variability could also bias relative abundance estimates, but unfortunately, a database does not yet exist that has information on the number of rRNA gene cluster copies in the genomes of oral mycobiome components and that could be used to normalize abundance estimates. A critical appraisal of which ITS-1 reads represent metabolically active fungi is also needed. For instance, *Boletus* are edible mushrooms. *Saccharomyces*, certain *Aspergillus, Fusarium*, and *Phoma* are associated with food. *Alternaria* and *Cladosporium* may represent inhaled spores from indoor environments. It is likely that only yeasts, such as *Candida, Pichia, Clavispora, Malassezia*, and *Rhodotorula*, are functional, metabolically active mycobiome components.

In summary, our work revealed 2 salivary fungal community types associated with specific host characteristics and metabolically distinct bacteria. Since mycotypes appear to be associated with distinct ecologic conditions, which in turn are related to specific oral diseases (Marsh 1994), the mycotype classification could represent a biomarker and warrants further evaluation in the context of salivary diagnostics.

Author Contributions

B.Y. Hong, A. Hoare, A. Cardenas, contributed to data acquisition and analysis, critically revised the manuscript; A.K. Dupuy, L. Choquette, A.L. Salner, P.K. Schauer, U. Hegde, contributed to data acquisition, critically revised the manuscript; D.E. Peterson, contributed to conception, design, and data acquisition, critically revised the manuscript; A. Dongari-Bagtzoglou, contributed to conception, design, and data interpretation, critically revised the manuscript; L.D. Strausbaugh, contributed to conception, design,

data acquisition, and interpretation, critically revised the manuscript; P.I. Diaz, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This work was supported by grant R01DE021578 from the National Institute of Dental and Craniofacial Research, National Institutes of Health. We also acknowledge the support of pilot grant M01RR006192 from the UConn Health General Clinical Research Center. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

ORCID iDs

A.K. Dupuy https://orcid.org/0000-0001-5626-6391
P.I. Diaz https://orcid.org/0000-0002-9501-1145

References

Abusleme L, Diaz PI, Freeman AF, Greenwell-Wild T, Brenchley L, Desai JV, Ng WI, Holland SM, Lionakis MS, Segre JA, et al. 2018. Human defects in STAT3 promote oral mucosal fungal and bacterial dysbiosis. JCI Insight. 3(17):122061.

Ainamo J, Barmes D, Beagrie G, Cutress T, Martin J, Sardo-Infirri J. 1982. Development of the World Health Organization (WHO) Community Periodontal Index of Treatment Needs (CPITN). Int Dent J. 32(3):281–291.

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, et al. 2011. Enterotypes of the human gut microbiome. Nature. 473(7346):174–180.

Bertolini M, Ranjan A, Thompson A, Diaz PI, Sobue T, Maas K, Dongari-Bagtzoglou A. 2019. *Candida albicans* induces mucosal bacterial dysbiosis that promotes invasive infection. PLoS Pathog. 15(4):e1007717.

Chiew YY. 1989. The dynamics of carbohydrate metabolism in *Candida albicans*. Exp Mycol. 13(1):49–60.

Costea PI, Hildebrand F, Arumugam M, Backhed F, Blaser MJ, Bushman FD, de Vos WM, Ehrlich SD, Fraser CM, Hattori M, et al. 2018. Enterotypes in the landscape of gut microbial community composition. Nat Microbiol. 3(1):8–16.

Diaz PI, Hong BY, Dupuy AK, Choquette L, Thompson A, Salner AL, Schauer PK, Hegde U, Burleson JA, Strausbaugh LD, et al. 2019. Integrated analysis of clinical and microbiome risk factors associated with the development of oral candidiasis during cancer chemotherapy. J Fungi (Basel). 5(2):E49.

Diaz PI, Hong BY, Dupuy AK, Strausbaugh LD. 2017. Mining the oral mycobiome: methods, components, and meaning. Virulence. 8(3):313–323.

Ding T, Schloss PD. 2014. Dynamics and associations of microbial community types across the human body. Nature. 509(7500):357–360.

Dupuy AK, David MS, Li L, Heider TN, Peterson JD, Montano EA, Dongari-Bagtzoglou A, Diaz PI, Strausbaugh LD. 2014. Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of *Malassezia* as a prominent commensal. PLoS One. 9(3):e90899.

Ene IV, Adya AK, Wehmeier S, Brand AC, MacCallum DM, Gow NA, Brown AJ. 2012. Host carbon sources modulate cell wall architecture, drug resistance and virulence in a fungal pathogen. Cell Microbiol. 14(9):1319–1335.

Falsetta ML, Klein MI, Colonne PM, Scott-Anne K, Gregoire S, Pai CH, Gonzalez M, Watson G, Krysan DJ, Bowen WH, et al. 2014. Symbiotic relationship between *Streptococcus mutans* and *Candida albicans* synergizes the virulence of plaque-biofilms in vivo. Infect Immun. 82(5):1968–1981.

Gajer P, Brotman RM, Bai G, Sakamoto J, Schutte UM, Zhong X, Koenig SS, Fu L, Ma ZS, Zhou X, et al. 2012. Temporal dynamics of the human vaginal microbiota. Sci Trans Med. 4(132):132ra152.

Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, Gillevet PM. 2010. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog. 6(1):e1000713.

Gregoire S, Xiao J, Silva BB, Gonzalez I, Agidi PS, Klein MI, Ambatipudi KS, Rosalen PL, Bauserman R, Waugh RE, et al. 2011. Role of glucosyltransferase b in interactions of *Candida albicans* with *Streptococcus mutans* and with an experimental pellicle on hydroxyapatite surfaces. Appl Environ Microbiol. 77(18):6357–6367.

Hong BY, Sobue T, Choquette L, Dupuy AK, Thompson A, Burleson JA, Salner AL, Schauer PK, Joshi P, Fox E, et al. 2019. Chemotherapy-induced oral mucositis is associated with detrimental bacterial dysbiosis. Microbiome. 7(1):66.

Knights D, Ward TL, McKinlay CE, Miller H, Gonzalez A, McDonald D, Knight R. 2014. Rethinking "enterotypes." Cell Host Microb. 16(4):433–437.

Koopman JE, Roling WF, Buijs MJ, Sissons CH, ten Cate JM, Keijser BJ, Crielaard W, Zaura E. 2015. Stability and resilience of oral microcosms toward acidification and *Candida* outgrowth by arginine supplementation. Microb Ecol. 69(2):422–433.

Koren O, Knights D, Gonzalez A, Waldron L, Segata N, Knight R, Huttenhower C, Ley RE. 2013. A guide to enterotypes across the human body: meta-analysis of microbial community structures in human microbiome datasets. PLoS Comput Biol. 9(1):e1002863.

Kraneveld EA, Buijs MJ, Bonder MJ, Visser M, Keijser BJ, Crielaard W, Zaura E. 2012. The relation between oral *Candida* load and bacterial microbiome profiles in Dutch older adults. PLoS One. 7(8):e42770.

Larsson B, Olivecrona G, Ericson T. 1996. Lipids in human saliva. Arch Oral Biol. 41(1):105–110.

Lofgren LA, Uehling JK, Branco S, Bruns TD, Martin F, Kennedy PG. 2019. Genome-based estimates of fungal rDNA copy number variation across phylogenetic scales and ecological lifestyles. Mol Ecol. 28(4):721–730.

Marsh PD. 1994. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res. 8(2):263–271.

Mira A. 2018. Oral microbiome studies: potential diagnostic and therapeutic implications. Adv Dent Res. 29(1):71–77.

Mishler JM, Emerson PM. 1977. Development of neutrophilia by serially increasing doses of dexamethasone. Br J Haematol. 36(2):249–257.

Monteiro-da-Silva F, Araujo R, Sampaio-Maia B. 2014. Interindividual variability and intraindividual stability of oral fungal microbiota over time. Med Mycol. 52(5):498–505.

Pereira Tdos S, Silva Alves Jde F, Gomes CC, Rocha do Nascimento A, Stoianoff MA, Gomez RS. 2014. Kinetics of oral colonization by *Candida* spp. during topical corticotherapy for oral lichen planus. J Oral Pathol Med. 43(8):570–575.

Rustchenko EP, Curran TM, Sherman F. 1993. Variations in the number of ribosomal DNA units in morphological mutants and normal strains of *Candida albicans* and in normal strains of *Saccharomyces cerevisiae*. J Bacteriol. 175(22):7189–7199.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12(6):R60.

Senczek D, Siesenop U, Bohm KH. 1999. Characterization of *Malassezia* species by means of phenotypic characteristics and detection of electrophoretic karyotypes by pulsed-field gel electrophoresis (PFGE). Mycoses. 42(5–6):409–414.

Silness J, Loe H. 1964. Periodontal disease in pregnancy: II. Correlation between oral hygiene and periodontal condtion. Acta Odontol Scand. 22:121–135.

Wu G, Zhao H, Li C, Rajapakse MP, Wong WC, Xu J, Saunders CW, Reeder NL, Reilman RA, Scheynius A, et al. 2015. Genus-wide comparative genomics of *Malassezia* delineates its phylogeny, physiology, and niche adaptation on human skin. PLoS Genet. 11(11):e1005614.

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, et al. 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science. 334(6052):105–108.

Xiao J, Grier A, Faustoferri RC, Alzoubi S, Gill AL, Feng C, Liu Y, Quivey RG, Kopycka-Kedzierawski DT, Koo H, et al. 2018. Association between oral *Candida* and bacteriome in children with severe ECC. J Dent Res. 97(13):1468–1476.

Xu H, Sobue T, Bertolini M, Thompson A, Vickerman M, Nobile CJ, Dongari-Bagtzoglou A. 2017. *S. oralis* activates the Efg1 filamentation pathway in *C. albicans* to promote cross-kingdom interactions and mucosal biofilms. Virulence. 8(8):1602–1617.

Young G, Resca HG, Sullivan MT. 1951. The yeasts of the normal mouth and their relation to salivary acidity. J Dent Res. 30(3):426–430.

Zhou Y, Mihindukulasuriya KA, Gao H, La Rosa PS, Wylie KM, Martin JC, Kota K, Shannon WD, Mitreva M, Sodergren E, et al. 2014. Exploration of bacterial community classes in major human habitats. Genom Biol. 15(5):R66.