



Site-Specific Profiling of the Dental Mycobiome Reveals Strong Taxonomic Shifts during Progression of Early-Childhood Caries

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ABSTRACT Dental caries is one of the most common diseases worldwide. Bacteria and fungi are both commensals in the oral cavity; however, most research regarding caries has focused on bacterial impacts. The oral fungal mycobiome associated with caries is not well characterized, and its role in disease is unclear. ITS1 amplicon sequencing was used to generate taxonomic profiles from site-specific supragingival plaque samples ($n = 82$) obtained from 33 children with different caries status. Children were either caries free (CF), caries active with enamel lesions (CAE), or caries active with dentin lesions (CA). Plaque samples were collected from caries-free surfaces (PF) and from enamel (PE) and dentin (PD) lesions. Taxonomic profiles representing the different categorizations (CF-PF, CAE-PF, CAE-PE, CA-PF, CA-PE, and CA-PD) were used to characterize the mycobiome and its change through disease progression. A total of 139 fungal species were identified. *Candida albicans* was the most abundant species, followed by *Candida dubliniensis*. We found that severely progressed plaque communities (CA-PD) were significantly different from healthy plaque communities (CF-PF). A total of 32 taxa were differentially abundant across the plaque categories. *C. albicans*, *C. dubliniensis*, *Nigrospora oryzae*, and an unclassified *Microdochium* sp. were correlated with caries, whereas 12 other taxa were correlated with health. *C. dubliniensis* increased steadily as caries progressed, suggesting that *C. dubliniensis* may play an important role in caries pathogenicity. In contrast, four health-associated fungal taxa have the potential to antagonize the cariogen *Streptococcus mutans* via xylitol production, suggesting a possible fungal mechanism that could contribute to maintenance of dental health.

IMPORTANCE Early-childhood caries is one of the most prevalent diseases in children worldwide and, while preventable, remains a global public health concern. Untreated cavities are painful and expensive and can lead to tooth loss and a lower quality of life. Caries are driven by acid production via microbial fermentation of dietary carbohydrates, resulting in enamel erosion. While caries is a well-studied disease, most research has focused on bacterial impacts, even though fungi are commensal organisms living within the plaque biofilm. There is very little known about how fungi impact caries pathogenicity. The elucidation of fungal taxa involved in caries disease progression is necessary for a more holistic view of the human oral microbiome. Data from this study will improve our understanding of how the fungal community changes as disease progresses and provide insight into the complex etiology of dental caries, which is necessary for the development of treatment plans and preventative measures.

KEYWORDS dental caries, mycobiome, ITS, amplicon, microbiome, fungi, oral, oral microbiology

Early-childhood caries (ECC) is one of the most prevalent diseases in children, and, while preventable, it remains a global public health problem (1–3). The incidence of ECC in the United States is estimated between 3 and 6%, and in less developed

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countries, ECC continues to grow rapidly, with an incidence of up to 70% (3). Often, ECC remains untreated and leads to adverse effects on the child's overall health, development, and quality of life (3). Due to the significant detrimental effects of ECC on quality of life and the substantial economic burden associated with this disease, preventative measures and early treatment are crucial.

Dental caries is a complex, multifactorial, polymicrobial, and biofilm-induced disease resulting in loss of tooth hard tissues. Caries is driven by acid production via bacterial metabolism of easily fermentable carbohydrates such as sucrose (4, 5). Despite the wealth of studies on ECC and caries, the ecological aspects of the disease are still not fully understood. The focus of many studies examining the microbiology of caries has been on bacterial members of the tooth microbiome (6–9). More recently, studies have highlighted the presence of fungi in the oral cavity and in supragingival plaque of individuals presenting either oral health, caries, or other oral diseases (10–18).

The oral fungal community is not well characterized; this is particularly true regarding caries. The development of next-generation sequencing (NGS) technologies has provided an affordable method to identify and quantify members of the oral mycobiome. The internally transcribed spacer (ITS) 1 and 2 regions have been widely used for amplicon sequencing of fungal communities due to their ability to distinguish between a wide array of fungal taxa (19). A study from 2010 used the ITS1 region to classify the health-associated oral fungal mycobiome and highlighted the vast diversity of fungal taxa in the mouth (10). Since then, over 150 fungal species have been identified in the oral cavity using the ITS1 and ITS2 regions. In almost all of these studies, *Candida* spp., particularly *Candida albicans*, have dominated the composition of the oral mycobiome (10, 13, 16–18). Evidence of the role that *C. albicans* plays in caries has been contradictory. Some studies have observed no significant differences between caries status (caries free or caries active) and *C. albicans* prevalence (20, 21), while another showed there was no correlation between caries risk and *C. albicans* presence (22). In contrast, a study using a rodent caries model found that *C. albicans* had the ability to cause caries when fed a high-sucrose and -glucose diet (23). Moreover, other studies have found that *C. albicans* is commonly detected in association with the caries pathogen *Streptococcus mutans* in plaque samples from children with ECC (24–26). In one of these studies, increased glucosyltransferase activity of *S. mutans* (a caries virulence factor) was significantly elevated in the presence of *C. albicans*, highlighting an important cross-kingdom association (26). *In vitro* testing with *Candida* sp. has shown increased adherence of bacterial organisms on teeth when *Candida* species are present. It has been proposed that the *Candida* species act as a bridge to which oral bacteria can adhere, providing more surface area for extracellular polysaccharide (EPS) formation while also making bacteria less susceptible to antibiotics and antimicrobials (27). Oral bacterial and fungal interactions may be important risk factors and indicators in caries disease (27–29). To begin to elucidate the complex microbial interactions contributing to caries, a better characterization of the mycobiome of supragingival dental plaque during the stages of caries progression is needed.

This study used high-throughput amplicon sequencing of the ITS1 region to characterize the oral mycobiome associated with dental health and ECC and is only the second study to characterize the oral mycobiome associated with caries. Furthermore, we used a site-specific sampling approach to measure changes in mycobiome composition through the progressive stages of the disease. We hypothesized that the community composition would change as disease progressed. A characterization of the fungal community as caries progresses will allow for further investigation into the complex interactions between the bacterial and fungal microbiomes and how these interactions impact dental health and caries disease development and progression.

RESULTS

Illumina sequencing. The total number of reads after Illumina MiSeq sequencing was 14,448,675, with an average number of reads per sample of 138,840. After completing an initial quality check (QC) on the samples to remove low-quality reads,

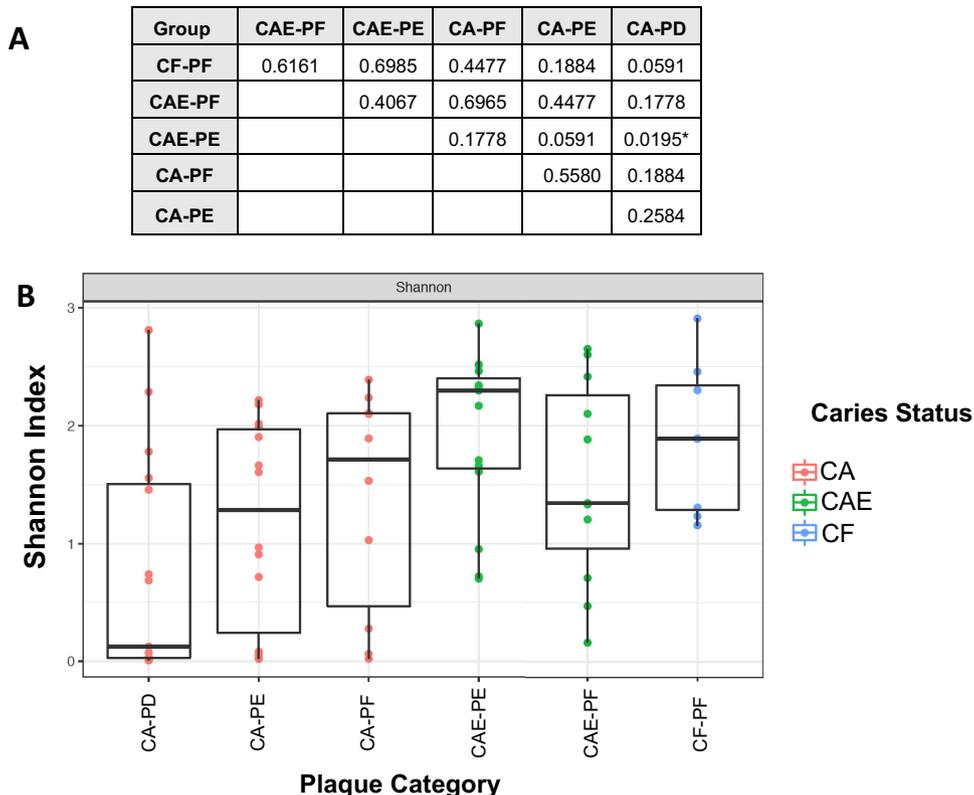


FIG 1 Shannon alpha diversity metrics of site-specific dental plaque mycobiome samples as dental caries progresses. (A) Pairwise Kruskal-Wallis test results. *P* values were corrected for multiple comparisons using FDR correction. *, statistical significance ($\alpha < 0.05$). (B) Box plot depicting Shannon alpha diversity measures for each sample category. Colors represent caries status of the child.

chimeric sequences, and PhiX reads, the total number of reads was reduced to 4,946,479. The average number of reads per sample was 67,759, with a minimum of 2,194 and a maximum of 168,228. Ten samples with low-quality reads, low numbers of reads, or low operational taxonomic unit (OTU) counts were removed from the analysis. A total of 412 taxonomic assignments were generated. OTUs with the same taxonomic classification were collapsed, and singletons were removed, resulting in a total of 214 unique taxonomic assignments composed of 2 phyla, 184 genera, and 139 species.

Children were either caries free (CF), caries active with enamel lesions (CAE), or caries active with dentin lesions (CA). Plaque samples were collected from caries-free surfaces (PF) and from enamel (PE) and dentin (PD) lesions. Significant differences in species richness were only observed between CAE-PE and CA-PD communities ($P = 0.019$) (Fig. 1A). Overall, fungal community richness decreased as caries progressed, with the highest alpha diversity observed in CF-PF communities and lowest alpha diversity observed in CA-PD communities (Fig. 1B).

Pairwise permutational multivariate analysis of variance (PERMANOVA) tests comparing beta diversity for samples of the same plaque category at different collection time points showed no significant differences (see File S1 in the supplemental material). Consequently, we grouped all samples of the same plaque category together for all subsequent analyses. Ordination analysis showed dissimilarity between communities representing the different types of plaque (Fig. 2A). CF-PF and CAE-PF samples were mainly clustered together in the bottom right quadrant, CAE-PE samples spanned across the right and top left quadrants, and CA-PF, CA-PE, and CA-PD samples clustered mostly in the left and top left quadrants. Significant differences in beta diversity were observed between CF-PF and CA-PD samples ($P = 0.015$). No significant differences were observed between the remaining community comparisons (Fig. 2B). A neighbor-

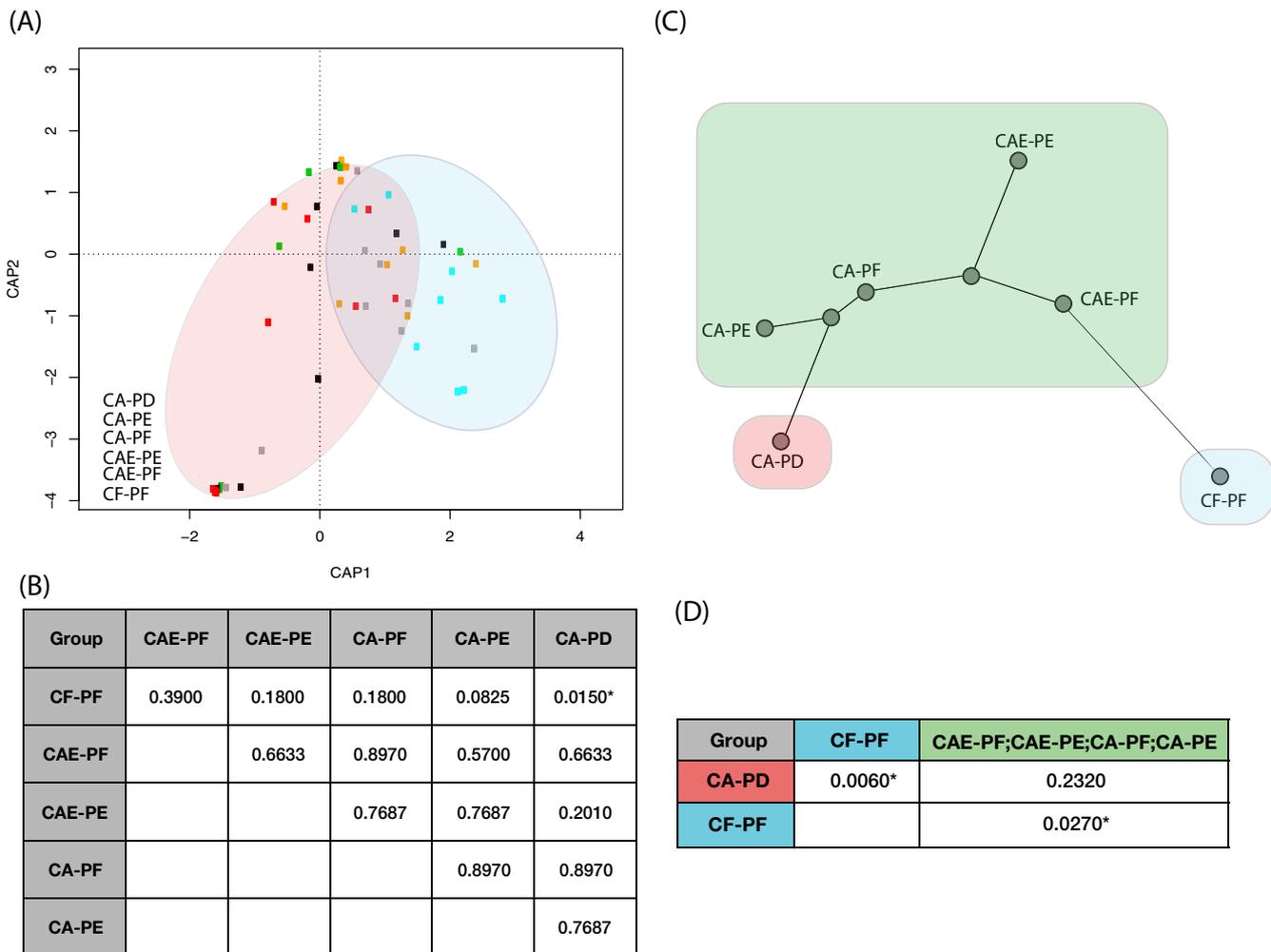


FIG 2 Beta diversity analyses of oral mycobiome samples as dental caries disease progresses. (A) Constrained analysis of principal coordinates (CAP) of the different sample categories using a distance-based redundancy analysis (db-RDA). A pairwise PERMANOVA detected a significant difference in beta diversity when CF-PF samples (blue) were compared to CA-PD (red). With one exception (CA-PD), the shaded ovals show the position of CF-PF and CA-PD samples. (B) Results of pairwise PERMANOVA tests using Bray-Curtis beta diversity distances across the six different categories. Values presented are Benjamini and Hochberg FDR-corrected *P* values. *, statistical significance. (C) Neighbor-joining phylogeny constructed using the variance components from the pairwise PERMANOVA tests. Branch lengths are representative of the degree of difference between communities. (D) Results of pairwise PERMANOVA tests across the three significantly different tooth classification groups delineated in the initial tests (shown with red, green, and blue shading). Values presented are again Benjamini and Hochberg FDR-corrected *P* values. *, statistical significance.

joining phylogeny built using pairwise PERMANOVA variance components showed increased dissimilarity in community composition as disease progressed, with CF-PF and CA-PD samples being the most dissimilar to each other (Fig. 2C). A second pairwise PERMANOVA was performed where samples categorized as CAE-PF, CAE-PE, CA-PF, and CA-PE were grouped together due to their nonsignificant differentiation in the first analysis (Fig. 2D). The community composition of CF-PF samples was significantly different from that of the grouped samples ($P = 0.027$), but the composition of CA-PD samples was not significantly different from the grouped samples ($P = 0.232$).

The oral mycobiome composition in caries and dental health. Two fungal phyla, *Ascomycota* and *Basidiomycota*, were detected in the plaque samples. All but one OTU were assigned taxonomies below the phylum level. A total of 86% of OTUs were assigned taxonomies to the genus level, and 65% of OTUs were classified to the species level. The average number of taxa per plaque sample was 21. The most abundant species across all plaque groups were *C. albicans*, *Candida dubliniensis*, *Debaryomyces* sp., and *Cladosporium exasperatum* (in order of prevalence; Fig. 3 and 4).

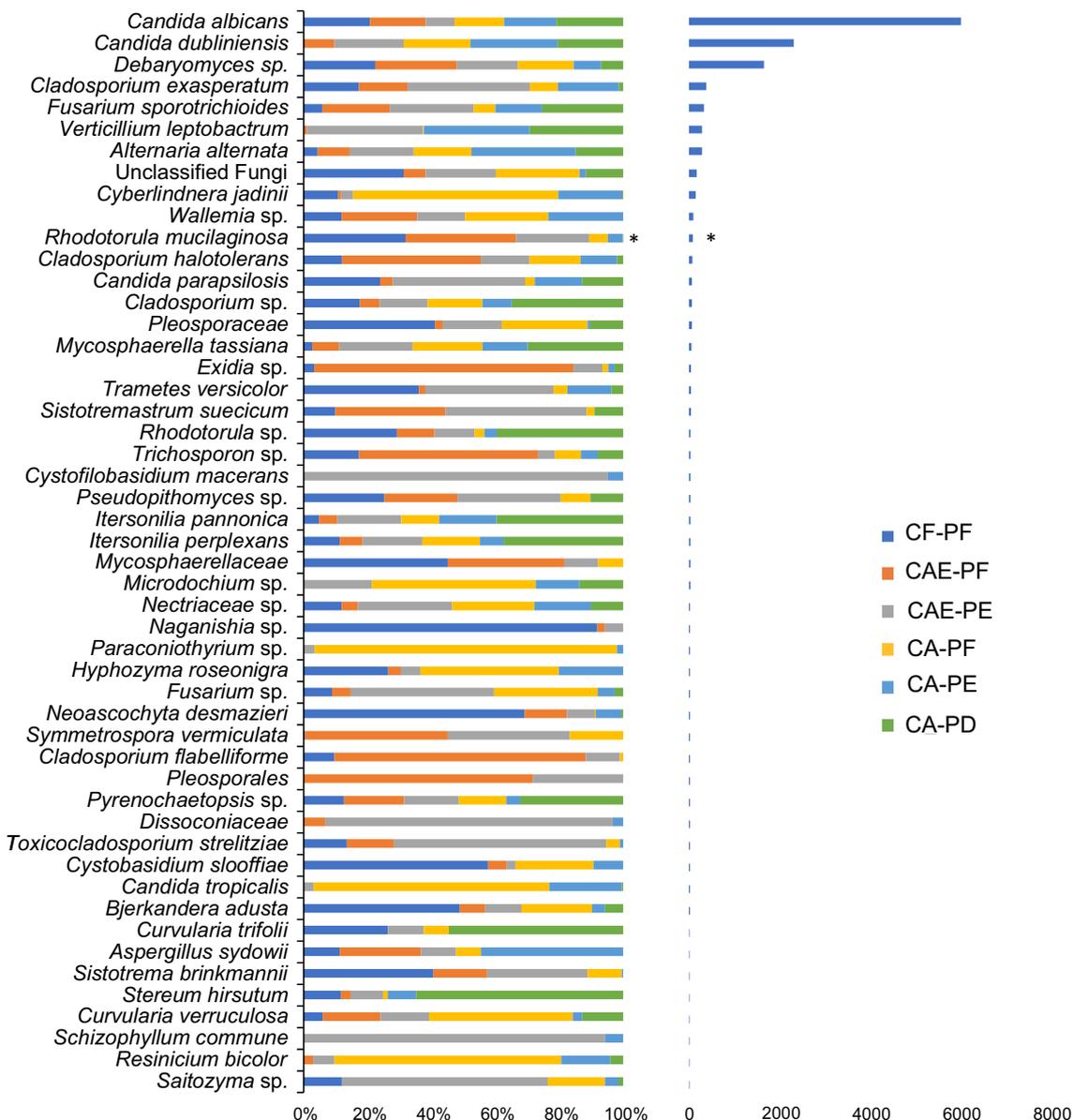


FIG 3 Distribution of the top 50 most abundant taxa across the six tooth classifications using rarefied taxon counts. The left chart shows mean taxon counts for each plaque category. Means are shown as relative proportions. The chart on the right depicts the sum total of the counts for each taxon; *, taxa whose frequency was significantly different among the plaque categories (determined using a *t* test and adjusted for multiple comparisons using Benjamini and Hochberg FDR-corrected *P* values).

Using rarefied taxon counts, the prevalence of two taxa, *Rhodotorula mucilaginosa* and an unidentified *Teratosphaeriaceae* family, were significantly different among the plaque groups (Fig. 3) (see File S2 in the supplemental material). Using the negative binominal distribution, 32 taxa were significantly differentially abundant for at least one comparison between the plaque groups (Fig. 4). In total, there were 200 significant comparisons between the plaque groups (see File S3 in the supplemental material). The comparison between CF-PF and CA-PD showed 26 differentially abundant taxa, which was the highest number across all the comparisons. The comparison between CA-PF and CA-PE showed the lowest, with only 6 differentially abundant taxa (Table 1). Of the 32 differentially abundant taxa, *C. albicans*, *C. dubliniensis*, *Nigrospora oryzae*, and an unidentified *Microdochium* sp. were strongly correlated with caries-active plaque samples, showing statistically significant increased abundance in CA-PF, CA-PE, and CA-PD communities compared to CF-PF communities (Fig. 5A; see also Fig. S4 in the

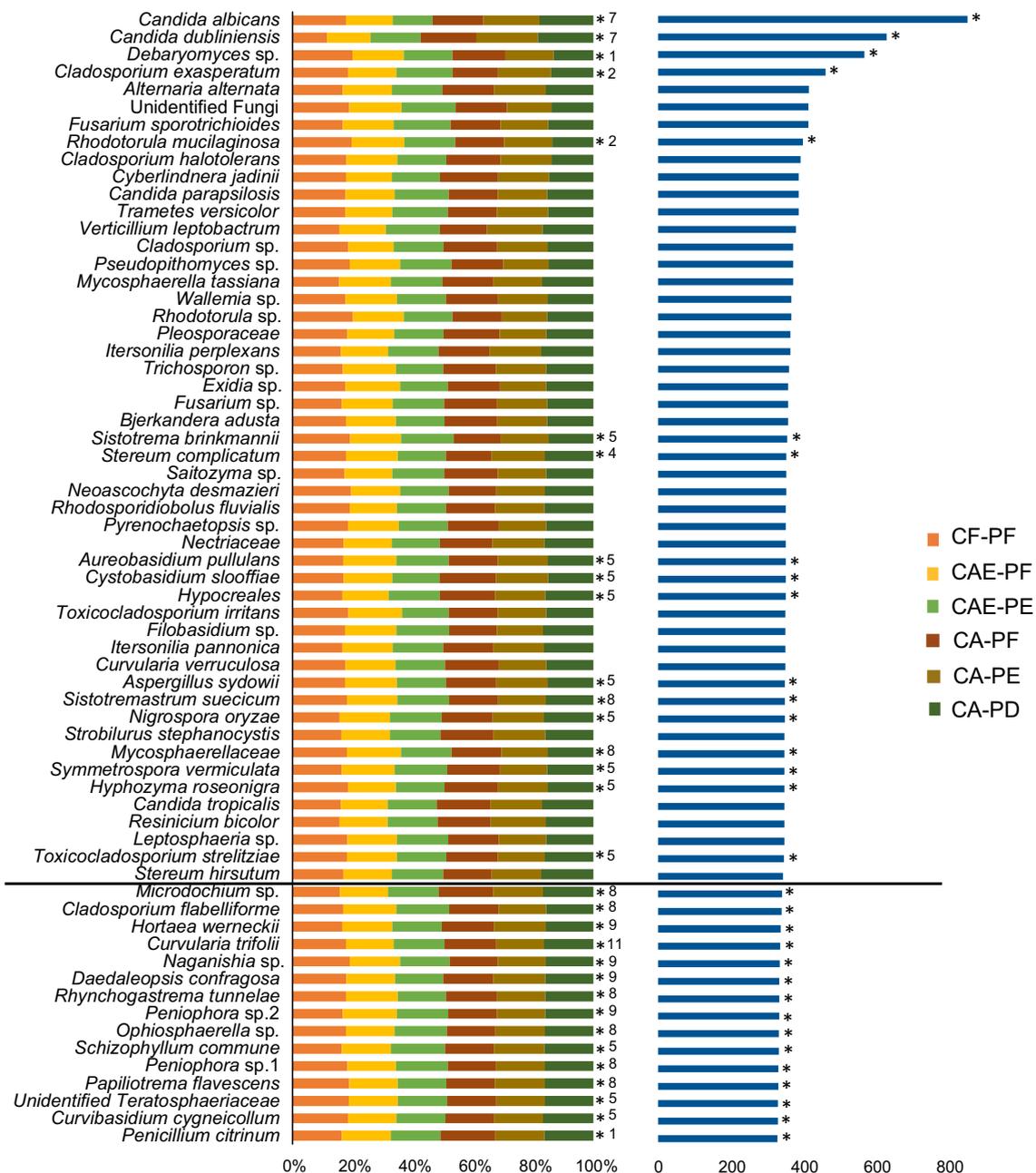


FIG 4 Distribution of the top 50 most abundant taxa across the six plaque categories using variance-stabilizing transformation of taxon counts. The left chart shows the mean taxon counts for each plaque category. All taxa below the bold line were not present in the top 50 most abundant taxa but were statistically significantly differentially abundant. The chart on the right depicts the sum total of the counts for each taxon. *, taxa whose frequency was significantly different among the plaque categories (determined using DESeq2 and adjusted for multiple comparisons using Benjamini and Hochberg multiple testing adjustment).

supplemental material [File S4]). *C. dubliniensis* showed a strong progressive increase in abundance moving from CF-PF to CA-PE, with a slight decrease moving from CA-PE to CA-PD. *C. albicans* showed a more variable pattern. Starting with a relatively high abundance in CF-PF, its abundance decreased until CAE-PE (this decrease from CF-PF to CAE-PE was not statistically significant [see File S4 in the supplemental material]). It then showed a similar pattern to *C. dubliniensis*, steadily increasing with disease progression, with a slight decrease moving from CA-PE to CA-PD. Although CF-PF abundance was relatively high, it was statistically significantly lower than both CA-PE and CA-PD. *N. oryzae* and *Microdochium* sp. did not show a steady increase with disease

TABLE 1 The number of significantly differentially abundant taxa for each plaque category comparison as determined using DESeq2^a

Group	No. of significantly differentially abundant taxa for:				
	CAE-PF	CAE-PE	CA-PF	CA-PE	CA-PD
CF-PF	11	9	13	16	26
CAE-PF		8	10	11	16
CAE-PE			11	13	19
CA-PF				6	14
CA-PE					12

^aP values were adjusted for multiple comparisons using Benjamini-Hochberg multiple testing adjustment.

progression as seen for *C. dubliniensis*. However, both were considerably more abundant in disease than in health. *N. oryzae* again showed a slight decrease moving from CA-PE to CA-PD. A total of 12 differentially abundant taxa showed strong correlations with dental health (CF-PF) and showed an overall decrease in abundance as caries

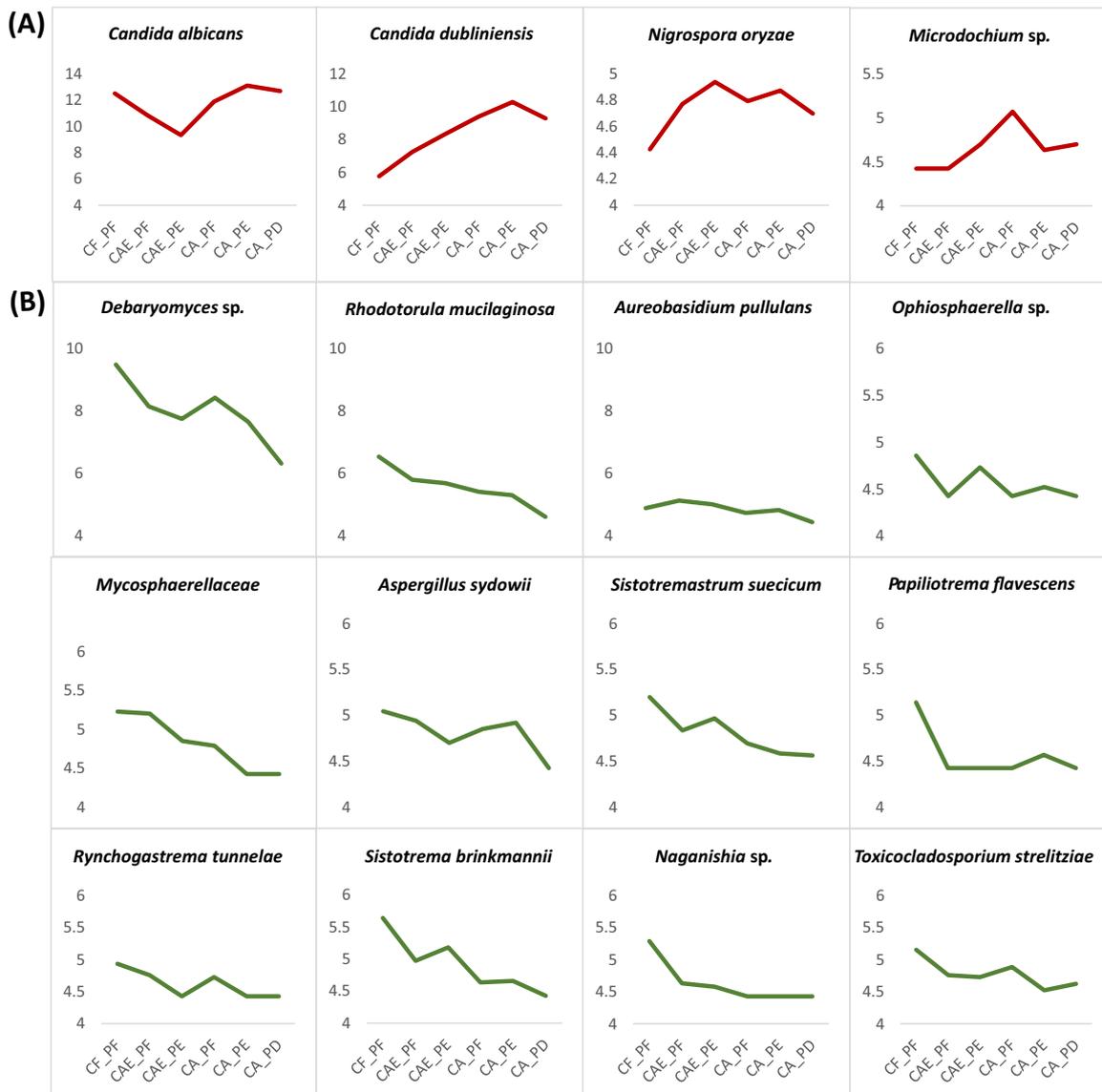


FIG 5 Significantly differentially abundant taxa enriched in dental caries and in health. (A) The four statistically significant taxa enriched in dental caries disease. (B) The 12 statistically significant taxa enriched in health. All graphs were generated from a variance-stabilizing transformation of counts within DESeq2.

disease progressed. For all of these 12 taxa, their lowest abundance was observed in CA-PD (Fig. 5B).

The remaining 16 taxa did not show strong correlations with health or disease; however, interesting patterns were observed for these taxa. Six of these 16 taxa showed an increase in abundance in CA-PF samples, while 2 other taxa showed an increased abundance in CA-PE, subsequently followed by a decrease in abundance in CA-PD samples (see Fig. S5 in the supplemental material [File S4]). In another seven taxa, an increased abundance in CAE-PF and CAE-PE samples was observed with decreased abundance in CA-PF, CA-PE, and CA-PD samples (see Fig. S6 in the supplemental material [File S4]).

DISCUSSION

This study characterized the oral mycobiome associated with the progressive stages of caries lesions using site-specific sampling. Supragingival dental plaque samples were collected from children and tooth sites of different caries status to obtain a better understanding of how the oral mycobiome changes as disease progresses. Our findings show that fungal community composition in health-associated plaque differed significantly from that associated with dentin caries (CF-PF versus CA-PD), which is the most advanced stage of caries. Importantly, plaque from a caries-free tooth in a caries-active child (CAE-PF and CA-PF) harbored a more similar community composition to caries-associated plaque in a caries-active child (CA-PD) than to plaque from a caries-free tooth of a caries-free child (CF-PF). This suggests that healthy teeth in caries-active children are at a high risk of developing caries lesions. A previous study of ours using site-specific sampling of the bacterial community in caries showed a similar pattern (9). These findings are also concordant with studies showing that previous caries experience is an important caries risk indicator (30).

A significant difference in alpha diversity was observed only between CAE-PE and CA-PD samples; however, there was a steady decrease in community diversity as caries worsened. This pattern of decreased microbial diversity with disease has been observed previously in relation to the bacterial communities associated with dental caries and other human microbiome-associated diseases such as Crohn's disease and ulcerative colitis (31, 32). In regard to caries, the shift toward decreased diversity is believed to be caused by increased carbohydrate consumption and fermentation leading to acid production and secretion. The low-pH environment is not optimal for the growth of many taxa, and therefore, the low pH drives the biofilm composition toward a higher abundance of acidogenic and aciduric taxa, which thrive under these conditions (32). It is possible that this is also occurring in the fungal microbiome community associated with dental caries, as some fungal taxa are known to be acidogenic and aciduric, while others are not capable of surviving in acidic environments (23, 29).

Previous studies examining the oral mycobiome in health and oral disease (caries and periodontal disease) did not observe significant differences in either alpha or beta diversity (17, 18). While their results were not statistically different, both of these studies showed a pattern of alpha diversity reduction in diseased samples. The high-resolution and site-specific nature of our sampling provides a more sensitive measure of fluctuations in community diversity, which may explain our results of statistically significant diversity compared to previous oral mycobiome studies (17, 18). It is well accepted that caries is driven by bacterial dysbiosis caused by environmental factors such as carbohydrate consumption, which leads to disruption of the plaque biofilm homeostasis toward a more pathogenic state (8, 33, 34). Our data support distinct shifts in fungal plaque communities as disease progresses, suggesting that fungal community dysbiosis is occurring and may be contributing to disease severity and progression, similar to what has been observed in bacterial dysbiosis (33–35). Furthermore, similar to bacterial communities (9), our results indicate that each stage of the disease has a characteristic taxonomic profile that is stable through time.

Only a few studies to date have utilized next-generation sequencing (NGS) of the ITS region to characterize the oral mycobiome associated with health and disease. In our

study, a total of 214 OTUs comprised of 139 fungal species were identified in the dental plaque samples. This fungal diversity is consistent with previous studies that have used the ITS1 region for sequencing of the oral mycobiome (10, 17). Of note, while the number of taxa between our study and previous oral mycobiome studies that utilized the ITS1 region are similar, these other studies used oral rinse samples and not dental plaque and are therefore not directly comparable. In contrast, Fechney et al. recently characterized the oral mycobiome associated with dental caries and health in children using dental plaque samples and found a much lower community diversity (a total of 46 fungal species); however, the ITS2 region was used for sequencing and could explain these differences (18). Further comparisons need to be completed to assess variations in taxonomic resolution between the ITS1 and ITS2 regions in dental caries.

Our results support previous work highlighting the vast diversity of fungal organisms present in the oral cavity (10, 11, 17). Despite this diversity, only a relatively small number of taxa comprised the bulk of the community. Specifically, *C. albicans*, *C. dubliniensis*, *Debaryomyces* sp., and *C. exasperatum* comprised 79% of the total community, with the remainder being comprised of a large diversity of low-frequency taxa. As in other oral mycobiome studies (10, 18), *C. albicans* was the most abundant species in the data set and was present in 68 of the 73 samples (93.2%). *C. albicans* is a commensal yeast in the oral cavity and is a known opportunistic pathogen that causes diseases such as oral candidiasis (15). *C. albicans* has recently been gaining traction as a possible cariogenic yeast and indicator species for ECC (36–39). In many recent studies, researchers have found that the prevalence of *C. albicans* in children with ECC is generally much higher than in children who are caries free and that, frequently, *C. albicans* is detected together with and in close proximity to *S. mutans* (25, 27, 29, 37). Because of the high prevalence of *C. albicans* in the oral mucosa and in ECC, more targeted studies have been completed to determine its cariogenic potential.

Studies have shown that *C. albicans* is aciduric and acidogenic and is capable of biofilm formation, especially when grown in culture with *S. mutans*, resulting in increased amounts of extracellular polysaccharides (EPS) pertinent for caries development (23, 29, 40, 81). Recent meta-omic analyses of *S. mutans* and *C. albicans* grown in mixed biofilms have provided molecular evidence to support the hypothesis that *C. albicans* enhances the cariogenic potential of *S. mutans* (28). These results are supported by a recent study by Sampaio et al., in which they found that a dual-species biofilm composed of *S. mutans* and *C. albicans* resulted in an overall increased virulence of cariogenic biofilms compared to monospecies biofilms (41). In contrast to these studies, Fechney et al. found that there was not a significant difference in *C. albicans* abundance between healthy children or in children with ECC, suggesting that *C. albicans* is not cariogenic (18).

Our results support increased prevalence of *C. albicans* in CA children, possibly highlighting its role in increased caries virulence. Interestingly, our results also show a relatively high frequency of *C. albicans* in CF-PF children, which decreased in frequency until caries progressed into the dentin tooth layer. These results highlight the importance of site-specific sampling to gain a high-resolution profile of the mycobiome throughout disease and enable fluctuations of the mycobiome to be monitored throughout all stages of disease progression rather than just in health versus disease. To explain the pattern observed above, it is possible that multiple strains of *C. albicans* are present in the tooth biofilm and that as conditions begin shifting toward a more cariogenic state, one strain dies off and the other begins to proliferate. Previous studies have identified the presence of multiple genotypes of *C. albicans* in the oral cavity whose relative proportions can continually change after host infection to allow for biofilm adaptations to the host environment (42–44). These studies have also shown a high prevalence of *C. albicans* genotype A in individuals with ECC or dental caries (43, 44). To further verify that different *C. albicans* strains have different virulence capabilities, whole-genome sequencing and targeted biochemical tests need to be completed (45). It is important to note that, while we observed a decreased prevalence of *C. albicans* in the early stages of caries, it is possible that *C. albicans* virulence factors are

still highly active and may be contributing to disease progression. *C. albicans* has been shown to have remarkable metabolic flexibility to utilize an array of nutrient sources and pH conditions. *C. albicans* is also capable of manipulating its environment to cause disease and avoid immune system detection (46). Further transcriptomic analysis is needed to fully understand the role that *C. albicans* contributes to caries development and progression. Our findings suggest that *C. albicans* may not be a reliable indicator of ECC, as it is present in high frequencies in both CF-PF children and CA-PF, CA-PE, and CA-PD children and shows a marked decrease during the initial stages of the disease.

Candida dubliniensis was the second most abundant species overall and was found in high frequency in disease. *C. dubliniensis* is the most closely related species to *C. albicans* and contains many of the same virulent traits, including the ability to produce germ tubes, chlamydoconidia, and hyphae (47, 48). In more recent studies, *C. dubliniensis* was identified in saliva samples from elderly, community-dwelling individuals and in oral wash samples from patients with chronic periodontitis, suggesting that this organism thrives when the host immune system is weakened (17). Indeed, the species is frequently found in individuals in an immunocompromised state (49).

Because of the morphological and biochemical similarities of *C. albicans* and *C. dubliniensis*, discriminating between these species in culture is problematic and, until recently, could not be done without the use of molecular technologies. It is possible that previous studies have misidentified *C. dubliniensis* as *C. albicans* and have therefore overlooked its importance in dental caries. It was only in 2015 that *C. dubliniensis* was first identified in plaque samples from dentinal carious lesions using scanning electron microscopy. In a study by Al-Ahmad and colleagues, *C. dubliniensis* and *C. albicans* were cultured from saliva and dental plaque samples of healthy children and children with active dentinal caries. They did not find any evidence of *C. dubliniensis* in CF children but found a statistically significant positive correlation between *C. dubliniensis* and severe childhood caries (15). Similarly, we detected low abundance/absence in CF samples, and, given that we also detected a steady increase in abundance as caries progressed, *C. dubliniensis* shows promise as an indicator species of ECC. However, biochemical studies and transcriptomics analyses are needed to fully elucidate the role that *C. dubliniensis* plays in dental caries progression and severity.

Along with *C. albicans* and *C. dubliniensis*, *N. oryzae* and an unidentified *Microdichium* sp. were also found to be associated with disease. Both of these taxa have been identified in previous studies examining the oral mycobiome of oral rinse samples. In a study by Mukherjee et al., an unidentified *Nigrospora* sp. was present in multiple people infected with HIV and was not observed in uninfected patients (11). In a more recent study examining the oral mycobiome in relation to periodontal disease, the taxa *Nigrospora* sp. and *Microdichium* sp. were found in higher frequency in samples from patients without periodontitis (17). Historically, based on their sexual stage, these taxa were named *Khuskia* sp. and *Monographella* sp., respectively. However, this naming system is no longer practiced (50). Future research is needed to clarify if these plant-associated pathogens are commensals in the oral cavity or if they are introduced via the environment, as well as to determine their role in dental caries.

In our study, a total of 12 taxa were associated with health and had a pattern of decreased frequency as disease progressed. Of these 12 taxa, 6 of the genera have been observed in previous oral mycobiome studies: *Debaryomyces*, *Rhodotorula*, *Aureobasidium*, *Aspergillus*, *Naganishia*, and *Toxicocladosporium*. Here, an unidentified member of the *Debaryomyces* genus was the third most abundant organism found overall. In previous studies, *Debaryomyces hansenii* has been isolated and identified from oral rinse samples, tongue scrapings, and supragingival plaque samples (13, 18). It was not statistically differentially abundant between health and disease in these studies; however, it was found in higher frequency in healthy samples. An important biochemical characteristic of *D. hansenii* is its ability to convert xylose (a monosaccharide in plants) to xylitol. Xylitol could potentially mitigate caries, as it negatively impacts the growth and proliferation of the cariogen *S. mutans* (51, 52). When xylitol is present as a carbohydrate source, *S. mutans* will convert it to xylitol-5-phosphate through the

phosphoenolpyruvate-phosphotransferase system (PEP:PTS system). The production and accumulation of xylitol-5-phosphate within the cells inhibits glycolytic enzymes, resulting in growth inhibition, decreased metabolism, decreased acid production, and sometimes cell death (51, 52). It is possible that *D. hansenii* helps to maintain a healthy oral microbiome through its xylitol production and may explain its increased frequency in health (53). Further biochemical testing and metatranscriptomic analysis are needed to determine if oral fungi produce sufficient xylitol to impact cariogenic bacteria and validate a role in dental health.

R. mucilaginosa was one of the most abundant species found in our data and was significantly enriched in health. *R. mucilaginosa* is generally considered an opportunistic pathogen that is capable of forming biofilms and can cause a variety of infections (54). *R. mucilaginosa* and other unclassified members of this genus have been identified as part of the oral mycobiome in multiple other studies (11, 17, 18). In the caries mycobiome study by Fechny et al., *R. mucilaginosa* was not differentially abundant between health or disease; however, it was present in a higher frequency in health than in caries. *R. mucilaginosa* is also capable of fermenting xylose and producing xylitol, which may contribute to a role in maintaining tooth health (55).

Aureobasidium pullulans was one of the top 50 most abundant fungal taxa in the data and is another species that was strongly and significantly associated with health (showing decreased abundance as disease progressed). Members in the *Aureobasidium* genus, including *A. pullulans*, have been found to be present in the oral mycobiome in previous studies (11, 17, 18). Furthermore, this species is able to produce bioactive compounds that are useful in industry, one of which includes the production of xylanase enzymes (56). Xylanase enzymes degrade xylan to xylose, which can be fermented by other fungal members of the oral biofilm to produce xylitol. Besides producing xylanases, *A. pullulans* has also been found to produce liamocin oils that have strong antibacterial activity against *Streptococcus* species, including *S. mutans* (57). All of these characteristics highlight the role that *A. pullulans* may play in health.

Aspergillus sydowii, another species in the top 50 most abundant taxa in our data, was found to decrease in frequency in dental caries and was present in the highest frequency in health. Other oral mycobiome studies have identified members of the *Aspergillus* genus; however, none have recorded the presence of *A. sydowii*. Interestingly, *A. sydowii* is also capable of producing xylanases and many active secondary metabolites, some of which have strong antimicrobial properties, which could point to a role in dental health (58, 59).

A total of 2 remaining genera, *Naganishia* and *Toxicocladosporium*, not present in the top 50 most abundant taxa, have been isolated from the oral mycobiome in previous studies. Organisms within the genus *Naganishia* have been observed in the human gut, skin, and scalp as commensals, but certain members of the genus are opportunistic pathogens capable of causing disease (60). *Naganishia diffluens* (previously *Cryptococcus diffluens*) was one of the most dominant species identified by Fechny et al. in their caries mycobiome study; however, it was not associated with either health or disease (18). Similar to our results, a study completed on human fecal mycobiomes in health, ulcerative colitis, and Crohn's disease found that *Naganishia* was more abundant in healthy controls than in disease (61).

The *Toxicocladosporium* genus was only recently described and is most often found growing on plants (62). In the Fechny et al. caries mycobiome study, they identified the presence of the fungus *Toxicocladosporium rubrigenum*. This species was found in higher frequencies in healthy samples (18). In our study, we identified *Toxicocladosporium strelitziae* in our samples, and it was also found in highest frequency in health. Future research is needed to elucidate the role that *Naganishia* sp. and *Toxicocladosporium* sp. play in caries disease; however, our results paired with previous dental caries mycobiome studies seem to implicate a role in tooth health.

The remaining taxa associated with health, *Sistotrema brinkmanii*, *Sistotremastrum suecicum*, *Papiliotrema flavescens*, and *Rynhogastrema tunnelae*, have not been identified in previous oral mycobiome studies. Most of these fungi have been isolated from

the environment or from plants. More research is needed to understand if these organisms play a role in dental caries or if they are transient members of the oral microbiota introduced via the external environment.

Finally, we observed an interesting pattern for several of the taxa associated with disease in which there was a noticeable decrease in the frequency of CA-PD. A possible explanation for this observation may be the influence of the bacterial members in the plaque biofilm. Previous research examining the bacterial microbiome associated with cavities showed that species such as *Veillonella parvula*, *Veillonella dispar*, *S. mutans*, *Lactobacillus salivarius*, and *Scardovia wiggsiae* increased drastically in abundance in dentinal carious lesions (9). Furthermore, both *Veillonella* spp. and some *Lactobacillus* spp. can produce acetic acid from the utilization of lactate or through heterolactic fermentation of pyruvate (63, 64). Acetic acid is known to inhibit the growth of many different types of fungi, including members of the *Candida*, *Aspergillus*, and *Fusarium* genera, especially when in a low-pH environment (65–67).

Another possible reason for the decreased abundance of many of the fungal species in CA-PD samples could be that they are susceptible to formic acid. Formic acid is a weak organic acid known to inhibit the growth of fungal species. Formic acid has been shown to inhibit the growth of members of the *Candida*, *Aspergillus*, and *Fusarium* genera at similar or greater levels than acetic acid (65, 67). A previous study examining gene expression of *S. mutans* when grown in culture with *C. albicans* showed a 5-fold increase in formate production when these two species were grown together (28). Again, it is possible that an increased abundance of *S. mutans* in CA-PD samples leads to increased formic acid production, creating an environment in which many fungal species cannot survive, explaining the observed pattern of decreased fungal abundance in dentinal carious lesions.

Our study utilized site-specific sampling and high-throughput amplicon sequencing to characterize the oral mycobiome through different stages of caries progression. A vast diversity of fungal organisms was identified from the supragingival plaque biofilms of children with and without ECC. We observed a strong shift in community structure for the dental mycobiome coupled with a loss of community diversity as caries progressed. *C. dubliniensis*, the second most abundant species in the samples, was highly correlated with disease and steadily increased in abundance as caries progressed. Our study highlights the important role it may play in disease progression and suggests that it may be a better option than *C. albicans* as a potential indicator species for ECC. Of note, several fungal taxa associated with health have the potential to antagonize the cariogen *S. mutans* via the production of xylitol and antimicrobial compounds, suggesting a possible fungal mechanism for the maintenance of dental health. Additional omic approaches, such as metatranscriptomics, and targeted biochemical studies will be required to fully explore these mechanisms and provide further insight into the complex interplay between the fungal and bacterial members of the plaque biofilm associated with caries.

MATERIALS AND METHODS

Site-specific plaque. A total of 82 supragingival plaque samples collected from 33 children ages 2 to 7 years were included in the present study. The sampling process and the demographics of the study population are described elsewhere (68). Informed consent was obtained from the parents or legal guardians of each child, and approval for the study was granted by the Institutional Review Board of the University of Florida Health Science Center. Briefly, caries lesions were detected and diagnosed by a single examiner using the International Caries Detection and Assessment System (ICDAS-II) visual criteria (69). Lesion activity was determined by clinical appearance, plaque stagnation, and tactile sensation. Children were grouped by caries status as caries free (CF), caries active with active enamel caries lesions only (CAE), and caries active with at least two active and unrestored dentin carious lesions (CA). The range of ICDAS scores (0 to 6) as a function of caries status group were CF (for no activity, ICDAS = 0), CAE (active lesions, ICDAS, 0 to 3), and CA (active lesions, ICDAS = 0 to 6). The threshold for the CA group was the presence of at least two ICDAS scores of 5 or 6 (cavitated dentin lesions). Supragingival plaque samples were collected from separate tooth surfaces and classified as follows: PF for samples collected from tooth surfaces with no active carious lesions (ICDAS score = 0), PE for samples collected from an enamel carious lesion (ICDAS score = 1 to 3), or PD for samples collected from dentin carious lesions (ICDAS score = 4 to 6). Multiple plaque samples were collected from children who had teeth with more

than one plaque category. Combining caries status with tooth classification produces plaque categories representing six progressive stages of the disease: CF-PF ($n = 8$), CAE-PF ($n = 12$), CAE-PE ($n = 16$), CA-PF ($n = 13$), CA-PE ($n = 16$), and CA-PD ($n = 17$) (see File S5 in the supplemental material).

DNA extraction and library construction. Fungal genomic DNA was extracted using the Qiagen DNeasy PowerBiofilm kit (Qiagen, USA) according to the manufacturer's protocol. An extraction blank was included in each set of extractions to monitor for external contamination.

The fungal ITS1 region was amplified using PCR. The forward primer used was ITS1F (CTTGGTCATT TAGAGGAAGTAA), and the reverse primer used was ITS2 (GCTGCGTCTTCATCGATGC) (10). Custom-barcoded primers were designed using the fungal ITS1 primers as outlined previously in Kozich et al. (70). Positive and negative control samples were run with each PCR. After PCR amplification, samples were run on 2.0% agarose Tris-acetate-EDTA (TAE) gels. The amplicon band was gel extracted and purified using Qiagen's QIAquick gel extraction kit (Qiagen, USA) according to the manufacturer's protocol. The concentration of the gel-extracted samples was measured using Qubit 3.0 (Thermo Fisher, USA). Samples were prepared for sequencing following Illumina's "Denature and Dilute Libraries Guide" (https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-denature-dilute-libraries-guide-15039740-10.pdf) and the Illumina "16S Metagenomic Sequencing Library Preparation Guide" (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Custom read 1, read 2, and index primers were spiked into the Illumina reagent cartridge as outlined previously (71).

Illumina sequencing, sequence analysis, and taxonomic classification. Fungal amplicons were sequenced on Illumina's MiSeq platform (Illumina, San Diego, CA) using a V2 500-cycle kit (250-bp paired-end reads). Demultiplexed paired-end reads were imported into QIIME2 (72) for sequence analysis. Sequence quality control was completed using the DADA2 pipeline incorporated into QIIME2. This program filtered and trimmed reads, removed chimeras, assigned reads to OTUs, and generated an OTU table. To obtain taxonomic assignment of the reads post-quality control, the dynamic UNITE fungal database with a 97 to 99% similarity threshold was used to fit and train a naive-Bayes taxonomic classifier. After taxonomic classification, multiple OTU IDs were often assigned to the same taxon. Using QIIME2, we collapsed OTUs with the same taxonomic classification at the species level.

Statistical analysis. Prior to completing diversity estimates, samples were rarefied to an even sampling depth. The samples did not undergo any further preprocessing to remove singletons or low-abundance OTUs before completing alpha diversity analysis. Prior to completing beta diversity analysis, sequences were filtered to remove singletons and OTUs present in less than 0.1% total abundance.

Statistical analysis of the sequences was completed using the QIIME2 pipeline (version 2019.1) (73), QIIME1 (version 1.9.0) (74), and R Studio (version 1.1.463) (75). The following packages were used in R Studio: phyloseq (version 1.26.1) (76), qiime2R (version 0.99.11), DESeq2 (version 1.22.2) (77), and microbiomeSeq (version 0.1) (73). The rarefied count table was used to estimate alpha and beta diversity metrics. Sampling depth was set to 2,194 reads, as this was the sample that had the lowest number of taxonomically assigned reads. Alpha diversity was estimated in QIIME2 using the Shannon index (78). Statistical significance of alpha diversity was calculated using nonparametric, pairwise Kruskal-Wallis tests. Adjusted P values were calculated using Benjamini and Hochberg false-discovery rate (FDR) correction for multiple comparisons (79). Alpha diversity plots were generated using the phyloseq package in R. Beta diversity was estimated in QIIME2 using the Bray-Curtis index (80). Pairwise PERMANOVAs were completed to determine statistical differences in beta diversity. Adjusted P values were calculated using Benjamini and Hochberg correction for multiple comparisons. Results were imported into QIIME1 to generate constrained analysis of principal coordinate (CAP) plots using distance-based redundancy analysis (db-RDA).

Differential abundance testing was completed using both QIIME1 and DESeq2 in phyloseq. In the QIIME1 analysis, Kruskal-Wallis tests were completed on rarefied data using the `compare_categories.py` script to determine significant differences in OTU frequency between different plaque categories. P values were generated using 10,000 permutations and were corrected for using the FDR. McMurdie and Holmes addressed the loss of statistical power when rarefying data for use in differential abundance testing and proposed a more sensitive method for detecting differentially abundant taxa through the use of the negative binomial distribution as implemented in DESeq2 (77). Here, we also take this approach to determine differentially abundant OTUs among different plaque sample categorizations. The nonrarefied and unfiltered read table generated in QIIME2 after DADA2 quality control was used for this analysis. The data were log transformed using variance-stabilizing transformation (VSD) to generate OTU frequencies. P values were corrected for using Benjamini and Hochberg FDR correction for multiple comparisons.

To generate taxonomic profiles for the most abundant taxa, both the rarefied OTU table from QIIME and the VSD normalized table from DESeq2 were used. The top 50 most abundant taxa were plotted using Excel for each analysis. The overall relative abundance of the top 50 taxa across the data set was also plotted.

Data availability. Sequence data have been deposited in the NCBI database under the BioProject accession number [PRJNA589851](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA589851).

Ethics approval and consent to participate. Informed consent was obtained from the parents or legal guardians for each child in the study, and the protocol was approved by the Institutional Review Board of the University of Florida Health Science Center (UF IRB, 201600154).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.03 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.3 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.7 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.01 MB.

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