ARTICLE

Candida albicans promotes tooth decay by inducing oral microbial dysbiosis

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Abstract

Candida albicans has been detected in root carious lesions. The current study aimed to explore the action of this fungal species on the microbial ecology and the pathogenesis of root caries. Here, by analyzing *C. albicans* in supragingival dental plaque collected from root carious lesions and sound root surfaces of root-caries subjects as well as caries-free individuals, we observed significantly increased colonization of *C. albicans* in root carious lesions. Further in vitro and animal studies showed that *C. albicans* colonization increased the cariogenicity of oral biofilm by altering its microbial ecology, leading to a polymicrobial biofilm with enhanced acidogenicity, and consequently exacerbated tooth demineralization and carious lesion severity. More importantly, we demonstrated that the cariogenicity-promoting activity of *C. albicans* was dependent on *PHR2*. Deletion of *PHR2* restored microbial equilibrium and led to a less cariogenic biofilm as demonstrated by in vitro artificial caries model or in vivo root-caries rat model. Our data indicate the critical role of *C. albicans* infection in the occurrence of root caries. *PHR2* is the major factor that determines the ecological impact and caries-promoting activity of *C. albicans* in a mixed microbial consortium.

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Introduction

Dental caries, also known as tooth decay, is one of the most common oral diseases that cause increasing socioeconomic burden to the humans. According to the reports from Global Burden of Diseases, Injuries, and Risk Factors Study 2016, dental caries ranked number one in prevalence and number two in incidence of the ten causes with highest prevalence/incidence [1]. Root caries is a subtype of dental caries that mainly affects elder populations. The prevalence of root caries in the elderly reaches 25–100% [2]. Root caries occurs in areas where the root surface is exposed to the oral environment. As a clinical subtype of dental caries, root caries is a polymicrobial infectious disease associated with the enrichment of acidogenic/aciduric species (e.g., *Streptococcus mutans*) and depletion of less aciduric commensal residents (e.g., *Streptococcus sanguinis*) in the microbial biofilm [3]. The “competitive exclusion” of *S. mutans* and *S. sanguinis* has been observed in both in vivo and in vitro studies [4–6]. In addition, a positive correlation between the risk of dental caries and *S. mutans*/*S. sanguinis* has been observed [7, 8], further suggesting the critical role of microbial disequilibrium in the course of dental caries. Consistently, a recent study has reported microbial dysbiosis in root-carious lesions [9], further

supporting the role of microbial ecology in the development of root caries. Another study on the microbial metatranscriptome in caries revealed that *S. sanguinis* was metabolically active in 3 types of caries lesions (i.e., noncavitated, open dentin, and hidden caries), while *S. mutans* was active only in noncavitated and open dentin caries [10], suggesting the role of oxygen availability in the interspecies interactions between these two bacteria.

Candida albicans is a commensal fungal species commonly colonizing the human mucosal surfaces. The oral carriage of *C. albicans* in healthy individuals is 18.5–40.9% based on cultivation [11–13]. Carriage rates are usually higher in individuals with compromised immunity, such as human immunodeficiency virus-positive individuals, diabetes patients, and infants and elder populations [11, 12, 14–16]. Hyphal formation and the ability to respond to extreme environmental pH levels are essential for *C. albicans* pathogenicity [17–19]. In addition, *C. albicans* can robustly interact with other oral microorganisms and impose significant impact on the virulence of polymicrobial biofilms. The microbial interaction and cross-kingdom feeding between *C. albicans* and oral bacteria such as *Streptococcus*, *Actinomyces*, and *Fusobacterium* species have been suggested being closely associated with the pathogenesis of oral infectious diseases for years [20–24]. Cross-kingdom interactions between *C. albicans* and *Streptococcus oralis* enhance biofilm virulence on mucosal surfaces [25–27]. In addition, a positive correlation between *C. albicans* prevalence/carriage and the severity of early childhood caries (ECC) has been noted [28–30], and *C. albicans* is associated with an increased abundance of plaque *S. mutans* in severe ECC [31, 32]. Findings by Falsetta et al. demonstrated that interactions between *C. albicans* and *S. mutans* not only increased bacterial–fungal carriage in the plaque but also enhanced the biofilm virulence by increasing the severity of carious lesions in a rodent model [33]. More importantly, *C. albicans* has been isolated from root caries lesions [34–36], and observed in carious dentin/dentine tubules [37]. Genes involved in metabolic activity, sugar transport, stress tolerance, invasion and pH regulation are upregulated in *C. albicans* colonized in root carious lesion as compared to *C. albicans* in sound root surface [38], further underscoring the potential role of *C. albicans* in the development of root caries.

Although the association of *C. albicans* with root caries has been suggested, the exact action of this fungal species on the microbial ecology and the pathogenesis of root caries is yet to be investigated. In addition, key factors that determine the successful colonization of *C. albicans* and the subsequent cross-kingdom interactions with other microorganisms in the polymicrobial biofilms are not clear. Here, we hypothesize that *C. albicans* may affect the microbial ecology and increase the cariogenicity of oral biofilms, and thus promote the development of root caries. Specifically, we

found increased colonization of *C. albicans* in root carious lesions. *C. albicans* colonization imposed significant ecological impact on oral microbiota, leading to a polymicrobial biofilm with enhanced cariogenicity. *PHR2* was identified as the major factor that determined the ecological impact and caries-promoting activity of *C. albicans*, which provided a promising target for the control of root caries.

Materials and methods

Clinical sample analyses

To investigate the association between *C. albicans* and root caries occurrence/microbial composition, we enrolled seventy volunteers aged 55–85 years old (35 root-caries subjects and 35 caries-free subjects) in this study. The study protocol was reviewed and approved by the Institution Review Board of West China Hospital of Stomatology, Sichuan University (WCHSIRB-D-2016-100). Written informed consent was obtained from all participants in the study. None of the volunteers had taken or received daily medicine, antibiotic treatment, hormone treatment or oral treatment within 3 months prior to the study. Dental plaque was collected from root carious lesions of root-caries molars and sound root surfaces of molars lacking carious lesions of root-caries patients, as well as the sound root surfaces of all molars of caries-free subjects.

Sample size was estimated using the following assumption: type I error equals 5%; type II error equals 10%; percentage of people infected oral *C. albicans* equals 30%; percentage of root-caries subjects infected oral *C. albicans* equals 72%. Based on these assumptions and using PASS 13.0 statistical software, the minimum required sample size in the root-caries subjects or caries-free controls was 30, so we included 35 subjects per group in our study.

Pooled saliva was additionally collected from ten caries-free healthy subjects without *C. albicans* infection (confirmed by PCR) for the establishment of saliva-derived biofilm. Each subject was instructed to spit 5 ml of saliva directly into a sterile collection tube. Saliva samples were then pooled together and centrifuged at $2600 \times g$ for 10 min to spin down large debris and eukaryotic cells. The supernatant was referred to as pooled saliva and used for the establishment of saliva-derived biofilm throughout this study. Part of the collected saliva was further filtered at 0.22 μm filters, and the resulting cell-free saliva was used to coat enamel blocks and glass coverslips prior to growing the in vitro biofilms. See Supplementary file for details.

Genomic DNA of dental plaque samples were isolated using QIAamp DNA micro kit (Qiagen, Valencia, CA, USA) with additional lysozyme (3 mg/ml, 1.5 h) treatment. The DNA quality was evaluated with a NanoDrop 2000

spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The detection rate of *C. albicans* was determined by PCR. *S. mutans*, *S. sanguinis*, *C. albicans* counts were quantified using qPCR. The quantification of specific microbe was further normalized by the total DNA amount of each clinical sample. See Supplementary file for details (Supplementary Tables S1 and 2).

In vitro biofilm model

S. mutans UA159, *S. sanguinis* ATCC10556, and *C. albicans* SC5314 were commercially obtained from the American Type Culture Collection, and the mutants of *C. albicans* used in this study were listed in Supplementary Table S3. The in vitro saliva-derived biofilm and three-species biofilm was incubated in SHI medium at 37 °C under anaerobic conditions for 24 h [39, 40]. For saliva-derived biofilm, 30 µl of pooled saliva and 2×10^2 cell/ml *C. albicans* or its mutants were inoculated into 1500 µl SHI medium. For three-species biofilm, inoculum for the experiment was adjusted to 2×10^6 CFU/ml of *S. mutans*, 2×10^7 CFU/ml of *S. sanguinis* and 2×10^4 cell/ml of *C. albicans* or its mutants (bacterial inoculum with similar ratio to which was detected in clinical samples).

After 24 h incubation, the biofilms were dispersed by sonication, and a dilution series of cell suspensions were prepared and spread on selective culture plates. The total colony-forming unit (CFU) of viable *S. mutans* and *S. sanguinis* was counted on Mitis Salivarius Agar plate (MSA, Hopebio, Qingdao, China) after 48 h anaerobic incubation at 37 °C, and the CFU of viable *S. mutans* was counted on the MSA plate supplemented with 0.2 U/ml bacitracin and 20% sucrose. *C. albicans* or its mutants were selectively cultured with CHROMagar Candida plate (Difco Laboratories) aerobically at 35 °C for 48 h before CFU counting. Genomic DNA of biofilms was also isolated using QIAamp DNA micro kit (Qiagen) with additional lysozyme (3 mg/ml, 1.5 h) treatment. Total RNA of yeast was isolated using Yeast RNAiso Kit (TaKaRa, Shiga, Japan). Total bacterial RNA from saliva-derived biofilm was isolated using TriZol Reagent (Invitrogen, Carlsbad, CA, USA) [41]. The DNA and RNA qualities were evaluated with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The amount of each microbe and the expression levels of *PHR1*, *PHR2*, *CRZ1*, *CRZ2*, *RIM101*, *RIM8*, *UME6*, *TPK1*, *TPK2*, *CPH1*, *CPH2*, *ldh*, *arcA*, and *ureC* were determined by qPCR. See Supplementary file for details (Supplementary Tables S2, 4, and 5).

16S rRNA sequencing and analyses

16s rRNA sequencing was performed to investigate the ecological impact of *C. albicans* or its *PHR1/PHR2* deletion

mutants on saliva-derived biofilm. PCR amplification of the nearly full-length bacterial 16S rRNA genes was performed using the forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and reversed primer (5'-ACCTTGTTACGACTT-3'). A unique 12-mer tag for each DNA sample was added to the 5'-end of both primers to pool multiple samples together. The amplicons were sequenced by the PacBio Sequel platform (Shanghai Personal Biotechnology Co., Ltd, Shanghai, China). An average of 5000 reads per sample was generated from the amplicon library. Sequence data analyses were mainly performed using QIIME (v1.8.0) and R packages (v 3.2.0), including quality control of raw data, taxonomic annotation according to NCBI database. The data were further analyzed as follows: (1) Shannon diversity index was calculated among groups. (2) The weighted UniFrac principal coordinates analysis (PCoA) and dissimilarity tests including Adonis and ANOSIM were used to examine the community differences between groups. (3) Co-occurrence analysis was performed by calculating Spearman's rank correlations between predominant taxa. Correlations with $\rho > 0.6$ and $P < 0.01$ were constructed into an association network and imported into Cytoscape software (<http://www.cytoscape.org/>) for visualization [42]. (4) The relative abundance difference of bacterial taxa between groups was compared by Kruskal–Wallis test at both the genus and species levels. (5) Microbial functions were predicted by Phylogenetic investigation of communities by reconstruction of unobserved states based on the 16S rRNA gene sequence data [43].

In vitro three-species biofilm imaging and structural analyses

To visualize the composition and structure of the biofilm, *C. albicans* or its mutants was stained with Calcofluor White (Sigma-Aldrich, St. Louis, MO, USA) [44], and fluorescent in situ hybridization (FISH) was performed as described previously to label *S. mutans* and *S. sanguinis* with species-specific probes (Supplementary Table S6) [45]. The microbe cells and extracellular polysaccharides (EPS) were labeled with SYTO 9 (Molecular Probes, Invitrogen) and Alexa Fluor 647-labeled dextran conjugate (Molecular Probes, Life Technologies, Grand Island, NY, USA) respectively [46]. Biofilm images were captured and analyzed with a laser scanning confocal microscope equipped with Fluoview SV1000 imaging software (Olympus FV1000, Tokyo, Japan). The population of microbes and EPS were analyzed based on integral optical density (IOD) with Image pro plus 6.0 (Media Cybernetics). See Supplementary file for details.

In vitro artificial caries model

To evaluate the demineralization-promoting capability of *C. albicans* and its mutants on saliva-derived biofilm, the

in vitro artificial caries model was established by seeding saliva-derived biofilm with/without *C. albicans* on human enamel blocks in SHI medium for 5 days and refreshed 1 ml medium every 24 h. The pH values of the spent media were measured with a pH meter (Thermo Fisher Scientific). The NH_3 level in the medium was determined with an ammonia assay kit (Jiancheng, Bioengineering institute, Nanjing, China) at the end of a 5-day period. After 5-day incubation, the enamel blocks were prepared for transversal micro-radiography (TMR) analysis [47]. See Supplementary file for details.

In vivo root-caries rat model

A root-caries rat model was used to further investigate the role of *C. albicans* and its mutants in the pathogenesis of root caries. The animal protocol was reviewed and approved by the animal research committee of West China School of Stomatology, Sichuan University (WCHSIRB-D-2017-113). The rat model was established as previously described with modifications (Supplementary Fig. S1) [48]. Male specific-pathogen-free Sprague-Dawley rats (aged 17 days and weighing 60 ± 5 g) were purchased from Dashuo Inc. (Chengdu, China), and were provided with cariogenic diet. Rats ($n = 5$ per group) were either infected with *C. albicans* or *S. mutans*/*S. sanguinis*, *S. mutans*/*S. sanguinis*/*C. albicans*, *S. mutans*/*S. sanguinis*/*phr2Δ/Δ*, *S. mutans*/*S. sanguinis*/*phr1Δ/Δ* with simple randomization. Rats without infection were also included as control. The rat mouths were infected by respective microbe with the same ratio as they were in the three-species biofilm as stated above. Gingivectomies were performed for all rats on day 10 after initial infection. Animals were sacrificed 6 weeks after initial infection. The dental plaque was collected for qPCR quantification, and the jaws were collected for root-caries scoring using Doff's system [49]. The determination of the root-caries score was blinded by codification of the jaws. See Supplementary file for details.

We estimated the sample size considering the variation and means of the samples calculated from the preliminary experiment. The type I error equals 5%, type II error equals 10%. Based on these assumptions and using PASS 13.0 statistical software, the minimum required sample size in each group was 5.

Statistical analyses

Each in vitro experiment was repeated three times. Statistical analysis of data, other than those obtained from microbiome sequencing, was performed with SPSS (v 16.0). Detection rates of *C. albicans* and quantitative analyses of microbial composition in the fluorescence-labeled three-species biofilm were analyzed with chi-squared test. Data obtained from

clinical samples, including quantification of *C. albicans*, *S. mutans*, *S. sanguinis* and *S. mutans*/*S. sanguinis* ratio were analyzed with Kruskal–Wallis test followed by post hoc Dunn's test due to asymmetrical distributions, and results are presented as median with interquartile range. Linear correlation and regression were also calculated between the level of *C. albicans* and *S. mutans* or *S. mutans*/*S. sanguinis* ratio in clinical samples. After test for homogeneity of variance, other data obtained from two independent groups were analyzed with *t*-test, and multiple group comparisons were performed by one-way analysis of variance test followed by Tukey's test to compare means of each group, and Dunnett *t*-test to compare the means of all other groups with the control group, and results are presented as mean $\pm$ standard deviation. Data were considered significantly different if the two-tailed *P* value was < 0.05 . Sample sizes for each experiment are detailed in the figure legends.

Results

Root carious lesions harbor increased amount of *C. albicans* and *S. mutans*

The microbial composition of supragingival dental plaque collected from root carious lesions was compared with that of sound root surfaces of root-caries subjects as well as caries-free controls. We found that *C. albicans* colonized more frequently at root carious lesions rather than at sound root surfaces of the same patients with root caries and the caries-free individuals (71.4%, 31.4%, and 28.6%, $P < 0.05$; Fig. 1a). Further quantification by qPCR showed that *C. albicans* was significantly enriched at root carious lesions [15.015 (27.386) copies/ng] compared to the sound root surfaces of root-caries patients [2.644 (8.738) copies/ng, $P < 0.05$] and the caries-free controls [0.135 (0.150) copies/ng, $P < 0.0001$; Fig. 1b]. In parallel, the root-caries lesions also showed an increased carriage of *S. mutans* [2.697×10^3 (6.284×10^3) copies/ng] as compared to the sound root surfaces of root-caries patients [5.419×10^1 (2.412×10^2) copies/ng, $P < 0.0001$] and the caries-free controls [1.060×10^1 (4.296×10^1) copies/ng, $P < 0.0001$; Fig. 1c]. A positive correlation was observed between the carriage of *C. albicans* and *S. mutans* ($r = 0.561$, $P < 0.01$; Fig. 1d). Conversely, a decreased amount of commensal *S. sanguinis* was observed in the root-caries lesions as compared to the caries-free controls (Fig. 1e). We further calculated the ratio of *S. mutans*/*S. sanguinis*, which was significantly higher in root carious lesions than that of the caries-free group ($P < 0.0001$), as well as that obtained from the sound root surfaces of root-caries patients ($P < 0.001$; Fig. 1f). More importantly, the *S. mutans*/*S. sanguinis* ratio showed a better positive correlation with the carriage of *C. albicans* detected at the same sites ($r = 0.759$, $P < 0.01$; Fig. 1g).

Fig. 1 Root carious lesions harbor increased amount of *C. albicans* and *S. mutans*. **a** The detection rate of *C. albicans* by PCR in the root carious lesions, as well as on the sound root surfaces of root-caries patients and caries-free individuals. **b**, **c** qPCR quantification of the amount of *C. albicans* and *S. mutans* in the collected clinical samples. **d** Linear correlation and regression between the amount of *C. albicans* and *S. mutans* in the supragingival plaque of all recruited subjects. **e** qPCR quantification of the amount of *S. sanguinis* in the collected

clinical samples. **f** The calculated *S. mutans*/*S. sanguinis* ratio of the corresponding samples. **g** Linear correlation and regression between the amount of *C. albicans* and *S. mutans*/*S. sanguinis* ratio in the supragingival plaque of all recruited subjects. The results are presented as median with interquartile range (**a–c**, **e**, **f**: $n = 35$ per group; **d**, **g**: $n = 105$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns not significant). RC root-caries group; CF caries-free group; *S. s* *S. sanguinis*; *S. m* *S. mutans*; *C. a* *C. albicans*.

C. albicans alters the microbial ecology of oral biofilm

To investigate the ecological impact of *C. albicans* on the polymicrobial oral biofilm, we inoculated *C. albicans* in a

saliva-derived biofilm. Data obtained from full-length 16S rRNA sequencing showed that *C. albicans* colonization imposed significant ecological impact on the oral biofilms. The α -diversity of oral biofilm was unaltered in the presence of *C. albicans* (Fig. 2a). However, the weighted PCoA

based on Bray–Curtis distance (Fig. 2b) and dissimilarity tests including Adonis and ANOSIM showed distinct microbial clusters with/without *C. albicans* (ANOSIM: $R = 0.5562$, $P = 0.019$; Adonis: $R^2 = 0.34198$, $P = 0.012$;

Table 1). We also constructed co-occurrence ecological networks at the species level to better delineate how saliva-derived biofilm assemble and whether *C. albicans* colonization made significant impact on the oral microbial

Fig. 2 *C. albicans* alters the microbial ecology of oral biofilms. **a** The Shannon indices and **b** the PCoA analysis on the polymicrobial oral biofilm in the absence/presence of *C. albicans*. **c** Network inferences of microbial relationships in biofilms. Each node represents an OTU, and each edge represents a significant pairwise association. Red lines indicate positive relationships while green lines represent negative relationships. **d** The relative abundance of the significantly different phyla and genera in the saliva-derived biofilm with/without *C. albicans*. **e** Relative abundance of the 20 most predominant oral bacteria at species level and **f** relative abundance of the significantly different species in the oral biofilm with/without *C. albicans*. **g** Prediction of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of oral biofilm in the absence/presence of *C. albicans*. The results are presented as mean $\pm$ standard deviation ($n = 4$ or 5 per group; * $P < 0.05$; ** $P < 0.01$; ns not significant). *S. s* *S. sanguinis*; *S. m* *S. mutans*; *C. a* *C. albicans*.

community network topology. Specifically, co-culture with *C. albicans* resulted in a salivary microbiome network of fewer nodes and links, and the negative relationship between species was slightly reduced (Fig. 2c).

At phylum level, *C. albicans* significantly increased the growth of *Firmicutes* and decreased the growth of *Proteobacteria* (Fig. 2d). At genus level, *C. albicans* significantly improved the growth of *Streptococcus* and decreased the growth of *Klebsiella*, *Haemophilus*, and *Rothia* (Fig. 2d). Consistently, at species level, *C. albicans* significantly decreased the abundance of *Klebsiella pneumoniae*, *Haemophilus parainfluenza*, and *Rothia dentocariosa* and increased the abundance of *Gemella sanguinis* (Fig. 2e, f). Furthermore, the saliva-derived biofilm when co-culture with *C. albicans*, showed increased representation of predicted Kyoto Encyclopedia of Genes and Genomes pathways involved in human infectious disease and cellular processes including cell growth and death as well as transport and catabolism, and cell motility-associated pathway was decreased (Fig. 2g). All these data indicate that *C. albicans* could impose significant ecological impact on the oral microbiome.

C. albicans robustly interacts with oral streptococci in the polymicrobial biofilm

As the sequencing data showed an increased abundance of *Streptococcus* in the presence of *C. albicans*, we further quantified the carriage of *C. albicans* and *S. mutans*/*S. sanguinis* in the oral biofilms. Co-culture of *C. albicans* with oral biofilms synergistically promoted the growth of both *C. albicans* and *S. mutans*, while the growth of *S. sanguinis* was unchanged as demonstrated in either saliva-derived biofilms (Fig. 3a, b) or three-species biofilms by FISH, qPCR quantification (Fig. 3c and Supplementary Fig. S2a, b) as well as viable CFU counting (Supplementary Fig. S2c, d). Accordingly, the ratio of *S. mutans*/*S. sanguinis* was increased in the presence of *C. albicans* (Fig. 3d,

Table 1 Dissimilarity tests (Adonis and ANOSIM) for the biofilm microbiota.

	ANOSIM		Adonis	
	<i>R</i>	<i>P</i>	<i>R</i> ²	<i>P</i>
Over all	0.2963	0.008	0.31515	0.003
Saliva VS Saliva + <i>C. albicans</i>	0.5562	0.019	0.34198	0.012
Saliva VS Saliva + <i>phr2</i> Δ/Δ	-0.136	0.914	0.05396	0.968
Saliva VS Saliva + <i>phr1</i> Δ/Δ	0.352	0.036	0.25467	0.016

Supplementary Fig. S2e, f). In addition, the biofilm generated more EPS in the presence of *C. albicans* as shown by laser scanning confocal microscopy (Fig. 3e). *EFG1*- and *CPH1*-deficient mutants of *C. albicans* were constructed to further investigate if the candidal–streptococcal interactions in the polymicrobial biofilm were hyphae-dependent. Deletion of either *EFG1* or *CPH1* did not affect the carriage of *S. mutans*, however, *C. albicans* and *S. sanguinis* in the polymicrobial biofilm were unchanged or slightly decreased as compared to the WT strain (Fig. 3f, g and Supplementary Fig. S2g–j). In parallel, the *S. mutans*/*S. sanguinis* ratio was unchanged by the deletion of *EFG1* and *CPH1* compared with the biofilm co-cultured with the *C. albicans* WT strain (Fig. 3h and Supplementary Fig. S2k, l).

C. albicans enhances the cariogenicity of oral biofilm and promotes the development of root caries

We further investigated if the altered microbial ecology in the presence of *C. albicans* could lead to increased cariogenicity of oral biofilm. *C. albicans* alone was not acidogenic in our culture conditions. However, it significantly promoted the acidogenicity of the saliva-derived biofilm as reflected by a lower supernatant pH along with decreased ammonia production relative to the *C. albicans*-free biofilm (Fig. 4a, b). Consistently, *C. albicans* significantly upregulated acid production-associated gene *ldh*, and downregulated ammonia production-associated genes *arcA* and *ureC* of the oral biofilm (Fig. 4c). In addition, data obtained from TMR showed that inoculation of *C. albicans* significantly increased the demineralized lesion depth and mineral loss of tooth hard tissue caused by the saliva-derived biofilm in the in vitro artificial caries model (Fig. 4d–f).

We employed a root-carries rat model to validate the role of *C. albicans* in the pathogenesis of root caries in vivo. Rats co-infected with *C. albicans*, *S. mutans*, and *S. sanguinis* showed increased microbial carriage in the dental plaque and an exacerbated development of root caries. Specifically, infection with *C. albicans* alone caused nearly no root caries, while co-infection with *C. albicans*/*S. mutans*/*S. sanguinis* caused significantly more severe root caries compared to the rats infected with *S. mutans*/*S. sanguinis* (Fig. 4g, h). A

Fig. 3 *C. albicans* robustly interacts with oral streptococci in the polymicrobial biofilm. **a**, **b** qPCR quantification of the amount of *C. albicans*, *S. mutans* and *S. sanguinis* in the saliva-derived biofilm. **c** Representative images of fluorescence-labelled *S. mutans* (green), *S. sanguinis* (red), and *C. albicans* (blue) within three-species biofilm and quantitative analyses of microbial composition. Scale bar, 60 μ m. **d** The *S. mutans*/*S. sanguinis* ratio in the saliva-derived biofilm quantified by qPCR. **e** Representative confocal images of double fluorescence-labelled three-species biofilm. Bacteria and *C. albicans*

were labelled green, EPS was labelled red, and quantitative analyses of microbe/EPS within biofilm. Scale bar, 100 μ m. **f–h** qPCR quantification of *C. albicans* or its *EFG1/CPH1* deletion mutants, *S. mutans* and *S. sanguinis* in the saliva-derived biofilm, and the corresponding *S. mutans*/*S. sanguinis* ratio. The control group of (**g**) was saliva-derived biofilm. The results are presented as mean $\pm$ standard deviation ($n = 5$ per group; * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; ns not significant). *S. s* *S. sanguinis*; *S. m* *S. mutans*; *C. a* *C. albicans*.

significant increased carriage of *C. albicans* (16.4-fold) was observed in rats co-infected with *C. albicans*/*S. mutans*/*S. sanguinis* as compared to the group only infected with *C. albicans* (Fig. 4i). In parallel, the *C. albicans*/*S. mutans*/*S.*

sanguinis co-infection group showed increased colonization of *S. mutans* as compared to the *S. mutans*/*S. sanguinis* infected group (Fig. 4j). In addition, the *S. mutans*/*S. sanguinis* ratio was also increased in the presence of *C. albicans*

Fig. 4 *C. albicans* enhances the cariogenicity of oral biofilm and promotes the development of root caries. **a** Dynamic pH values of the spent media of saliva-derived biofilm in the absence/presence of *C. albicans*. **b** NH_3 production by the saliva-derived biofilm on the 5th day after inoculation. **c** Relative expression of *ldh*, *arcA*, and *ureC* of the 24 h saliva-derived biofilm. **d** Representative transversal micro-radiography (TMR) images of human enamel blocks demineralized by saliva-derived biofilm in the absence/presence of *C. albicans*. Scale bar, 100 μm . **e**, **f** Quantitative analyses of the lesion depth and mineral

loss of the demineralized human enamel blocks by TMR. **g** Representative photographs of mandibular molars from gingivectomized rats infected with *S. mutans*, *S. sanguinis*, and/or *C. albicans* for 6 weeks. Black arrows indicated root carious lesions. **h** Root-caries scores according to Doff's system. **i**, **j** Levels of *C. albicans*, *S. mutans*, and *S. sanguinis* in in vivo root-caries rat model quantified by qPCR. Data are presented as mean $\pm$ standard deviation ($n = 5$ per group; $^{*}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns not significant; nd not detectable). *S. s* *S. sanguinis*; *S. m* *S. mutans*; *C. a* *C. albicans*.

as compared to the group only infected with *S. mutans*/*S. sanguinis* (Supplementary Fig. S3).

PHR2 is the major contributor to the successful colonization of *C. albicans* in the polymicrobial biofilm under cariogenic condition

Since response to extreme environmental pH is critical for the pathogenicity of *C. albicans*, the pH-related genes of *C. albicans* were further investigated in the polymicrobial biofilms. *PHR2* gene expression was significantly upregulated among the pH-related genes of *C. albicans* in the three-species biofilms under cariogenic conditions (Fig. 5a). Deletion of *PHR2*

significantly suppressed the candidal–streptococcal interactions. Deletion of *PHR2* significantly reduced the growth of *C. albicans* in the polymicrobial biofilms as quantified by qPCR and FISH (Fig. 5b–d). In addition, *S. mutans*/*S. sanguinis* ratio, as well as the growth of *S. mutans* and *S. sanguinis* in the *S. mutans*/*S. sanguinis*/*phr2* Δ/Δ three-species biofilm was comparable to those in the *S. mutans*/*S. sanguinis* dual species biofilm (Fig. 5c–e and Supplementary Fig. S4a). Deletion of other pH-related genes such as *PHR1*, *CRZ1*, *CRZ2*, *RIM101*, *RIM8*, *UME6*, *TPK1*, *TPK2*, *CPH1*, and *CPH2* also showed a similar trend, but to a lesser extent, regarding the growth of *C. albicans* and streptococcal composition in the three-species biofilms (Fig. 5b, e and Supplementary Fig. S4a). Consistent

data regarding the growth of each species in the three-species biofilm were observed by CFU counting (Supplementary Fig. S4b–d).

Consistent with data obtained from three-species biofilm, *PHR2* gene expression was also significantly upregulated by 13.8-fold when *C. albicans* was co-cultured with saliva-

Fig. 5 *PHR2* contributes to the success colonization of *C. albicans* in the polymicrobial biofilm under cariogenic condition. **a** The pH-relative genes expression of *C. albicans* in the *S. mutans*/*S. sanguinis*/*C. albicans* three-species biofilm. The control group was *C. albicans*-only biofilm. **b** qPCR quantification of *C. albicans* and its mutants in the three-species biofilm. The control group was *C. albicans*-only biofilm. **c** Representative images of fluorescence-labelled *S. mutans* (green), *S. sanguinis* (red), and *C. albicans* or its *PHR1*/*PHR2* mutants (blue) within three-species biofilm. Scale bar, 60 μ m. **d** Quantitative analyses of microbial composition in the fluorescence-labelled three-species biofilm based on IOD. **e** *S. mutans*/*S. sanguinis* ratio in the three-species biofilm quantified by qPCR. The control group was *S. mutans*/*S. sanguinis* two-species biofilm. **f** PCoA analysis of the polymicrobial oral biofilm in the absence/presence of *C. albicans* or its *PHR1*/*PHR2* deletion mutant. **g** Relative abundance of the 20 most predominant oral bacteria in the absence/presence of *C. albicans* at genus level. **h** The relative abundance of *Streptococcus* in saliva-derived biofilm in the absence/presence of *C. albicans* or its mutants. **i** Quantification of *S. mutans* and *S. sanguinis* in oral biofilm by qPCR. The control group was saliva-derived biofilm. Data are presented as mean $\pm$ standard deviation (**a**, **b**, **e**: $n = 3$ per group; **c**, **d**, **i**: $n = 5$ per group; **f**–**h**: $n = 5$ or 4 per group; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns not significant). *S. s* *S. sanguinis*; *S. m* *S. mutans*; *C. a* *C. albicans*.

derived biofilm, while *PHR1* gene expression was unchanged (Supplementary Fig. S4e). Of note, although deletion of *PHR2* did not affect the growth of *C. albicans* when grown alone, the growth of *phr2* Δ/Δ was significantly reduced when co-cultured with saliva-derived biofilm as compared to its WT strain (Supplementary Fig. S4f). The impact of *C. albicans* on the microbial ecology of oral biofilm was also *PHR2*-dependent. Both PCoA (Fig. 5f) and dissimilarity tests (ANOSIM: $R = -0.1360$, $P = 0.914$; Adonis: $R^2 = 0.05396$, $P = 0.968$; Table 1) showed no significant differences in the microbial community structure in oral biofilm with/without *phr2* Δ/Δ . *phr2* Δ/Δ also showed little impact on the microbial composition of the oral biofilm, particularly the relative abundance of *Streptococcus* (Fig. 5g, h and Supplementary Fig. S4g). More importantly, the abundance of *S. mutans*, as well as the *S. mutans*/*S. sanguinis* ratio, was unchanged when the saliva-derived biofilms were grown with *phr2* Δ/Δ , as compared with the biofilm free of *C. albicans* (Fig. 5i and Supplementary Fig. S4h). However, *phr1* Δ/Δ showed similar synergistic interactions with streptococci in the saliva-derived biofilm as compared to its WT strain (Fig. 5f–i and Supplementary Fig. S4f–h).

PHR2 deletion deprives *C. albicans* of the cariogenicity-promoting ability on oral biofilm

To further investigate whether the cariogenicity-promoting ability of *C. albicans* on oral biofilm is dependent of *PHR2* or not, an in vitro artificial caries model and a root-caries rat model were further used. When the *PHR2* was deleted, the cariogenicity-promoting effects of *C. albicans* was

abolished, as reflected by the unchanged supernatant pH, ammonia production as well as *ldh*, *arca*, or *ureC* gene expression of saliva-derived biofilms with/without *phr2* Δ/Δ (Fig. 6a, b and Supplementary Fig. S5). Consequently, the saliva-derived biofilm, when co-cultured with *phr2* Δ/Δ , induced less demineralization on the tooth hard tissue as compared to the biofilm co-cultured with WT *C. albicans* (Fig. 6c–e). Consistently, the synergistic interactions between *C. albicans* and streptococci on the development of root caries in vivo were also abolished when *PHR2* gene was deleted (Fig. 6f–i and Supplementary Fig. S6). Conversely, deletion of *PHR1* did not alter the cariogenicity-promoting effect of *C. albicans* on oral biofilms (Fig. 6a–i, and Supplementary Figs. S5 and S6).

Discussion

Dental caries is a result of microbial dysbiosis characterized by the enrichment of acidogenic pathogens and depletion of alkali-generating commensal microbes within the plaque biofilm [3, 50]. Root caries is a subtype of dental caries that specifically affects the exposed root of tooth, and a significant microbial dysbiosis in the root carious lesions has also been reported [9]. However, factors that drive the microbial alteration which predisposes an individual to root caries are still unclear. We found increased carriage of *C. albicans* in the root-caries lesions, which was positively correlated with increased number of *S. mutans* as well as *S. mutans*/*S. sanguinis* ratio. Further validation with in vitro polymicrobial biofilm models demonstrated that *C. albicans* colonization caused significant microbial dysbiosis characterized with distinct microbial composition and structure as compared to the biofilm in the absence of *C. albicans*. These findings are in agreement with previous studies showing that *C. albicans* influenced the microbial composition of saliva biofilms [51, 52]. In addition, recent study by Delaney et al. also showed that candida species had a subtle impact on the bacterial microbiome of denture patients and significantly influenced specific genera [53]. Our data showed that *C. albicans* enriched *Streptococcus* in the polymicrobial biofilms, particularly increased the ratio of *S. mutans*/*S. sanguinis*, leading to a more cariogenic biofilm that promoted the development of root caries.

Previous studies showed that *C. albicans* could robustly interact with oral streptococci but with distinct mechanisms. Cell-cell adhesion mediates *C. albicans*/Mitis Group Streptococci interactions [23, 24, 33, 54–58], while EPS plays a critical role in the *C. albicans*/*S. mutans* interactions [24, 33, 54, 55, 57–59] and also contributes to the antifungal resistance of *C. albicans* in the mixed biofilm [60]. Deletion of genes responsible for hyphae formation of *C. albicans* neither reduces microbial carriage nor inhibits

Fig. 6 *PHR2* deletion deprives *C. albicans* of the cariogenicity-promoting ability on oral biofilm. **a** Dynamic pH values of the spent media of saliva-derived biofilm in the absence/presence of *C. albicans* or its *PHR1/PHR2* deletion mutants. **b** NH_3 production by the saliva-derived biofilm on the 5th day after inoculation. **c** Representative TMR images of human enamel blocks demineralized by saliva-derived biofilm in the absence/presence of *C. albicans*. Scale bar, 100 μm . **d**, **e** Quantitative analyses of the lesion depth and mineral loss of the demineralized human enamel blocks by TMR. **f** Representative photographs of mandibular molars from gingivectomized rats infected

with *S. mutans*, *S. sanguinis*, and/or *C. albicans*/its mutants for 6 weeks. Black arrows indicated root carious lesions. **g** Root-caries scores according to Doff's system. **h**, **i** Amount of *C. albicans*/its mutants and *S. mutans*/*S. sanguinis* in the root-caries rat model quantified by qPCR. The control group of (i) was rats infected with *S. mutans* and *S. sanguinis*. Data are presented as mean $\pm$ standard deviation ($n = 5$ per group; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns not significant; nd not detectable). *S. s* *S. sanguinis*; *S. m* *S. mutans*; *C. a* *C. albicans*.

cariogenicity of *C. albicans*/*S. mutans* mixed biofilm [57]. Consistently, the current study also showed that deletion of *EFG1* and *CPH1* (key hyphae-associated morphological regulators) [61] did not reduce the number of fungal cells in the polymicrobial biofilm under cariogenic condition as compared to the wild-type *C. albicans*. This is likely due to the fact that the robust production of extracellular α -glucans by *S. mutans* via glycosyltransferases (GTFs) under sugar-rich condition plays a major role in mediating the coaggregation and biofilm formation of *C. albicans*, which

in turn promotes the growth and GTFs expression of *S. mutans* by secreting polysaccharides, quorum sensing molecules as well as metabolic cross-feedings [62–66]. The filamentation-independent synergistic interactions between *C. albicans* and *S. mutans* under cariogenic condition ultimately lead to a biofilm with enriched *C. albicans* and acidogenic *S. mutans*, and the latter competes the growth of *S. sanguinis* and counters the co-adherence of *C. albicans*/*S. sanguinis*, leading to an unchanged amount of *S. sanguinis* in the mixed microbial consortium. Deletion of *EFG1* and

CPH1 can compromise the co-adherence of *C. albicans*/*S. sanguinis* but still promote the accumulation of *S. mutans* in the polymicrobial biofilm, and this likely explains the reduced amount of *S. sanguinis* observed in the presence of *EFG1* and *CPH1* mutants.

Although the deletion of hyphae-related morphological regulators of *C. albicans* such as *EFG1* and *CPH1* did not affect its co-existence and synergistic interactions with *S. mutans* in the polymicrobial biofilm, the current study found that deletion of pH-relative genes, particularly *PHR2* which is associated with acid adaptation of *C. albicans*, significantly reduced the number of fungal cells in the cariogenic biofilm. *PHR1* and *PHR2* are functional homologs regulated by extracellular pH through the *Rim 101*-dependent pathway in opposite ways. *PHR1* is expressed at pH values of 5.5 or higher, whereas *PHR2* has the reverse expression pattern [67–69]. They are critical factors that determine environment adaptation of *C. albicans* under differed pH conditions [69–71]. The current study demonstrated that *PHR2*, rather than *PHR1* and other pH-relative genes, was significantly upregulated when *C. albicans* was co-cultured with oral biofilms under cariogenic conditions. More importantly, deletion of *PHR2* partially restored the microbial ecology of the polymicrobial biofilm, resulting in a biofilm with decreased acidogenicity, compromised demineralizing capability and reduced cariogenicity as recapitulated by the root-caries rat model. It is noteworthy that deletion of *PHR2* gene only abolished the cariogenicity-promoting effects of *C. albicans* on the biofilms, but the innate cariogenicity of oral biofilms under cariogenic conditions still existed. These findings suggest that the robust interactions between *C. albicans* with oral streptococci under cariogenic conditions make the traditional hyphae-related virulence genes not the optimal target for the reduction of biofilm cariogenicity at root-caries lesions. However, *PHR2* that adapts the co-existence of *C. albicans* with oral streptococci under acidic/cariogenic conditions provides a potential target for the disruption of candidal–streptococcal interactions and their synergistic pathogenicity in the development of root caries.

Taken together, our data demonstrated the critical role of *C. albicans* infection in development of root caries. *C. albicans* colonization caused significant microbial dysbiosis characterized with an increased abundance of acidogenic/aciduric *S. mutans*, leading to a more cariogenic biofilm that promoted the development of root caries. *PHR2* is the major factor that determines the successful colonization and ecological impact of *C. albicans* in a mixed microbial consortium under cariogenic conditions, and thus may represent a promising target for the control of root caries with candidal involvement. Further study is warranted to investigate the molecular target(s) and signaling pathways via which *C. albicans* binds to and interacts with oral bacteria and

consequently elevates the virulence of polymicrobial oral biofilm.

Data availability

Microbiome sequencing data have been deposited in public database Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/Traces/sra>) with accession no. PRJNA613946. All data sets generated and/or analyzed in the current study are available from the corresponding author on reasonable request.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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