APMIS JOURNAL OF PATHOLOGY, MICROBIOLOGY AND IMMUNOLOGY

DOI 10.1111/apm.13327

Investigating the role of *Candida albicans* as a universal substrate for oral bacteria using a transcriptomic approach: implications for interkingdom biofilm control?

CHRISTOPHER DELANEY,[1,2,*] SUSANTH ALAPATI,[1,2] MUHANNA ALSHEHRI,[1,2] DOMINIKA KUBALOVA,[1,2] CHANDRA LEKHA RAMALINGHAM VEENA,[1,2] SUMAYA ABUSREWIL,[1,2] BRYN SHORT,[1,2] DAVID BRADSHAW[3] and JASON L. BROWN[1,2,*]

[1]Oral Sciences Research Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, College of Medical, Veterinary and Life Sciences, University of Glasgow; [2]Glasgow Biofilm Research Network (www.glasgowbiofilms.ac.uk), Glasgow; and [3]R&D Innovation, Haleon, Weybridge, UK

Delaney C, Alapati S, Alshehri M, Kubalova D, Veena CLR, Abusrewil S, Short B, Bradshaw D, Brown JL. Investigating the role of *Candida albicans* as a universal substrate for oral bacteria using a transcriptomic approach: implications for interkingdom biofilm control?. APMIS. 2023.

Candida albicans is frequently identified as a colonizer of the oral cavity in health and has recently been termed a "keystone" commensal due to its role on the bacterial communities. However, the role that *C. albicans* plays in such interactions is not fully understood. Therefore, this study aimed to identify the relationship between *C. albicans* and bacteria associated with oral symbiosis and dysbiosis. To do this, we evaluated the ability of *C. albicans* to support the growth of the aerobic commensal *Streptococcus gordonii* and the anaerobic pathogens *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in the biofilm environment. RNA-Sequencing with the Illumina platform was then utilized to identify *C. albicans* gene expression and functional pathways involved during such interactions in dual-species and a 4-species biofilm model. Results indicated that *C. albicans* was capable of supporting growth of all three bacteria, with a significant increase in colony counts of each bacteria in the dual-species biofilm ($p < 0.05$). We identified specific functional enrichment of pathways in our 4-species community as well as transcriptional profiles unique to the *F. nucleatum and S. gordonii* dual-species biofilms, indicating a species-specific effect on *C. albicans*. *Candida*-related hemin acquisition and heat shock protein mediated processes were unique to the organism following co-culture with anaerobic and aerobic bacteria, respectively, suggestive that such pathways may be feasible options for therapeutic targeting to interfere with these fungal-bacterial interactions. Targeted antifungal therapy may be considered as an option for biofilm destabilization and treatment of complex communities. Moving forward, we propose that further studies must continue to investigate the role of this fungal organism in the context of the interkingdom nature of oral diseases.

Key words: Biofilms; *Candida albicans*; *Streptococcus gordonii*; *Fusobacterium nucleatum*; *Porphyromonas gingivalis*; fungal-bacterial interactions; RNA-sequencing.

Christopher Delaney and Jason L Brown, Oral Sciences Research Group, Glasgow Dental School, School of Medicine, Dentistry and Nursing, University of Glasgow, 378 Sauchiehall Street, Glasgow G2 3JZ, UK. e-mail: christopher.delaney@glasgow.ac.uk and jason.brown@glasgow.ac.uk

Fungal microorganisms have long been recognized as inhabitants of the oral cavity in upwards of ~50% of healthy individuals using culture-based methodologies [1–3]. A collation of studies from this time predicted that the oral fungal carriage rates can be as high as ~70% [4]. Fungal colonization of various oral niches has further been confirmed in recent decades using sequencing platforms [5, 6]. These observations are suggestive that most oral fungal microorganisms are largely commensal in nature: indeed, *Candida* spp. such as *Candida albicans*, a commonly identified colonizer of various oral tissues [5], exists as a pathobiont that has the ability to shift its phenotype to drive infection [7, 8]. This phenotypic shift is largely evident in immunosuppressed individuals and those undergoing treatment for cancer, which can lead to diseases such as oral candidiasis [9]. The

Received 3 March 2023. Accepted 21 April 2023

Candida genus has readily been detected in cases of oral candidiasis, with the incidence increasing in patients undergoing cancer therapy [10, 11].

Most infections of the oral cavity seldom arise from mono-species colonization. As such, *C. albicans* has been co-isolated alongside oral microbial communities in biofilm-related diseases such as denture stomatitis [12], dental caries [13], periodontitis [14], and angular chelitis [15, 16]. Such interactions are largely beneficial for a variety of commensal and pathogenic bacterial species, with the fungi providing a mechanical support structure for adhesion and colonization: a fungal yeast cell biovolume is almost 150-fold larger (~70 μM) than an average bacterial cell (~0.5 μM), thereby providing an ideal substrata for bacterial attachment [17]. Furthermore, it is proposed that the surface area of a hyphal cell is approximately 2- fold greater than that of a yeast cell, offering an even greater landscape for bacterial colonization [18]. As such, cell–cell interactions between *C. albicans* and bacterial species associated with the above diseases such as *Streptococcus mutans*, *Porphyromonas gingivalis* and *Staphlyococcus aureus* have all been reported in the literature [19–21]. For example there is evidence to suggest that *S. mutans* and *P. gingivalis* all actively bind to *C. albicans* through expression of adhesions such as GtfB [22, 23] and InlJ [24], respectively. Physical interactions between *C. albicans* and *S. aureus* also exist, with the bacteria adhering to the fungal hyphae to facilitate tissue invasion and dissemination during biofilm infection [25–27].

Beyond physical adhesion mechanisms, *Candida*-bacterial interactions also result in important mutualistic relationships that are critical for key biological processes in the microbial community at a transcriptional, metabolite and protein level, as well as driving physiological changes in the immediate microenvironment of the cells. For example, GftB secretion by *S. mutans* drives carbohydrate breakdown providing a carbon source for *C. albicans* [22], while also upregulating expression of key virulence and adhesion genes such as *HWP1*, *ALS1* and *ALS3* in the fungus [28]. Conversely, *C. albicans* can further support *S. mutans* growth aside from its adhesive capabilities by upregulating gene pathways related to sugar metabolism [29] and driving increased bacterial growth independent of cell–cell contact through metabolite production [30]. Other bacterial species of the oral cavity also possess similar symbiotic associations with *C. albicans* beyond direct cellular interactions. For example, *Streptococcus gordonii*, an oral commensal bacteria, and *C. albicans* develop biofilms together through a combination of both physical interactions and production of chemical or protein "signals" such as quorum sensing molecules and secretory proteases [31–33]. Similar pathways are present in interactions between *P. gingivalis* and *C. albicans*: Direct contact between these two microorganisms resulted in *P. gingivalis* survival in oxic conditions that would be otherwise lethal to the bacterium, likely arising from a dense hyphal network that resulted in O_2 depletion by *C. albicans* [34]. Such protective "shielding" in these dual-species communities can also promote *P. gingivalis* invasion of the host *via* immune evasion [35].

Due to all these interactions discussed above, it has recently been proposed that *C. albicans* acts as a "keystone" commensal [17]. This can be explained by the organism being a minority with respect to the overall bioburden (~0.1% of the total microbial load), but still providing an important physical scaffold and influencing the local microenvironment within the biofilm bringing changes to micronutrient availability, oxygen consumption, and pH buffering [17]. We have confirmed that such *Candida*-bacterial interactions are present in more complex biofilm communities, demonstrating that *C. albicans* enhances bacterial colonization in a 6- and 11-species soft and hard oral tissue models, while buffering the pH of the spent media [36]. However, the effects that such mixed communities have on *Candida* at a cellular level are largely unknown.

Taken together, the aforementioned interplay between fungi and bacterial species in the oral cavity presents such interactions as a possible avenue for therapeutic target. Given the potent role that fungal microorganisms such as *C. albicans* play in providing a substrata for bacterial attachment, it could be postulated that destruction of this microbial scaffold could have huge implications in the pathogenesis of various oral diseases. This could be achieved through directing treatment against the fungal element of these biofilms, using antifungal therapies or targeting specific adhesion molecules [37]. In recent years, the development of molecular techniques such as CRISPR-Cas9 technologies and increased availability of omics approaches for in-depth analyses has made targeting specific genetic pathways and/or biological processes a feasible, yet exploitable, treatment strategy [38, 39]. However, the role that bacteria play in regulating such pathways in the fungal species is understudied.

The purpose of this study was to investigate the transcriptional response in *C. albicans* following co-culture with three orally relevant bacterial species when grown as a dual-species or a 4-species biofilm model using a transcriptomic approach. We envisage that such analysis may highlight gene pathways at work arising from such interactions, with the hope of identifying key "genes" that could be

targeted to remove this "keystone" commensal, and thus preventing such challenging fungal-bacterial interkingdom interactions.

MATERIALS AND METHODS

Microbial culture

Yeast and bacterial cultures were prepared according to our previously standardized methods [40]. Briefly, *C. albicans* SC5314 type strain was first cultured on solid agar (Sabouraud; SAB) for 24–48 h, then propagated overnight in Yeast Peptone Dextrose liquid culture at 30 °C. Cells were then retrieved by centrifugation for 5 min and washed twice with phosphate buffered saline (PBS) before being standardized to 1×10^7 cells/mL by counting *via* hemocytometer. Bacterial cultures for *P. gingivalis* ATCC 33277 *and F. nucleatum* ATCC 10953 were prepared anaerobically firstly in Fastidious Anaerobic Agar (FAA) for 48–72 h and then sub-cultured in Schaedlers broth for 24 h. *S. gordonii* ATCC 35105 was initially grown on Columbia Blood Agar (CBA) then propagated in Tryptic Soy Broth in 5% CO_2 overnight. All bacterial cultures were centrifuged for 5 min and washed twice with PBS before being standardized by spectrophotometry at 550 nm. Bacteria were standardized to 1×10^8 cells/ml at an OD of 0.2 for *P. gingivalis and F. nucleatum* and 0.5 for *S. gordonii*, respectively. Yeast and bacteria were used for biofilm growth as described below following preparation of standardized cells.

Biofilm preparation

All biofilms were prepared in 1:1 media comprised of Roswell Park Memorial Institute (RPMI-1640) and Todd Hewitt Broth (THB) supplemented with 0.01 mg/mL hemin and 2 μg/mL menadione to support the growth of bacterial and fungal biofilms as previously described [40, 41]. For colony counting and compositional analysis, all biofilms were prepared in 24-well polystyrene plates at an initial concentration of 1×10^7 cells/mL for bacteria and 1×10^6 cells/mL for yeast. For the dual-species or 4-species model, bacteria were added following 4 h of *C. albicans* biofilm growth. Biofilms that were grown with a preformed 4-h *Candida* biofilm followed by the introduction of either single or all three bacteria were also replenished with 1:1 media containing the standardized bacteria before being grown for a further 20 h. For RNA-sequencing experiments, biofilms were grown in 75 cm^2 cell culture flasks to provide enough bioburden for RNA extraction. All biofilm models were grown for a total of 24 h in 5% CO_2 at 37 °C.

Colony counting and composition analysis

Colony counts and/or biofilm compositional analysis for single, dual, or multi-species biofilms prepared by initial growth of *Candida* for 4 h followed by 20 h with bacteria as described above was performed using validated methods for Colony Forming Equivalents (CFE) and Colony Forming Units (CFU) [42]. Conditions remained the same as previously stated with the amendment of the growth of biofilms on Thermanox™ coverslips allowing for the removal of coverslips and sonication for 15 min in 1 mL of PBS at 35 hz. For CFU counting, the sonicate was serially diluted in 1/10 dilutions before each dilution was plated on SAB (for *Candida* counts) and FAA (*P. gingivalis* or *F. nucleatum*) or CBA (*S. gordonii*) according to the Miles and Misra method [43].

For CFE analysis, propidium monoazide (PMA) was added to 500 μL of the sonicate in PBS and incubated in the dark for 10 min prior to treatment of the sample with a 650 W halogen light. DNA extraction was performed by QIAamp DNA mini kit according the manufacturers protocol. Real-time qPCR was then used to determine the live and total cell number. Each reaction mixture contained 10 μL of Fast SYBR® Green Master Mix, 1 μL of each forward and reverse species primers at 10 μM, 1 μL of either PMA treated or untreated DNA, and 7 μL nuclease free water. PCR cycles were performed on the Step-One plus machine using the following stages 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. CFEs were then calculated using standard curves for each species using serially diluted DNA. All primers used were as previously described [40].

RNA-sequencing

Following biofilm growth, the supernatant was removed from the biofilms before being stored in 1 mL of RNA*later* at −80 °C. RNA was extracted from all single species fungal, dual and multi-bacterial species biofilms by the RiboPure™ Purification kit according the manufacturers guidance. RNA quantity was determined using the NanoDrop spectrophotometer ND-1000 with a total of 2.5 μg deemed adequate for sequencing, and RNA integrity number (RIN) was determined using the Bioanalyser with a RIN of > 7 being necessary for sequencing. *Candida* transcripts were prepared by PolyA selection and sequenced on the Illumina NovaSeq6000 at the Edinburgh Genomics sequencing facility (http://genomics.ed.ac.uk/).

Paired end 100 bp reads were retrieved from the Illumina sequencing performed by Edinburgh Genomics. Preprocessing was performed on Raw data using our in house pipeline. The reads were first quality controlled by the removal of low quality sequences and removal of adapters using Trimmomatic (v0.38) [44]. A haploid version of the *Candida* genome database (CGD) genome (SC5314_A22) was prepared by removal of the B variants of the chromosomes before the resulting fasta file was indexed using Hisat2 (v2.1.0) [45, 46]. Hisat2 was then again used to align the remaining trimmed reads to the haploid genome. The aligned SAM files were converted to BAM compressed format using samtools (v1.7) before HTSeq-count (v0.11.0) was implemented to count the occurrence of each transcript [45]. HTSeq-count files for each sample were compiled and imported into R using DESeq2 (v1.26) [47]. The counts were compiled into one data frame before differential analysis was performed using DESeq2. Subsequently, the differentially expressed genes between our single species fungal biofilm and interkingdom biofilms with a $\log_2FC > 1.5$ and FDR adjusted p-value < 0.05 were selected for further analysis. Differentially expressed transcripts between each condition were represented in an UpsetPlot using UpsetR (v1.4.0) which displays data in a Venn diagram like manner for larger numbers of samples.

Upregulated *Candida* genes in the dual- or multi-species biofilms compared to *Candida* only biofilms were compared to find unique and overlapping genes between the dual- and multi-species biofilms. Over representation analysis (ORA) was performed using the hypergeometric analysis in the R package ClusterProfiler (v3.14.3) for the unique and overlapping genes in each comparison based upon the *C. albicans* Gene Ontological terms.

Data presentation and statistical analysis

RNA-sequencing data were plotted in R using a combination of the packages listed above, base R and ggplot2 (v.3.3.0). Graph generation related to the CFU- and CFE-derived data was achieved in GraphPad Prism 9. When it was pertinent a t-test was performed to compare between the CFU counts in the biofilms grown as mono- or dual-species models. Statistical significance was achieved if $p < 0.05$.

RESULTS

Candida as a scaffold

Initially, we sought to determine the capability of *C. albicans* in supporting the growth of oral bacterial species in a biofilm. We enumerated the viable *Candida* and bacterial cells for *P. gingivalis, S. gordonii*, and *F. nucleatum* grown alone for 24 h in a single species biofilm and also grown in the presence of a pre-established *C. albicans* biofilm. We hypothesized that the preformed *Candida* biofilm would behave as a scaffold and provide an environment that promotes the growth of the oral bacterial species. *Candida* counts remained unchanged following co-culture with the bacterial species (Fig. 1A). Conversely, there was an increase in bacterial CFUs for all three bacteria in the biofilm model with *C. albicans* (Fig. 1B). This is indicative that *C. albicans* supports or promotes the growth of oral pathogens in the biofilm; however, bacteria do not provide the same impact on *C. albicans*. There is a particularly pronounced effect on *P. gingivalis*, as there is little to no biofilm formation, on average, in the *P. gingivalis* single species biofilms, however, there is consistently greater than 1×10^6 viable cells/mL in the dual-species biofilm. This was found to be a significant increase ($p = 0.0068$; Fig. 1B). Compositionally for

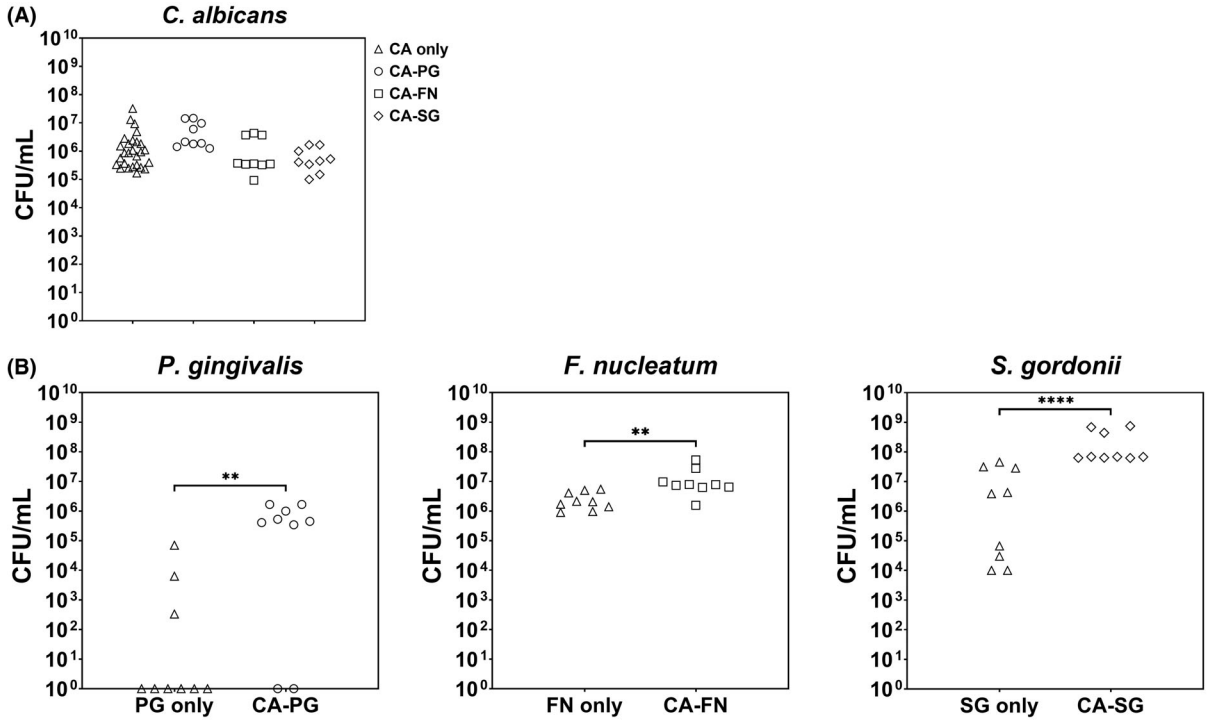

Fig. 1. Microbial analysis from dual-species biofilms. Colony forming units per ml of *C. albicans* (A) or *P. gingivalis*, *S. gordonii*, and *F. nucleatum* (B). Comparison is made from growth of mono-species biofilms of *C. albicans* only or bacterial only biofilms compared to dual-species biofilms formed by initial growth of *C. albicans* for 4 h followed then by 20 h with each bacterial species. T-tests were performed between dual and single species biofilms for each comparison (**$p < 0.01$, ****$p < 0.0001$). All individual points representative of biofilms generated from three technical replicates and three biological replicates ($n = 9$). A total of 27 points are visible for *C. albicans* only biofilms as these have been collated from individual controls (*e.g.*, 9) for each bacterial co-culture experiment. No significant differences were observed between the *C. albicans* mono-species biofilms compared to each dual-species biofilms with *P. gingivalis*, *F. nucleatum*, and *S. gordonii*.

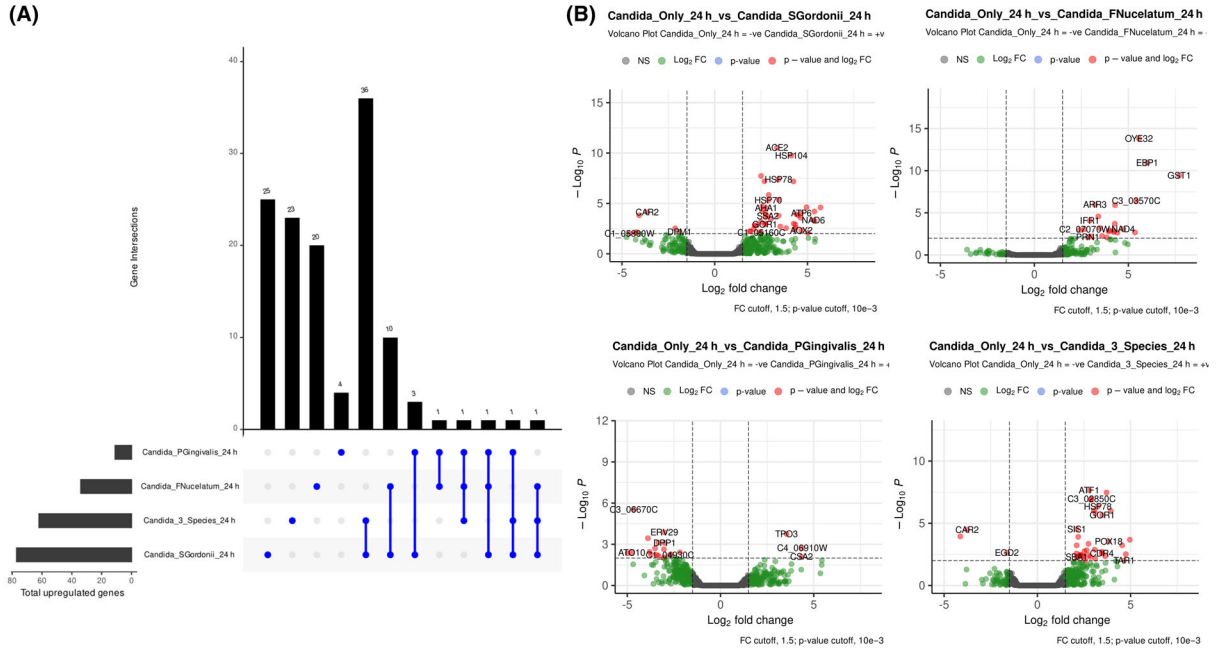

Fig. 2. Differential expression of *C. albicans* genes in response to co-culture with bacteria. UpSet plot depicting the overlap of upregulated *C. albicans* transcripts in dual- and multi-species biofilms of *P. gingivalis, S. gordonii, F. nucleatum* or all three species compared to *Candida* only biofilms. Biofilms were all mature biofilms grown for a total of 24 h. Within the UpSet plot bars depict the number of overlapping upregulated genes between each of the interkingdom biofilms, depicted by the cross sectional blue lines (A). Volcano plots depict the individual upregulated genes in each of the fungal vs interkingdom biofilms following co-culture (B). Genes with a $\log_2$FC greater than 1.5 (green) and a p-value < 0.01 (red) are shown within the plot. The inverted $\log_{10}$ p-value is depicted on the *y*-axis along with the Log_2FC on the *x*-axis. The top most prominent features are also overlayed with gene symbols or CGD identifiers.

the multi-species biofilms we see that there was an increased competition from the *S. gordonii* and *F. nucleatum* resulting in reduced numbers of *P. gingivalis* by ~1 log compared to the dual species whereas the CFE numbers of *S. gordonii and F. nucleatum* remained consistent in the different models (Figure S1). Nevertheless, viability of the bacterial strains used remained high when grown in dual- and 4-species biofilms.

Transcriptional response of *C. albicans* to oral pathogens

From our RNA-Seq data, we discerned the upregulated and downregulated *C. albicans* genes in response to dual- and multi-species biofilms. When comparing the effect of bacteria on *Candida* transcription, we observed the most pronounced changes in the dual-species biofilms containing *S. gordonii* and the multi-species containing all three bacterial species and *C. albicans* (Fig. 2). It is also noteworthy that the multi-species and *S. gordonii* dual-species biofilms had the largest overlap in expressed genes. There were 36 upregulated genes shared between the two biofilm models (Fig. 2A). We had already noted that *S. gordonii* was the most prevalent member of the multi-species models (Figure S1), while here it also stands apart as the dominant driver of the *C. albicans* transcriptional response. However, in each of our comparisons there was a species-specific response from the *C. albicans*, with 25, 20, and 4 genes being unique to the *S. gordonii, F. nucleatum, and P. gingivalis* dual-species biofilm models, respectively (Fig. 2B). It is apparent from the UpSet plot and volvano plots that *S. gordonii* exhibits the largest response from *C. albicans* and *P. gingivalis* the least (Fig. 2). Additionally, there were 20 unique transcripts from the multi-species biofilm indicating an influence on the *C. albicans* transcript which is unique to the combined impact of all three bacterial species.

Next, we performed Gene Ontology (GO) Over Representation Analysis (ORA) on the unique and overlapping genes in each of our dual-species biofilms compared to the multi-species. All the genes included in the ORA were the upregulated genes in each of the conditions compared to the *C. albicans* biofilm only control. This approach was chosen to elucidate and separate the species-specific interactions compared to those from the complex biofilm

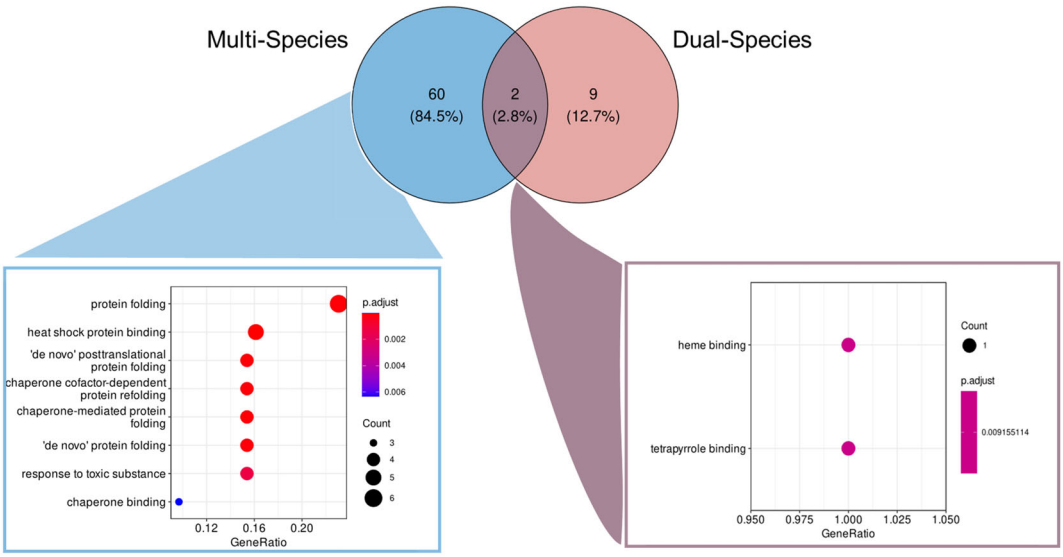

Fig. 3. Comparative analysis of the enriched Gene Ontology terms in multi-species compared to *P. gingivalis* dual-species biofilms. Upregulated genes in dual or multi-species biofilms after 24 h were selected based upon a $\log_2$FC > 1.5 and a FDR corrected p-value < 0.05. The unique and overlapping genes in the dual-species biofilm (with *P. gingivalis*) vs the multi (4-species) were then compared and depicted in a Venn Diagram. The unique and overlapping genes then were included in an over representation analysis to determine enriched functional pathways. The dot plots depict significantly over represented pathways from each of the gene subsets and the overall gene ratio of the pathway is shown on the *x*-axis, the weight of the dots is proportional to the number of genes within the analysis and the color is indicative of the FDR adjusted p-value from the hypergeometric testing.

containing all bacterial consortia. The *C. albicans*-*P. gingivalis* biofilms presented only 11 upregulated genes in comparison with the control and 9 of these were not present in the multi-species biofilm. Very few of the upregulated genes were over represented in functional pathways, and those that were contained very few genes within the network (Fig. 3). There was no significantly enriched pathways for the 9 unique *P. gingivalis* genes and only 2 genes that were significant in the overlapping pathways. The over represented terms included genes involved in heme binding and tetrapyrrole binding networks from *C. albicans* in response to *P. gingivalis*.

Fusobacterium nucleatum exhibited a more pronounced response from *C. albicans* in the dual-species biofilms with significant enrichment (FDR $p < 0.05$) in the 3 oxidoreductase pathways. The overlapping genes within the multi-species biofilm included the same two genes involved in iron acquisition that were expressed by *C. albicans* in response to *P. gingivalis* (Fig. 4). This is indicative of a shared interaction with *C. albicans* by the two anaerobes in dual-species and multi-species conditions in relation to potential heme acquisition and/or iron metabolism.

Finally when comparing the unique and shared genes in the *S. gordonii* dual species compared to the 4 species model, we observed the largest overlap of upregulated genes. It is apparent that *S. gordonii* produces a much larger transcriptional response from *C. albicans* than the two anaerobic pathogens. Within the comparison in Fig. 5, there are significantly over represented pathways that are comprised of 38 overlapping genes. Notably, many of these overlapping pathways are between *S. gordonii* dual-species and multi-species biofilms, which are largely related to protein processing related processes, heat shock, and toxic substance response pathways (Figs 3 and 4). Taken together, our commensal *S. gordonii* was the largest driver of differential gene expression in the multi-species biofilm. The unique *S. gordonii*-derived transcriptome contained 39 genes which were specifically related to heat shock protein binding. There were also 24 genes that were only expressed in the multi-species biofilm when compared directly to the *S. gordonii* dual- species. These genes were over represented in the GO terms involved in amino acid biosynthesis. These are likely genes that are in response specifically to the combined effects of all three microorganisms when cultured with *C. albicans*.

DISCUSSION

Increasing evidence in the literature suggests that the opportunistic pathogen, *C. albicans*, may play important, yet largely undefined roles in

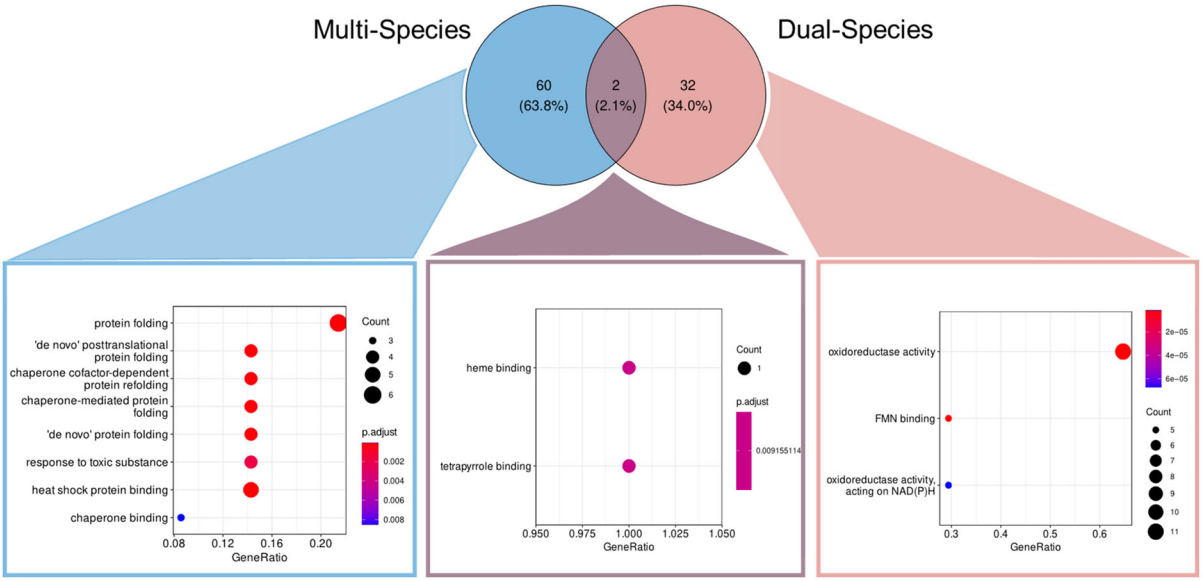

Fig. 4. Comparative analysis of the enriched Gene Ontology terms in multi-species compared to *F. nucleatum* dual-species biofilms. Upregulated genes in dual or multi-species biofilms after 24 h were selected based upon a $\log_2$FC > 1.5 and a FDR corrected p-value < 0.05. The unique and overlapping genes in the dual-species biofilm (with *F. nucleatum*) vs the multi (4-species) were then compared and depicted in a Venn Diagram. The unique and overlapping genes then were included in an over representation analysis to determine enriched functional pathways. The dot plots depict significantly over represented pathways from each of the gene subsets, and the overall gene ratio of the pathway is shown on the *x*-axis, the weight of the dots is proportional to the number of genes within the analysis and the color is indicative of the FDR adjusted p-value from the hypergeometric testing. There were no significantly overrepresented pathways for the dual species only pathways.

pathogenesis of oral diseases such as dental caries and periodontitis [13, 14]. Conversely, due to the presence of *Candida* spp. in the oral cavity of healthy individuals [4, 5], *C. albicans* has been proposed as a "keystone" commensal pertaining to its role as a microbial scaffold for bacterial species and the ability of the organism to alter the local microenvironment to support bacterial growth [17]. However, studies investigating the effects that bacteria have on the fungus are limited. Thus to further investigate this "keystone" commensal phenomena, the role of *C. albicans* as a microbial scaffold to three oral bacterial species was first confirmed. Following this, the transcriptomic response in the fungal organism was assessed when grown as mono-species biofilms, or grown as dual-species and 4-species biofilms with the oral bacteria, *S. gordonii*, *F. nucleatum*, and *P. gingivalis*. Results from these RNA-sequencing experiments are suggestive that the fungal response was species-dependent, with transcriptional regulation of *C. albicans* dictated by the presence of the bacterial species, and predominantly the oral commensal, *S. gordonii*.

Initial microbiological results showed that *C. albicans* provided a substrata for bacterial attachment, increasing cell counts for all three bacterial species when grown as dual-species biofilms compared to grown alone. This is in line with previous observations, emphasizing the idea that *C. albicans* is a structural scaffold to the oral bacterial species, *S. gordonii* and *P. gingivalis* [31, 35]. Interestingly, results for the *C. albicans*-*F. nucleatum* co-incubation showed that numbers of the bacteria increased when cultured with the fungi which is in contrast with observations elsewhere [48]. Bor *et al.* (2016) showed that although *C. albicans* adhered with *F. nucleatum*, aggregation was detrimental to growth and hyphal morphogenesis for the fungus. This difference between these observations might be explained by experimental design, with *C. albicans* added first here for 4 h, allowing the organism to form hyphae before addition of the bacteria for a further 20 h. Alternatively, as *Candida* biofilms display a degree of heterogeneity, strain variability might explain the differences in results between the studies [49]. The presence of *C. albicans* was also essential to support growth of *P. gingivalis* in the 5% CO_2 conditions. As an obligate anaerobe, *P. gingivalis* cannot survive in oxygen levels of above 6% unless grown in excess hemin [50, 51]. Thus to survive, *P. gingivalis* must attach to other colonizers of the oral cavity

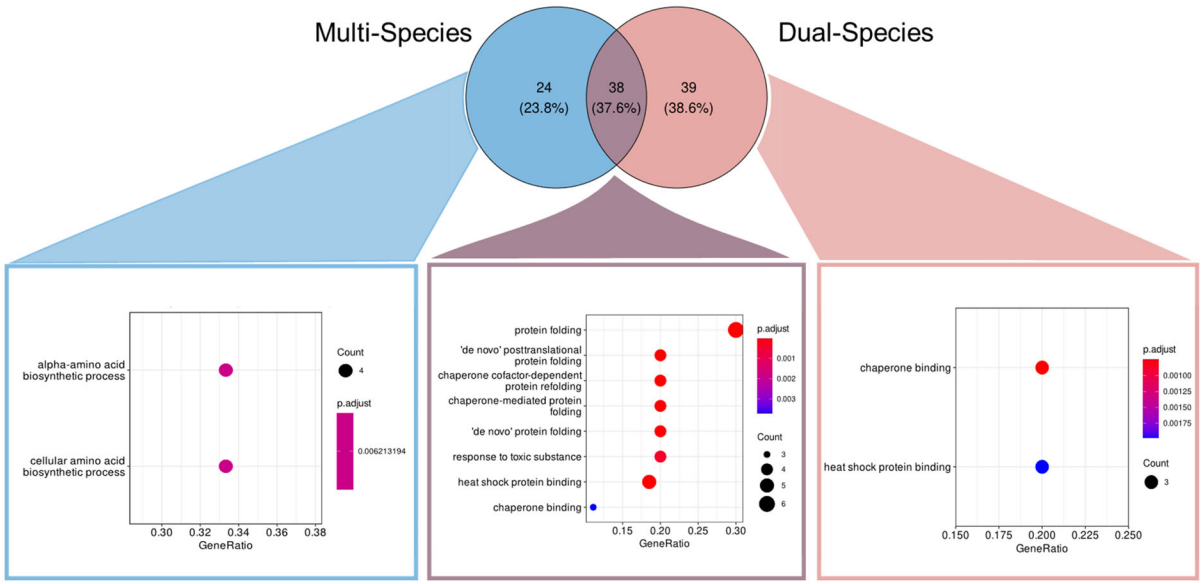

Fig. 5. Comparative analysis of the enriched Gene Ontology terms in multi-species compared to *S. gordonii* dual-species biofilms. Upregulated genes in dual- or multi-species biofilms after 24 h were selected based upon a $\log_2$FC > 1.5 and a FDR corrected p-value < 0.05. The unique and overlapping genes in the dual-species biofilm (with *S. gordonii*) vs the multi (4-species) were then compared and depicted in a Venn Diagram. The unique and overlapping genes then were included in an over representation analysis to determine enriched functional pathways. The dot plots depict significantly over represented pathways from each of the gene subsets and the overall gene ratio of the pathway is shown on the *x*-axis, the weight of the dots is proportional to the number of genes within the analysis and the color is indicative of the FDR adjusted p-value from the hypergeometric testing.

such as *Enterococcus faecalis* and *F. nucleatum* to persist in oxic conditions [52–54]. In line with previous observations, results here show that *P. gingivalis* can use *C. albicans* as a substrata to survive oxygenated conditions [34]. Although we observed that *Candida* was able to support the growth of our obligate anaerobes in 5% CO_2 in our dual-species biofilm models, within the 4-species model *S. gordonii* was the most competitive member. This may in part be due to these conditions favouring *S. gordonii* growth thus highlighting a potential limitation of this study. Future work may warrant investigations into other conditions (*e.g.*, an anaerobic microenvironment) when growing these biofilm models to further support *F. nucleatum* and *P. gingivalis* growth. However such investigations could be influenced by the inability of *C. albicans* to form biofilms under such anaerobiosis, while depleted O_2 conditions can also interfere with hyphal formation [55, 56]. Careful considerations are required for such future work.

Previous studies have shown that *S. gordonii* interactions with *C. albicans* are largely synergistic in nature [31–33]. One study by Bamford *et al* (2009) reported that contact-dependent interactions between the bacterial cells and *Candida* hyphae were necessary for mixed-species biofilm development. As well as modulating *C. albicans* biofilm formation *S. gordonii* has also been shown to co-aggregate and interact with *P. gingivalis and F. nucleatum* within the oral cavity [57, 58], and therefore was selected for incorporation into our mixed species model. Whether such transcriptional responses are similar in *C. albicans* when cultured alongside other oral *Streptococcus* species such as *S. oralis*, would be an interesting follow-up study. Here, *S. gordonii* cell counts were increased in the presence of *C. albicans*, although assessment of hyphal formation and cell–cell interactions were not pursued in this study. Nevertheless, results from our RNA-sequencing study highlighted an increase in expression of protein processing machinery (*e.g.*, chaperone binding) and heat shock pathways (*e.g.*, *HSP70* and *HSP78* in dual species, and *HSP78* in the 4-species model) in *C. albicans* when grown alongside bacterial species. Heat shock proteins have previously been linked to biofilm formation and hyphal development in the organism [59, 60]. Additionally, the chaperone network involving heat shock proteins have been shown to play a role in drug resistance, morphogenesis, and virulence [61]. Here, increases in *Candida* counts were not observed between mono- and dual-species or 4-species biofilms, suggestive that bacterial presence may simply be driving

hyphal elongation or other biological processes which was not investigated. Nevertheless, these results highlight that heat shock proteins may be a feasible target not only for *C. albicans* virulence pathways (reviewed in [62]) but also key cell–cell interactions with bacterial communities of the oral cavity.

When investigating the transcriptome of *C. albicans* grown in mixed-species biofilms with *F. nucleatum* and *P. gingivalis*, unique pathways relating to heme binding were identified in the fungal organism. For example, there was an upregulation of the gene responsible for the extracellular heme binding protein CSA2 in dual-species biofilms and the complex 4-species model. This hemophore can sequester heme and subsequently iron from human hemoglobin [63]. The ability of *C. albicans* to utilize heme as an iron source is documented in the literature [64, 65], with hemin uptake linked with hyphal formation in the fungi [66]. Further work would need to be performed to elucidate the heme-related interaction between *Candida* and the anaerobes. *P. gingivalis* has the ability to accumulate heme on the surface of the cell, giving the organism its black pigmentation [67]; thus, the upregulation of the candidal hemophore may relate to an increased heme availability in adjacent or adhered cells. Alternatively, it could be postulated that this is a stress based response to anaerobic bacteria to negate the "competition" for micronutrients. To this end, *P. gingivalis* has been shown to upregulate expression of HmuR, HmuY, and HusA, three key proteins responsible for heme acquisition systems, when grown alongside *C. albicans* [68]. Nevertheless, the mutualistic relationship of *C. albicans*-bacterial interactions merits much further consideration.

A number of genes relating to oxidative stress pathways were upregulated in *C. albicans* grown alongside *F. nucleatum*. The ability of *F. nucleatum* to produce reactive oxygen species (ROS) is well known [69, 70] and has been linked to the progression of oral cancers through this mechanism. Within the dual-species biofilm of *Candida* and *F. nucleatum*, we observed a marked upregulation of the oxidoreductase based processes including genes, *OYE2*, *GST1*, and *EBP1*. Interestingly, similar genes have previously been shown to be upregulated in *C. albicans* during DNA damage response [71]. Given that ROS are potent inducers of DNA damage in yeast [72], *Candida* may be undergoing protective measures to prevent *F. nucleatum*-mediated damage. Such mechanisms might explain previous antagonistic relationships between the two organisms, even in the presence of cell–cell attachment [48]. Also upregulated were the *NAD* family of genes related to mitochondrial respiration. Superoxide scavengers such as NADH are known to reduce oxidative stress in eukaryotes such as *C. albicans* with the mitochondria being central to this antioxidant response [73]. This oxidoreductase response is only apparent in the dual species and not the multi-species model suggestive this phenomena is potentially being buffered or otherwise controlled by the other bacterial communities. Alternatively, this could be a specific *C. albicans*-*F. nucleatum* interaction, the dynamics of which are unclear. It is evident that further interrogation is necessary to decipher this interaction. A further limitation of our results is that it is unknown whether there is a conserved response with other *Candida* species, or if it is specific to *C. albicans*; to this end, there is evidence that there are shared pathways between the species, such as the oxidoreductase and iron acquisition systems (*e.g.*, CSA2) in other *Candida spp.* such as *Candida glabrata* [74].

CONCLUSIONS

In conclusion, we have documented an in-depth transcriptomic study looking at the interactions between *C. albicans* and three orally-relevant bacterial species. Results are indicative that although *Candida* provides a structural and supportive substrata for bacteria to attach, critical for anaerobes such as *P. gingivalis* to survive in oxic conditions, the transcriptional profile of the fungal organism can vary in a bacterial species-dependent manner. Admittedly, these results may raise more questions than they answer, but highlights that the ideology of "one size fits all" is not applicable to such fungal-bacterial interactions.

We deem it pertinent for future studies to continue to consider the role that this fungus plays in the dynamics of the oral microbiome. Given the reported presence of *C. albicans* in the oral cavity of healthy and diseased individuals, and its potential role as a "keystone" commensal, we postulate that the organism could be a potent target for therapeutic treatment to control interkingdom oral diseases.

FUNDING INFORMATION

We would like to acknowledge the funding support from the GlaxoSmithKline BBSRC Industrial CASE PhD studentship for CD (BB/P504567/1).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

REFERENCES

1. Young G, Resca HG, Sullivan MT. The yeasts of the normal mouth and their relation to salivary acidity. J Dent Res. 1951;30(3):426–30.
2. Knighton HT. A study of Monilia and other Yeastlike organisms found in the oral cavity. J Dent Res. 1939;18(2):103–25.
3. Arendorf TM, Walker DM. The prevalence and intra-oral distribution of *Candida albicans* in man. Arch Oral Biol. 1980;25(1):1–10.
4. Scully C, el-Kabir M, Samaranayake LP. Candida and oral candidosis: a review. Crit Rev Oral Biol Med. 1994;5(2):125–57.
5. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog. 2010;6(1):e1000713.
6. Dupuy AK, David MS, Li L, Heider TN, Peterson JD, Montano EA, et al. Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of Malassezia as a prominent commensal. PLoS One. 2014;9(3):e90899.
7. Soll DR. The role of phenotypic switching in the basic biology and pathogenesis of *Candida albicans*. J Oral Microbiol. 2014;6. doi:10.3402/jom.v6.22993
8. Chow EWL, Pang LM, Wang Y. From Jekyll to Hyde: the yeast-hyphal transition of *Candida albicans*. Pathogens. 2021;10(7):859.
9. Williams D, Lewis M. Pathogenesis and treatment of oral candidosis. J Oral Microbiol. 2011;3. doi:10.3402/jom.v3i0.5771
10. Lalla RV, Latortue MC, Hong CH, Ariyawardana A, D'Amato-Palumbo S, Fischer DJ, et al. A systematic review of oral fungal infections in patients receiving cancer therapy. Support Care Cancer. 2010;18(8):985–92.
11. Diaz PI, Hong BY, Dupuy AK, Choquette L, Thompson A, Salner AL, et al. Integrated analysis of clinical and microbiome risk factors associated with the development of oral candidiasis during cancer chemotherapy. J Fungi (Basel). 2019;5(2):49.
12. O'Donnell LE, Alalwan HKA, Kean R, Calvert G, Nile CJ, Lappin DF, et al. Candida albicans biofilm heterogeneity does not influence denture stomatitis but strongly influences denture cleansing capacity. J Med Microbiol. 2017;66(1):54–60.
13. Du Q, Ren B, He J, Peng X, Guo Q, Zheng L, et al. *Candida albicans* promotes tooth decay by inducing oral microbial dysbiosis. ISME J. 2021;15(3):894–908.
14. Peters BA, Wu J, Hayes RB, Ahn J. The oral fungal mycobiome: characteristics and relation to periodontitis in a pilot study. BMC Microbiol. 2017;17(1):157.
15. Ohman SC, Dahlén G, Möller A, Ohman A. Angular cheilitis: a clinical and microbial study. J Oral Pathol. 1986;15(4):213–7.
16. McCormack MG, Smith AJ, Akram AN, Jackson M, Robertson D, Edwards G. Staphylococcus aureus and the oral cavity: an overlooked source of carriage and infection? Am J Infect Control. 2015;43(1):35–7.
17. Janus MM, Willems HM, Krom BP. Candida albicans in multispecies oral communities; a keystone commensal? Adv Exp Med Biol. 2016;931:13–20.
18. Mukaremera L, Lee KK, Mora-Montes HM, Gow NAR. Candida albicans yeast, Pseudohyphal, and hyphal morphogenesis differentially affects immune recognition. Front Immunol. 2017;8:629.
19. Delaney C, Kean R, Short B, Tumelty M, McLean W, Nile CJ, et al. Fungi at the scene of the crime: innocent bystanders or accomplices in oral infections? Curr Clin Microbiol Rep. 2018;5(3):190–200.
20. Montelongo-Jauregui D, Lopez-Ribot JL. Candida interactions with the oral bacterial microbiota. J Fungi (Basel). 2018;4(4):122.
21. Bertolini M, Dongari-Bagtzoglou A. The relationship of *Candida albicans* with the oral bacterial microbiome in health and disease. Adv Exp Med Biol. 2019;1197:69–78.
22. Falsetta ML, Klein MI, Colonne PM, Scott-Anne K, Gregoire S, Pai CH, et al. Symbiotic relationship between *Streptococcus mutans* and *Candida albicans* synergizes virulence of plaque biofilms in vivo. Infect Immun. 2014;82(5):1968–81.
23. Hwang G, Liu Y, Kim D, Li Y, Krysan DJ, Koo H. *Candida albicans* mannans mediate *Streptococcus mutans* exoenzyme GtfB binding to modulate cross-kingdom biofilm development in vivo. PLoS Pathog. 2017;13(6):e1006407.
24. Sztukowska MN, Dutton LC, Delaney C, Ramsdale M, Ramage G, Jenkinson HF, et al. Community development between *Porphyromonas gingivalis* and *Candida albicans* mediated by InlJ and Als3. mBio. 2018;9(2):e00202-18.
25. Peters BM, Ovchinnikova ES, Krom BP, Schlecht LM, Zhou H, Hoyer LL, et al. *Staphylococcus aureus* adherence to *Candida albicans* hyphae is mediated by the hyphal adhesin Als3p. Microbiology (Reading). 2012;158(Pt 12):2975–86.
26. Schlecht LM, Peters BM, Krom BP, Freiberg JA, Hänsch GM, Filler SG, et al. Systemic *Staphylococcus aureus* infection mediated by *Candida albicans* hyphal invasion of mucosal tissue. Microbiology (Reading). 2015;161(Pt 1):168–81.
27. Van Dyck K, Viela F, Mathelié-Guinlet M, Demuyser L, Hauben E, Jabra-Rizk MA, et al. Adhesion of *Staphylococcus aureus* to *Candida albicans* during Co-infection promotes bacterial dissemination through the host immune response. Front Cell Infect Microbiol. 2020;10:624839.
28. Ellepola K, Liu Y, Cao T, Koo H, Seneviratne CJ. Bacterial GtfB augments *Candida albicans* accumulation in cross-kingdom biofilms. J Dent Res. 2017;96(10):1129–35.
29. He J, Kim D, Zhou X, Ahn SJ, Burne RA, Richards VP, et al. RNA-seq reveals enhanced sugar metabolism in *Streptococcus mutans* co-cultured with *Candida albicans* within mixed-species biofilms. Front Microbiol. 2017;8:1036.
30. Kim D, Sengupta A, Niepa TH, Lee BH, Weljie A, Freitas-Blanco VS, et al. *Candida albicans* stimulates *Streptococcus mutans* microcolony development via cross-kingdom biofilm-derived metabolites. Sci Rep. 2017;7:41332.
31. Bamford CV, d'Mello A, Nobbs AH, Dutton LC, Vickerman MM, Jenkinson HF. *Streptococcus gordonii* modulates *Candida albicans* biofilm formation through intergeneric communication. Infect Immun. 2009;77(9):3696–704.
32. Ricker A, Vickerman M, Dongari-Bagtzoglou A. *Streptococcus gordonii* glucosyltransferase promotes

biofilm interactions with *Candida albicans*. J Oral Microbiol. 2014;6. doi:10.3402/jom.v6.23419
33. Dutton LC, Jenkinson HF, Lamont RJ, Nobbs AH. Role of Candida albicans secreted aspartyl protease Sap9 in interkingdom biofilm formation. Pathog Dis. 2016;74(3):ftw005.
34. Bartnicka D, Karkowska-Kuleta J, Zawrotniak M, Satała D, Michalik K, Zielinska G, et al. Adhesive protein-mediated cross-talk between *Candida albicans* and *Porphyromonas gingivalis* in dual species biofilm protects the anaerobic bacterium in unfavorable oxic environment. Sci Rep. 2019;9(1):4376.
35. Bartnicka D, Gonzalez-Gonzalez M, Sykut J, Koziel J, Ciaston I, Adamowicz K, et al. Candida albicans shields the periodontal killer *Porphyromonas gingivalis* from recognition by the host immune system and supports the bacterial infection of gingival tissue. Int J Mol Sci. 2020;21(6):1984.
36. Young T, Alshanta O-A, Kean R, Bradshaw D, Pratten J, Williams C, et al. Candida albicans as an essential "keystone" component within polymicrobial oral biofilm models? Microorganisms. 2021;9(1):59.
37. Martin H, Kavanagh K, Velasco-Torrijos T. Targeting adhesion in fungal pathogen *Candida albicans*. Future Med Chem. 2021;13(3):313–34.
38. Uthayakumar D, Sharma J, Wensing L, Shapiro RS. CRISPR-based genetic manipulation of Candida species: historical perspectives and current approaches. Front Genome Ed. 2020;2:606281.
39. Kuloyo O, Fourie R, Cason E, Albertyn J, Pohl CH. Transcriptome analyses of *Candida albicans* biofilms, exposed to arachidonic acid and fluconazole, indicates potential drug targets. G3 (Bethesda). 2020;10(9):3099–108.
40. Abusrewil S, Brown JL, Delaney C, Butcher MC, Tiba M, Scott JA, et al. Chitosan enhances the antibiofilm activity of biodentine against an interkingdom biofilm model. Antibiotics. 2021;10(11):1317.
41. Montelongo-Jauregui D, Srinivasan A, Ramasubramanian AK, Lopez-Ribot JL. An In vitro model for Oral mixed biofilms of *Candida albicans* and *Streptococcus gordonii* in synthetic saliva. Front Microbiol. 2016;7:686.
42. Sherry L, Lappin G, O'Donnell LE, Millhouse E, Millington OR, Bradshaw DJ, et al. Viable compositional analysis of an eleven species oral polymicrobial biofilm. Front Microbiol. 2016;7:912.
43. Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. J Hyg (Lond). 1938;38(6):732–49.
44. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for illumina sequence data. Bioinformatics. 2014;30(15):2114–20.
45. Binkley J, Arnaud MB, Inglis DO, Skrzypek MS, Shah P, Wymore F, et al. The Candida genome database: the new homology information page highlights protein similarity and phylogeny. Nucleic Acids Res. 2013;42(D1):D711–6.
46. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat Biotechnol. 2019;37(8):907–15.
47. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
48. Bor B, Cen L, Agnello M, Shi W, He X. Morphological and physiological changes induced by contact-dependent interaction between *Candida albicans* and *Fusobacterium nucleatum*. Sci Rep. 2016;6:27956.
49. Kean R, Delaney C, Rajendran R, Sherry L, Metcalfe R, Thomas R, et al. Gaining insights from Candida biofilm heterogeneity: one size does not fit all. J Fungi (Basel). 2018;4(1):12.
50. Diaz PI, Rogers AH. The effect of oxygen on the growth and physiology of *Porphyromonas gingivalis*. Oral Microbiol Immunol. 2004;19(2):88–94.
51. Lewis JP, Iyer D, Anaya-Bergman C. Adaptation of *Porphyromonas gingivalis* to microaerophilic conditions involves increased consumption of formate and reduced utilization of lactate. Microbiology (Reading). 2009;155(Pt 11):3758–74.
52. Bradshaw DJ, Marsh PD, Watson GK, Allison C. Role of *Fusobacterium nucleatum* and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration. Infect Immun. 1998;66(10):4729–32.
53. Diaz PI, Zilm PS, Rogers AH. *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. Microbiology (Reading). 2002;148(Pt 2):467–72.
54. Tan HC, Cheung GSP, Chang JWW, Zhang C, Lee AHC. *Enterococcus faecalis* shields *Porphyromonas gingivalis* in dual-species biofilm in oxic condition. Microorganisms. 2022;10(9):1729.
55. Biswas SK, Chaffin WL. Anaerobic growth of *Candida albicans* does not support biofilm formation under similar conditions used for aerobic biofilm. Curr Microbiol. 2005;51(2):100–4.
56. Fox EP, Cowley ES, Nobile CJ, Hartooni N, Newman DK, Johnson AD. Anaerobic bacteria grow within *Candida albicans* biofilms and induce biofilm formation in suspension cultures. Curr Biol. 2014;24(20):2411–6.
57. Mutha NVR, Mohammed WK, Krasnogor N, Tan GYA, Choo SW, Jakubovics NS. Transcriptional responses of *Streptococcus gordonii* and *Fusobacterium nucleatum* to coaggregation. Mol Oral Microbiol. 2018;33(6):450–64.
58. Chawla A, Hirano T, Bainbridge BW, Demuth DR, Xie H, Lamont RJ. Community signalling between *Streptococcus gordonii* and *Porphyromonas gingivalis* is controlled by the transcriptional regulator CdhR. Mol Microbiol. 2010;78(6):1510–22.
59. Fiori A, Kucharikova S, Govaert G, Cammue BP, Thevissen K, Van Dijck P. The heat-induced molecular disaggregase Hsp104 of *Candida albicans* plays a role in biofilm formation and pathogenicity in a worm infection model. Eukaryot Cell. 2012;11(8):1012–20.
60. Mayer FL, Wilson D, Jacobsen ID, Miramon P, Slesiona S, Bohovych IM, et al. Small but crucial: the novel small heat shock protein Hsp21 mediates stress adaptation and virulence in *Candida albicans*. PLoS One. 2012;7(6):e38584.
61. O'Meara TR, Robbins N, Cowen LE. The Hsp90 chaperone network modulates Candida virulence traits. Trends Microbiol. 2017;25(10):809–19.
62. Horianopoulos LC, Kronstad JW. Chaperone networks in fungal pathogens of humans. J Fungi (Basel). 2021;7(3):209.

63. Okamoto-Shibayama K, Kikuchi Y, Kokubu E, Sato Y, Ishihara K. Csa2, a member of the Rbt5 protein family, is involved in the utilization of iron from human hemoglobin during *Candida albicans* hyphal growth. FEMS Yeast Res. 2014;14(4):674–7.
64. Andrawes N, Weissman Z, Pinsky M, Moshe S, Berman J, Kornitzer D. Regulation of heme utilization and homeostasis in *Candida albicans*. PLoS Genet. 2022;18(9):e1010390.
65. Santos R, Buisson N, Knight S, Dancis A, Camadro JM, Lesuisse E. Haemin uptake and use as an iron source by *Candida albicans*: role of CaHMX1-encoded haem oxygenase. Microbiology (Reading). 2003;149(Pt 3):579–88.
66. Casanova M, Cervera AM, Gozalbo D, Martinez JP. Hemin induces germ tube formation in *Candida albicans*. Infect Immun. 1997;65(10):4360–4.
67. Smalley JW, Silver J, Marsh PJ, Birss AJ. The periodontopathogen *Porphyromonas gingivalis* binds iron protoporphyrin IX in the mu-oxo dimeric form: an oxidative buffer and possible pathogenic mechanism. Biochem J. 1998;331(Pt 3):681–5.
68. Guo Y, Wang Y, Wang Y, Jin Y, Wang C. Heme competition triggers an increase in the pathogenic potential of *Porphyromonas gingivalis* in *Porphyromonas gingivalis*-Candida albicans mixed biofilm. Front Microbiol. 2020;11:596459.
69. McIlvanna E, Linden GJ, Craig SG, Lundy FT, James JA. *Fusobacterium nucleatum* and oral cancer: a critical review. BMC Cancer. 2021;21(1):1212.
70. Kedziora A, Lesiow MK, Krupa K, Korzeniowska-Kowal A, Adamski R, Komarnicka UK, et al. Protocol of proceedings with *Fusobacterium nucleatum* and optimization of ABTS method for detection of reactive oxygen species. Future Microbiol. 2020;15:259–71.
71. Feng Y, Zhang Y, Li J, Omran RP, Whiteway M, Feng J. Transcriptional profiling of the *Candida albicans* response to the DNA damage agent methyl Methanesulfonate. Int J Mol Sci. 2022;23(14):7555.
72. Yao S, Feng Y, Zhang Y, Feng J. DNA damage checkpoint and repair: from the budding yeast Saccharomyces cerevisiae to the pathogenic fungus *Candida albicans*. Comput Struct Biotechnol J. 2021;19:6343–54.
73. Mao X, Yang L, Liu Y, Ma C, Ma T, Yu Q, et al. Vacuole and mitochondria patch (vCLAMP) protein Vam6 is involved in maintenance of mitochondrial and vacuolar functions under oxidative stress in *Candida albicans*. Antioxidants (Basel). 2021;10(1):136.
74. Gerwien F, Safyan A, Wisgott S, Brunke S, Kasper L, Hube B. The fungal pathogen Candida glabrata does not depend on surface ferric reductases for iron acquisition. Front Microbiol. 2017;8:1055.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Bacterial composition analysis of dual and multi-species biofilms. Bacteria were grown in either a dual species with C. albicans or in a multi-species comprised of each of the three bacteria (*P. gingivalis*, *F. nucleatum*, and *S. gordonii*). Colony forming equivalents for live and total counts was calculated for each of the three bacteria in either the dual species biofilm (A) or the multi-species biofilm following 20 h of incubation with a 4 h preformed *C. albicans* biofilm for a total of 24 h.

