## Title

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# Anaerobic Bacteria Grow within *Candida albicans* Biofilms and Induce Biofilm Formation in Suspension Cultures

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## Summary

The human microbiome contains diverse microorganisms, which share and compete for the same environmental niches [1, 2]. A major microbial growth form in the human body is the biofilm state, where tightly packed bacterial, archaeal and fungal cells must cooperate and/or compete for resources in order to survive [3–6]. We examined mixed biofilms composed of the major fungal species of the gut microbiome, *C. albicans*, and each of five prevalent bacterial gastrointestinal inhabitants: *Bacteroides fragilis*, *Clostridium perfringens*, *Escherichia coli*, *Klebsiella pneumoniae* and *Enterococcus faecalis* [7–10]. We observed that biofilms formed by *C. albicans* provide a hypoxic microenvironment that supports the growth of two anaerobic bacteria, even when cultured in ambient oxic conditions that are normally toxic to the bacteria. We also found that co-culture with bacteria in biofilms induces massive gene expression changes in *C. albicans*, including upregulation of *WOR1*, which encodes a transcription regulator that controls a phenotypic switch in *C. albicans*, from the “white” cell type to the “opaque” cell type. Finally, we observed that in suspension cultures, *C. perfringens* induces aggregation of *C. albicans* into “mini-biofilms,” which allow *C. perfringens* cells to survive in a normally toxic environment. This work indicates that bacteria and *C. albicans* interactions modulate the local chemistry of their environment in multiple ways to create niches favorable to their growth and survival.



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## Results

### The fungal species *C. albicans* forms mixed biofilms with five bacterial species

*C. albicans* with or without *C. perfringens*, *B. fragilis*, *E. faecalis*, *E. coli* or *K. pneumoniae* cells were adhered to a bovine serum coated, polystyrene well for 90 minutes and allowed to develop into biofilms for 24 hours, a standard procedure for producing *C. albicans* biofilms [11, 12]. Confocal scanning laser microscopy (CSLM) images confirmed that in all cases, both fungal and bacterial species incorporated into the biofilm (Figure 1). The bacteria adhered to both *C. albicans* hyphal and yeast-form cells (Figure 1; Figure S1A – F). While *B. fragilis*, and *C. perfringens* had minimal effect on the biofilm architecture, incorporation of *E. coli*, *E. faecalis* and *K. pneumoniae* reduced the overall biofilm thickness (Figure S1G). We designed a colony forming unit (CFU) assay as a readout for live bacterial and *C. albicans* cells present, and found that both bacteria and *C. albicans* were incorporated into the biofilms over time (Figure 2A – D, S2A – C).

### *C. perfringens* and *B. fragilis* proliferate in co-cultured biofilms with *C. albicans* under ambient oxic conditions

*C. albicans* and/or *C. perfringens* or *B. fragilis* cells were co-cultured in biofilms for 4, 24, 48, or 72 h, under ambient oxic or anoxic conditions. Growth of each species over time was measured by plating for CFUs (Figure 2A – D). The adherence and growth of *C. albicans* was unaffected by the presence or absence of bacterial cells; however the initial adherence of *C. perfringens* and *B. fragilis* increased ten-fold in the presence of *C. albicans.* In mixed biofilms, after adherence, *C. perfringens* showed substantial growth, from ~$5\times10^5$ CFU/ml to ~$1\times10^7$ CFU/ml in 24 h, regardless of whether the biofilm was grown under ambient oxic or anoxic conditions (Figure 2A, C). Without *C. albicans*, viable *C. perfringens* cells decreased below detection (<10 CFU/ml) after 24 h in ambient oxic conditions (Figure 2A). *B. fragilis* showed the same trend (Figure 2B, D). In addition to the standard laboratory strain of *C. albicans* (SC5314), we tested two other clinical isolates of *C. albicans* and found they are also able to support anaerobe growth (Figure S2D, E). Our data demonstrate that incorporation into a *C. albicans* biofilm grown under ambient oxic conditions enables growth of the anaerobes *C. perfringens* and *B. fragilis*; without the protective biofilm, the viability of both bacterial species rapidly declines.

### *C. albicans* biofilms create a hypoxic microenvironment

To test the hypothesis that biofilms create locally hypoxic environments which enable the growth of anaerobic bacteria, we measured oxygen levels in biofilms using a miniaturized, Switch-able Trace Oxygen Sensor (STOX-Sensor), an instrument capable of measuring oxygen concentrations as low as 10 nM [13]. Measurements with the STOX-Sensor revealed a gradient of oxygen concentration throughout the depth of the biofilm, decreasing from ~300 μM (ambient oxygen) near the top of the biofilm to less than 50 μM near the bottom (Figure 2E). The oxygen gradient remained the same whether *C. albicans* was grown in monoculture or was co-cultured with *C. perfringens* or *B. fragilis.*

### Co-culture in biofilms with bacteria alters gene expression in *C. albicans*

To determine whether *C. albicans* was responding to bacteria in the mixed-species biofilm, we measured gene expression changes in *C. albicans* by microarray (Figure 3A; Dataset 1). Relative to the *C. albicans* biofilm formed in the absence of bacteria, many genes were up- and down-regulated in the presence of bacteria. Some genes changed expression in response to all of the bacterial species, while others were specific to a few species.

Among the most differentially regulated genes were those encoding the transcription regulators controlling the white-opaque switch in *C. albicans*, a transition between two cell types, each of which is heritable for many generations [14–17] (Figure 3B). In particular, *WOR1*, which encodes the "master" regulator of white-opaque switching, was strongly upregulated by co-culture with *K. pneumoniae*, *E. coli*, and *E. faecalis*. Co-culture with *K. pneumoniae* also induced upregulation of several other transcription regulators known to play roles in the white-opaque switch, in a *WOR1*-independent manner (Figure S3, Dataset 2) [16, 18–21].

Although a number of opaque-specific genes were upregulated, the full opaque-specific gene expression pattern was not observed, and when removed from this condition, the *C. albicans* cells revert to "classical" white cells. We propose that co-culture with bacterial cells poises *C. albicans* to switch from white to opaque, but that additional signals are required for full switching.

### *C. perfringens* is protected by and induces aggregation of *C. albicans* in suspension culture

To further explore interactions between *C. albicans* and the bacterial microbiome members, we co-cultured them in suspension cultures, and observed that some of the bacteria induced co-aggregation with *C. albicans* cells (Table S1, Figure 4A – D). The most dramatic effect occurred with *C. perfringens* in ambient oxic conditions. Light microscopy revealed that the aggregates induced by *C. perfringens* were composed of dense clumps containing both *C. albicans* and *C. perfringens* cells and resembling miniature biofilms (Figure 4G). By monitoring CFUs/ml of *C. perfringens* grown in suspension cultures over time (Figure 4H, I), we observed that the presence of *C. albicans* enabled survival of *C. perfringens* in oxic suspension conditions to levels of ~$1\times10^6$ CFU/ml; in the absence of *C. albicans*, *C. perfringens* CFUs dropped at least five orders of magnitude, to undetectable levels (<10 CFU/ml) by 24 h (Figure 4H).

Although the mini-biofilms are too small to directly probe for oxygen concentration, we note that *C. albicans* gene expression under these conditions was significantly enriched for genes regulated during hypoxic conditions ($P = 1.4\times10^{-5}$) [22] (Figure S4A, Dataset 3), suggesting that the mini-biofilms, like conventional, surface-adhered biofilms, provide a hypoxic environment. Consistent with this idea, we found that *C. perfringens* cells also stimulate aggregation in early stages of conventional *C. albicans* biofilm formation on a solid surface (Figure S4B).

We repeated the suspension growth experiment with cell-free supernatant or heat-killed *C. perfringens* cells, and observed that both are able to induce aggregation of *C. albicans*

(Figure 4E, F). We blindly screened a library of 205 deletion strains in *C. albicans* [23] (Table S2), and identified eight transcription regulator-encoding genes and two other genes that are required for the observed interspecies aggregation (Figure 4K–R; Figure S4C). Notably, six of the transcription regulators (Brg1, Tec1, Rob1, Bcr1, Ndt80, and Efg1) found in our screen were previously identified "master regulators" of conventional biofilm formation [12], providing strong evidence that *C. perfringens* induces aggregate formation via the biofilm genetic program. The other two regulator mutants deficient in aggregation were *rim101*Δ/Δ and *flo8*Δ/Δ, which have not been reported to be required for conventional biofilm formation. *DEF1*, which regulates hyphal extension [24], and *ALS3*, which encodes an adhesin important for biofilm formation and plays a role in interacting with many bacterial species [25–29], were also required for aggregation (Figure S4C). As described in supplemental materials, we quantified aggregation using a sedimentation assay and verified that the deletion strains were complemented by gene "add-backs" (Figure S4D, E).

These results support a model whereby in ambient oxic suspension culture, *C. perfringens* induces *C. albicans* to form protective aggregates, which depend on the *C. albicans* biofilm genetic program. These mini-biofilms, which contain both *C. albicans* and *C. perfringens*, allow *C. perfringens* to survive in oxic conditions that are normally toxic.

## Discussion

In this work we uncovered multiple interactions between *C. albicans*, a major fungal species of the human microbiome, and several bacterial members of the microbiome.

### *C. albicans* biofilms: a microenvironment supporting anaerobic bacterial growth

It has been known for some time that bacterial biofilms are able to generate hypoxic microenvironments, supporting the growth of anaerobic bacterial species [30, 31], and it has been speculated that biofilms formed by *Candida* species may also be hypoxic, based on gene expression data and mutant phenotypes [30, 32–34]. Our work directly demonstrates, for the first time, that *C. albicans* biofilms create a hypoxic internal microenvironment when grown under ambient oxygen conditions. We also show that the microenvironment within the *C. albicans* biofilm is sufficient to support the growth of two different anaerobic species, *C. perfringens* and *B. fragilis*, and it is likely that decreased oxygen concentration plays a major role in anaerobe survival. Different strains of *C. perfringens* and *B. fragilis* have been reported to grow in oxygen levels as high as 3–5% (~40–70 μM) [35, 36], and we have shown that *C. albicans* biofilms provide an environment where the oxygen concentration is as low as ~50 μM. This finding suggests that *C. albicans* may permit the growth of anaerobes in oxic areas of the host that would otherwise be uninhabitable by those species. This idea may be especially important for the establishment of *C. perfringens* infection, which causes a wide variety of illnesses, including enterotoxemia, gas gangrene, and wound infections, many of which are life-threatening [37, 38].

The fact that oxygen concentration decreases steadily from the top to the bottom of a *C. albicans* biofilm adds to our understanding of the heterogeneous nature of biofilms. *C. albicans* biofilms are composed of multiple cell types (yeast, pseudohyphae, hyphae, persister/dormant cells and dispersing cells) that express different genetic programs [39–43]

due to their precise location within the biofilm. The oxygen concentration gradient is one critical variable that structures the biofilm microenvironment and suggests that metabolism and gene expression vary between cells at different levels throughout the biofilm.

## Partial induction of the white/opaque switch program in *C. albicans*

We monitored the transcriptional response of *C. albicans* to bacterial species in mixed biofilms, and found there was significant overlap between the genes upregulated by co-culture with *K. pneumoniae* and genes enriched in opaque cells compared to white cells ($p = 8.4\times10^{-20}$). There is also significant overlap between genes upregulated by co-culture with *K. pneumoniae* and genes enriched in a strain overexpressing *WOR1* after passage through the mouse gut, compared to a wild type strain ($p = 3.4\times10^{-9}$) [44]. We propose that induction of *WOR1* by bacteria may prime *C. albicans* for white-opaque switching, but that additional environmental cues are needed to fully induce the switch to the opaque form. An alternative hypothesis is that partial induction of the opaque program is an adaptive response to exposure to bacteria.

## Aggregation induction by co-culture in suspension

We found that *C. perfringens* induces aggregation of *C. albicans* in ambient oxic suspension cultures and that the aggregates, which contain both fungi and bacteria, allow *C. perfringens* to survive in a normally toxic environment. Induction of aggregation may be similar to induction of biofilm formation, as aggregation requires the same master regulators needed for *C. albicans* to form a “conventional” biofilm on a solid surface. Moreover, the cells in the aggregates resemble cells in biofilms on solid surfaces. These observations indicate that the biofilm “program” in *C. albicans* does not require a solid surface to become activated, and the definition of a *C. albicans* biofilm may be expanded from a substrate-attached community to include suspended aggregates. *E. coli*, *Pediococcus damnosus*, and several other bacterial species were previously found to induce aggregation when co-cultured with several yeast species, including *Candida utilis*, *S. cerevisiae*, and *Schizosaccharomyces pombe* [45]. The evidence suggests that many microbial species are able to co-aggregate, and our work has demonstrated that adherence between fungi and bacteria can allow the survival of the bacteria.

## Interspecies Interactions

We have shown that *C. albicans* interacts in a variety of ways with several representative species of the gut microbiome. These microbes are clearly able to sense one another; for example *C. albicans* responds through large changes in adherence and gene expression. We have provided new evidence of antagonistic (reduction of *C. albicans* biofilm thickness by the presence of *K. pneumoniae*) and beneficial (protection of *C. perfringens* by *C. albicans* biofilms) relationships, and have begun to uncover the genes involved in these interactions. These findings highlight the importance of considering the microenvironments encountered by microbiome members. The strategy of studying pairwise interactions between fungi and bacteria in the context of heterogeneous microenvironments can be expanded to better understand the complex community of thousands of species that encounter one another in the host.

## Experimental Procedures

### Co-cultures in suspension or biofilms

*C. albicans* and/or bacteria were grown in suspension or in biofilms adhered in 6-well polystyrene plates, in Brain Heart Infusion (BHI) medium, supplemented with 5% fetal bovine serum (BHI-FBS). Additional details in Supplement.

### Colony Forming Units (CFUs) Assay

CFUs were plated from serial dilutions of either biofilms or suspension cultures. Dilutions were plated on YPD agar, LB agar, or blood agar at 30°C or 37°C, depending on the species. Additional details in Supplement.

### Oxygen measurement

Oxygen concentration in biofilms was measured with a Unisense STOX-Sensor microelectrode, with measurements obtained every 10 μm from top to bottom. Additional details in Supplement.

### Gene expression microarrays

Cy3 or Cy5-labeled cDNA was hybridized to custom Agilent microarrays, analyzed in GenePix Pro, and normalized with LOWESS. Additional details in Supplement.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Highlights

- *C. albicans* biofilms are hypoxic and support anaerobic bacteria survival
- Bacteria induce part of the *C. albicans* opaque genetic program in mixed biofilms
- *C. perfringens* induces biofilm formation in *C. albicans* in suspension co-culture

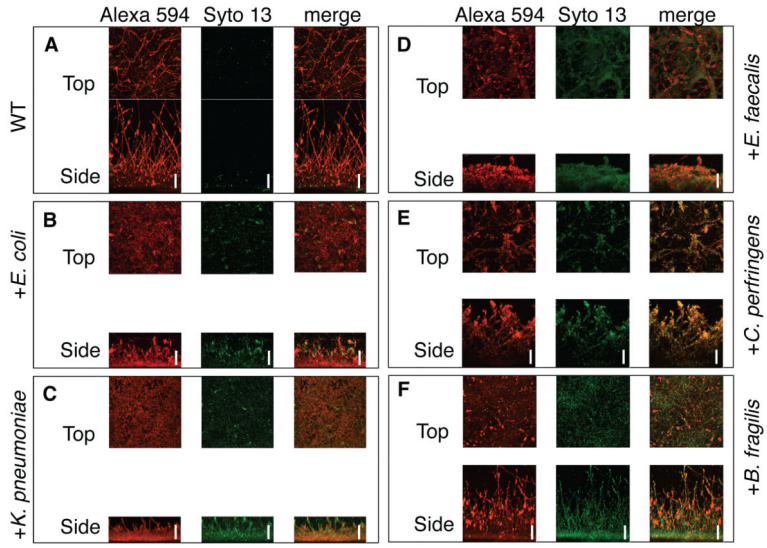


**Figure 1. *C. albicans* forms biofilms with five different species of bacteria *in vitro***

*C. albicans* was grown in biofilms for 24 h either alone (A), or with *E. coli* (B), *K. pneumoniae* (C), *E. faecalis* (D), *C. perfringens* (E), or *B. fragilis* (F). Biofilms were stained with conconavalin A – Alexa 594 and Syto 13 dyes, then imaged by CSLM. Images are maximum intensity projections of the top and side view. Representative images of at least three replicates are shown. Scale bars are 50 μm. See also Figure S1.

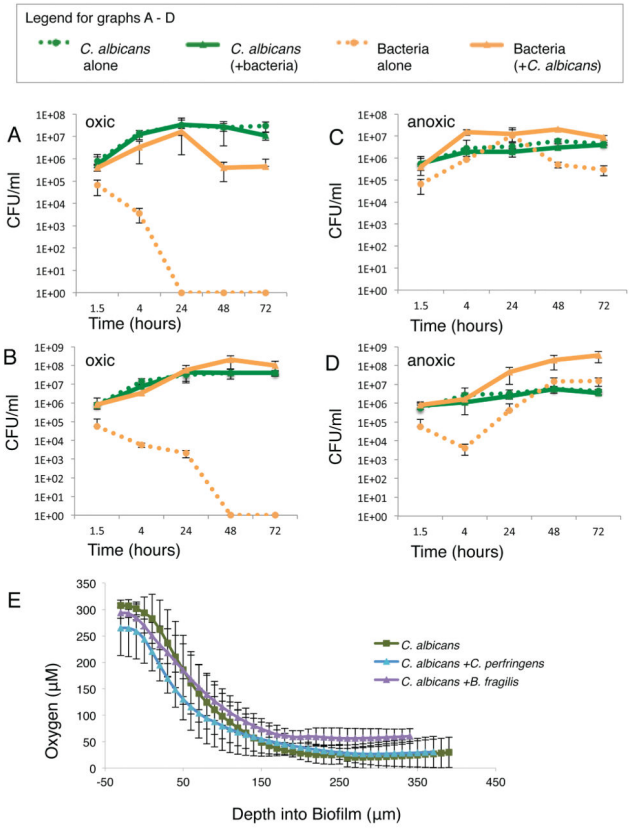


**Figure 2. Mixed-species biofilms provide a niche for growth of anaerobic bacteria**

(A–D) CFU/ml of indicated species grown in biofilms in monoculture or co-culture under oxic or anoxic conditions. Cells were collected from biofilms only (not from the media above the biofilm) at 1.5, 4, 24, 48, and 72 h, and plated for CFUs. A) *C. albicans* and/or *C. perfringens* in oxic conditions. B) *C. albicans* and/or *B. fragilis* in oxic conditions. C) *C. albicans* and/or *C. perfringens* in anoxic conditions. D) *C. albicans* and/or *B. fragilis* in anoxic conditions. E) Oxygen was measured in biofilms composed of the indicated species using a STOX-Sensor. Readings were taken every 10 μm from the top to the bottom of the biofilm. For all graphs, the mean of at least two replicates is shown, with error bars representing standard deviation. See also Figure S2.

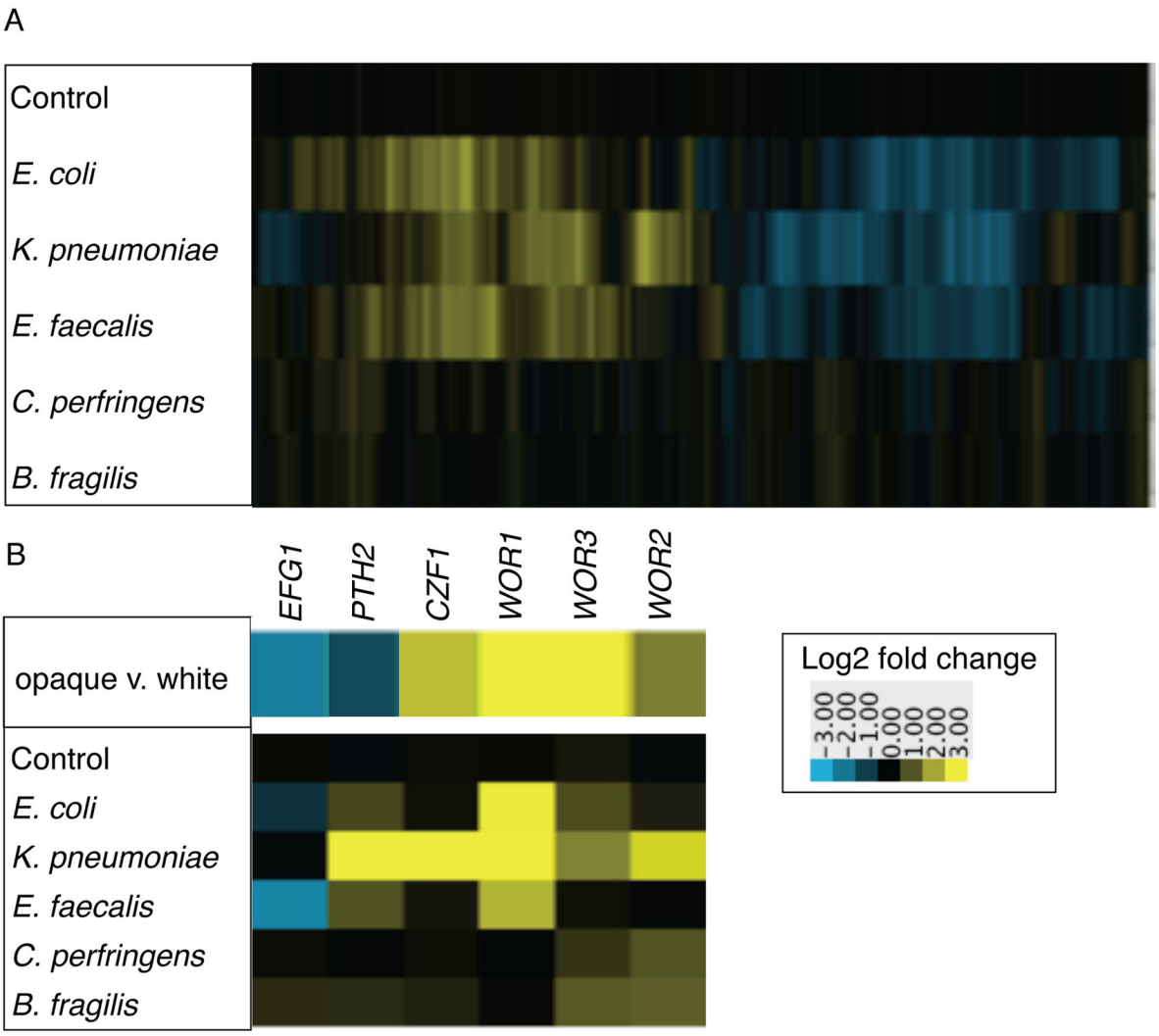


**Figure 3. Co-culture with bacteria in biofilms induces differential gene expression in *C. albicans***

A) Heat map of gene expression in *C. albicans* when co-cultured with the indicated species in biofilms, compared to *C. albicans* alone. Shown are the median values of at least two biological replicates. Control refers to *C. albicans* with media added to mimic the inoculum with bacteria, compared to *C. albicans* alone. 2863 genes differentially regulated at least twofold in at least one condition are displayed along the x-axis. Upregulated genes are yellow, downregulated genes are blue. B) Gene expression pattern of genes encoding transcription regulators that control the white-opaque switch circuit. The top panel shows expression levels measured in opaque vs. white cells from [19]. The bottom panel shows expression levels when *C. albicans* is co-cultured in biofilms with the indicated bacterial species, compared to *C. albicans* alone. See also Figure S3.

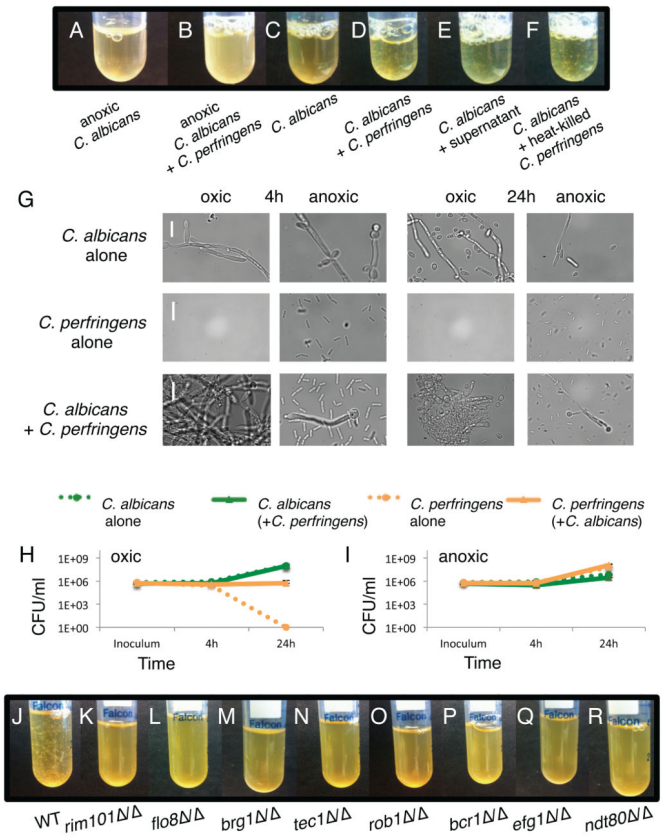


**Figure 4. *C. perfringens* induces aggregation of *C. albicans* during ambient oxic, suspension co-culture**

Suspension cultures of *C. albicans* with or without *C. perfringens*, grown for 4 h or 24 h at 37°C, in anoxic or ambient oxic conditions. A–F) 4 h growth. A) *C. albicans* alone, anoxic. B) *C. albicans* + *C. perfringens*, anoxic. C) *C. albicans* alone, oxic. D) *C. albicans* + *C. perfringens*, oxic. E) *C. albicans* + cell-free supernatant from *C. perfringens* culture. F) *C. albicans* + heat-killed *C. perfringens* cells. G) *C. albicans* and/or *C. perfringens* imaged by light microscopy. Representative images are shown. Scale bars are 20 μm. H–I) CFU/ml of indicated species grown in monoculture or co-culture, in suspension cultures under ambient oxic or anoxic conditions. H) *C. albicans* and/or *C. perfringens* in ambient oxic conditions. I) *C. albicans* and/or *C. perfringens* in anoxic conditions. Shown is the mean of at least two replicates, error bars are standard deviation. J–R) *C. albicans* wild type or mutant strains grown in suspension, in ambient oxygen, for 4 h with *C. perfringens*. J) WT. K) *rim101*Δ/Δ. L) *flo8*Δ/Δ. M) *brg1*Δ/Δ. N) *tec1*Δ/Δ. O) *rob1*Δ/Δ. P) *bcr1*Δ/Δ. Q) *efg1*Δ/Δ. R) *ndt80*Δ/Δ. Assay was performed at least twice for each condition or mutant strain. See also Figure S4.