

Candida albicans alters the bacterial microbiome of early *in vitro* oral biofilms

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ABSTRACT

The yeast *Candida albicans* is an oral commensal microorganism, occurring in the oral cavity of 50–70% of healthy individuals. Its effect on oral ecology has mostly been studied using dual-species models, which disregards the complex nature of oral biofilms. The aim of this study was to culture *C. albicans* in a complex model to study its effect on oral biofilms. Biofilms, inoculated using pooled stimulated saliva with or without addition of *C. albicans*, were grown under anaerobic, aerobic, or aerobic +5% CO₂ conditions. Red autofluorescence was quantified using a spectrophotometer and visualized in fluorescence photographs. The microbiome of 5 h biofilms was determined using 16S rDNA sequencing. *C. albicans* was only able to proliferate in biofilms grown under aerobic conditions. After 48 h, *C. albicans* did not induce differences in total biofilm formation, lactic acid accumulation (cariogenic phenotype) or protease activity (periodontitis phenotype). *In vitro*, anaerobically grown biofilms developed red autofluorescence, irrespective of inoculum. However, under aerobic conditions, only *C. albicans*-containing biofilms showed red autofluorescence. Facultative or strict anaerobic *Veillonella*, *Prevotella*, *Leptotrichia*, and *Fusobacterium* genera were significantly more abundant in biofilms with *C. albicans*. Biofilms without *C. albicans* contained more of the aerobic and facultative anaerobic genera *Neisseria*, *Rothia*, and *Streptococcus*. The presence of *C. albicans* alters the bacterial microbiome in early *in vitro* oral biofilms, resulting in the presence of strictly anaerobic bacteria under oxygen-rich conditions. This *in vitro* study illustrates that *C. albicans* should not be disregarded in healthy oral ecosystems, as it has the potential to influence bacteria significantly.

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Introduction


The healthy oral cavity represents a very diverse niche [1] that is colonized by >500 bacterial species [2]. In addition, >100 fungal species were identified in the oral cavity of healthy individuals [3,4]. With oral diseases being among the most prevalent in Western society [5], it is not surprising that the relation between microbial colonization and the development of oral diseases is well-established both for bacteria and for fungi. Oral diseases develop due to an imbalance between microbial colonization and the host. According to the ecological plaque hypothesis [6,7], changes in environmental conditions can lead to long-term changes in microbial ecology. Such an imbalance can lead to tooth decay (i.e. caries) or inflammation of the soft tissues of the mouth (e.g. gingivitis). Ecological balance, the net result of all inter-microbe and host-microbe interactions, is essential in maintaining health.

Candida albicans is a polymorphic yeast and an opportunistic pathogen. In the oral cavity, it is associated with early childhood caries [8], and it can cause infections of the oral soft tissues, ranging from superficial overgrowth to deep-seeded

invasion, resulting in disseminated disease [9]. Nevertheless, *C. albicans* colonizes the oral cavity as a commensal in 50–70% of individuals [3,4]. It has the ability to interact with many bacterial species on different levels [10]. For instance, the physical interaction between *C. albicans* and *Streptococcus gordonii* enhances hyphal formation of *C. albicans* and increases the biomass of the dual-species biofilms [11]. *C. albicans* was also found to have a complex interaction with the cariogenic organism *S. mutans*. The latter produces glucan that binds to the cell wall of *C. albicans*. The yeast thus provides adhesion sites for the bacterium, resulting in increased biofilm formation of the dual-species biofilms [12]. In a rodent model, these dual-species biofilms resulted in higher numbers of severe caries lesions compared with infection with either of the species alone [12].

In addition to the physical interaction, *C. albicans* also interacts with bacteria via chemical signals in a process called quorum sensing. For instance, *Aggregatibacter actinomycetemcomitans* produces autoinducer-2 (AI-2), which is a small signaling molecule. AI-2 is sensed by *C. albicans*, which then

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responds to this signal, resulting in lower biofilm formation [13]. Similarly, *S. mutans* produces a small peptide called competence-stimulating peptide (CSP), which inhibits *C. albicans* hyphal formation in the early stages of biofilm formation [14].

Finally, metabolic interactions are found between *C. albicans* and *S. mutans*. Willems et al. showed that despite higher abundance of *S. mutans* in 72 h dual-species biofilms, the pH of the spent medium remained higher compared with *S. mutans* biofilms alone [15]. The lactic acid produced by *S. mutans* can be metabolized by *C. albicans* [16], and *C. albicans* can increase the external pH by producing ammonia [17], leading to less cariogenic conditions.

Interactions between *C. albicans* and bacteria have been studied in dual-species biofilm models. It is clear that these interactions affect the behavior of both microbes involved. While studies on bacterial biofilms have evolved to multi-species or even microcosm model systems, mixed kingdom biofilm studies have not. The aim of this study was to introduce *C. albicans* in an *in vitro* model for complex oral biofilm in order to study its effect on the microbiome and pathology-related phenotypes of these biofilms.

Materials and methods

Inoculum collection and strains

Saliva used in this study was obtained in accordance with the ethical principles of the 64th WMA Declaration of Helsinki. The Medical Ethical Committee of the VU Medical Center, The Netherlands (2011/236) determined that Dutch law concerning Medical Research Involving Human Subjects Act (WMO) was not applicable. Stimulated saliva from 10 self-reported systemically and orally healthy donors was collected on ice and pooled [18]. *C. albicans* SC5314 [19] was grown overnight, centrifuged, and resuspended in fresh buffered semi-defined McBain medium [20] to $OD_{600} = 5.0$. An inoculum was created by diluting pooled saliva 50-fold in fresh McBain medium supplemented with 0.2% sucrose, or by diluting both pooled saliva and *C. albicans* 50-fold in fresh McBain medium supplemented with 0.2% sucrose (mixed inoculum).

Biofilm formation

Biofilms were grown *in vitro* in buffered semi-defined McBain medium [20] supplemented with 0.2% sucrose using the Amsterdam Active Attachment Model (AAA model) [20], with 12 mm glass coverslips (Menzel, Braunschweig, Germany). Glass cover slips were used as model substrates because glass resembles the physical properties (smoothness and surface characteristics) of

teeth without demineralization related to microbial acid production during growth. Biofilms were generated with medium refreshment twice a day [20]. The initial microbial seeding used pooled saliva, a mixed inoculum, or *C. albicans* alone. Biofilms were incubated at 37°C anaerobically, aerobically, or aerobically with 5% CO₂.

For the dynamic analysis of red autofluorescence, biofilms were grown up to 72 h with medium refreshment twice a day.

Confocal scanning laser microscopy

Prior to confocal scanning laser microscopy (CLSM), 48 h biofilms were fixed for 30–60 min with 4% formaldehyde, washed with phosphate-buffered saline (PBS), and stained with SYTO9 (Life Technologies Europe BV, Bleiswijk, The Netherlands) for 15 min in the dark. CLSM images of two biofilms per condition were captured with a LEICA TCS SP2 microscope using a 20× objective and the FITC filter settings (excitation 488 nm, emission 510–540 nm).

DNA isolation and quantitative polymerase chain reaction of *Candida*

DNA isolation and purification were performed as previously described [18]. To determine the amount of *Candida* in the biofilms, quantitative polymerase chain reaction (qPCR) was performed using four biofilms per condition. Primers and a probe for the 28S large-subunit ribosomal RNA gene were used (F: GCA TAT CAA TAA GCG GAG GAA AAG, R: TTA GCT TTA GAT GAT TTAC CAC C, Probe: 6FAM-CGG CGA GTG AAG CGG SAA RAG CTC-BHQ1) [21]. Reactions contained LightCycler® 480 Probes Master (Roche Diagnostics, Basel, Switzerland), 0.5 pmol primers, 0.3 pmol probe, and 3 µL DNA in a total reaction volume of 20 µL. qPCR was performed using the Light cycler 480-II (Roche Diagnostics) with the following protocol: 10 min pre-incubation at 95°C, followed by 50 cycles of denaturation (95°C, 10 s), annealing (60°C, 30 s), extension (72°C, 30 s), and cooling at 37°C for 10 s. The concentration of *Candida* DNA (ng/µL) was determined from standard curves derived from *C. albicans* genomic DNA.

Quantifying biofilm formation

Biofilms (48 h) were dispersed in PBS by sonication [18]. To estimate the amount of biofilm formation, total anaerobic colony forming units (CFUs) were determined [20] for four biofilms per condition.

Cariogenic potential

Lactic acid production is a key virulence factor of oral biofilms with regard to caries [6,22]. To assess the cariogenic potential of the 48 h biofilms, lactic acid accumulation of four biofilms per condition was determined prior to harvesting [20]. Biofilms were incubated anaerobically for 3 h at 37°C in sterile Buffered Peptone Water (BPW) supplemented with 0.2% sucrose. Lactic acid accumulation (in mM per biofilm) was determined using a colorimetric assay [23].

Protease activity

Increased protease activity, and especially gingipain activity, is related to periodontitis [24]. Total and gingipain-specific protease activity was measured to determine the periodontitis activity of the 48 h biofilms. The protease activity was measured using a fluorescence resonance energy transfer (FRET) assay, as described previously [18,24]. Briefly, spent medium of four biofilms per condition was filter sterilized and mixed 1:1 with TRIS-buffered saline (TBS). A FRET-probe PEK-054 ([FITC]-NleKkkkVLPiQLNAATDK-[KDbc]) [25] was used to measure total protease activity and gingipain-specific protease activity [24]. The probe was added at a final concentration of 16 µM and fluorescence (excitation at 485 nm and emission at 530 nm) was measured every 2 min for 2 h using a fluorescence microplate reader (Fluostar Galaxy; BMG Laboratories, Offenburg, Germany). Protease activity was defined in relative fluorescence per min (RF/min).

Red autofluorescence

Red autofluorescence is a fast and direct, non-invasive, quantifiable biofilm phenotype. This autofluorescence is related to older oral biofilms [26,27]. It has been suggested that red autofluorescence is also related to oral disease, especially gingival inflammation [28] and caries [26,27,29].

Red autofluorescence of four biofilms per condition was assessed after 8, 24, 32, 48, 56, and 72 h by measuring the emission spectra (excitation 405 ± 20 nm) with a fiber-optic spectrophotometer (USB4000-FL, reflection probe, Ø 600 µm; Ocean Optics, Inc., Dunedin, FL), which consists of six illumination fibers around one read fiber. Measurements were performed in a dark room 3 mm from the surface of the glass coverslips (three measuring points per biofilm) [27]. Thereafter, biofilms were photographed for visual fluorescence assessment using a quantitative light-induced fluorescence digital (QLF-D) SLR camera system (Inspektor Research Systems BV, Amsterdam, The

Netherlands). The photographs were taken with fixed camera settings (white light photographs: shutter speed 1/30th; aperture value 8.0; ISO 1600; QLF-D photographs: shutter speed 1/30th; aperture value 5.6; ISO 1600).

Analysis of initial biofilm formation

To increase total biomass of the initial biofilms compared with initial studies, a roughly 5× larger surface area was used for the 5 h biofilms. Sterilized microscope slides (Menzel-Gläser, 7.6 × 2.6 cm) were placed in a 50 mL Greiner tube, filled with 50 mL pooled saliva inoculum or mixed inoculum, and incubated under aerobic +5% CO₂ conditions at 37°C.

After 5 h, the slides were aseptically transferred to a Petri dish filled with 25 mL BPW, and attached microbes were dislodged using a cell scraper. Subsequently, 5 mL BPW was serially diluted for CFU determination, and microbes in the remaining volume were harvested by centrifugation and frozen at -20°C for DNA isolation, qPCR, and sequencing.

Microbiome analysis

Seven 5 h-old initial biofilms for both saliva and mixed inoculum were used for microbiome analysis. Bacterial DNA concentration after purification was determined by qPCR using a universal primer-probe set to target the 16S rRNA gene [30].

The V4 hypervariable region of the 16S rRNA gene was amplified using 100 pg DNA, as described previously [30], except that 33 amplification cycles were performed. The generated amplicons were pooled in equimolar quantities and then purified from agarose gels (Qiagen, Roermond, The Netherlands) to remove non-specific PCR products prior to sequencing. The Illumina MiSeq platform and Illumina MiSeq reagent kit V2 (Illumina, Inc., San Diego, CA) were used for paired-end sequencing of the amplicons to generate 200-bp paired-end reads.

Sequence processing and analysis

The sequence data were processed as described previously [31].

Data normalization and statistical analysis

Fluorescence spectra were normalized to the amplitude and angle of inclination of the excitation peak (420–430 nm) using dedicated software (SP1 v1.0.0.10; Inspektor Research Systems BV; zero compensation 345–380 nm). Gaussian curve fitting was performed to calculate the separate fluorescence peaks for peak wavelength, amplitude, and full width at half maximum [27].

A two-sided t -test was used for statistical comparison of lactate production, protease activity, or biofilm formation in different groups. qPCR data were \log_{10} -transformed prior to performing a two-sided unpaired t -test. Groups were considered statistically different at $p < 0.05$.

The OTU table was randomly subsampled at 10,500 reads/sample. The Shannon diversity index was calculated using PAST software v3.01 [32]. The OTU data set was \log_2 transformed and ordinated by principal component analysis (PCA) into two dimensions using PAST. One-way permutational multivariate analysis of variance was performed on the Bray–Curtis Similarity Index to calculate the significance of the compositional differences between the saliva biofilms and mixed biofilms. Groups were considered statistically different at $p < 0.05$.

Linear discriminant analysis effect size (LEfSe) [33] was used to identify OTUs that differed in relative abundance between the saliva and mixed biofilms. The alpha values were kept at the default of 0.05, and the LDA (spell in full) threshold was kept at 2.0.

Results

C. albicans integrates in complex in vitro oral biofilms in the AAA model

The AAA biofilm model was inoculated using saliva or saliva + *C. albicans* and cultured at 37°C under anaerobic, aerobic, and aerobic +5% CO₂ conditions. Under both aerobic conditions, the presence of *C. albicans* was readily observed using CLSM (Figure 1 (a and b)). Under elevated CO₂ conditions, *C. albicans* formed hyphae, while under aerobic conditions, hyphal formation was less extensive. In contrast, anaerobic growth conditions resulted in biofilms without visible *C. albicans* (Figure 1(c)).

Since biofilms are three-dimensional structures, it is possible that *C. albicans* was present yet undetectable using CLSM. Therefore, the presence of *C. albicans* was analyzed using qPCR. As expected, the saliva-inoculated biofilms showed no detectable presence of *C. albicans* in all conditions (Figure 1(d)). In line with the CLSM data, significant presence of *C. albicans* was observed in biofilms cultured in the presence of oxygen. Under anaerobic conditions the

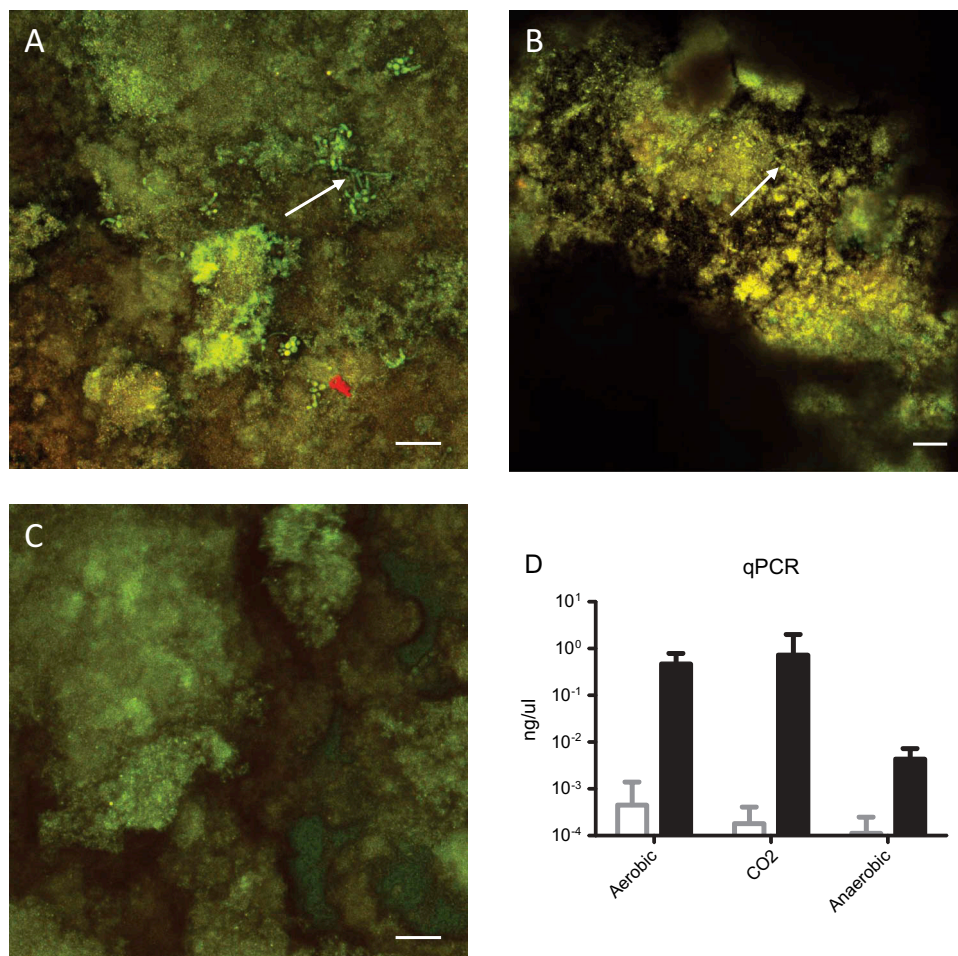


Figure 1. Confocal images of (a) aerobic biofilm, (b) aerobic +5% CO₂ biofilm, and (c) anaerobic biofilm. White arrows indicate visible *Candida albicans* cells; the size bar indicates 25 μm. (d) Quantitative polymerase chain reaction (qPCR) data in ng/μL, where white bars are saliva biofilms and black bars are mixed biofilms. The differences in *C. albicans* presence between the saliva only and mixed inoculum were all significant ($p < 0.05$) within the same condition. *C. albicans* was integrated in the microcosm biofilm when O₂ was available.

presence of *C. albicans* was 100-fold lower than under either aerobic condition. In conclusion, *C. albicans* can be introduced in the AAA model for oral microcosm biofilms when cultured under aerobic conditions.

Effect of *C. albicans* on phenotypes of oral biofilms

Oral biofilms consist mainly of oral bacteria with fungi probably representing <0.1% of viable cells. Therefore, the effect of *C. albicans* on total biofilm formation was evaluated by counting bacterial CFUs. For both aerobic conditions, the mixed biofilms resulted in similar total CFUs compared to biofilms inoculated with saliva alone (Figure 2(a)), indicating a similar amount of biofilm formation. For the anaerobic biofilms, the total CFUs were statistically different (1.3×10^9 vs. 1.6×10^9 , $p = 0.36$). Since the difference is much less than one log, this difference is most likely not biologically relevant. Pathology-related phenotypes [18] were assessed by measuring lactic acid production (cariogenic phenotype) and protease activity (periodontitis phenotype). The presence of *C. albicans* did not affect lactic acid accumulation (Figure 2(b)), and neither did it affect total or specific protease activity of the 48 h biofilms (Figure 2(c and d)).

Dynamic analysis of red autofluorescence

In vivo oral biofilms produce red autofluorescence under certain conditions [28]. *In vitro* saliva biofilms, grown for 48 h in the presence of oxygen, showed reduced red autofluorescence in some experiments (end-point measurement) compared with mixed biofilms (data not shown). To obtain more insight into the development of this red autofluorescence, the dynamics of its development with time were studied.

Red autofluorescence was analyzed after 8, 24, 32, 48, 56, and 72 h for all biofilms. Anaerobically grown biofilms always showed red autofluorescence from 32 h onwards, irrespective of the presence of *C. albicans* in the inoculum (Figure 3(c and f)). This was in contrast to both aerobic conditions, where clear differences appeared in red autofluorescence between the saliva and mixed biofilms. In saliva biofilms, red autofluorescence was absent or considerably reduced for all time points, while mixed biofilms were red autofluorescent from 32 h onwards (Figure 3(a, b, d, and e)). *C. albicans* thus induces red autofluorescence in mixed biofilms under aerobic growth conditions, mimicking the anaerobically grown biofilms.

The pattern of the red autofluorescence in the biofilms was also different between the three conditions. In anaerobic biofilms, red autofluorescence was always evenly distributed throughout the biofilms, while in both aerobic conditions, spots of red autofluorescence

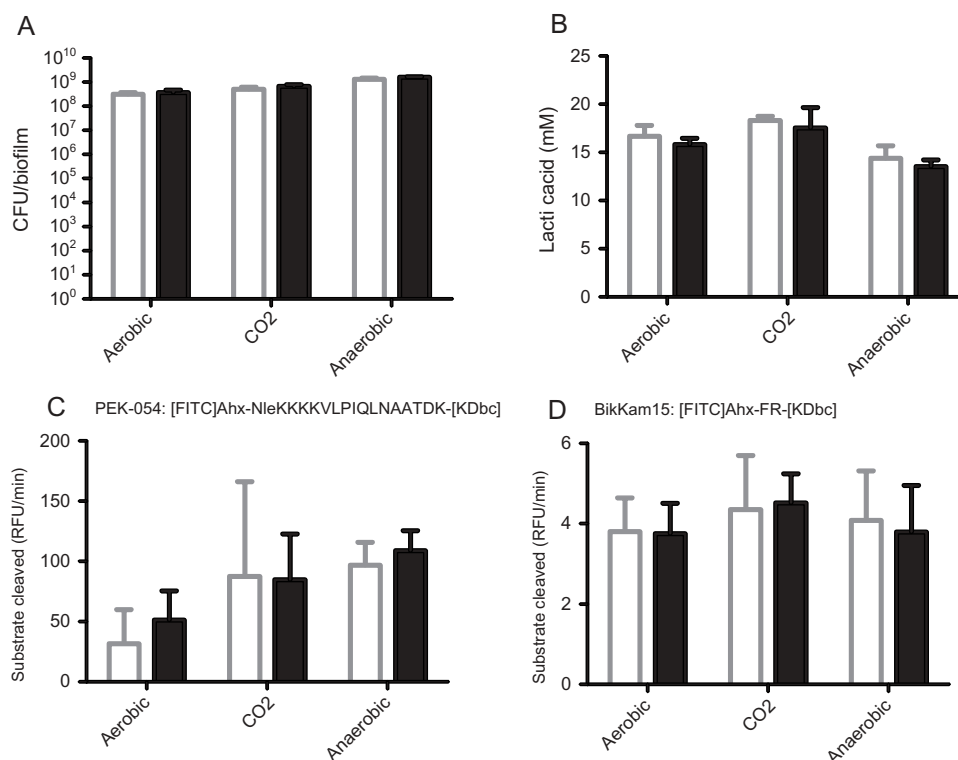


Figure 2. Phenotypes of 48 h biofilms. White bars represent saliva biofilms; black bars represent mixed biofilms. (a) Colony forming units (CFU) counts in CFU per biofilm. (b) Lactic acid accumulation in mM per biofilm. (c) Total protease activity in RFU/min. (d) Specific protease activity in RFU/min. No statistical differences were found between mixed and saliva biofilms. All conditions resulted in statistically different CFUs.

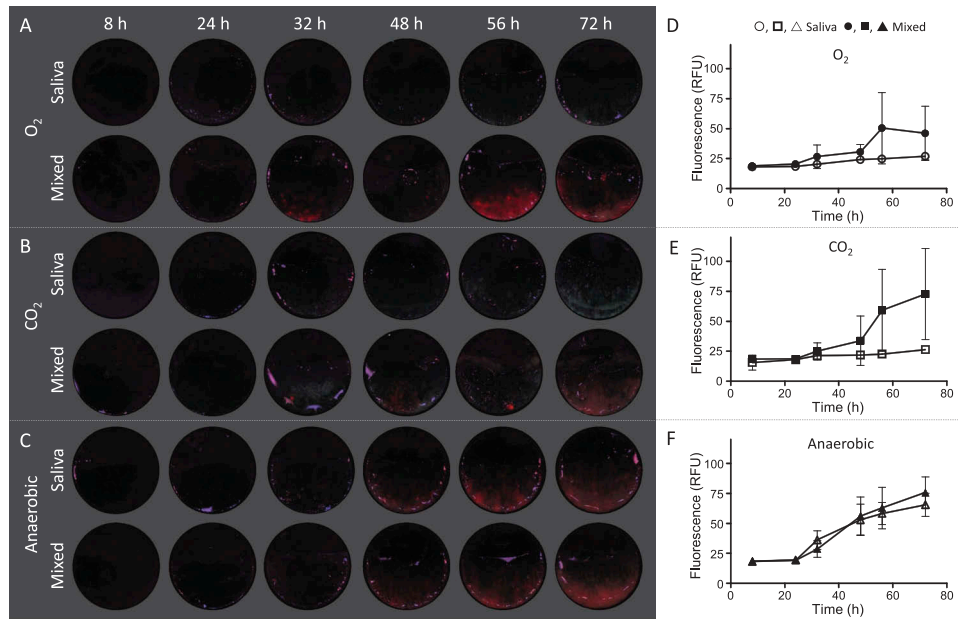


Figure 3. Red autofluorescence of biofilms grown under aerobic, anaerobic, and CO₂ conditions in time. The biofilms were inoculated with pooled stimulated saliva + *C. albicans* (mixed) or with saliva alone. Representative quantitative light-induced fluorescence pictures of (a) biofilms grown aerobically, (b) biofilms grown aerobically +5% CO₂, and (c) biofilms grown anaerobically. Red autofluorescence in RFU after curve fitting for (d) biofilms grown aerobically, (e) biofilms grown aerobically +5% CO₂, and (f) biofilms grown anaerobically.

were visible, explaining the high standard deviations in the red fluorescence spectra (Figure 3(d, e, and f)).

Effect of *C. albicans* on microbiome of early biofilms

Hyphae provide sites of adhesion for many bacteria that interact with *C. albicans*. Since hyphae were present predominantly in the CO₂ grown conditions (as

observed by CLSM, *vide supra*), the effect of *C. albicans* presence during initial (5 h) biofilm formation was analyzed for CO₂ conditions. Based on qPCR, the saliva biofilms resulted in hardly detectable *C. albicans*, very close to the detection limit of the qPCR. In the mixed biofilms, *C. albicans* was a detectable part of the biofilm (Figure 4(a)) and more than 4 log₁₀ higher than in the saliva control. The same amount of *C. albicans* was found in the 5 h biofilms as in the 48 h biofilms

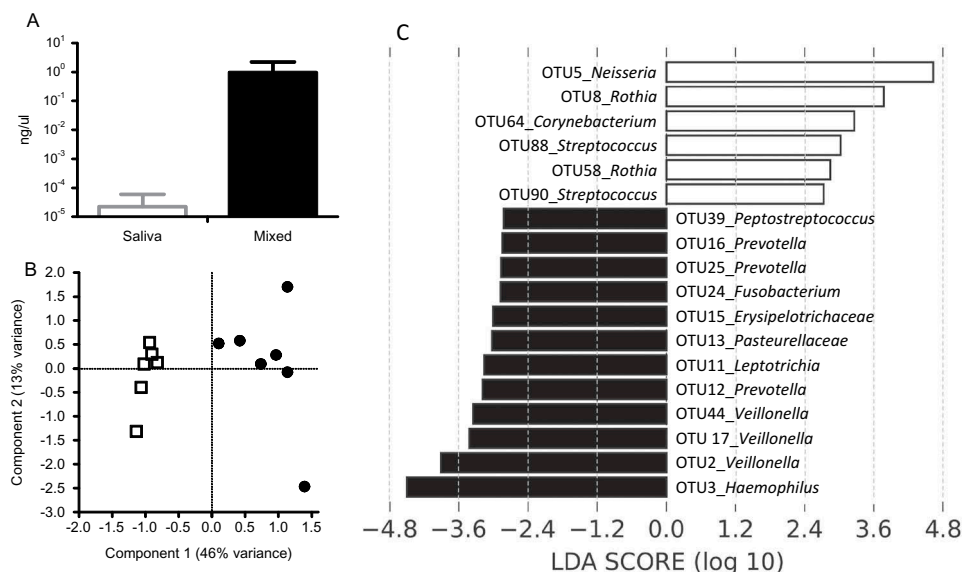


Figure 4. Microbiome analysis of initial (5 h) biofilms. (a) qPCR of *C. albicans* in ng/μL. White bar represents saliva biofilms; black bar represents mixed biofilms. (b) Principal component analysis plot of initial (5 h) biofilms where □ are saliva biofilms and ● are mixed biofilms. The data were randomly subsampled and log₂ transformed. (c) Visualization of most significant OTUs that differentiate between saliva and mixed biofilms, ranked by the effect size in LEfSe. White bars represent OTUs more abundant in saliva biofilms; black bars represent OTUs more abundant in mixed biofilms.

(0.73 ng/ μ L vs. 9.8 ng/ μ L). Since the surface covered by the 5 h biofilms was roughly 25 times larger than the surface covered by the 48 h biofilms, this indicates that *C. albicans* not only blends in the biofilm, but also is able to proliferate.

Since not all bacterial species interact with *C. albicans*, the introduction of this yeast during initial biofilm formation potentially leads to differences in the bacterial microbiome of the biofilm. This microbiome was analyzed using 16S rDNA sequencing of the 5 h biofilms. The complete OTU list is available in [Supplementary Table S1](#). The Shannon diversity index was statistically significantly higher ($p = 0.02$) for mixed biofilms (3.6) than it was for saliva biofilms (3.4). A clear difference in species composition was observed between the saliva biofilms and mixed biofilms ($p = 0.0004$, $F = 8.4$), which is also visible in the PCA plot ([Figure 4\(b\)](#)). LEfSe [33] was used to detect the OTUs responsible for this separation, and the relative abundance of the OTUs uncovered by LEfSe is plotted in [Supplementary Figure S1](#). A total of 18 OTUs were significantly different in relative abundance between the 5 h saliva and mixed biofilms ([Figure 4\(c\)](#)). The most prominent differences were *Neisseria* (OTU 5), *Rothia* (OTU 8, OTU 58), and *Streptococcus* (OTU 90) that were more abundant in saliva biofilms. Biofilms inoculated with *C. albicans* and saliva contained relatively more *Haemophilus* (OTU 3), *Veillonella* (OTU 2, OTU 17, OTU 44), *Prevotella* (OTU 12, OTU 16, OTU 25), *Leptotrichia* (OTU 11), and *Fusobacterium* (OTU 24). These OTUs are in agreement with the first component of the PCA, which explains 46% of the variance and separates the two groups. Concluding, biofilms inoculated with a mixture of saliva and *C. albicans* showed a significantly different bacterial species composition and a higher diversity compared with biofilms inoculated with saliva alone.

Discussion

Oral biofilms are very complex, diverse communities with >500 species of bacteria, most of which are unculturable [2]. *Candida* is present in low abundance in the oral cavity of 50–70% of healthy individuals [3,4], and thus can be considered a commensal microorganism. This study aimed to introduce *C. albicans* in a microcosm *in vitro* model to study the effect of *C. albicans* on the phenotype and microbiome of complex oral biofilms.

In order to study the fundamentals of oral biofilm development, one should aim to use *in vitro* models that mimic the oral situation as well as possible. The AAA model, inoculated with saliva, grown in artificial saliva medium, is such a model [20]. Usually, this model, like many other oral biofilm models, is incubated anaerobically to preserve the strictly anaerobic

species typical for oral biofilms. Since the oral cavity is not strictly anaerobic [7], this approach may result in a biased microbial population with aerobic species being underrepresented. For example, while generally present in the initial inoculum, *C. albicans* was not present in biofilms grown in the AAA model under standard anaerobic conditions. Although *C. albicans* is able to grow under anaerobic conditions, growth rates are very low [34]. *C. albicans* is probably out-competed by (facultative) anaerobic bacteria that grow more efficient in the absence of oxygen. In the present study, this problem was overcome by culturing the AAA model in the presence of oxygen. *C. albicans* successfully integrated in the microcosm biofilms in the aerobically incubated AAA model. When cultured under aerobic conditions, *C. albicans* was detectable both by microscopy and qPCR. Under aerobic conditions with 5% CO₂, hyphae were formed, which is consistent with reports showing that CO₂ is a potent inducer of hyphae formation [35]. It may be expected that aerobic incubation will prevent anaerobic bacteria from colonizing the model, which indeed seemed to occur in the saliva biofilms. However, this did not happen in the presence of *C. albicans*, where strict anaerobic bacteria colonized the AAA model.

The presence of *C. albicans* did not affect the total number of culturable microorganisms (CFU counts) in the biofilm or the pathology-related phenotypes of oral biofilms that were defined previously [18]. Lactic acid accumulation in the biofilms was not different between saliva and mixed biofilms. This was unexpected, as *C. albicans* has been suggested to be a cariogenic microbe, especially in early childhood caries [36]. It was also previously reported that *C. albicans* enhanced the virulence of *S. mutans* [15]. The discrepancy between the study by Willems et al. [15] and this study is probably related to the complex nature of the biofilms compared with previously used dual-species models (give at least a couple of references). In complex biofilms, metabolic interaction of its members is not uncommon [37]. One example is the production of lactic acid by saccharolytic bacteria, which is consumed by *Veillonella* [38]. End products of a dual-species biofilm are therefore not necessarily waste products to a complex biofilm, making the produced metabolites of complex biofilms very different from dual-species biofilms. The findings support the importance of using more complex, realistic models to study the role of microbes in a biofilm.

The occurrence of red autofluorescence of the *in vitro* biofilms was affected by the presence of *C. albicans*. *C. albicans* is known to autofluoresce orange/red when excited with 405 nm (blue-violet) light [29]. However, no studies have reported the effect of *C. albicans* presence on red autofluorescence

of oral biofilms. This is, to the authors' knowledge, the first study describing red autofluorescence of biofilms grown under different oxygen conditions. *In vivo*, red autofluorescent plaque is indicative of older plaque [26,27], which contains more anaerobic species than young plaque does [37]. In line with this, red autofluorescence was observed *in vitro* when saliva biofilms were cultured under anaerobic conditions, and it was rarely detected when cultured under aerobic conditions. However, the presence of *C. albicans* induced red autofluorescence under aerobic conditions. The induced red autofluorescence might therefore be an indication that *C. albicans* supports the growth of anaerobic bacteria in the early stage of biofilm formation.

Interactions between *C. albicans* and bacteria can occur on physical, chemical, and metabolic levels [10]. In many cases, physical interactions (e.g. adhesion) occur with the hyphae [39,40]. Therefore, 5 h biofilms were grown with 5% CO₂ to stimulate hyphae formation. Community analysis of 5 h-old biofilms confirmed the presence of more anaerobic bacteria in mixed biofilms. In the presence of *C. albicans*, more strict anaerobic genera were found, namely *Veillonella*, *Leptotrichia*, *Prevotella*, and *Fusobacterium*, compared with more aerobic genera in saliva biofilms, namely *Neisseria* and *Rothia*. This is in agreement with a recent study reporting that *C. albicans* biofilms allow anaerobic bacteria to grow under aerobic culture conditions [41]. It should, however, be noted that in contrast to the Fox study [41], in the present study, *C. albicans* biofilms were not preformed. The depletion of oxygen seems to be rapid and not necessarily biofilm dependent. Oxygen consumption by *C. albicans* is high and rapid [42], and the presence of *C. albicans* could quickly create anaerobic micro-niches. In these micro-niches, strict anaerobic bacteria could survive and proliferate. As a consequence of the presence of *C. albicans*, young oral biofilms that would normally consist of aerobic or facultative anaerobic bacteria can now also contain strict anaerobes. This is supported by the induced red autofluorescence observed in the present study. The amount of oxygen removed, and the extent of anaerobic niches formed, would be dependent on the number of *C. albicans* cells present and their individual respiratory rate.

All OTUs that determine the difference between saliva and mixed biofilms were identified as part of the healthy oral core microbiome [2]. However, many of these organisms are to some extent associated with oral infectious diseases. For example, *Leptotrichia* and *Prevotella* were reported to be related to the presence of active caries lesions [43,44]. *Prevotella* and *Fusobacterium* species are part of the so-called orange complex, which is a consortium of bacteria related to periodontitis [45]. *Veillonella* species are also associated with caries lesions, presumably as a result of high

concentrations of lactic acid [6]. Yet, they are mostly considered beneficial to the oral cavity due to their lactic acid consumption [46], which reduces the caries activity.

Concluding, a model was established to incorporate *C. albicans* in microcosm oral biofilms. Using this model, it was shown that *C. albicans* does not elevate disease-associated phenotypes (i.e. lactic acid accumulation and proteolytic activity) of the biofilms, but it did induce survival and proliferation of strictly anaerobic bacteria under aerobic culture conditions. As such, the presence of *C. albicans* in initial *in vitro* oral biofilms changes the community composition significantly.

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Disclosure statement

MHvdV is a co-inventor of several patents related to quantitative light-induced fluorescence. The authors declare that there is otherwise no conflict of interest pertaining to the data presented in this article.

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microbial communities in relation to the role these communities play in maintaining oral health and causing oral infections.

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