Molecular characterization of Candida in the oral cavity and factors involved in biofilm formation and virulence
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Chapter 3

The effect of *Streptococcus mutans* and *Candida glabrata* on *Candida albicans* biofilms formed on different surfaces


Although *Candida* containing biofilms contribute to the development of oral candidosis, the characteristics of multi-species *Candida* biofilms and how oral bacteria modulate these biofilms is poorly understood. The aim of this study was to investigate interactions between *Candida albicans* and either *Candida glabrata* or *Streptococcus mutans* in biofilms grown on various surfaces, with or without saliva. Hydroxyapatite (HA), polymethylmetacrylate (PMMA) and soft denture liner (SL) discs were used as substratum. Counts of viable micro-organisms in the accumulating biofilm layer were determined and converted to colony forming units per unit surface area. Confocal laser scanning microscopy was used to characterize biofilms and to quantitate the number of hyphae in each condition tested. Viable counts of *C. albicans* and *C. glabrata* per mm$^2$ decreased in the order HA > PMMA > SL (p<0.05). Biofilms grown on saliva-coated specimens harboured fewer *C. glabrata* than uncoated specimens (p<0.05). Glucose and the presence of *S. mutans* suppressed *C. albicans* hyphal formation. Dual *C. species* biofilms did not show competitive interaction between the two species. We conclude that *Candida* biofilms are significantly affected by saliva, substratum type and by the presence of other micro-organisms.
Candida species are the main pathogens responsible for the development of denture stomatitis, which is the most common infection in denture wearers (1). Poorly fitting dentures and poor oral hygiene are the most frequent cause of this opportunistic infection (2). Especially in elderly patients, several predisposing factors may accumulate (i.e. dietary factors, malignancies, use of broad-spectrum antibiotics, smoking, age, diabetes mellitus, iron and vitamin deficiencies, and salivary gland dysfunction (2,3), which often leads to candidal infections.

Although Candida albicans is the predominant isolate in these infections (4), other non-albicans species, in particular Candida glabrata, are also frequently isolated from acrylic surfaces and the palatal mucosa (5). Moreover, while Candida species are identified as the major pathogens, bacteria from denture biofilms are generally also involved (6).

Denture biofilms are composed mainly of bacteria (7,8), with Streptococcus mutans showing a high prevalence, while yeast constitutes a minor part of the total microbial flora (9,10). On a given surface, the formation of multi-species biofilms increases the chance of survival for many micro-organisms in the oral environment. Although the oral cavity consists of many habitats, in terms of growth sites and growth conditions, each of which favours a specific group of bacteria, micro-organisms interact to ensure their individual survival (11,12).

C. albicans virulence is attributed to its ability to grow in the full range of vegetative morphologic forms: yeast, pseudohyphae and true hyphae. The observations that elongated hyphae evade or escape phagocytic cells and that yeast cells disseminate in the tissue and bloodstream suggest that morphology contributes as a major factor in the survival of C. albicans at various sites or conditions (13,14). Hyphae formation is required for robust biofilm formation, as well as cell–substrate and cell–cell interactions, and extracellular matrix production, which are key steps in biofilm development (15,16). Not only C. albicans properties but also interactions with co-habitating oral micro-organisms may determine C. albicans virulence characteristics and together this justifies studying multi-species biofilms on surfaces.

Very little is known on substratum effects on the interactions between Candida species and other oral micro-organisms, specifically denture liners substrata surfaces containing or releasing antifungals. Fungal growth is known to
destroy the surface properties of denture liners and this may lead to irritation of the oral tissues. This observation is the rationale why attempts have been undertaken to incorporate antifungal agents or antiseptics in these materials. The use of denture liners for denture prostheses is needed in clinical situations in which patients have thin, sharp, or resorbed residual alveolar ridges, chronic tissue irritation from dentures or have received implant treatment (17). Even though these materials show excellent tissue tolerance, one of the problems is the colonization of *Candida* spp. on and in the material. Similarly, the role of saliva during the initial colonization and subsequent multi-species biofilm formation is poorly understood. Several studies have demonstrated that pre-treatment of samples with whole saliva decreased the initial adherence of *C. albicans* (18-22), while other studies showed either an increased adherence (23,24), or no effect (25).

While bacterial biofilms are currently being extensively studied, few studies have addressed fungal-bacterial biofilms. The complex interactions between yeasts, substratum surfaces, presence of saliva and oral bacteria have been studied superficially (10,22,26), but many questions have remained unanswered. Since colonization, growth and differentiation of *Candida* spp. in the oral cavity are of significant clinical importance, the purpose in our study was to analyse single and dual-species biofilm formation on various substratum types (one containing an antifungal agent), and to determine the effects of whole saliva and *S. mutans* on this process.

**Material and methods**

**Experimental design**

This *in vitro* study had a completely randomized and blinded design (regarding CFU counts), with substratum type (hydroxyapatite - HA, polymethylmetacrylate - PMMA or soft denture liner - SL), saliva (coated or uncoated), biofilm type (single species biofilms: *Candida albicans* and *Candida glabrata*; and dual species biofilms: *C. albicans* plus *Streptococcus mutans*, *C. glabrata* plus *S. mutans* and *C. albicans* plus *C. glabrata*) and type of carbohydrate (glucose or sucrose) as factors. CFU counts of *C. albicans* and *C. glabrata* and number of hyphae (*C. albicans*) were the dependent variables. Scanning electron microscopy (SEM) was used to characterize substratum surfaces and confocal scanning laser microscopy
(CLSM) was used to visualize the biofilm structure and to quantify hyphae formation.

HA, PMMA and SL discs were used as substrata, using 24-well polystyrene tissue culture plates. Discs without yeast or bacterial cells served as controls. Single and dual species biofilms were formed for 24 hours. After this period, discs with biofilms were removed from the wells and CFU counts of each microorganism were calculated.

Preparation of PMMA and SL discs
Soft denture liner (Coe Soft, GC America, Alsip, IL, USA) and polymethylmetacrylate (Rebaron, GC Dental Products Corp., Aichi, Japan) discs were prepared according to the manufactures specifications at room temperature (20 ± 1.0 ºC and 50 ± 5% relative humidity), under aseptic conditions, using a Teflon mould (10.6 mm in diameter and 1.5-2.0 mm in thickness). A uniform surface was ensured by placing glass slides on both sides of the mould and firmly fixing both ends, and separating the glass slides after curing, after preparation (27). Discs were used immediately. The soft denture liner contained undecylenic acid (1-5%) as the antifungal ingredient.

Inoculum and media
The micro-organisms used in this study were *S. mutans* PDM15 (28): a mutant of *S. mutans* UA159 containing a green fluorescent protein (GFP) coding gene fragment, *C. albicans* ATCC 90028 and *C. glabrata* ATCC 90030. To prepare the inocula, *S. mutans* was first grown anaerobically on Todd-Hewitt yeast extract (THY; Difco, Sparks, MD, USA) agar plates, supplemented with 10 μg/ml erythromycin, for 2 days. *C. albicans* and *C. glabrata* were both grown aerobically on CHROMagar™ (CHROMagar™ *Candida*, Paris, France) plates for 24 hours. The modified semi-defined medium (pH 7.0) used in this study (29) contained 76 mM K₂HPO₄, 15 mM KH₂PO₄, 10 mM (NH₄)₂SO₄, 35 mM NaCl, and 2 mM MgSO₄ ·7H₂O and was supplemented with filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 μM riboflavin, 0.3 μM thiamine HCl, and 0.05 μM D-biotin), amino acids (4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, and 0.1 mM L-tryptophan), 0.3% (w/v) yeast extract. This medium was selected for its constant pH (6.8), as pH is known to affect hyphal development. Also, the medium allows both species to
grow together (data not shown) and diminishes background interference on CLSM. As it was one of our aims to check the role of the carbohydrates, we selected a medium with a single added source of carbon.

Subsequently, single colonies were inoculated into 10 ml of the semi-defined medium (18 mM glucose-enriched) individually for each micro-organism and incubated anaerobically for *S. mutans* and aerobically for *Candida* species at 37 °C overnight. Cells were harvested in the late exponential growth phase, washed with phosphate buffered saline (PBS; pH 7.2) and resuspended spectrophotometrically to a concentration of 10^8 cells/ml (0.35 at 600nm) for bacteria and 10^7 cells/ml for *Candida* species (0.38 at 520 nm). A standard curve of turbidity against colony forming unit (CFU) was used to obtain the number of cells (25).

**Biofilm assays**

Biofilm assays were performed with single-species biofilms of *C. albicans* or *C. glabrata*, and dual-species biofilms of *S. mutans* plus *C. albicans*, *S. mutans* plus *C. glabrata* and *C. albicans* plus *C. glabrata*. Discs of the three materials, prepared as previously described, were placed on the bottom of 24-well (15 mm diameter each well) polystyrene tissue culture plates (bio-one; Greiner, Frickenhausen, Germany). Subsequently, 2 ml of each cell suspension (10^8 CFUs *S. mutans* and/or 10^7 CFUs *C. albicans/C. glabrata* in the semi-defined medium (18 mM glucose or 24.35 mM sucrose), was added to each well.

Biofilms were formed on saliva-coated or non-coated hydroxyapatite discs (sHA or nHA), polymethylmethacrylate discs (sPMMA or nPMMA) and/or soft denture liner discs (sSL or nSL). Disc surface areas were 2.7 ± 0.2 cm². The sHA, sPMMA and sSL discs were prepared by incubation with clarified human whole saliva for 1 hour at 37°C. Human whole saliva was collected from a single healthy volunteer during masticatory stimulation with Parafilm M (American Can Co., Greenwich, CT, USA) in an ice-chilled polypropylene tube and clarified by centrifugation at 10,000g for 10 minutes at 4°C (30). For every experiment the saliva sample was collected at the same time of day and the volume limited to 50 ml per collection period, such as to account for the circadian rhythm in saliva composition (31). The supernatant was removed and immediately used.

All biofilm assays were performed in duplicate in at least four independent experiments on different days. The organisms were grown undisturbed (i.e. no
dynamic growth condition such as the use of a rotary/orbital shaker or other source of shear forces that would disturb the biofilms was used) during 24 hours to allow biofilm formation. Additional biofilms were grown for biofilm analysis by means of CLSM.

**Biofilm analyses**

In all experiments, after the biofilm development phase (24 h), each disc was aseptically removed and washed twice with PBS in a standard fashion to remove loosely adherent material, by gentle insertion in a new well containing 2mL of sterilized PBS for 2 seconds. Discs were subsequently processed and vortexed for 1 minute in cysteine peptone water (CPW), to dissociate chains and aggregates of micro-organisms, as described elsewhere (4). The suspensions were subsequently serially diluted in PBS and 20 μl samples were plated in triplicate on Trypticase Yeast-Extract Cysteine Sucrose Bacitracin agar (TYCSB), CHROMagar™, and blood agar, (the latter to rule out possible contamination). The plates were incubated at 37 °C, under anaerobic (blood agar and TYCSB agar), or aerobic (CHROMagar™) conditions for 24–72 h. Colony-forming units (CFU) were counted using a stereomicroscope, and the results were expressed in colony-forming units per area.

**Scanning Electron microscopy and Confocal Scanning Laser Microscopy**

For SEM, discs of all materials tested were mounted on a stub, air-dried, sputter-coated with gold (Balzers Union MED 010 evaporator) and examined with a Zeiss (Thornwood, NY) DSM940A scanning electron microscope at an accelerating voltage of 20.0 kV for surface characterization prior to the biofilms assays.

For CLSM, the discs were carefully removed from the wells (after 24 h of biofilm formation), placed (face down) on a 35-mm-diameter glass-bottom Petri-dish (MatTek Corp., Ashland, MS, USA) containing 1 ml of PBS and 25 μg/ml of concanavalin A conjugate (ConA-rhodamine, Invitrogen, The Netherlands) and incubated for 15 min at 37°C. ConA binds to glucose and mannose residues of the yeast cell wall polysaccharides as indicated by red fluorescence in CLSM. Biofilms were observed by CLSM (LSM510, Carl Zeiss, Jena, Germany) mounted on an inverted microscope (20x objective lens, Axiovert100 M, Zeiss). This microscope was equipped with an Ar-ion laser tuned at 488 nm and a 543 nm HeNe laser for simultaneous measurement of GFP (green; 505-530 nm
bandpass) and conA (red; 560 nm longpass) in multitrack mode. To assess the structure of the biofilms, a series of optical sections was taken throughout the full depth of the biofilm. All images were captured by direct acquisition with Z-step ranging from 0.5 to 2 μm. Data were subsequently processed using ImageJ and ObjectJ (for display of 3D images; maximum pixel intensity for projection of Z series), and the number of hyphae was counted. Individual hyphal elements were enumerated within the 3D image sections by marking each element. This marking step allowed counting without repetition, as each counted element would appear with a mark generated by the computer program and therefore, avoided overlapping count. Using the Z-step, each hyphal element was verified in order to allow hyphal branching counts. As a parameter to standardize the counts, if in the 3D movement of the step, the image was characterized by branched hyphae without any separation, this was counted as a single element.

Statistical analysis
Statistical analyses were done using SAS software (SAS Institute Inc., version 9.0, Cary, N.C., USA) employing a significance level fixed at 5%. The null hypothesis assumed no differences among sugars, saliva, substrata or dual or single species biofilms. Data that violated the assumptions of equality of variances and normal distribution of errors were transformed. Data of hyphae and CFU counts were analyzed by ANOVA, followed by Tukey test.

Results
Assessment of the various materials with SEM showed different degrees of surface irregularities. Remarkably, large amounts of porosities and irregularities were observed in the soft liner samples, while HA and PMMA surfaces were smoother (Figure 1).

All tested biofilms displayed significantly higher growth on HA (p<0.002), followed by PMMA and SL respectively, irrespective of the sugar type (glucose or sucrose) or the biofilm combination (single or dual-species; Tables 1 and 2; p<0.0001). C. glabrata showed higher CFU counts compared to C. albicans under all experimental conditions (p<0.05).
Figure 1. SEM images showing the materials’ surfaces. (A) Note the irregularities on the materials (x40); (B) Details of the samples, showing typical examples of maximum irregularities/holes (x500).
Saliva coating resulted in lower CFU counts only for some of the conditions chosen for *C. glabrata* biofilm growth (*p*<0.05). *C. albicans* biofilms were not affected by saliva (Table 1; *p*>0.05).

When compared to the other types of biofilms, *C. albicans* co-cultured with *S. mutans* showed higher counts for all substrata tested (*p*<0.001). Dual *Candida* species, however, did not differ from single-species *C. albicans* biofilms with respect to *C. albicans* counts (*p*>0.05). Both *C. albicans* and *C. glabrata* biofilms grown with glucose showed higher CFU counts when compared with the sucrose-grown biofilms (Tables 1 and 2; *p*<0.001). Under all experimental conditions *C. glabrata* showed higher counts when grown together with *C. albicans*, when compared with the other biofilms under study (*p*<0.05).

Hyphae counts differed depending on sugar type and the presence of *S. mutans* for all experimental conditions (Table 3; *p*<0.05) and depended on saliva coating for PMMA discs (*p*<0.001). Regarding CLSM, it interestingly revealed that the holes in the SL material harboured many yeast and bacterial cells (Fig. 2A). The dual-species biofilm (*C. albicans* plus *S. mutans*) formed on the soft liner was composed of two layers: one near the material surface consisting almost completely of *S. mutans* cells (Fig 2B), and the second, the outer/top surface of the biofilm, containing only *Candida* cells (Fig. 2C; the layer of *S. mutans* cells is still visible under the yeast layer).

**Figure 2.** CLSM images showing the structure of *C. albicans* (red)/ *S. mutans* (green) biofilms on SL. (A) Confocal image at the surface of the soft liner material; note the colonization of micro-organisms in the holes; (B) Confocal image right above the surface of the soft liner material, containing almost solely *S. mutans* (10μm from the surface); (C) Similar image taken at the water biofilm interface containing almost solely *C. albicans* (57μm from the surface).
Figure 3. CLSM images showing the biofilms’ structure under various conditions, after 24 h of growth. All images were taken at 50-60 μm from the surface. (A) *C. albicans* plus *S. mutans* on HA-discs after growth with sucrose, without saliva; (B) *C. albicans* plus *S. mutans* on HA after growth with glucose, without saliva; (C) *C. albicans* plus *S. mutans* on HA after growth with glucose, with saliva, displaying a less compact structure. (D) Single-species *C. albicans* biofilm on HA with glucose; (E) *C. albicans* plus *S. mutans* on PMMA after growth with sucrose. *C. albicans* and *S. mutans* are shown in red and green, respectively.
Effects of carbon source and saliva on biofilm structure were also observed (Fig. 3). Comparing panels A and B revealed that when biofilms were grown on glucose the formation of hyphae was suppressed in comparison with sucrose-grown biofilms (Table 3; p<0.05). Comparing Figure 3 panels B and D (yeast single and yeast-bacteria dual species), showed that hyphal inhibition by glucose was dependent also on the presence of *S. mutans*.

Saliva coated specimens showed a less dense biofilm structure, harbouring fewer micro-organisms, (Fig. 3C and Fig 4A). Dual *Candida* species biofilms showed a compact structure when grown on HA discs (Fig 4B). Grown on sucrose containing medium, hyphal elements were seen in the dual *Candida* species biofilm, as previously seen with *S. mutans* (Fig. 4A).

![Figure 4. CLSM images showing the biofilm structure (without saliva) of *C. albicans* plus *C. glabrata* on (A) SL with sucrose (80 μm from the surface); (B) HA with glucose (46 μm from the surface). Note the hyphal elements.](image-url)
<table>
<thead>
<tr>
<th>Material</th>
<th>Type of Biofilm</th>
<th>Glucose Uncoated</th>
<th>Saliva Coated</th>
<th>Sucrose Uncoated</th>
<th>Saliva Coated</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>C. albicans</td>
<td>210.71 ± 19.33 Ab</td>
<td>312.46 ± 38.74 Aa</td>
<td>115.21 ± 11.56 Aa</td>
<td>54.92 ± 4.12 Ab</td>
</tr>
<tr>
<td></td>
<td>C. albicans + S. mutans</td>
<td>395.00 ± 33.03 Aa</td>
<td>273.00 ± 19.27 Aa</td>
<td>124.44 ± 5.13 Aa</td>
<td>173.33 ± 5.20 Aa</td>
</tr>
<tr>
<td></td>
<td>C. albicans + C. glabrata</td>
<td>121.11 ± 10.29 Ac</td>
<td>127.78 ± 5.59 Ab</td>
<td>175.56 ± 14.68 Aa</td>
<td>61.33 ± 6.08 Ab</td>
</tr>
<tr>
<td>SL</td>
<td>C. albicans</td>
<td>29.5 ± 4.22 Cb</td>
<td>50.51 ± 5.05 Cb</td>
<td>2.00 ± 0.22 Cb</td>
<td>0.78 ± 0.05 Cc</td>
</tr>
<tr>
<td></td>
<td>C. albicans + S. mutans</td>
<td>41.24 ± 4.97 Cab</td>
<td>78.67 ± 5.93 Ca</td>
<td>1.82 ± 0.09 Cb</td>
<td>3.42 ± 0.08 Ca</td>
</tr>
<tr>
<td></td>
<td>C. albicans + C. glabrata</td>
<td>60.67 ± 11.26 Ba</td>
<td>28.13 ± 1.46 Cc</td>
<td>4.58 ± 0.45 Ca</td>
<td>2.00 ± 0.17 Cb</td>
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<tr>
<td>PMMA</td>
<td>C. albicans</td>
<td>101.50 ± 27.60 Bb</td>
<td>113.78 ± 11.13 Ba</td>
<td>32.17 ± 5.86 Ba</td>
<td>15.00 ± 1.91 Bb</td>
</tr>
<tr>
<td></td>
<td>C. albicans + S. mutans</td>
<td>143.79 ± 23.83 Ba</td>
<td>94.28 ± 32.51 Ba</td>
<td>30.38 ± 1.71 Ba</td>
<td>46.50 ± 5.96 Ba</td>
</tr>
<tr>
<td></td>
<td>C. albicans + C. glabrata</td>
<td>38.89 ± 3.02 Cc</td>
<td>47.22 ± 7.58 Bb</td>
<td>19.61 ± 3.74 Bb</td>
<td>11.78 ± 0.59 Bc</td>
</tr>
</tbody>
</table>

Distinct upper case letters represent statistically significant differences among materials. Distinct lower case letters represent differences among types of biofilms (microbial combination). No significant effects of saliva were observed among experimental groups. All groups were statistically different regarding the sugar used (ANOVA; p<0.05).
Table 2. Average ± standard error of Candida glabrata CFU enumeration (x 10^6).

<table>
<thead>
<tr>
<th>Material</th>
<th>Type of Biofilm</th>
<th>Glucose</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uncoated</td>
<td>Saliva Coated</td>
</tr>
<tr>
<td>HA</td>
<td>C. glabrata</td>
<td>339.58 ± 34.49 Ac 263.75 ± 16.12 Ab</td>
<td>251.33 ± 56.22 Ab</td>
</tr>
<tr>
<td></td>
<td>C. glabrata + S. mutans</td>
<td>901.67 ± 75.49 Aa 487.62 ± 37.85 Aa*</td>
<td>304.76 ± 13.07 Aab</td>
</tr>
<tr>
<td></td>
<td>C. glabrata + C. albicans</td>
<td>533.33 ± 43.95 Bb 453.33 ± 22.80 Aa*</td>
<td>414.44 ± 37.88 Aa</td>
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<tr>
<td>SL</td>
<td>C. glabrata</td>
<td>94.88 ± 6.16 Bb 116.92 ± 7.92 Ba</td>
<td>5.53 ± 0.42 Cb</td>
</tr>
<tr>
<td></td>
<td>C. glabrata + S. mutans</td>
<td>109.76 ± 12.43 Cb 103.33 ± 11.09 Ca*</td>
<td>65.44 ± 9.25 Ca</td>
</tr>
<tr>
<td></td>
<td>C. glabrata + C. albicans</td>
<td>411.73 ± 201.92 Aa</td>
<td>114.00 ± 7.98 Ba*</td>
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<tr>
<td>PMMA</td>
<td>C. glabrata</td>
<td>304.67 ± 57.06 Aa 233.33 ± 51.37 Ab</td>
<td>64.17 ± 2.61 Bb</td>
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<tr>
<td></td>
<td>C. glabrata + S. mutans</td>
<td>172.50 ± 32.40 Bb 130.11 ± 9.01 Bc*</td>
<td>81.25 ± 7.38 Ba</td>
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<td>C. glabrata + C. albicans</td>
<td>232.91 ± 25.88 Cab 358.94 ± 176.94 Aa</td>
<td>69.04 ± 6.64 Bab</td>
</tr>
</tbody>
</table>

Distinct upper case letters represent statistically significant differences among materials. Distinct lower case letters represents differences among types of biofilms (microbial combination). (*) represents experimental groups that differed regarding saliva coating (p<0.001). All groups were statistically different regarding the sugar used (ANOVA; p<0.05).
Table 3. Average ± SD of *Candida albicans* hyphae counts per field (mean value of 8-10 fields analyzed in each sample – 65 x 65 μm; z-step ranging from 0.5 to 2 μm).

<table>
<thead>
<tr>
<th>Material</th>
<th>Type of Biofilm</th>
<th>Glucose</th>
<th></th>
<th>Sucrose</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Uncoated</td>
<td>Saliva Coated</td>
<td>Uncoated</td>
<td>Saliva Coated</td>
</tr>
<tr>
<td>HA</td>
<td><em>C. albicans</em></td>
<td>15.50 ± 8.66Aa</td>
<td>14.25 ± 9.98Aa</td>
<td>9.80 ± 6.37Ab</td>
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<tr>
<td></td>
<td><em>C. albicans</em> + <em>S. mutans</em></td>
<td>4.00 ± 2.91Ab</td>
<td>5.00 ± 2.00ABb</td>
<td>11.33 ± 6.82Ba§</td>
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<tr>
<td></td>
<td><em>C. albicans</em> + <em>C. glabrata</em></td>
<td>10.25 ± 6.13Aa</td>
<td>9.00 ± 6.68Aa</td>
<td>2.75 ± 1.50Cai</td>
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<tr>
<td>SL</td>
<td><em>C. albicans</em></td>
<td>13.80 ± 8.40Aa</td>
<td>15.00 ± 7.28Aa</td>
<td>12.2 ± 5.87</td>
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<td></td>
<td><em>C. albicans</em> + <em>S. mutans</em></td>
<td>3.70 ± 3.16 Ab</td>
<td>2.40 ± 1.26Bb</td>
<td>8.10 ± 3.98Aa§</td>
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<td></td>
<td><em>C. albicans</em> + <em>C. glabrata</em></td>
<td>6.50 ± 1.73Bb</td>
<td>9.25 ± 6.50Aa</td>
<td>12.00 ± 4.24Ba</td>
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<tr>
<td>PMMA</td>
<td><em>C. albicans</em></td>
<td>4.00 ± 2.91Ba</td>
<td>13.40 ± 8.85Aa*</td>
<td>4.70 ± 4.19Ba</td>
<td>9.10 ± 6.85Aa*</td>
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<td></td>
<td><em>C. albicans</em> + <em>S. mutans</em></td>
<td>3.00 ± 2.36Aa</td>
<td>8.40 ± 7.75Aab*</td>
<td>5.40 ± 5.13Ba§</td>
<td>11.40 ± 6.29Aa§</td>
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<td></td>
<td><em>C. albicans</em> + <em>C. glabrata</em></td>
<td>2.75 ± 1.50Ca</td>
<td>4.75 ± 1.50Bb*</td>
<td>4.50 ± 0.70Ba</td>
<td>6.00 ± 4.24Ab*</td>
</tr>
</tbody>
</table>

Distinct upper case letters represent statistically significant differences among materials. Distinct lower case letters represents differences among types of biofilms (microbial combination). * Indicates differences between saliva coating and uncoating; § Indicates differences between sugars (ANOVA; p<0.05).
**Discussion**

Our study has shown that *C. albicans* biofilm formation is influenced by a multitude of interacting environmental conditions. The extent and morphology of biofilm formation were found to depend on the sugar used for growth, the substratum type, the presence of other micro-organisms and saliva. These findings emphasize the necessity to study these interactions in complex systems mimicking the oral cavity. We have shown that *S. mutans* increases *Candida* biofilm formation, and that *C. albicans* displays synergism with *C. glabrata*. Our study is the first to show formation of two layers (surface associated *S. mutans* cells separate from *C. albicans* cells) on a substratum containing an antifungal agent. The understanding of biofilm formation under different conditions, especially considering the presence of other micro-organisms may be a factor key in the development of therapies to prevent *Candida*-related diseases (9,10, 32).

Novel assays on quantification of *Candida* biofilms are based on assessing metabolic activity rather than viability (25,32,33). Of these, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium salt and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) used in reduction assays are colorimetric methods that quantify metabolic activity. We nevertheless still preferred CFU counts for several reasons. First, MTT or XTT can only be used for comparing conditions while using one yeast strain (33). Since it was one of our aims to compare biofilm growth of *C. albicans* and *C. glabrata*, these assays were not applicable; moreover inclusion of the prokaryotic *S. mutans* would further complicate interpretations. Second, although quantification of CFU of resuspended biofilm cells is time-consuming and laborious, it directly enumerates the cell numbers, which unlike XTT/MTT assays, are not influenced by their metabolic status (25).

We used Confocal Laser Scanning Microscopy to gain understanding of (dual-species) biofilms formed on the different materials. We chose this technique instead of SEM because the fixation and dehydration required for SEM severely distorts biofilm architecture and shrinks any aqueous phase, whereas live-cell CLSM preserves the intact structure of biofilms (34). Although light penetration depth is restricted to about 100 μm in single photon CLSM, this was not a limitation for our thin biofilms. The CLSM observations revealed that other
micro-organisms, not sensitive to undecylenic acid (or other antifungal), can first attach and grow on the SL after which *C. albicans* was able to colonize on this layer of *S. mutans*. We presume that other ‘early colonizers’ may also serve this purpose. Evaluation of (novel) denture liners should take this finding into account.

The soft denture liner with antifungal used in this study resulted in a lower amount of viable *C. albicans* cells in the surface biofilms under all conditions tested, corroborating studies where denture liners showed an inhibitory effect on *Candida* accumulation (27). However, conflicting results on single species *Candida* are found in the literature reporting both inhibitory and no antifungal effect (27,35,36,37,38). Colonization and growth of a certain species on biological surfaces is an indicator for this species’ pathogenic potential. Even though previous studies showed a similar trend on the initial growth effects of antifungals on *C. albicans* and *C. glabrata* (21,22,39,40), the degree of growth found in our study, specifically in the case of the denture liner, indicates lack of activity of undecylenic acid against *C. glabrata*. Differences in growth inhibition may be explained by different models used to study fungal biofilms (single or multi-species), and by the complex phenotypic heterogeneity of a *Candida* population in the oral cavity. This heterogeneity is displayed by a variable surface hydrophobicity, the absence or presence of secreted extracellular proteinases, hyphae formation and/or thigmotropism (5,39), all directly influencing *Candida* adherence. Although our results should be interpreted with care, since the nutrient-rich environment of the oral cavity does not (fully) match the *in vitro* nature of our study, they do point towards important clues on how *Candida* biofilms behave in the presence of an antifungal. Specific attention should be given to *C. glabrata*, which formed biofilms with higher cell counts than *C. albicans* under most of the experimental conditions and used materials.

Proportions of yeasts and hyphal cells have been shown to be dependent on the nutrient source in single species (*Candida* spp.) biofilms (6,25,32). Comprehensive studies on the effect of dietary sugars on modulation of oral *Candida* colonization and biofilm formation have already been reported on (25,41). The current multi-species study, showed that higher yeast counts were found in the presence of glucose when compared with sucrose.
From our data it is evident that the relationship between saliva pellicle on different substrata and *Candida* colonization is complex. Innate defence mechanisms, such as the flushing effect of saliva, and anti-*Candida* salivary components affect *Candida* physiology and decrease *Candida* adherence to oral surfaces (42). Other components in whole saliva have been reported to adsorb to *C. albicans* thereby increasing adherence to saliva-coated resins and resilient materials (24). Antimicrobial properties of saliva may contribute to the lower counts of micro-organisms. In addition, the nature of the substratum may influence the composition and the formation of the pellicle, which may be more important than the surface properties of the dental materials (43). Another observation is that the use of a saliva coating may in fact have little effect on biofilm formation (25), which agrees with our results, where saliva had no effect on *C. albicans* counts.

To study *Candida*-bacteria interactions we selected *S. mutans* because this bacterium is regularly found in denture plaque and is directly related to dental caries (44). We observed that *S. mutans* increased growth of both *Candida* species under all experimental conditions. These results not only suggest that there may be mutual growth stimulation of these micro-organisms, but also that they can co-aggregate with each other, which may enhance the adhesion process (45). In contrast to the previously reported competition between *C. albicans* and *C. dubliniensis* (46) we observed a stimulatory effect when *C. glabrata* was co-cultured with *C. albicans*.

An important observation is the fact that *S. mutans* leads to suppression of hyphae formation of *C. albicans*. Most likely this is a result of the biofilm-growth benefits that *S. mutans* displays on *C. albicans*. This finding that *S. mutans* affects prominent virulence parameters of *C. albicans* should be considered in studies dealing with prevention of oral manifestations of *C. albicans*.

Our null hypothesis tested was rejected since the combined results show that all factors under study influenced yeast counts. It is justified to speculate on the importance of the rehabilitation material in clinical situations. Since yeasts may reside metabolically dormant in more remote sites of this material, the oral cavity will be quickly re-colonized after antimycotic treatment in patients with oral candidosis. In most societies the use of such rehabilitation materials cannot be avoided, so special care to avoid oral cavity re-colonization is mandatory.
Further studies with a larger number of yeast strains and more oral bacterial species are needed to further increase our understanding of the oral ecosystem and the clinically important micro-organisms/materials interactions.

References


