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In vitro study of biofilm formation and effectiveness of antimicrobial treatment on various dental material surfaces

L. Li[1], M.B. Finnegan[2], S. Özkan[1], Y. Kim[1], P.B. Lillehoj[3], C.-M. Ho[3], R. Lux[1], R. Mito[1], Z. Loewy[2] and W. Shi[1,4,5]

1 UCLA School of Dentistry, University of California, Los Angeles, CA, USA
2 GlaxoSmithKline Consumer Healthcare, Parsippany, NJ, USA
3 UCLA School of Engineering and Applied Science, Los Angeles, CA, USA
4 UCLA Molecular Biology Institute, University of California, Los Angeles, CA, USA
5 Department of Oral Biology, Peking University Dental School, Beijing, China

Correspondence: Wenyuan Shi, UCLA School of Dentistry, 10833 LeConte Avenue, Los Angeles, CA 90095-1668, USA
Tel.: +1 310 825 8356; fax: +1 310 794 7109; E-mail: wenyuan@ucla.edu

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SUMMARY

Elevated proportions of *Candida albicans* in biofilms formed on dentures are associated with stomatitis whereas *Streptococcus mutans* accumulation on restorative materials can cause secondary caries. *Candida albicans*, *S. mutans*, saliva-derived and *C. albicans*/saliva-derived mixed biofilms were grown on different materials including acrylic denture, porcelain, hydroxyapatite (HA), and polystyrene. The resulting biomass was analysed by three-dimensional image quantification and assessment of colony-forming units. The efficacy of biofilm treatment with a dissolved denture cleansing tablet (Polident®) was also evaluated by colony counting. Biofilms formed on HA exhibited the most striking differences in biomass accumulation: biofilms comprising salivary bacteria accrued the highest total biomass whereas *C. albicans* biofilm formation was greatly reduced on the HA surface compared with other materials, including the acrylic denture surface. These results substantiate clinical findings that acrylic dentures can comprise a reservoir for *C. albicans*, which renders patients more susceptible to *C. albicans* infections and stomatitis. Additionally, treatment efficacy of the same type of biofilms varied significantly depending on the surface. Although single-species biofilms formed on polystyrene surfaces exhibited the highest susceptibility to the treatment, the most surviving cells were recovered from HA surfaces for all types of biofilms tested. This study demonstrates that the nature of a surface influences biofilm characteristics including biomass accumulation and susceptibility to antimicrobial treatments. Such treatments should therefore be evaluated on the surfaces colonized by the target pathogen(s).

INTRODUCTION

The adherence of microbial species to denture and other dental restorative materials and the subsequent formation of biofilms on these surfaces are contributory factors to plaque-related oral and systemic diseases. Numerous studies have established strong correlation between *Candida albicans* and denture stomatitis (Bergendal & Isacsson, 1983; Arendorf & Walker, 1987), a disease that affects up to 65% of

patients wearing dentures (Dorey *et al.*, 1985). *Streptococcus mutans*, the major pathogen that causes dental caries (Islam *et al.*, 2007), has also been implicated in causing secondary caries and the degradation of dental restorative material (Willershausen *et al.*, 1999; Mjor *et al.*, 2000) making it an important pathogen in patients who wear partial dentures. Sumi *et al.* (2002) reported that respiratory pathogens including *Streptococcus pneumoniae* colonize the dentures of dependent elderly and their presence is correlated with an elevated incidence of respiratory diseases. Lack of oral care, including denture treatment, was suggested to increase the risk of infection in the elderly or otherwise dependent population. Although these plaque-related diseases are the results of a combination of both local and systemic factors, the effective removal of dental plaque from dentures and dental restorative materials plays an important role in maintaining both oral and systemic health.

In this study, we tested four different types of surfaces to examine their effects on biofilm formation of *C. albicans*, *S. mutans*, salivary bacteria, and salivary bacteria mixed with *C. albicans.* Based on our observations, we found that these individual materials exhibited different biomass accumulation depending on the various microbial biofilm inocula. Furthermore, we observed significant surface-material-dependent differences in the susceptibility of these biofilms to an antimicrobial treatment. These findings underscore the importance of considering the influence of the relevant surface materials when studying biofilm characteristics or testing antimicrobial agents.

METHODS

Dental material fabrication and sterilization

The fabrication of dental materials used in this study is described below. Unsintered hydroxyapatite (HA) discs (16-mm diameter) were purchased from Himed (Old Bethpage, NY). One hundred denture acrylic resin discs were made using lucitone 199® resin base and lucitone liquid (Densply, York, PA) with a 3 : 1 powder to liquid ratio. A coin (nickel) coated with a thin film of wax was used to make the stone mold. The resin mix was pressure packed into the stone mold and cured for 6 h (4 h at 65.5°C and 2 h at 100°C). The acrylic discs were then retrieved and trimmed. The discs were polished with pumice and buffed with a cloth. Porcelain discs were made using ceramco® II (Densply) by shaping the dentin porcelain mix into discs of 16-mm diameter and 2-mm thickness which were placed on a glass slab. The discs were subjected to a firing program of 5 min dry and 5 min preheat time. The idle and high temperatures were set at 650 and 965°C, respectively, and the heat rate at 37°C min^{-1} (Phoenix Quick Cool dental porcelain furnace; Densply Ceramco, York, PA). The discs were cooled slowly to avoid cracks. The porcelain discs were glazed with Universal glazing medium (Pentron, Wallingford, CT) and subjected to a firing program of 3 min dry and 3 min preheat time. The idle and high temperatures were set at 650 and 935°C, respectively, at a heat rate of 52°C min^{-1}.

All samples were sterilized with 95% ethanol and 1 hr ultraviolet illumination (UV crosslinker; Thermo Fisher Scientific Inc., Waltham, MA) on each side before biofilm inoculation.

Strains and growth conditions

Streptococcus mutans

Streptococcus mutans (UA140) cells were grown anaerobically (85% N_2, 10% H_2, and 5% CO_2) in brain–heart infusion (BHI) medium (Difco, Sparks, MD) overnight at 37°C, and diluted to approximately 0.5×10^5 cells ml^{-1} in BHI supplemented with 1% sucrose for biofilm growth.

Candida albicans

Candida albicans strain 5296 (clinical isolate obtained from the University of California, Los Angeles hospital) cells were grown in yeast extract, bacto-peptone, and dextrose (YPD) overnight at 37°C in a 5% CO_2 atmosphere, and diluted in Ham's F-12 medium (Thermo Fisher Scientific Inc.) to approximately 0.5×10^3 cells ml^{-1} for biofilm growth (Murillo *et al.*, 2005).

Saliva

Unstimulated saliva was collected from four to six subjects who had refrained from cleaning their teeth for more than 6 h (University of California Los Angeles IRB #09-08-068-01). This saliva was pooled in equal proportions and diluted 1 : 4 into Ham's F-12

medium supplemented with 1% glucose, 1% mannose, and 1% sucrose. This suspension was subjected to a low-speed centrifugation (10 min at 1400 ***g***) to remove eukaryotic cells and large debris to prepare the inoculum for biofilm growth. Saliva samples from subjects were plated onto BHI plates supplemented with 800 μg ml^{-1} spectinomycin and 100 μl ml^{-1} kanamycin, only samples with no viable colonies observed after overnight incubation were chosen for further studies.

To identify a mixed biofilm condition that closely resembles the ratio of *C. albicans* to salivary bacteria present in stomatitis patients, a range of approximately 1×10^2 to 1×10^6 *C. albicans* cells was added to the processed saliva suspension before biofilm inoculation.

Biofilm growth

Unless otherwise noted, 2 ml of the respective diluted cultures for the different type of inocula was added for biofilm growth into each well of 12-well plates (Thermo Fisher Scientific Inc.) that contained discs made of different sterile dental materials. A well without such a disc was also included in the experiments and denoted as polystyrene. Biofilms for *S. mutans*, salivary bacteria alone, and supplemented with *C. albicans* were allowed to form for 20 h under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) at 37°C, while single-species *C. albicans* biofilms were grown for 20 h aerobically in a 5% CO_2 atmosphere at 37°C.

Determination of biomass by confocal laser scanning microscopy

The biofilms used in this study were grown on the various dental material surfaces and stained with SYTO59 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Biofilms were examined with a PASCAL 5 confocal laser scanning microscope (Zeiss, Jena, Germany). Samples were viewed through a 40× oil-immersion objective (Plan-Neofluar/N.A. 1.3) or 20× objective (Plan-Neofluar/N.A. 0.5) (for *C. albicans* single-species biofilm only). An excitation of 633 nm with a helium–neon laser and a 650-nm long-pass emission filter was applied for SYTO59-staining. The *z*-stacks of five randomly chosen locations were taken per sample for processing with the COMSTAT software (Heydorn *et al.*, 2000) to determine the average biomass of the biofilms.

Analysis of treatment efficacy

Biofilms were grown overnight on the various dental material surfaces examined in this study and washed three times with phosphate-buffered saline (PBS) to remove non-adherent cells. A Polident® denture cleansing tablet (GlaxoSmithKline, Brentford, UK) was dissolved in H_2O according to the manufacturer's instructions and each biofilm sample was treated with 1 ml of the solution. Treatment was stopped immediately after 5 min of incubation through removal of the treatment solution and three subsequent washes with 2 ml PBS. The treated biofilms were subjected to mechanical disruption, serial dilution, and plating. Treatment efficacy was assessed by the surviving colony-forming units on BHI plates (for *S. mutans* and salivary bacterial cells) and BHI plates supplemented with 800 μg ml^{-1} spectinomycin and 100 μg ml^{-1} kanamycin (for *C. albicans* cells) in comparison with PBS-treated controls.

RESULTS

Growth of four different types of biofilms including *C. albicans* incorporation into saliva-derived biofilm

In this study, we assessed the formation of four types of biofilm on four different surfaces. In addition to single-species *S. mutans* and *C. albicans* and complex saliva-derived biofilms, we evaluated mixed biofilms comprising saliva and *C. albicans* to identify the condition allowing biofilm formation with proportions of *C. albicans* similar to plaque isolated from patients with denture stomatitis. According to Radford *et al.* (1999), *C. albicans* comprised 0.3% of total plaque cells in patients with denture stomatitis. We tested the incorporation of *C. albicans* into salivary bacterial communities by adding different amounts of *C. albicans* cells to the inoculum (Fig. 1). The addition of approximately 1×10^4 *Candida* cells resulted in biofilms containing approximately 0.4% *C. albicans*, similar to the level reported in stomatitis patients. For this reason, this inoculation ratio of *C. albicans* and salivary bacteria was used to assess the effects of surface on saliva and *C. albicans* mixed biofilms.

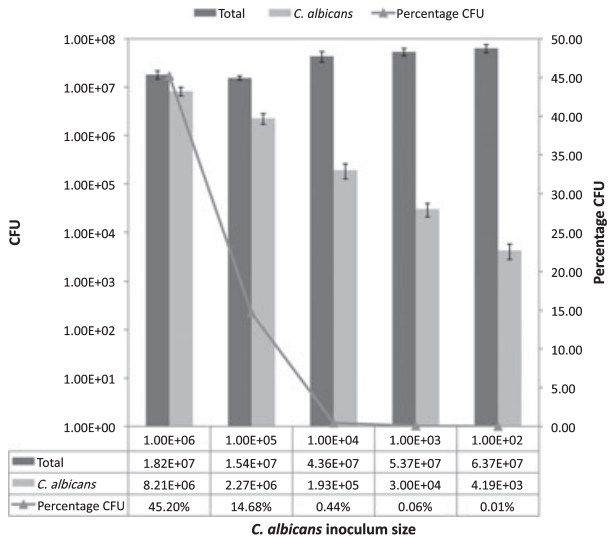

Figure 1 *Candida albicans* incorporation into saliva-derived biofilms. Various amount of *C. albicans* (approximately 1×10^2 to 10^6) cells were added to the inoculum together with processed saliva to form a mixed biofilm. The resulting biofilms were mechanically disrupted and subject to plating. Total biofilm cells were assessed by colony-forming units (CFU) on brain–heart infusion plates, and *C. albicans* cells were assessed by CFU on brain–heart infusion/kanamycin/spectinomycin plates. Standard error of nine replicates was presented.

Total biomass of biofilms formed on different test surfaces

Surface features influence the adherence of microorganisms so we determined the biomass accumulation of the different types of biofilms on each test surface (Fig. 2). Interestingly, *S. mutans* cells did not exhibit differential adherence and the total biomass of *S. mutans* biofilms was independent of the type of surface. However, on the HA surface, the overall biofilm morphology of *S. mutans* appeared to be different and more compact microcolonies were observed compared with the other test surfaces (Fig. 2A). In contrast to *S. mutans* biofilms, *C. albicans* adhered to HA with the lowest affinity ($P < 0.002$ when comparing *C. albicans* biofilms formed on HA with other surfaces), whereas salivary bacteria accrued the highest total biomass in biofilms formed on these surfaces independent of exogenously added *C. albicans* ($P < 0.0003$ for saliva-derived biofilms and $P < 0.02$ for mixed biofilms) (Fig. 2A,B). The mechanism for the different characteristics of biofilm formation of *C. albicans*, *S. mutans*, and salivary bacteria on HA surfaces is unclear.

Effects of different test surfaces on biofilm sensitivity to antimicrobial treatment

The observed differences in biofilm morphology and total biomass accumulation provide a strong indication that the nature of the surface is an important determinant in biofilm properties. To evaluate their susceptibility to an antimicrobial agent as an additional parameter, the biofilms were treated with a denture-cleansing agent (Polident). The tablet was dissolved in water as described above, and used to treat all types of biofilms formed on the four test surfaces. After removing the treatment by several washes with PBS, the colony-forming unit count, which reflects cell viability, was used as a read-out rather than the biomass determination applied in the previous section (Fig. 2), which does not allow distinction of live and dead cells. *Streptococcus mutans* and *C. albicans* single-species biofilms grown on polystyrene were most sensitive to the treatment, and were completely eliminated whereas the same type of biofilms formed on other surfaces maintained 10^3–10^6 surviving colony-forming units (Fig. 3A,B). In addition, biofilms formed on HA were generally more resistant to treatment (Fig. 3A–D). Moreover, in saliva-derived biofilms mixed with *C. albicans* (0.4% of total colony-forming units), the condition that closely mimics the situation found in stomatitis patients (Radford *et al.*, 1999), the Polident denture cleansing tablet was able to reduce *C. albicans* viability by three to four orders of magnitude on the relevant acrylic denture material ($P < 0.01$) (Fig. 3D).

DISCUSSION

Dental plaque accumulation on oral prostheses and restorative materials has long been recognized as an important health concern. Fungal growth not only leads to denture liner degradation and subsequent tissue irritation, but is also responsible for denture stomatitis (Bergendal & Isacsson, 1983; Arendorf & Walker, 1987). Accumulation of *S. mutans* on restorative materials such as porcelain and resin composite often results in material degradation and secondary caries (Willershausen *et al.*, 1999; Mjor *et al.*, 2000). The presence of denture biofilms has also been associated with malodor, aspiration pneumonia, infectious endocarditis, and other systemic conditions

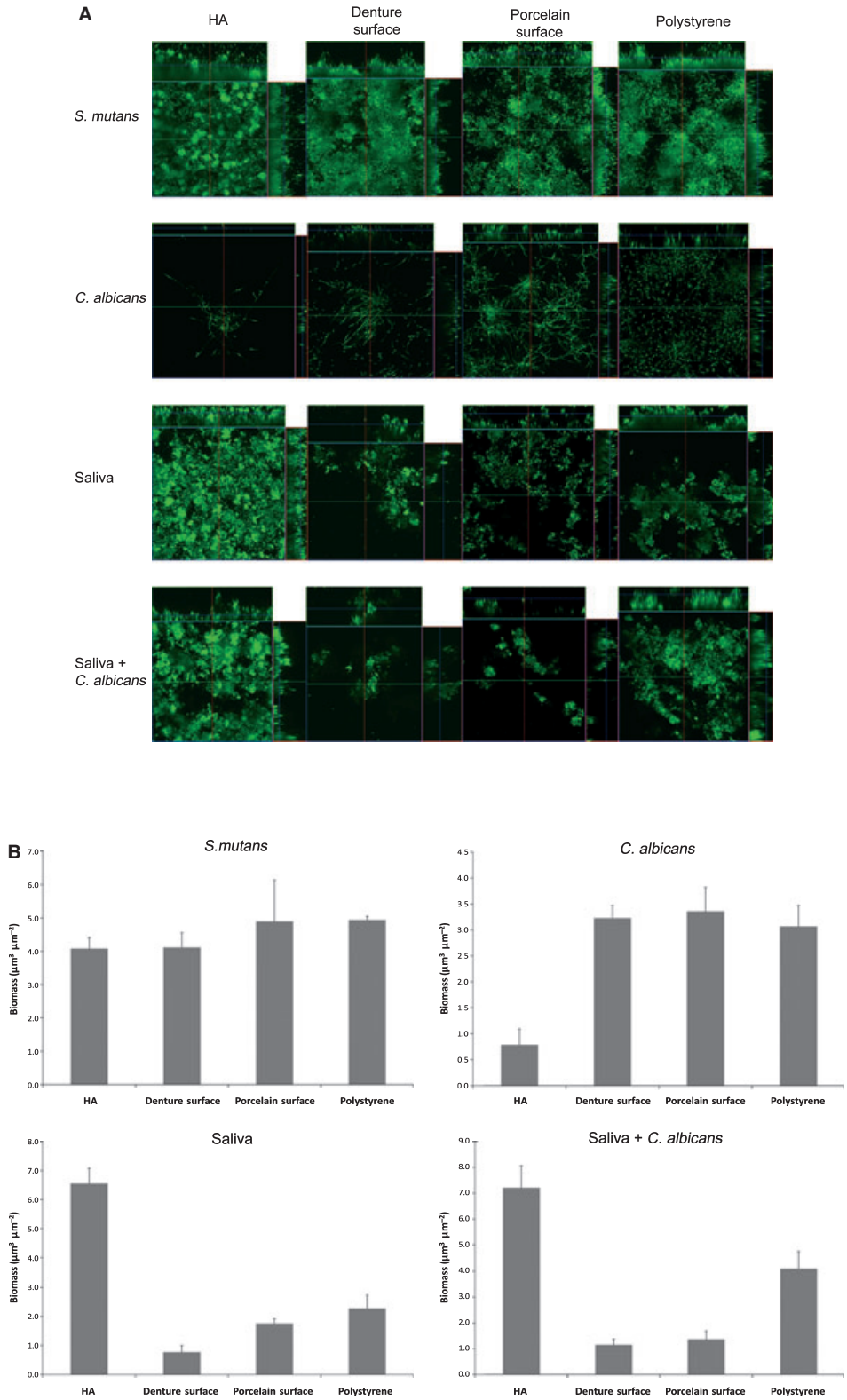

Figure 2 Biofilm formation on various surfaces. Representative images of *Streptococcus mutans*, *Candida albicans*, saliva, and saliva mixed with *C. albicans* biofilm formed on hydroxyapatite (HA), denture, porcelain, and polystyrene surfaces are presented (A). The quantification of the total biomass of these biofilms are also presented (B), standard error of five quantified scans is included.

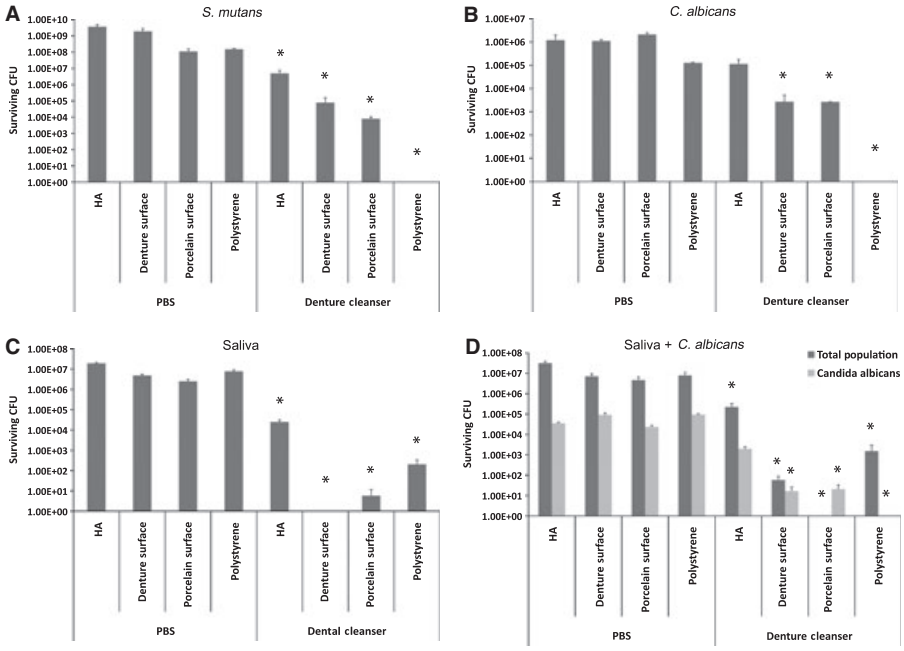

Figure 3 Antimicrobial treatment efficacy against biofilms formed on various surfaces (all significant differences with $P < 0.05$ are marked with an asterisk). Biofilms were treated with denture cleanser for 5 min followed by mechanical disruption and plating. The viability of biofilm after treatment was assessed by colony-fomring units (CFU) on brain–heart infusion plates (*Streptococcus mutans* and salivary bacteria) and brain–heart infusion/kanamycin/spectinomycin plates (*Candida albicans*), standard error of at least three replicates is presented.

especially affecting elderly and dependent populations (Goldberg *et al.*, 1997; Taylor *et al.*, 2000; Coulthwaite & Verran, 2007; Parahitiyawa *et al.*, 2009).

In this study, we presented a comprehensive assessment of materials used in restoration procedures including dentures for their effect on oral biofilms. The biomass of *S. mutans* was very similar on all surfaces but *C. albicans* attached and grew well on denture material and porcelain but not HA. The biomass accumulation pattern for salivary bacteria with and without addition of *C. albicans* was very similar. These types of biofilms seemed to favor HA surfaces. Importantly, the biofilms formed by *C. albicans* under single-species conditions and mixed with salivary species growth conditions, appeared to exclusively grow as hyphae. It was previously reported that the ability to transition between the yeast form and the filamentous hyphal form is important for the virulence of *C. albicans* (Lo *et al.*, 1997) In addition, the mixed biofilms contained similar levels of *C. albicans* (0.4%) as plaque recovered from stomatitis patients.

More importantly, the same species biofilms formed on various surfaces exhibited significant differences in sensitivity to antimicrobial treatment (Fig. 3). As the nature of the surface can influence the outcome by several orders of magnitude, choosing a surface relevant for treatment of the target species is essential for the testing of antimicrobial efficacies. In addition to considering the most appropriate surface, it is also important to closely mimic the anticipated *in vivo* situation (biofilms grown on a certain surface) that will be targeted by the antimicrobial agent (Fig. 3). For the commercially available denture-cleansing tablet tested in this study, we found that treatment of the most relevant situation (saliva and *C. albicans* mixed biofilms formed on the denture materials) led to a three to four orders of magnitude reduction in viability.

Taken together, these results support the clinical relevance of this product and suggest that the Polident denture-cleansing tablet is an effective tool in maintaining good denture hygiene. The use of this convenient denture-cleansing approach could potentially obviate dentures as a reservoir for *C. albicans* and correspondingly decrease denture-associated stomatitis.

Furthermore, these biofilm models are not limited to use in testing antifungal products; they can be easily expanded to other pathogens causing local or systemic infections associated with poor oral hygiene, such as the respiratory pathogen *S. pneumoniae*. In

addition to incorporating other relevant pathogens, the model system can be easily expanded to include other dental materials (e.g. resin composite or orthodontic wires and adhesives). In summary, this study represents a comprehensive assessment of the influence of surface material on oral biofilm formation and most importantly, treatment efficacy of oral care products. These findings strongly indicate that these factors should be considered for product evaluation and can serve as a design template for future screening approaches to improve target specific development of antimicrobial formulations.

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