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Development of a New Model System to Study Microbial Colonization on Dentures

Tingxi Wu, DDS,[1,2] Wei Hu, PhD,[3] Lihong Guo, DDS, PhD,[4] Marybeth Finnegan, PhD,[5#] David J. Bradshaw, PhD,[6] Paul Webster, PhD,[7] Zvi G. Loewy, PhD,[8] Xuedong Zhou, DDS, PhD,[9] Wenyuan Shi, PhD,[10] & Renate Lux, PhD[11]

[1]PhD student, West China School of Stomatology, Sichuan University, Chengdu, Sichuan, China
[2]Visiting Student, Section of Oral Biology in the Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, CA
[3]Visiting Scholar, Section of Oral Biology in the Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, CA
[4]Scientist, Section of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, CA
[5]Principal Scientist, Denture Care, GlaxoSmithKline Consumer Healthcare, Parsippany, NJ
[6]Principal Scientist, New Product Research, GlaxoSmithKline, Family Oral Health, Weybridge, UK
[7]Professor, House Ear Institute, Los Angeles, CA
[8]Professor, Department of Pharmaceutical and Biomedical Sciences, Touro College of Pharmacy, New York, NY
[9]Professor and Dean, West China School of Stomatology, Sichuan University, Chengdu, Sichuan, China
[10]Professor and Chair, Section of Oral Biology in the Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, CA
[11]Assistant Professor, Section of Oral Biology in the Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, CA

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Correspondence
Dr. Renate Lux, UCLA School of Dentistry, 10833 Le Conte Avenue, Los Angeles, CA 90095-1668. E-mail: lux@ucla.edu

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Abstract

Purpose: Dentures are often colonized with a variety of microorganisms, including *Candida albicans*, that contribute to denture stomatitis. Several in vitro models have been previously established to study denture-related microbial colonization and evaluate treatment efficacy of denture cleansers; however, those models typically fail to appreciate the complex topology and heterogeneity of denture surfaces and lack effective ways to accurately measure microbial colonization. The purpose of this study was to study microbial colonization with a new model system based on real dentures, to more realistically mimic in vivo conditions.
Materials and Methods: Scanning electron microscopy was used to observe topological structures among surfaces from different parts of the denture. Employing *C. albicans* as a model microorganism, we established microbial colonization on different denture surfaces. Moreover, we applied a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) colorimetric assay to quantify *C. albicans* colonization on dentures without the necessity of biofilm removal and to evaluate treatment efficacy of denture cleansers.
Results: There were significant variations in topological structures among surfaces from different parts of the denture, with the unpolished side having the highest amounts of indentations and pores. The distinct denture surfaces support microbial colonization differently, with the unpolished side containing the highest level of microbial colonization and biofilm formation. Furthermore, the modified MTT colorimetric assay proved to be an accurate assay to measure biofilm formation on dentures and evaluate treatment efficacy of denture cleansers.
Conclusion: This new denture model system in conjunction with the MTT colorimetric assay is a valuable tool to study denture-related microbiology and treatment approaches.

Dentures are prosthetic devices commonly used in adults to replace missing teeth.[1-4] Although dentures enhance the patient's ability to chew, their use is strongly associated with several diseases, including local as well as systemic infections. Local infections include denture stomatitis, angular cheilitis, and contact allergy to denture material. Systemic diseases associated with dentures include chronic respiratory disease, cardiovascular diseases, diabetes, and arthritic disorders.[4-5] Denture

stomatitis is the most common disease that manifests as inflammation of oral mucosa in contact with dentures.[6] *Candida albicans* is known to be the main contributor to this disease, as it colonizes and subsequently forms a biofilm on the denture surface.[7]

To study denture-related microbial colonization and determine the efficacy of different denture cleansers, a variety of in vitro bacterial or fungal biofilm models have been established using microtiter plates, acrylic strips, and discs.[8-14] While these are simple, convenient, economic models to study microbial colonization and test the antimicrobial efficacy of denture cleansers, they have intrinsic limitations. The model systems using acrylic strips and discs do not account for the complex topology of denture structures. In addition, treatment efficacies are mainly determined by scraping, vortexing, or sonicating microbes off the strips or discs. This is ineffective and often leads to inaccuracies in CFU counting. To address this problem, investigators have applied 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based colorimetric assays to measure the metabolic activities of total microbial flora and demonstrated that this assay was a more accurate and convenient way to evaluate microbial viability than scraping and CFU counting.[14-16] This approach, however, has not yet been used to study microbial colonization on real dentures. Furthermore, all dentures have a polished side (called smooth surface in this study) and an unpolished side (called rough surface in this study). In clinical applications, the smooth surfaces are typically exposed to the oral cavity, while the rough surfaces are in contact with the oral mucosa. Most previously described model systems do not consider the possible differences in microbial colonization between these two sides.[10-14]

In this study, we aimed to develop a new model system in which microbial biofilms were grown on real dentures taking into consideration their complex topological structures and surface features. The MTT colorimetric assay was employed to validate its utility to measure microbial colonization on real dentures compared to the traditional methods of scraping, vortexing, or sonication and to evaluate treatment efficacies of denture cleansers. *C. albicans* was chosen as the model microorganism for this study.

Materials and methods

Strains and growth conditions

C. albicans strain 5296 is a clinical isolate obtained from the University of California, Los Angeles Hospital. The cells were inoculated in YDP medium (10 mg/ml yeast extract, 20 mg/ml dextrose, 20 mg/ml peptone) and incubated in a 5% (v/v) CO_2 chamber at 30°C.[17]

Denture materials

The acrylic denture discs used in this study were manufactured as previously described.[13] The partial dentures were provided by GlaxoSmithKline and were manufactured by commercial vendors following standard procedures. All denture specimens were disinfected with 75% (v/v) ethanol treatment prior to microbial inoculation.

Growth of *C. albicans* biofilms on various surfaces

A previously described protocol was applied with modifications.[13] *C. albicans* biofilms were grown on dentures, denture discs, and plastic wells. For these experiments, the overnight cultured *C. albicans* cells were diluted in Ham's F-12 medium (Fisher Scientific, Raleigh, NC) to approximately 10^4 cells/ml as inoculum.

C. albicans biofilms formed in plastic wells were used to generate standard curves for the MTT assay. In these experiments, 2 ml of serial diluted inoculum (10^3 to 10^8 cells/ml) was added into each well of 12-well plates (Fisher Scientific) and incubated aerobically for 3 hours at 37°C to form the biofilm on polystyrene surfaces.

C. albicans biofilms formed on acrylic denture discs were used in some of the experiments for comparison and analysis. Diluted inoculum (2 ml; 10^4 cells/ml) and a sterile acrylic denture disc with a rough or smooth surface were added into each well of 12-well plates and cultured aerobically on a shaker at 140 rpm for 24 hours at 37°C to form the biofilms on denture discs.

For our new model system, sterilized acrylic dentures were submerged in 80 ml of inoculum (10^4 cells/ml) in a 250 ml conical flask, and aerobically cultured on a shaker at 140 rpm for different time periods at 37°C to form biofilms.

Crystal violet staining of biofilms on denture surfaces

C. albicans biofilms formed on dentures, and denture discs were stained with crystal violet according to a published protocol with some modifications.[18] Specimens were washed twice with phosphate-buffered saline (PBS), air dried for 30 minutes, and stained with 0.4% (w/v) crystal violet in distilled water (at 0.22 μm filtered before use) for 20 minutes. The stained specimens were gently washed three times with sterilized distilled water and air dried for 30 minutes before being photographed. Images were taken with a D50 digital camera (Nikon, Tokyo, Japan).

Examination of biofilms on denture surfaces with confocal laser scanning microscopy

C. albicans biofilms grown on both rough and smooth surfaces of dentures were stained with 10 μM SYTO 9 and 10 μM SYTOX orange (Invitrogen, Grand Island, NY) in PBS buffer for 30 minutes at room temperature in the dark.[13] The specimens were observed through a PASCAL 5 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with a 20× objective (Plan-Neofluar/NA 0.5). Excitation at 488 nm with an argon laser in combination with a 505 to 530 nm band-pass emission filter was used for SYTO 9 fluorescence imaging. SYTOX orange signals were visualized using 543 nm excitation with a helium-neon laser and a 560 to 615 nm band-pass emission filter.

Analysis of denture surfaces with SEM

Specimens were coated with a 10-nm thick film of platinum in a sputter coater (EMS 575 high resolution sputter coater with thin film monitor, EMS Inc, Hatfield, PA). Images were

obtained using an XL30 S FEG scanning electron microscope (FEI Inc, Hillsboro, OR) operating at 5kVa.

Mechanical disruption of *C. albicans* biofilm on partial denture

C. albicans biofilms grown on denture surfaces were disrupted and removed by scraping, vortexing, or sonication. For scraping, each biofilm on a denture was scraped thoroughly with a scraper for 3 minutes. For vortexing, dentures with biofilms were vortexed in 20 ml PBS buffer containing 3 mm glass beads (Fisher) for 3 minutes. For sonication, dentures with biofilms were placed in a 20 ml PBS solution and sonicated at 7 W energy level for 3 minutes using a Sonic Dismenbrator (Fisher). The residual biofilms remaining on treated dentures were visualized with crystal violet staining (as described above).

MTT colorimetric assay

The MTT colorimetric assay is based on the cleavage of MTT into a blue formazan by living cell enzymes. The amount of formazan formed is correlated to the number of viable cells. The MTT assay was performed as previously described with modifications.[19-21] The MTT assay solution was prepared by dissolving 1 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Biosynth, Itasca, IL), 1% (w/v) glucose, and 10 μM menadione (Acros, Pittsburgh, PA) in PBS buffer. The solution was filtered at 0.22 μm and pre-warmed to 37°C prior to use. The MTT solution was added to *C. albicans* biofilm samples, incubated at 37°C for 3 hours, and then carefully removed. The formazan crystals formed in viable cells were dissolved by acid-isopropanol (5 ml 3N HCI in 95 ml isopropanol). The optical density of this solution was determined at 540 nm using a microplate reader (Bio-Rad, Hercules, CA). Solutions without biofilms were used as blank controls.

The MTT standard curve was generated using *C. albicans* biofilms grown on polystyrene surfaces (as described above). The biofilms formed by different numbers of viable cells (10^3 to 10^8 cells/ml) were obtained by inoculating serially diluted *C. albicans* cells in Ham's F-12 medium prior to cultivation. After 3 hours of incubation, medium was removed from each well, and the biofilms were washed three times with PBS buffer, then incubated with 2 ml MTT solution for 3 hours. The formation of formazan crystals by viable cells was determined as described above. The viable cell numbers in the respective biofilm samples formed by different initial inocula were also assessed by colony-forming units (CFU) on BHI agar plates supplemented with 800 μg/ml spectinomycin and 100 μg/ml kanamycin as previously described.[13] The MTT standard curve was determined by plotting the number of viable cells (CFU counts obtained from different inocula) on the *x*-axis against the optical density on the *y*-axis (MTT assay readout of different inocula) using log/log power regression analysis. Viable cell numbers were evaluated through the formula obtained from the standard curve. Individual standard curves were generated for each experiment.

To assay the viable cells in *C. albicans* biofilms formed on denture surfaces, the medium was removed, and each denture was incubated with 18 ml MTT solution for 3 hours. The biofilms containing formazan crystal were submerged in 5 ml acid-isopropanol and vortexed with glass beads for 10 minutes. Two hundred μl of this acid-isopropanol solution was removed and transferred into the wells of a 96-well plate to measure the optical density. The corresponding viable cell numbers were calculated using the formula obtained from the MTT standard curve (see above). The growth curve of a biofilm on a denture was generated by graphing the dependent variable number of cells on the *x*-axis versus optical density on the *y*-axis.

Evaluation of the treatment efficacy of an antifungal cleanser

C. albicans 24-hour biofilms grown on both denture discs and dentures were washed three times with PBS buffer to remove non-adherent cells. A Polident® denture cleansing tablet (GlaxoSmithKline, Weybridge, UK) was dissolved in 150 ml distilled water according to the manufacturer's instructions. Ethanol, 75% (v/v), was used as a positive treatment control, and PBS was used as the corresponding negative control. Each specimen was treated with 50 ml of the treatment solution for 3 minutes, 5 minutes, and 10 minutes, respectively. After treatments, solutions were removed, and dentures were washed three times with PBS buffer. The treatment efficacy was evaluated by the MTT assay described above.

Results

Analysis of topological structures of different denture surfaces and their influence on biofilm formation of *C. albicans*

Our goal was to create a more realistic denture biofilm model that can mimic in vivo conditions. In this study, we used real dentures to support microbial biofilm formation to better appreciate the complex topology and heterogeneity of denture surfaces derived by standard manufacturing processes. As the first step, we decided to better understand their physical structures. Different parts of the denture were viewed by SEM (Fig 1). The polished side (Fig 1A) showed an extremely smooth surface devoid of pores and indentations (Fig 1C), while the unpolished side (Fig 1B) displayed a rough surface with many irregularities and pores (Fig 1D).

The variations in physical structures present in smooth and rough denture surfaces (Fig 1) may lead to differences in microbial colonization. To test this hypothesis, we used *C. albicans* as the model microbe to grow biofilms on manufactured dentures as described above. Assayed with crystal violet staining (Fig 2), we found that different surfaces had different abilities to support biofilm formation under these growth conditions. Biofilms on both sides of the dentures were unevenly distributed, and much more biomass accumulated on rough surfaces (Fig 2B) than smooth surfaces (Fig 2A). To further examine the differences in biofilm grown on both rough and smooth surfaces, we also grew biofilms on both surfaces of the denture discs similar to the procedures applied to real dentures. A dense, tightly attached biofilm formed on the rough surface of the denture disc (Fig 2D), whereas a less dense, loosely attached biofilm was found on the smooth surface of the denture disc (Fig 2C), similar to our observations on real dentures (Fig 2A, 2B). We then used laser scanning confocal microscopy to examine the

architectural differences between the biofilms formed on both rough and smooth surfaces. A thicker biofilm with tight aggregates was observed on rough surfaces (Fig 2F), while only thin biofilms with loose aggregates were observed on smooth surfaces (Fig 2E). Most interestingly, using SEM, we found that microbes on the rough surfaces often colonized within the cracks and pores (Fig 2G).

Evaluation of standard procedures for measuring microbial colonization on real dentures

Based on the above data, the new model system using real dentures showed different microbial colonization patterns on different sides of a denture, similar to what had been observed with dentures obtained from patients. Despite this advantage, the use of real dentures also created new problems, especially the difficulty in accurately measuring the number of bacteria colonizing the denture surfaces. Standard approaches used to count microbial CFUs on denture strips or discs, such as scraping, vortexing, or sonication, proved to be ineffective for this denture model (Fig 3) since substantial amounts of biofilms remained on the dentures after the treatments, especially on the rough sides (Figs 3B, 3D, 3F).

Adaptation of the MTT assay to determine microbial colonization of the new denture model system

Recognizing the deficiency of the standard procedures used above, we decided to adapt the MTT assay for this new model system for the reasons described in the Materials and Methods section. As the first step, a standard curve was constructed to evaluate the correlation between viable cell numbers and optical readings from the MTT assay (Fig 4A). After establishing a good power relationship ($R^2 > 0.9$) between viable cell numbers and optical readings, we applied the MTT assay to measure *C. albicans* 24-hour biofilm developed on dentures. As expected, the viable cell numbers determined by the MTT assay were significantly higher than the inaccurate CFU counts produced by standard procedures ($p < 0.01$) (Fig 4B).

Furthermore, we also demonstrated that the MTT assay was suitable for monitoring the dynamic process of biofilm formation on dentures. The accumulation of viable cells on dentures was consistent with previous findings (Fig 4C) and exhibited logarithmic growth in the first 24 hours, followed by a stationary phase from 24 hours to 72 hours and a decrease in cell viability after 72 hours.

Evaluation of antifungal efficacy with the new denture model system in conjunction with the MTT colorimetric assay

In this section, we further validated the utility of this new denture model system and determined its ability to evaluate the treatment efficacy of a denture cleanser in comparison with the denture disc assay. The *C. albicans* biofilms formed on denture discs and dentures were treated with Polident® according to the manufacturer's instructions. The Polident solution was able to completely eliminate viable *C. albicans* biofilms on denture discs after 5 minutes of treatment, while it only reduced *C. albicans* viability by three to four orders of magnitude on the dentures ($p < 0.01$) (Fig 5). The elimination of viable *C. albicans* biofilms on dentures was achieved with a 10-minute treatment of Polident solution ($p < 0.01$).

Discussion

In this study, we established a new in vitro denture biofilm model system using real dentures to form microbial biofilms under shaking conditions. Our studies showed that the polished (smooth) and unpolished (rough) surfaces of dentures had great difference in their topological structures, which led to different outcomes in biofilm formation. In particular, the rough surfaces were found to contain extensive indentations

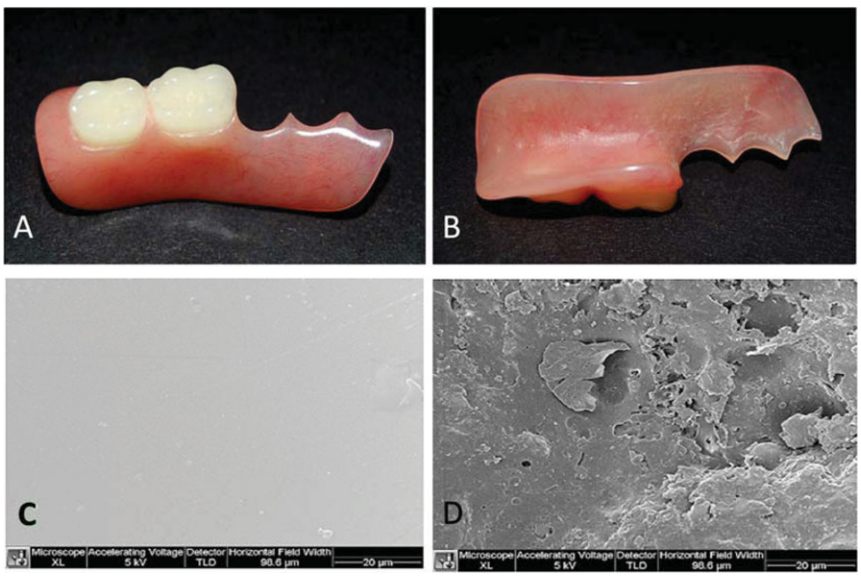

Figure 1 Representative images of denture surfaces. Shown are the (A) polished (smooth) and (B) unpolished (rough) surfaces of the partial dentures. SEM images (5000× magnification) of the (C) smooth and (D) rough surfaces of the partial dentures. Four random fields of view were examined for each sample.

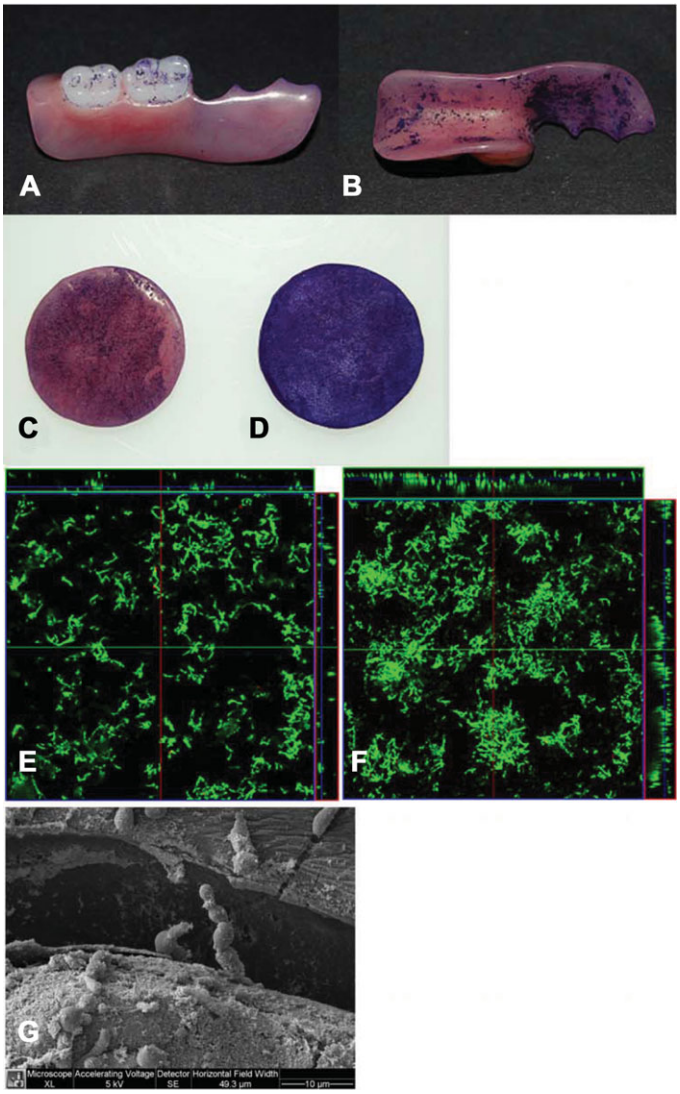

Figure 2 *C. albicans* biofilm formation on dentures and denture discs. (A and B) *C. albicans* biofilms formed on the smooth (A) and rough (B) denture surfaces were stained by crystal violet shown in blue. (C and D) *C. albicans* biofilms formed on the smooth (C) and rough (D) surfaces of denture discs stained by crystal violet. (E and F) *C. albicans* biofilms formed on the smooth (E) and rough (F) surfaces of denture discs examined with CLSM after staining with SYTO 9 and SYTOX Orange. (G) *C. albicans* cells colonizing the surface imperfections of the rough denture surface. Four random fields of view were examined by SEM and CLSM for each sample.

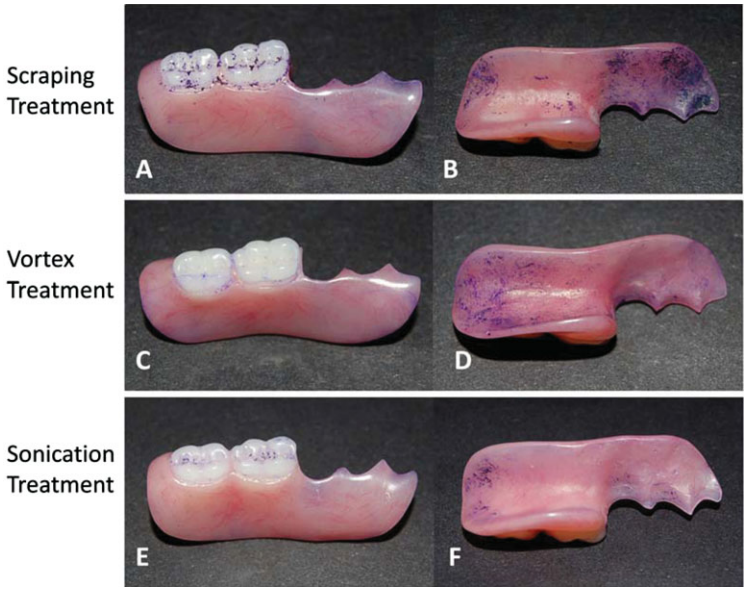

Figure 3 Biofilms remaining on dentures after removing *C. albicans* with scraping (A and B), vortexing (C and D), or sonication (E and F). The remaining biofilms on the denture were visualized by crystal violet (shown in blue). Panels A, C, and E are smooth surfaces, while panels B, D, and F are rough surfaces. The experiments were repeated three times.

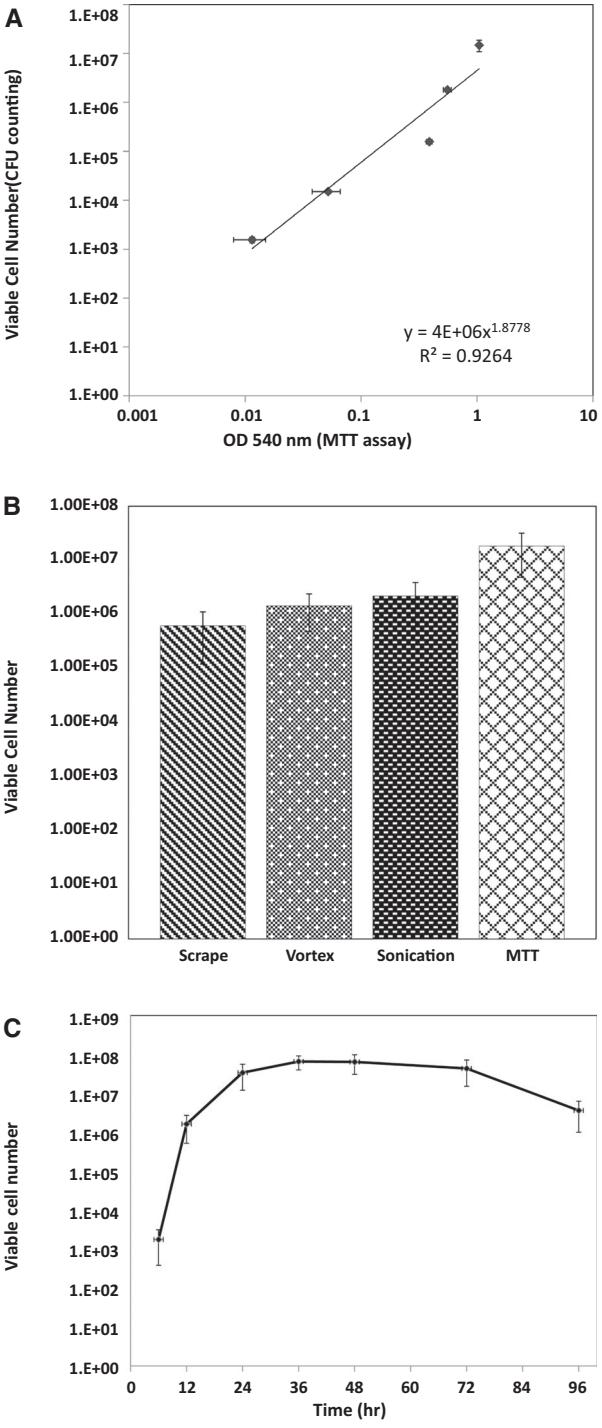

Figure 4 Quantification of viable cell number of *C. albcians* biofilms on denture surfaces. (A) Standard curve of the MTT assay was obtained for quantification of viable cell numbers of *C. albicans* biofilms. (B) Determination of viable cell number in *C. albicans* biofilm on dentures by CFU count through scraping, vortexing, sonication (three columns on the left), and MTT assay (the right column). The difference between scraping/vortexing/sonication and MTT is significant ($p < 0.01$). (C) The growth curve of *C. albicans* biofilm grown on dentures was determined by MTT assay. All errors are within the 10% to 15% range, and experiments were repeated at least three times.

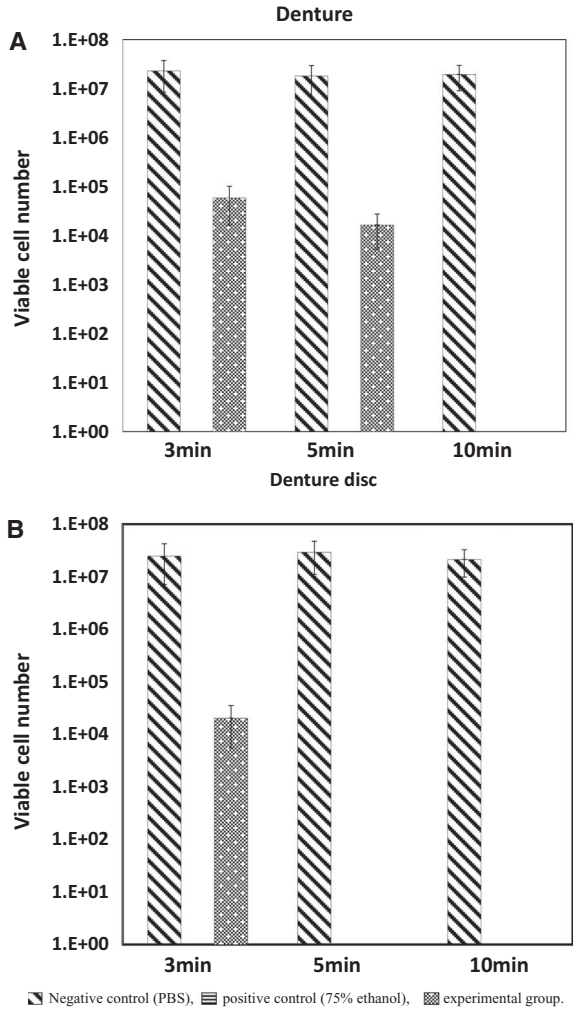

Figure 5 Antifungal treatment efficacy against *C. albicans* biofilms on denture discs and dentures. Biofilms on (A) dentures and (B) denture discs were treated with denture cleanser for 3 minutes, 5 minutes, and 10 minutes. The treatment efficacy was determined by the MTT assay. The errors are within 15%, and the differences between control groups and treated groups are significant ($p < 0.01$). All experiments were repeated three times.

and pores that provided niches for increased *C. albicans* colonization. It should be noted that because in clinical applications the rough side of a denture is in direct contact with the oral mucosa, this enhanced ability to support *C. albicans* colonization on a rough surface could thus contribute to denture-associated stomatitis. The finding also suggested that this new model overcomes many limitations of previous model systems and more realistically mimics in vivo conditions.[8-14]

Since this new model uses real dentures containing complex topological structures, standard biofilm quantification methods are not suitable due to the difficulties of biofilm removal from the surface. Our data show that the MTT assay is a good alternative and is suitable for this model system. In addition to its

ability to measure biofilms on denture surfaces, the MTT assay is also useful in monitoring the dynamic process of biofilm formation, creating the possibility for many future applications to use this model system (in conjunction with MTT) to study denture biofilm related biological questions.

The data reported in this article also demonstrated that this model system is a useful tool to evaluate the treatment efficacy of antimicrobial cleansers for dentures. It is particularly interesting to note the variability in treatment efficacy when we tested the same denture cleanser with a denture-disc-based in vitro model as compared to the real denture based new model system. We found that it took 10 minutes to kill all *Candida* cells on the denture, while 5-minute treatment was sufficient to kill all *Candida* cells on the denture discs. This difference could be due to differences in the properties of the biofilms formed on actual dentures, further validating the positive features of this new model system for future applications in screening antimicrobial products, since it may more realistically reflect in vivo conditions.

Conclusion

In conclusion, the present study describes a more realistic denture biofilm model system, which reflects the real topology and surface properties of dentures and their associated abilities for microbial colonization and biofilm formation. In conjunction with the MTT colorimetric assay, it is a convenient, cost-effective, and high-throughput tool to study denture-related microbiological and treatment questions. Furthermore, this denture biofilm model is not limited to *C. albicans*-related stomatitis; it could easily be modified and adapted to various other biofilms associated with denture-related local or systemic diseases, including respiratory pathogens such as *Streptococcus pneumoniae* and *Enterobacter cloacae*. This model system could also be modified and adapted to other denture materials such as porcelain and composite resins for additional studies.

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