Reducing the Incidence of Denture Stomatitis: Are Denture Cleansers Sufficient?

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Abstract

Purpose: *Candida albicans* is the predominant oral yeast associated with denture stomatitis. With an increasing population of denture wearers, the incidence of denture stomatitis is increasing. Effective management of these patients will alleviate the morbidity associated with this disease. The aim of this study was to examine the capacity of four denture cleansers to efficiently decontaminate and sterilize surfaces covered by *C. albicans* biofilms.
Materials and Methods: Sixteen *C. albicans* strains isolated from denture stomatitis patients and strain ATCC 90028 were grown as mature confluent biofilms on a 96-well format and immersed in Dentural, Medical™ Interporous®, Steradent Active Plus, and Boots Smile denture cleansers according to the manufacturers' instructions or overnight. The metabolic activity and biomass of the biofilms were then quantified, and scanning electron microscopy (SEM) used to examine treated biofilms.
Results: Dentural was the most effective denture cleanser, reducing the biomass by greater than 90% after 20 minutes. Steradent Active plus was significantly more effective following 10-minute immersion than overnight ($p < 0.001$). All cleansers reduced the metabolic activity by greater than 80% following overnight immersion; however, Boots Smile exhibited significantly reduced metabolic activity following only a 15-minute immersion ($p < 0.001$). SEM revealed residual *C. albicans* material following Dentural treatment.
Conclusions: This study showed that denture cleansers exhibit effective anti-*C. albicans* biofilm activity, both in terms of removal and disinfection; however, residual biofilm retention that could lead to regrowth and denture colonization was observed. Therefore, alternative mechanical disruptive methods are required to enhance biofilm removal.

Oropharyngeal candidosis (OPC) is a common infection among the immuno-compromised and elderly, associated with significant morbidity, including oral pain and burning, altered taste sensation, and nutritional compromise.[1] One of the most common clinical presentations of OPC is the erythematous form of denture-induced stomatitis, which is often recurrent and characterized by inflammation or erythema on the oral mucosa of denture-bearing mucosa. In the majority of these cases, the denture wearer is unaware of any underlying problem.[2-5] Considering that there are currently some 15 million denture wearers within the United Kingdom, denture-induced stomatitis represents a significant clinical and socioeconomic burden.[6]

The onset and severity of denture stomatitis is of multifactorial origin, being influenced by factors such as salivary flow, denture cleanliness, age of prosthesis, denture base material, denture trauma, continuous denture wearing, smoking, and nutritional intake.[7-10] Nevertheless, fungal biofilms play the most important role clinically.[11,12] Denture-induced stomatitis is primarily caused by the opportunistic fungal pathogen *Candida albicans*; however, an increasing proportion of other Candidal species are being implicated in pathogenesis, including *C. glabrata.*[13] Although not life threatening *per se*, the collective presence of *Candida* species within the saliva, adhesion to the oral mucosa, and the colonization and development of biofilms on the denture surface are associated with mild-to-severe pathophysiological effects, according to Newton's criteria.[14-17] Once formed, cells within the biofilm undergo profound phenotypic changes. Most notably, they exhibit increased resistance to antifungal agents.[18,19] It has also been demonstrated that formation of biofilms in the cracks and imperfections of denture bases

makes the biofilm resilient to physical forces, most notably removal by brushing.[13,17,20] These studies highlight the inherent difficulties experienced by denture wearers in minimizing the fungal burden of their dentures, thereby preventing the onset of denture-induced stomatitis.

Recent studies have established that sonication significantly reduces the fungal burden upon removable dentures, and that microwave technology may offer a potential method of denture disinfection;[21,22] however, these technologies have limited applicability due to either excessive costs or the capacity to damage the denture base material.[23] Denture wearers therefore have to rely on the use of over-the-counter oral hygiene products, which has increased based on the large consumer base in this specialized healthcare market.[6] This study aims to examine the efficacy of four over-the-counter denture cleansers to establish their respective capacities to remove and/or kill *C. albicans* biofilms.

Materials and methods

Organisms

C. albicans-type strain ATCC 90028 and 16 clinical strains of *C. albicans* isolated from a recent denture stomatitis study were used in these investigations.[13] All the isolates were stored on Sabouraud dextrose (SAB) agar plates (Oxoid, Cambridge, UK) at 4°C.

Culture conditions and standardization

C. albicans were propagated on SAB agar plates at 37°C overnight. A colony of each isolate was inoculated into 10 ml of yeast peptone dextrose (YPD, Oxoid) and placed in a shaker at 30°C overnight. The cells were washed by centrifugation in sterile phosphate-buffered saline (PBS; pH 7.4, Oxoid). The yeast cells were then counted using a Neubauer hemocytometer and adjusted to the required concentration in RPMI 1640 medium (Sigma, Dorset, UK). All procedures were carried out in a laminar flow cabinet (Microflow Biological Safety Cabinet, Bioquell, Hants, UK).

Denture cleansers

Four denture cleansers (Boots Smile, Medical™ Interporous®, Steradent Active Plus, Dentural; Martindale Pharmaceuticals Ltd., UK) were used in this study. These were prepared as described in the manufacturer's instructions (Table 1).

Treatment of biofilms with denture cleansers

C. albicans biofilms were formed on commercially available presterilized polystyrene, flat-bottomed, 96-well microtiter plates (Corning, Corning, NY), as described previously.[24] Briefly, biofilms were formed by adding 200 μl of standardized cells (1×10^6 cells/ml) to each well and incubating at 37°C overnight. The biofilm was subsequently washed three times with sterile PBS to remove nonadherent cells. Each of the four denture cleanser products were added independently to ten replicate biofilms across adjacent wells of each of four rows. Positive (untreated) and negative (no biofilm) controls were included. Biofilms were then immersed for the time indicated by the manufacturer (Table 1) (recommended) or for 18 hours (overnight) at room temperature. Following each defined treatment, the denture cleansers were decanted and replaced with European neutralizing solution (1% [v/v] phosphate buffer, 0.1% [w/v] l-histidine, 0.5% [w/v] sodium thiosulphate, 0.3% [w/v] lecithin [soya refined] and 10% [v/v] Tween 80) for 5 minutes. Biofilms were then washed three times with sterile PBS prior to quantification of the biofilm metabolic activity and biomass. These experiments were performed on three separate occasions.

Quantification of biofilm activity

A semiquantitative measure of each biofilm was calculated using a formazan salt-based XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide) reduction assay, adapted from previous studies to quantify anti-*C. albicans* biofilm activity.[24-26] Briefly, XTT (Sigma) was prepared as a saturate solution of 0.5 g/l of sterile PBS. The solution was then filtered through a 0.22 μm filter, aliquoted and stored at −80°C. Prior to use, XTT was thawed and menadione (Sigma; 10 mM prepared in acetone) added to a final concentration of 1 μM. A 100-μl aliquot of the XTT/menadione solution was then added to each prewashed biofilm and to control wells (for measurement of background XTT-reduction levels). Plates were then incubated in the dark for up to 2 hours at 37°C. A colorimetric change in the XTT-reduction assay, a direct correlation of metabolic activity of the biofilm, was then measured in a microtiter plate reader (FluoStar Omega, BMG Labtech, Aylesbury, UK).

Table 1 Denture cleaning products used

Product name	Manufacturer	Active ingredient	Directions for use
Boots Smile denture cleanser	Boots, Nottingham, UK	EDTA, sodium perborate	15 min
Medical™ Interporous®	MSI Laboratories-AG, Liechtenstein	EDTA, sodium biocarbonate	15 min
Steradent	Reckitt & Colman Pharmaceuticals, Glostrop, UK	Tetraacetylethylenediamine, sodium carbonate peroxide	10 min
Dentural	Martindale Pharmaceuticals Ltd., Essex, UK	Sodium hypochlorite (1.5%, w/v), sodium hydroxide (1.7%, w/v)	20 min

Quantification of biofilm biomass

The biofilm biomass following denture cleanser immersion was assessed using a crystal violet assay described by Mowat et al, adapted from Christensen's original method.[27,28] For the purpose of this study, the stain acts in an analogous manner to a plaque-disclosing agent.[29] XTT was removed from each biofilm, and these were washed with PBS. Biofilms were air dried, and then 100 μl of 0.5% (w/v) crystal violet solution added for 5 minutes. The solution was then removed by carefully rinsing the biofilms under running water until excess stain was removed. These were de-stained by adding 100 μl of 95% (v/v) ethanol into each well. The ethanol was gently pipetted to completely solubilize the crystal violet for 1 minute. Subsequently, the ethanol was transferred to a clean 96-well microtiter plate and absorbance read at 570 nm. The absorbance values are proportional to the quantity of biofilm biomass, which is comprised of hyphae and extracellular polymeric material (the greater the quantity of biological material, the greater the quantity of staining and absorbance measured).[28]

Scanning electron microscopy

The biofilm removal effects of Dentural were examined by SEM on three denture materials. Self-curing (SC, PalaXpress® autopolymerizing acrylic resin, Heraeus Kulzer, Hanau, Germany), conventional pressure-packed (CPP, Trevalon®, Dentsply Ltd., Addleston, UK), and injection-molded (IM, PalaXpress®, Heraeus Kulzer) acrylic resins were prepared in accordance with each manufacturer's instructions. Acrylic specimens were adjusted to remove excess material flash and trimmed into 10 mm^2 sections. Specimens were then placed in sterile water to remove residual monomer for 3 days. Immediately prior to use in the study, specimens were sterilized using an ultraviolet light unit for 10 minutes. Sterile specimens of IM, CPP, or SC denture base materials were placed into a Costar 12-well tissue culture plate (Corning), and *C. albicans* (ATCC 90028) biofilms were formed as described earlier. These were then treated with Dentural, as recommended by the manufacturer, washed in PBS, and processed for SEM. Untreated positive controls were also included. Briefly, the denture base materials were fixed in 2% *para*-formaldehyde, 2% glutaraldehyde, 0.15M sodium cacodylate, and 0.15% Alcian Blue, pH 7.4, and prepared for SEM as previously described.[30] The fixed and dried denture base specimens were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope.

Statistical analysis

The statistical analysis of biofilm formation was performed using SPSS® Software (Chicago, IL). For multigroup comparisons, the Kruskal–Wallis test and chi-square statistic were used to determine if any groups exhibited a statistically significant different percentage of biofilm viability or biomass. If the Kruskal–Wallis test demonstrated at least one of the groups to be statistically different, a *post hoc* analysis using the Mann-Whitney U test and Bonferroni correction was used to adjust the significance value (p) for the number of comparisons. Differences between conventional and overnight treatments were compared using a Mann-Whitney U test, and a Bonferroni correction was used to adjust the significance value (p) for the number of comparisons.

Results

The anti-biofilm activity of four commercially available mouthwashes was examined to assess whether any of the four products tested provided complete biofilm eradication and whether any of these offered an overall benefit in comparison to one another. All *C. albicans* strains were initially tested for their ability to form biofilms on conventional pressure-packed acrylic discs. The resultant biofilms were shown to have equivalent biomass to those formed on polystyrene microtiter plates (results not shown). Therefore, for simplicity and the potential to investigate the large quantity of permutations required within this study, the 96-well microtiter plate high throughput model was used.

The ability to remove *C. albicans* biofilm from the substrate was investigated following treatment with each of the four denture cleansers, either as recommended by the manufacturer or following overnight incubation (Fig 1). Overall, the most effective product tested was Dentural, which reduced the biomass by greater than 90% after 20 minutes and 18 hours immersion, with no significant differences observed between them. This exhibited significantly improved biomass removal capacity than did the other three products when used as per the manufacturer's instructions ($p < 0.001$). Steradent Active Plus showed a significant difference between recommended and overnight immersion, with improved biofilm removal at 10 minutes (84%) compared to overnight (76%) ($p < 0.001$). Medical™ Interporous® produced a mean reduction of 80% and 75% after 15 minutes and 18 hours immersion,

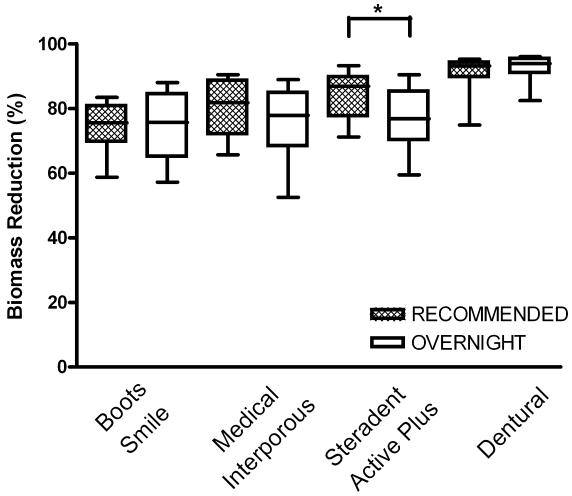

Figure 1 Effects of denture cleansers on biomass reduction. Reduction in the biofilm biomass of 16 *C. albicans* denture stomatitis strains and the *C. albicans*-type strain NCTC 90028. The box and whiskers plot illustrates the mean and variation of all strains tested. Statistical analysis shows any differences between the recommended and overnight treatment regimens (*$p < 0.001$).

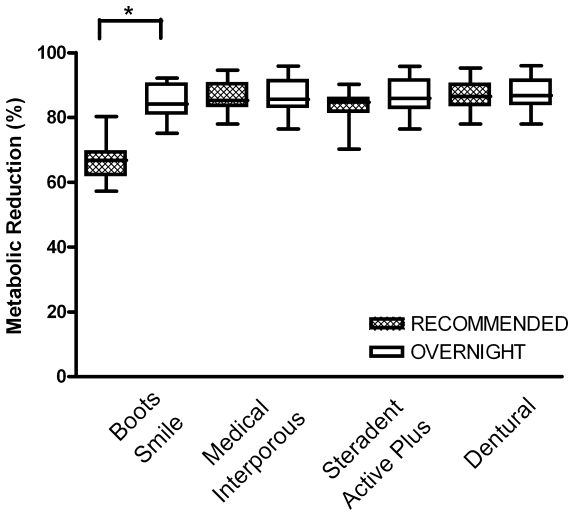

Figure 2 Effects of denture cleansers on metabolic reduction. Reduction in the biofilm metabolism of 16 *C. albicans* denture stomatitis strains and the *C. albicans*-type strain NCTC 90028. The box and whiskers plot illustrates the mean and variation of all strains tested. Statistical analysis shows any differences between the recommended and overnight treatment regimens ($^*p < 0.001$).

respectively, and was more significantly active than Steradent Active Plus at either time point. Boots Smile was the poorest denture cleanser overall, with a mean reduction of 73% at both 15 minutes and 18 hours following immersion, which appeared to have decreased biofilm removal activity at 15 minutes in comparison to Medical™ Interporous® ($p < 0.05$), but the difference was not statistically significant after a Bonferroni correction was applied. In comparison to Steradent Active Plus and Dentural, Boots Smile had significantly decreased biofilm removal activity at 15 minutes ($p < 0.001$), but was significantly different only from Dentural following overnight immersion ($p < 0.001$).

Metabolic activity of *C. albicans* biofilms was also determined following treatment with each of the four denture cleansers, either as recommended by the manufacturer or following overnight incubation (Fig 2). No significant differences in reduction of biofilm activity were observed between Medical™ Interporous® (86%), Steradent Active Plus (83%), and Dentural (86%); however, Boots Smile was significantly less effective (66%) ($p < 0.0001$). Following overnight immersion, all four cleansers showed no significant difference from one another, demonstrating metabolic reductions in the range of 85 to 87%. Boots Smile ($p < 0.001$) was significantly more effective at reducing the metabolism following an 18-hour immersion compared to disinfection times recommended by the manufacturer.

The biofilms were also examined on three clinically relevant denture base acrylics treated with Dentural, the most effective denture-cleansing agent. SEM analysis of SC, CPP, and IM acrylics showed the presence of residual hyphae adhering and aggregating upon the surfaces of the three materials (Fig 3). These cells tend to be found in areas of imperfection and enhanced surface topography. In both treated and untreated biofilms, yeast cells are scant, as these tend to be removed during the SEM processing.

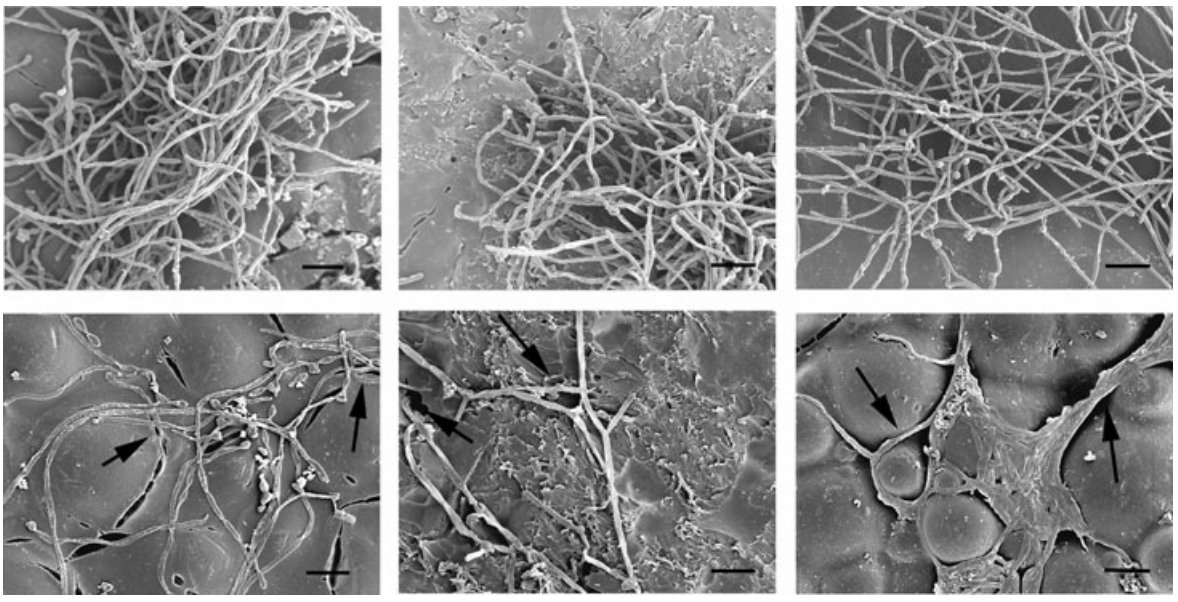

Figure 3 SEM of Dentural treated biofilms on three denture acrylics. *C. albicans* biofilms formed on SC (left), CPP (middle), and IM (right) acrylics were (upper row) untreated material matched controls or (lower row) treated with Dentural. Note the expansive biofilms comprised of intertwined hyphae for untreated controls. The treated biofilms have significantly reduced quantities of hyphae; however, the hyphae tend to aggregate and adhere to areas of surface irregularities (denoted by arrows). Scale bars represent 20 μm.

Discussion

Disinfection of removable dentures is an essential process in reducing the overall incidence of denture stomatitis.[31] The data described herein indicates that all four denture cleansers have the capacity to substantially reduce the fungal bioburden by chemical disinfection alone, thus highlighting their utility against *C. albicans* biofilms. Nevertheless, complete biofilm destruction was not achieved for any product. This illustrates that when used as a sole means of denture sterilization, they are not entirely adequate and require the combination of physical disruption, as advocated by the British Dental Association through their bdasmile program (http://www.bdasmile.org). Coco et al recently demonstrated that as many as 100 times more yeast cells could be removed from a denture surface by sonication than rinsing alone.[13] This same study also demonstrated that those patients with the greatest quantities of yeast upon their removable prostheses were those with poor compliance to oral hygiene, and each of these patients displayed high levels of oral mucosal inflammation.

This study aimed to determine whether any of the denture cleansing products tested herein, and currently available within the UK, was superior to one another with respect to effectiveness against biofilms formed by *C. albicans*, the leading cause of denture-induced stomatitis. The panel of *C. albicans* isolates used in this in vitro study were selected from a recent denture stomatitis study to provide clinical relevance.[13] Of all the agents tested, the sodium hypochlorite-containing Dentural was significantly more effective than the other compounds, even after 20 minutes immersion. Indeed, sodium hypochlorite has been shown to be the most effective denture disinfectant in other studies;[32] however, both the biomass and metabolic data indicate that residual biofilm material was retained on the substrate surface and was viable, as assessed by XTT measurements. This has direct implications for subsequent fungal regrowth and shedding of *C. albicans* cells to distal sites within the oral cavity.

Interestingly, we observed that overnight biomass readings were greater than during short exposure in some instances, whereas decreased metabolic activity was noted in corresponding samples. This suggests that although there is retention of biomass, the cells within it are largely dead. Overnight soaking with these agents, although significantly improving overall biofilm disinfection, may lead to deleterious effects on the denture material, which has been indicated previously with lining materials.[33] For example, sodium hypochlorite can have adverse effects on dental materials and oral tissues.[34,35] In addition, it is recognized that some denture cleansers may contribute to biofilm formation through the deterioration of material.[36] Therefore, although significant differences were observed between the recommended and overnight regimens, these differences were minimal on the whole, suggesting that the manufacturer's guidelines can be adhered to. Nevertheless, SEM analysis of several denture acrylics did show the presence of hyphae and yeast cells adhering, irrespective of their surface finish, confirming the quantitative biofilm data. It also demonstrates that although *C. albicans* initial adhesion is governed by surface topography and roughness,[37,38] the resultant biofilm remains tenacious once colonization is established. Therefore, residual viable material adherent to the substrate indicates that physical methods of decontamination are a necessary adjunct to chemical means.

Previous studies have highlighted the importance of using mechanical intervention to remove the clinically relevant biofilm.[39] Brushing is used to physically remove biofilm plaque within the oral cavity and on dentures; however, this may actively contribute toward subsequent *C. albicans* adhesion and biofilm formation through damage to the denture surface.[20,40] It has also been established that dentures can support candidal biofilms within the cracks and imperfections generated through wear and aging.[17] Although the active ingredients within the denture cleansers are of an antimicrobial nature, *C. albicans* biofilms are associated with high levels of antifungal resistance.[18,41] In this study, the denture cleansers consisted of a range of antimicrobial agents (Table 1), including EDTA, sodium bicarbonate, sodium perborate, hydrogen peroxide, and sodium hypochlorite. Although in previous studies these have been demonstrated to be useful in inhibiting biofilm development, they have minimal or reduced efficacy against mature *C. albicans* biofilms.[42-45] Therefore, it was not surprising that complete biofilm eradication and killing was not observed in this study.

Conclusion

Commercially available denture disinfectants used within this study were capable of reducing *C. albicans* biofilms in vitro; however, none of the tested products were effective in completely eliminating established *C. albicans* biofilms. Further research is required to help develop new denture disinfectant products capable of effectively reducing adherent microorganisms upon denture bases, which subsequently contribute toward denture-induced stomatitis. The development of a commercially available sonic bath analogous to a sonic toothbrush, or toothbrushes that do not impact the topography of the acrylic, is perhaps an option in reducing preventable cases of oral candidosis.

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