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A Comparative In Vitro Study of Two Denture Cleaning Techniques as an Effective Strategy for Inhibiting *Candida albicans* Biofilms on Denture Surfaces and Reducing Inflammation

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Abstract

Purpose: *Candida albicans* is the predominant oral yeast associated with denture-induced stomatitis, and with an increasing population of denture wearers its incidence is increasing. Maintaining good oral and denture hygiene, through chemical and/or mechanical intervention, is essential to reducing this disease. The aim of this study, using a robust adherent *C. albicans* cell model system, was to evaluate and compare the efficacy of a novel denture cleanser to the efficacy of a commonly used dentifrice coupled with brushing.

Materials and Methods: Four *C. albicans* strains isolated from individuals diagnosed as having denture-induced stomatitis, were adhered to denture acrylic resin sections (1 cm^2 by 1 mm thickness) and after 4 hours of growth, challenged daily sequentially for 4 days with a denture cleanser (Polident) or intermittently with denture cleanser (day 1), then dentifrice (Colgate Cavity Protection Toothpaste) and brushing (days 2 and 3) and denture cleanser (day 4). Colony forming units were evaluated for each treatment, as were the levels of regrowth. Scanning electron microscopy (SEM) was also performed. Microbial susceptibility testing and time-kill studies were performed on biofilms. A coculture model was also used to assess interleukin-8 (IL-8) production from treated biofilms.

Results: It was shown that sequential treatment with the denture cleanser killed and inhibited regrowth each day. Intermittent treatment showed that viable *C. albicans* biofilms were only retained rather than being dispersed, which could be visualized by SEM. Time-kill studies demonstrated that the novel denture cleanser was highly active and killed quickly, unlike the dentifrice. IL-8 was expressed in greater levels in 24-hour biofilms than in 4-hour biofilms, but treatment with denture cleanser reduced IL-8 output.

Conclusions: The data indicate that maintaining good oral health for denture wearers requires daily use of a denture cleanser rather than an alternating regimen. The inability of the denture cleanser to sterilize during intermittent treatments demonstrates the difficulty in controlling established biofilm. Moreover, the presence of mature biofilm may result in high levels of inflammation, but this can be controlled through denture cleansing.

Oropharyngeal candidosis (OPC) is caused by the opportunistic yeast *Candida* spp., of which *C. albicans* is the most prolific. This condition typically presents with white pseudomembranous lesions covering large areas of the oral mucosa, tongue, and palate.[1] Although OPC is rarely associated with mortality, significant morbidity is often experienced, heavily burdening both the individual and the healthcare system.[2] Symptoms of OPC include changes in taste and oral pain, which is often referred to as a burning sensation.[3] The consequence of such generalized symptoms is that the condition is often underdiagnosed, and appropriate treatment is therefore not administered.[4] However, even after prescription of antifungal treatments, colonization is often reestablished.[5] Thereafter, problems can arise regularly if the underlying reason for immunosuppression is not identified or cannot be treated.[6]

One of the most common types of OPC is the erythematous form, which when associated with wear of oral prosthetic appliances, such as dentures, often leads to denture-induced stomatitis, an inflammation of the oral mucosa.[1] *C. albicans* is implicated as the main causative organism of denture-induced stomatitis, primarily due to its ability to readily adhere to, and form, resilient biofilms on oral cavity soft and hard tissues, which are resistant to antifungal agents.[7,8] It has been suggested that in terms of its role in denture-induced stomatitis *C. albicans* preferentially adheres to and forms a biofilm on poly (methyl methacrylate) (PMMA) acrylic resin, compared with other dental materials.[9]

This biofilm growth upon acrylic resin denture material produces a significant challenge for effective microbial removal by chemical and physical methods.[10] A number of mechanical and chemical denture cleansers are available in the market with claims for their various efficacies. It is hardly surprising, therefore, that denture wearers are confused as to the optimum denture cleansing regimen. A recent systematic review suggested a lack of evidence about the comparative effectiveness of the different denture cleaning methods, where it was surmised from analysis of six randomized control trials that there was lack of information relating to the comparison of chemical and mechanical denture cleaning studies.[11] This point was echoed in a parallel analysis, which also concluded that it was unclear as to whether the optimum denture cleansing approach should be chemical, mechanical, or a combination of both methods.[12] It is, however, accepted that it is important to disinfect dentures regularly to ensure efficient removal of microbial biofilm, as there was significant variation of microbial load upon dentures when brushed with different denture cleansers.[13] The aim of this study was to evaluate denture cleansing regimens, rather than denture cleanser type, by comparing the efficacy of two denture cleansing regimens: daily soak for 4 days with a denture cleanser or intermittently with a denture cleanser (day 1), then dentifrice and brushing (days 2 and 3) and denture cleanser (day 4), using an adherent *C. albicans* cell model system. The null hypothesis was that there is no difference in the effectiveness between the two denture cleansing regimens in controlling the *C. albicans* biofilm.

Materials and methods

Culture conditions and strain characterization

Four *C. albicans* clinical strains from a previous study, isolated from patients with denture-induced stomatitis, were selected for use in this study.[7] These strains represented isolates from different levels of Newton's type (NT) classification of inflammation:[14] GDS25 (NT0), GDS18 (NT1), GDS3 (NT2), and GDS71 (NT3). *C. albicans* strains 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, Cambridge, UK) 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 1×10^6 cells/ml in RPMI (Roswell Park Memorial Institute) 1640 medium (Sigma, Gillingham, UK). All procedures were carried out in a laminar flow cabinet (Microflow Biological Safety Cabinet).

Preparation of acrylic resin specimens (denture base material)

For all quantitative and microscopic analyses, 1 cm^2 specimens of PMMA acrylic resin (Chaperlin and Jacobs Ltd, Surrey, UK) were used. Specimens were prepared according to manufacturer's instructions. All sections used in the study were decontaminated prior to use by submerging in sterile PBS and sonicating at 35 kHz for 5 minutes (Ultrasonic bath, Fisherbrand, Fisher Scientific, UK) and then sterilized under an ultraviolet light for 15 minutes per section side.

Treatment with denture cleansers

A commercially available denture cleanser (Polident, GlaxoSmithKline, Parsippany, NJ) and a commercially available dentifrice (Colgate Cavity Protection Toothpaste, Colgate Palmolive, New York, NY) were used throughout the study. Each *C. albicans* isolate was inoculated onto a 1 cm^2 acrylic resin specimen placed within a 24-well tissue culture plate (Costar, Corning Inc, Corning, NY) at an optimized concentration of 1×10^6 cells/ml in RPMI for 4 hours at 37°C, as previously described.[15] An inoculum of 1×10^6 cells/ml from each isolate was added to a 1 cm^2 denture acrylic specimen and incubated for 4 hours to form an early biofilm. Sterilized uninoculated and inoculated discs served as negative and positive controls, respectively, throughout. The treatment regimens were as follows: Treated directly with (A) denture cleanser and sequentially treated daily treatment thereafter (24, 48, and 72 hours) for 3 minutes as per the manufacturer's recommendations, and (B) treated initially with denture cleanser (4 hours) for 3 minutes and then with dentifrice and brushing at 24 and 48 hours, followed by a further denture cleanser treatment (72 hours). The specimens were brushed with a 10% w/v slurry of dentifrice (25°C) in double-distilled H_2O for approximately 2 seconds using a soft bristle toothbrush (Oral-B, Procter and Gamble, Cincinnati, OH). The 2-second time was calculated based on

the proportional surface area of an upper denture being brushed for an average of 2 minutes.

Total viable cell counts were performed following each treatment as follows: Following treatment, sections were transferred to bijou tubes (Sterilin Ltd, Newport, UK) containing 1 ml of sterile PBS, and sonicated at 35 kHz for 5 minutes (Ultrasonic bath, Fisherbrand). Total viable counts were then quantified using the Miles and Misra plate counting technique onto SAB agar plates. In parallel, acrylic resin specimens treated at each time point were reinoculated into RPMI, and the levels of regrowth quantified. Untreated biofilms were also quantified throughout the investigation. All experiments were performed on all strains on at least two separate occasions in triplicate.

Scanning electron microscopic examination

Representative specimens following the denture cleansing treatment regimens were retained for visual analysis using scanning electron microscopy (SEM) as previously described.[16] Briefly, the acrylic resin specimens were washed in PBS, fixed in 2% paraformaldehyde, 2% gluteraldehyde, and 0.15% w/v Alcian Blue in 0.15 M sodium cacodylate (pH 7.4). The fixed and dried denture base specimens were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope.

Antifungal susceptibility testing

Antifungal testing to determine planktonic minimum inhibitory concentrations (PMICs) was performed using the Clinical Laboratories Standards Institute (CLSI) M-27A broth microdilution method,[17] and sessile minimum inhibitory concentrations (SMICs) were performed as previously described.[18,19] RPMI buffered with 3-(N-morpholino)propanesulfonic acid (MOPS) was used for these studies. For PMICs, microtiter trays were incubated at 37°C, and endpoints read visually at 48 hours. For SMICs, biofilms were grown overnight, then treated for 24 hours at 37°C. SMICs were determined at 80% inhibition using an 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.[19-21]

Time-kill studies

To further evaluate the antimicrobial activity of each product, biofilms from each strain were prepared in 96-well plates, as described previously.[19] These were either challenged with a 10% w/v slurry of dentifrice (25°C) or with the denture cleanser (40°C) in ddH_2O for 0.5, 1, 3, 5, and 10 minutes. Biofilms were washed and the metabolic activity quantified using an XTT assay, as previously described.[18,21] Appropriate positive and negative controls were included. The activity of the products was evaluated by subtracting the background levels of XTT and calculating the proportional decrease in metabolic activity compared to the untreated control. Experiments were performed in quadruplicate on three occasions.

Biofilm host cell inflammation

A biofilm epithelial cell coculture model was developed to assess host cell response to treated biofilms. OKF6-TERT2 epithelial cells, provided by the Rheinwald laboratory (Brigham and Woman's Hospital, Boston, MA), were grown as adherent monolayers in 5% CO_2 at 37°C in keratinocyte serum-free medium (KSFM). These are a keratinocyte cell line immortalized through forced expression of telomerase that have been shown to resemble primary oral keratinocytes in cytokine induction studies.[22] To assess cytokine responses, cells were seeded at a density of 1×10^5 cells in KSFM in a 24-well culture plate and grown until 90% to 100% confluent. To assess the inflammatory response of OKF6-TERT2 cells to biofilms ± denture cleanser treatment, IL-8 protein release was quantified using ELISA technology. Biofilms were grown for 4 or 24 hours on denture acrylic resin specimens, treated with the denture cleanser and then washed, as described above. Acrylic resin specimens containing treated and untreated biofilms were attached to hanging inserts and placed into wells containing epithelial cells with the biofilm inverted. This model allowed a 0.5 mm gap between the biofilm and the cells and avoided direct media starvation of the epithelial cells. Zymosan A (ZYM), which is a glucan cell wall component from *S. cerevisiae* that activates Toll-like receptor 2 (TLR2), was used as a positive control for IL-8 induction. OKF6/TERT2 cells were stimulated for 24 hours in 5% CO_2 at 37°C. Cell supernatants were then removed and used to perform an IL-8 ELISA in accordance with the manufacturer's instructions (BioSource, Invitrogen, Paisley, UK). The results were calculated using a 4-parameter curve fit, quantifying colometric changes at 450 nm (FLUOstar Omega, BMG LabTech, Ortenberg, Germany).

Data analysis

All analyses were performed and figures produced using GraphPad PRISM (v5, La Jolla, CA). Two-tailed *t*-tests were performed to analyze for statistical differences of $p < 0.05$.

Results

The effect of the denture cleansing regimens on biofilm growth is presented in Figure 1. Treatment of immature 4-hour biofilms with the denture cleanser alone was shown to completely inhibit the biofilm throughout the study, with no growth detected for any of the treated acrylic resin specimens at 24, 48, and 72 hours posttreatment. No regrowth following treatment was detected at each treatment phase (Fig 1A). Treatment of immature 4-hour biofilms with denture cleanser was shown to completely inhibit *C. albicans* biofilms after 4 hours, but following sequential brushing with dentifrice, residual levels were detected. The residual levels increased to 0.6% and 50% of their time-matched controls at 24 and 48 hours, respectively. Regrowth of the biofilms was also observed at 24 (8%) and 48 hours (343%). A final denture cleanser treatment reduced the biofilm viability by 90%, but regrowth was observed from the residual cells, resulting in 434% of the 4-hour-untreated biofilm (Fig 1B).

The results of the SEM analysis of 24-hour-treated acrylic resin specimens demonstrated that irrespective of treatment, residual yeasts and hyphal cells were retained, particularly within cracks and imperfections of the material surface. However, the visible number of cells was lower than the untreated control sections (Fig 2).

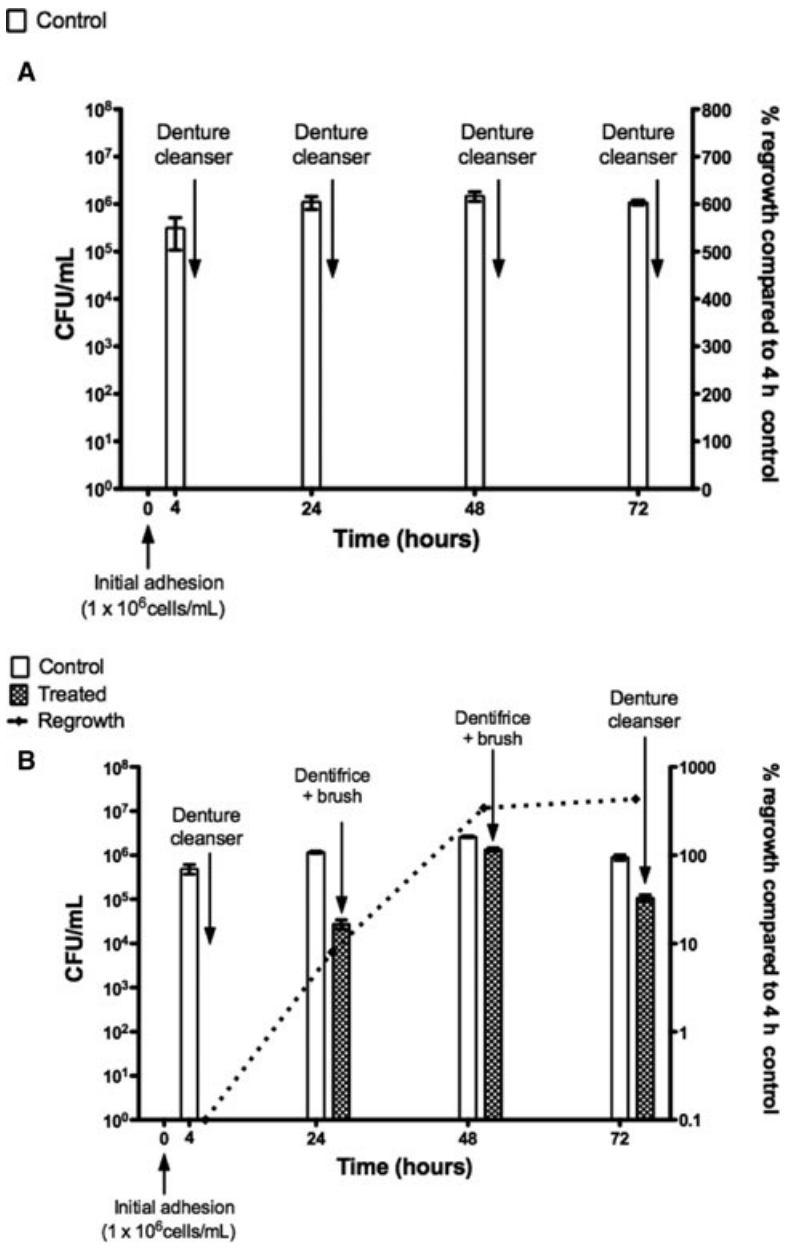

Figure 1 Daily sequential treatment with denture cleanser inhibits biofilm regrowth. An inoculum of 1×10^6 cells/ml from each isolate was added to a 1 cm^2 denture acrylic section and incubated for 4 hours to form an early biofilm, then treated directly with (A) denture cleanser and sequential daily treatment thereafter (24, 48, and 72 hours) and (B) treated initially with denture cleanser (4 hours) and then with dentifrice and brushing at 24 and 48 hours, followed by a further denture cleanser treatment (72 hours). Total viable cell counts were performed following each treatment. In parallel, acrylic resin specimens treated at each time point were reinoculated into RPMI, and the levels of regrowth enumerated. Untreated biofilms were also enumerated throughout the experiment (positive control). It was shown that denture cleanser inhibited *C. albicans* growth and subsequent repopulation, maintaining apparent sterility throughout, whereas for intermittent treatment resulted in significant repopulation of the acrylic resin. All experimental time points were performed twice on quadruplicate specimens, with triplicate technical replicates for each section processed.

The effect of the denture cleansers on inhibiting biofilm metabolism is presented in Table 1. The PMIC and SMIC were performed on each agent, where it was shown that the denture cleanser was highly inhibitory against planktonic cells (PMIC = 6.25%) compared to the dentrifice (PMIC = 25%). Against biofilms, the denture cleanser was again the most effective (SMIC = 25%) compared to the dentifrice (SMIC = 50%). For time-kill studies, it was shown that dentifrice slurry was minimally fungicidal, reducing the metabolism by only 53% after 10 minutes of exposure. The denture cleanser was highly active, inhibiting the metabolism by 96.5% after only 30 seconds, remaining around 97% after 10 minutes (Fig 3).

In terms of the inflammatory response induced by biofilms that can be controlled using denture cleansers (Fig 4), biofilms (4- and 24-hour) coincubated with oral epithelial cells showed that 24-hour biofilms induced significantly greater levels of IL-8 (~1500 pg/ml) than 4-hour immature biofilms (~600 pg/ml [$p < 0.001$]). Treatment of biofilms with the denture cleanser significantly reduced IL-8 release from oral epithelial cells stimulated with 24-hour biofilms (~200 pg/ml, $p < 0.001$), but not significantly for 4-hour immature biofilms (~400 pg/ml).

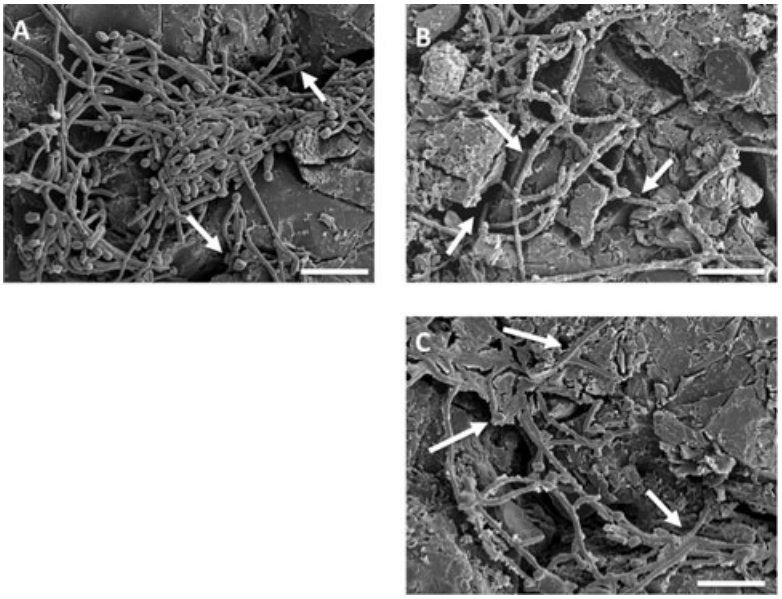

Figure 2 Scanning electron microscopy (SEM) of treated *Candida albicans* on denture acrylic. *C. albicans* were grown on acrylic resin specimens for 24 hours and treated with (A) phosphate buffered saline, (B) dentifrice and brushing, and (C) denture cleanser. Following defined treatments, the specimens were prepared for SEM analysis. Note the expansive biofilms comprised intertwined hyphae for untreated controls. Both the dentifrice- and denture cleanser-treated biofilms have significantly reduced quantities of hyphae; however, the hyphae tend to aggregate and adhere to areas off surface irregularities (denoted by arrows). Scale bar is equal to 20 μm.

Table 1 Planktonic and sessile minimum inhibitory concentrations of denture cleanser and dentifrice

	Denture cleanser		Dentifrice	
Strain	PMIC	SMIC	PMIC	SMIC
ATCC90028 (C)	6.25%	25%	25%	50%
BC052 (NT0)	6.25%	25%	25%	50%
BC030 (NT1)	6.25%	25%	25%	50%
BC015 (NTII)	6.25%	25%	25%	50%
BC071 (NTIII)	6.25%	25%	25%	50%

PMIC = planktonic minimum inhibitory concentration; SMIC = sessile minimum inhibitory concentration.

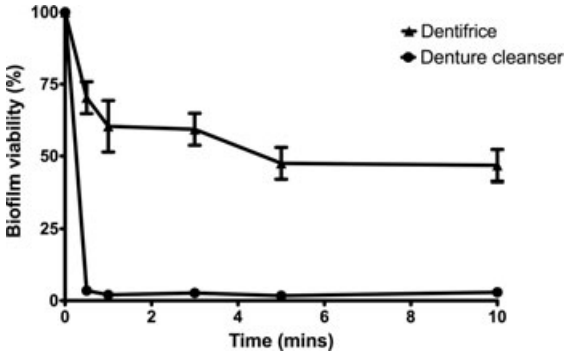

Figure 3 Time-kill analysis of *Candida albicans* biofilms treated with denture cleanser and dentifrice. Biofilms were grown in 96-well microtitre plates for 24 hours before treatment at time points of 0.5, 1, 3, 5, and 10 minutes. Metabolic activity of the treated biofilm was assessed by an XTT assay, and the proportional decrease in viability was compared to the untreated biofilms. Note the quick and active action of the denture cleanser compared to the dentifrice. All experimental time points were performed on three separate occasions with all four strains on ten independent samples.

Discussion

Decontamination of dentures is a key aspect for effective oral hygiene, as retention of microbes upon the denture may lead to excessive biofilm growth and subsequent oral disease. *C. albicans* is the major yeast species isolated from patients with oral candidosis (including denture-induced stomatitis), with its ability to form biofilms on a variety of surfaces.[23-25] In addition to those clinically diagnosed with the disease, a number of infected denture wearers are asymptomatic. Clinical management is required in all cases when possible. This is especially important given the possibility of *C. albicans* to proliferate unchecked within the oral cavity, a situation implicated to be associated with oral malignancies.[26] Therefore, in addition to daily oral hygiene practices, denture decontamination is pivotal to reducing and eliminating *Candida* spp. retained upon the denture as adherent biofilm communities. It has been shown that denture cleansers, while killing and disrupting *C. albicans* biofilms in an in vitro study, are not able to entirely decontaminate.[18]

This study compared the effectiveness of two commonly used denture hygiene regimens at controlling candidal biofilms. Using a robust in vitro model, it was shown that sequential daily treatments with a novel denture cleanser significantly reduced *C. albicans* retention upon denture base acrylic resin when compared with intermittent treatment with denture cleanser combined with a dentifrice and brushing. In contrast, it was previously shown that the combination of mechanical and chemical disinfection was more effective than chemical disinfection of *C. albicans* alone.[27] However, these analyses were performed on a stainless steel substrate rather than rough acrylic, which would facilitate efficient mechanical removal of *C. albicans* due to the smooth surface. It was found that daily sequential chemical treatment of colonized acrylic resin specimens was able to

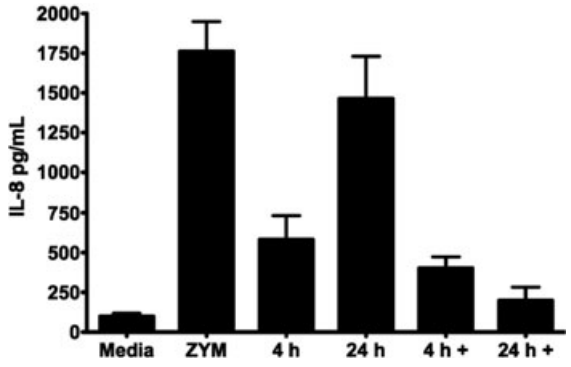

Figure 4 Biofilms induce a pronounced inflammatory response that can be controlled using denture cleansers. A biofilm epithelial cell coculture model was developed to assess host cell response to treated biofilms' IL-8 release (pg/ml), quantified using an ELISA from 4- and 24-hour biofilms ± denture cleanser treatment. Zymosan used as a positive control for cell stimulation and a media control as a negative control. Experiments were performed in duplicate on three independent occasions. Mature biofilms induced IL-8 expression in greater levels than immature biofilms, and treatment of both biofilms maintained levels of IL-8 not significantly different from the negative control. This experiment was performed three times on triplicate independent samples, with duplicate technical replicates for each supernatant processed.

maintain *C. albicans*-free acrylic resin, whereas an intermittent regimen of chemical and mechanical cleansing was highly ineffective. The denture cleanser was therefore significantly more effective compared to the dentifrice. A possible explanation for the superior cleansing qualities of the novel cleanser is that the oxidizing ability of the cleanser provided enhanced fungicidal activity, therefore limiting *C. albicans* regrowth and biofilm formation. This was confirmed by the MIC and time-kill studies, which provided validation on how quickly and effectively the cleanser killed *C. albicans* biofilms compared to the dentifrice, which showed limited fungicidal activity. One would expect that the limited fungicidal effects of the dentifrice should be augmented by the associated mechanical brushing; however, regrowth of candidal cells upon the acrylic resin on days 2 and 3 confirmed that this was not the case.

Previous studies have suggested that it is difficult to eradicate *Candida* completely from the denture using a variety of soak-type chemical cleansers. SEM of a selection of specimens confirmed this and showed retention of *Candida* within cracks and crevices of the acrylic resin. It is therefore possible that residual organisms are able to survive in low numbers following even a chemical challenge, which when replaced into an environment with nutrients allows the biofilm to repopulate. These cells may exhibit the persister cell phenotype, which has been shown to be present in individuals with long-term carriage of *C. albicans*.[28] In addition, the extrapolymeric matrix may further protect the cells from chemical damage, as recent data have shown how released extracellular glucans can sequester different classes of antifungal agents and decrease their overall sensitivity.[29] Efflux pumps have also been shown to be differentially expressed during *C. albicans* growth,[30] so these may also augment the biofilms' capacity to resist denture cleansers.

These factors may explain why previous studies have reported the ineffectiveness of the exclusive use of chemical methods for denture decontamination in studies of intact biofilms.[18,27] The results of this study suggest that there is a genuine possibility of controlling *C. albicans* on dentures using a sequential daily chemical approach by preventing excessive candidal growth that can support the growth and colonization of other oral microbiota and lead to oral disease, such as denture-induced stomatitis. It was also shown that the frequent use of the novel denture cleanser was able to suppress the release of the proinflammatory chemokine IL-8, which is a further benefit to oral health.

A caveat to these data is that they should be interpreted in the context of how the experiments were performed, that is, in vitro modeling. The study was performed over a relatively short timeframe compared to the average duration of denture use, so investigating these sampling methodologies in vivo under controlled conditions would provide more robust data to support regular chemical denture cleansing. It is important that daily use of the soak-type denture cleanser is used according to manufacturer's instructions, with particular reference to the temperature of the water, to prevent deterioration of the denture base material.[31]

Conclusions

Using a robust in vitro model, it was shown that sequential daily treatments with a novel denture cleanser significantly reduced *C. albicans* retention upon denture base acrylic resin when compared with intermittent treatment with denture cleanser combined with a dentifrice and brushing.

Acknowledgments

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