

## Long-term efficacy of denture cleansers in preventing *Candida* spp. biofilm recolonization on liner surface

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**Abstract:** This study evaluated the long-term efficacy of denture cleansers against *Candida* spp. biofilm recolonization on liner surface. Specimens were fabricated of a poly(methyl methacrylate)-based denture liner and had their surface roughness evaluated at baseline and after cleansing treatments. *C. albicans* or *C. glabrata* biofilms were formed on liner surface for 48 h, and then the specimens were randomly assigned to one of cleaning treatments: two alkaline peroxides (soaking for 3 or 15 min), 0.5% sodium hypochlorite (10 min) or distilled water (control; 15 min). After the treatments, the specimens were sonicated to disrupt the biofilm, and residual cells were counted (cell/mL). Long-term effectiveness of the cleaning processes was determined by submitting a set of cleaned specimens to biofilm growth conditions for 48 h followed by estimation of cell counts. The topography of specimens after cleaning treatments was analyzed by SEM. Data were analyzed by ANOVA and Tukey's test ( $\alpha = 0.05$ ). Results of cell count estimation showed significant differences in cleanliness among the treatments ( $p < 0.001$ ), and it could be observed by SEM. However, no significant difference ( $p > 0.05$ ) was observed among the *Candida* species regarding the recolonization condition. Alkaline denture cleansers showed similar cleaning performance and both differed from the control ( $p < 0.001$ ). Sodium hypochlorite was the only treatment that removed biofilm efficiently, since no viable cells were found after its use. In conclusion, alkaline peroxide denture cleansers were not effective in removing *Candida* spp. biofilm from denture liner surfaces and preventing biofilm recolonization.

**Descriptors:** Denture, complete; Biofilms; Candidiasis.

### Introduction

Denture liners are important in clinical practice, considering that their use provide relief for sharp bony undercuts or extreme sensitivity due to submucosal exposure of the inferior alveolar nerve.<sup>1</sup> Although denture liners are commonly used, their physical characteristics make them susceptible to sorption, which results in dimensional changes that favor biofilm formation on their surfaces, leading to easy colonization and infection by *Candida* spp.<sup>2</sup>

The occurrence of *Candida* spp. biofilm on denture base material and its consequences for removable denture wearers, such as chronic erythematous candidosis (CEC), have been shown in the literature.<sup>3,4</sup>

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Although *C. albicans* is commonly associated with CEC, other non-*albicans* species have been isolated from removable denture surfaces and palatal mucosa, in particular *C. glabrata*, an emerging fungal pathogen.<sup>5</sup>

Denture cleansers are increasingly used by the large consumer base in this specialized healthcare market,<sup>6</sup> mainly due to the increase number of elderly people and the use of liners. Usually indicated as an auxiliary denture care method, denture cleansers can also be indicated as the main method for elderly patients in long-term care hospitals, who are unable to brush their dentures adequately because of disease, dementia, poor dexterity and visual acuity.<sup>7</sup>

Classified into different groups according to their main components,<sup>8,9</sup> effervescent tablets are classified as chemical soak-type products. When dissolved in water, the sodium perborate readily decomposes to form an alkaline peroxide solution that subsequently releases oxygen, thus enabling a mechanical cleaning by oxygen bubbles as well as chemical cleaning.<sup>8,10</sup> Although microorganism elimination by denture cleansers has been evaluated,<sup>7,11</sup> it is suggested that denture cleansers are not effective in preventing their initial adherence to the denture liners.<sup>12</sup> Another aspect of denture cleaners that is not fully understood is related to recolonization of the host surface by microbial biofilm after using these products. Considering that denture cleansers are applied in an attempt to remove biofilm, it is important for these products to be capable of preventing, or at least, delaying surface recolonization.

This study evaluated the efficacy of denture cleansers against *Candida* spp. biofilm developed on liner surface and their long-term effect on biofilm surface recolonization.

## Materials and Methods

### Experimental design

This *in vitro* study was approved by the local Research Ethics Committee, and the volunteer who donated the saliva used in the study signed a written informed consent.

The present study had a randomized and blinded design regarding cell counts. Treatments with chemical cleansers (enzymatic cleanser solution, cleanser

solution or 0.5% sodium hypochlorite) or distilled water (as control), surface roughness and *Candida* specie (*C. albicans* or *C. glabrata*) were considered as factors under study. Surface roughness and cell counts (*C. albicans* and *C. glabrata*) were the test variables. Analyses were performed immediately after treatments and in a long-term, after treatments and recolonization.

Acrylic resin specimens relined with a layer of a permanent denture liner were fabricated according to the manufacturer's instructions. *C. albicans* or *C. glabrata* biofilms were formed for 48 h, and specimens were then assigned to one of the 4 treatments. Remaining adherent microorganisms were removed from the treated specimens by sonication and cell counts of each microorganism were calculated (immediately). The long-term efficacy of the denture cleansers was determined using another set of specimens covered with biofilm and cleaned by the same treatments. After, the specimens were inserted in new fresh culture medium and biofilm was allowed to develop for 48 h. After this time, the specimens were sonicated and the cells were quantified (cell/mL). Cell count data were analyzed statistically. Scanning electronic microscopy (SEM) was used to evaluate the liner surface after cleansing treatment.

### Specimen preparation

The specimens were prepared according to the manufacturers' instructions at room temperature, under aseptic conditions. Cylindrical wax pattern discs (10 mm in diameter and 1.5 mm thick) were prepared using an aluminum matrix. Discs were invested in metallic flasks and subsequently the wax was softened and eliminated with boiling water. The heat-polymerized poly(methyl methacrylate) (PMMA) (Lucitone 550, Denstply, Rio de Janeiro, Brazil) resin was then packed and the flasks were placed in a hot water bath at 74°C during 9 h. Once processed, all flasks were allowed to bench cool for 2 h. The specimens were then removed and immersed in distilled water at 37°C for 12 h for residual monomer release.<sup>13</sup> Next, these discs were relined with a PMMA permanent resin liner (Kooliner, GC America; Alsip, USA) and polymerized at room tem-

perature. A uniform 1.5-mm-thick liner layer was applied by inserting each disc into a glass mould, pouring in the denture liner, placing glass slides over and both ends of the mold are firmly fixing them. The glass slides were separated after the material was polymerized and the specimens were removed from the moulds,<sup>12,14</sup> finished and used immediately.

Surface roughness ( $\mu\text{m}$ ) of the relined specimens was measured at baseline and after the cleansing treatments, using a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan) accurate to the nearest 0.01-mm, calibrated at a specimen length of 0.8 mm, 2.4-mm percussion of measure, and 0.5 mm/s. Three readings were made for each specimen and a mean value was obtained.<sup>15</sup> After, the specimens were ultrasonically cleansed (Thornton T 740; Thornton-Inpec Eletronica Ltda., Vinhedo, SP, Brazil) in sterile distilled water for 20 minutes prior to biofilm formation to remove any contaminants and artifacts from their surfaces.<sup>16</sup> Subsequently, these specimens were randomly divided into two groups according to the *Candida* strains (*C. albicans* - ATCC 90028 or *C. glabrata* - ATCC 2001) and were exposed to human whole saliva for acquired pellicle formation.

### Inoculum and growth conditions

Prior to each experiment, the *Candida* strains were aerobically cultured at 37°C for 24 h on Sabouraud Dextrose Agar (SDA; Difco Laboratories, Detroit, MI, USA) and a loopful of yeast cultures growth was inoculated into Yeast Nitrogen Base (YNB) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 50 mM glucose. After 18 to 20 h of incubation, cells were washed twice with PBS and suspended in YNB supplemented with 100 mM glucose and standardized to  $10^7$  cells/mL ascertained spectrophotometrically (Bausch & Lomb Spectronic 20, San Pablo, Calif, USA) at 520 nm.<sup>13</sup>

### Biofilm assays

Each specimen was placed in 24-well polystyrene tissue culture plates (TPP, Trasadingen, Switzerland). Subsequently, 2 ml of each cell inoculum was added to each well. Biofilms were formed on saliva-coated relined discs by incubation with clarified

and sterilized by 0.22  $\mu\text{m}$  membrane filtration (TPP, Trasadingen, Switzerland) human whole saliva for 30 minutes at 37°C.<sup>13</sup> All biofilm assays were performed in duplicate in at least 4 independent experiments on different days. The organisms were grown at 37°C at 75 rpm in an orbital shaker (model NT 151; Kline Shaker; Nova Técnica Laboratório, Sao Paulo, Brazil) for 48 h to allow biofilm formation. The medium was changed every 24 h.<sup>17</sup>

### Cleansing treatment

Each specimen was individually placed in a sterile beaker containing 8 ml of one of the treatment solutions:<sup>12</sup> POL (alkaline peroxide containing enzyme; Polident 3-minutes, GlaxoSmithKline; Philadelphia, PA, USA); EFF (alkali peroxide; Efferdent, Warner Lambert Co., Morris Plains, NJ, USA); HYP (0.5% sodium hypochlorite, Proderma Pharmacy, Piracicaba, Brazil) or DW (distilled water - control). POL and EFF were prepared with distilled water following the manufacturer's directions. The immersion periods were: 3 min for POL and 15 min for EFF, in accordance with the manufacturers' directions; for HYP the immersion time was 10 min and for DW the immersion time was 15 min, as a reference for the longest time used for EFF.

### Biofilm cell counts immediately after the treatments

After the cleansing procedure, the specimens were immersed in sterile PBS and sonicated (7 W, for 30 s) to disrupt the biofilm structure.<sup>18</sup> The sonicated solutions were serially diluted in PBS and 20  $\mu\text{L}$  specimens were plated in triplicate onto SDA. The plates were incubated at 37°C, under aerobic conditions for 24-48 h. Yeast cells were counted using a stereomicroscope (Coleman Comp. Imp., Santo André, SP, Brazil), and the results were expressed in cell counts/mL.<sup>17</sup>

### Long-term efficacy of the treatments

To evaluate the long-term efficacy of the treatments, a different set of specimens covered with biofilms were submitted to the cleansing process in the same manner as previously described. Afterwards, the specimens were washed twice with sterile PBS

and transferred to a new sterile 24-well plate containing 2 ml YNB supplemented with 100 mM glucose. The plates were incubated for 48 h at 37°C at 75 rpm in an orbital shaker. At each 24 h incubation period, all specimens were washed with PBS followed by the addition of 2.0 ml of fresh medium. After 48 h, cell count estimation was performed.<sup>17</sup>

### Scanning electron microscopy

After the treatments, the specimens were rinsed with sterile PBS and prepared for SEM performance. The surface features of the biofilm were visualized with a SEM (JEOL JSM5600LV; Tokyo, Japan) in high vacuum mode at 15 kV.

### Statistical analysis

All analyses were performed using the SAS software (SAS Institute Inc., version 9.0, Cary, USA) with the level of significance set at 5%. The normality of error distribution and degree of non-constant variance were checked for the response variables and data were transformed as suggested by the software.

**Table 1** - Surface roughness ( $\mu\text{m}$ ) of relined specimens at baseline and after the treatments (Mean  $\pm$  SD; n = 8).

| Treatments   | Baseline        | After treatments |
|--------------|-----------------|------------------|
| DW (Control) | 3.8 $\pm$ 0.5 a | 4.0 $\pm$ 0.3 b  |
| HYP          | 3.5 $\pm$ 0.9 a | 4.0 $\pm$ 0.9 b  |
| EFF          | 3.2 $\pm$ 0.4 a | 3.8 $\pm$ 0.6 b  |
| POL          | 3.2 $\pm$ 0.5 a | 3.4 $\pm$ 0.4 b  |

Different lowercase letters indicate significant differences between baseline and after treatments ( $P = 0.013$ ).

**Table 2** - Cell counts/mL for *Candida* spp. immediately after cleansing treatments (Mean  $\pm$  SD; n = 8).

| Treatments | <i>Candida</i>                    |                                 |
|------------|-----------------------------------|---------------------------------|
|            | <i>C. albicans</i>                | <i>C. glabrata</i>              |
| DW         | 5.8 $\pm$ 5.4 $\times 10^6$ A,a   | 5.0 $\pm$ 2.3 $\times 10^6$ A,a |
| EFF        | 0.17 $\pm$ 0.23 $\times 10^6$ A,b | 4.8 $\pm$ 3.2 $\times 10^6$ B,a |
| POL        | 0.07 $\pm$ 0.1 $\times 10^6$ A,b  | 5.7 $\pm$ 7.0 $\times 10^6$ B,a |
| HYP        | 0.0                               | 0.0                             |

Different uppercase letters in rows and different lowercase letters in columns indicate statistically significant differences between *Candida* spp., and among denture cleansers, respectively.

The cell count data were transformed by logarithm [ $\log_{10}(\chi)$ ]. All data were analyzed using two-way ANOVA and Tukey's test.

### Results

After the treatments, the surface of relined specimens was rougher when compared with the baseline values ( $P = 0.013$ ; Table 1).

After the treatments, denture cleansers showed similar cleaning performance and both presented lower counts compared to the control ( $P < 0.001$ ) for *C. albicans*. However, no differences ( $P > 0.05$ ) were found for *C. glabrata* after the treatments using DW or alkaline peroxides (Table 2).

Regarding surface recolonization, alkaline peroxides and DW treatments showed statistically similar results for *C. albicans* ( $P > 0.05$ ), while for *C. glabrata*, both alkaline peroxides showed higher counts when compared to the control ( $P < 0.001$ ) (Table 3).

The only effective treatment to clean the liner surfaces was the use of HYP, since no *Candida* cell growth was observed under both conditions and for both strains (Tables 2 and 3).

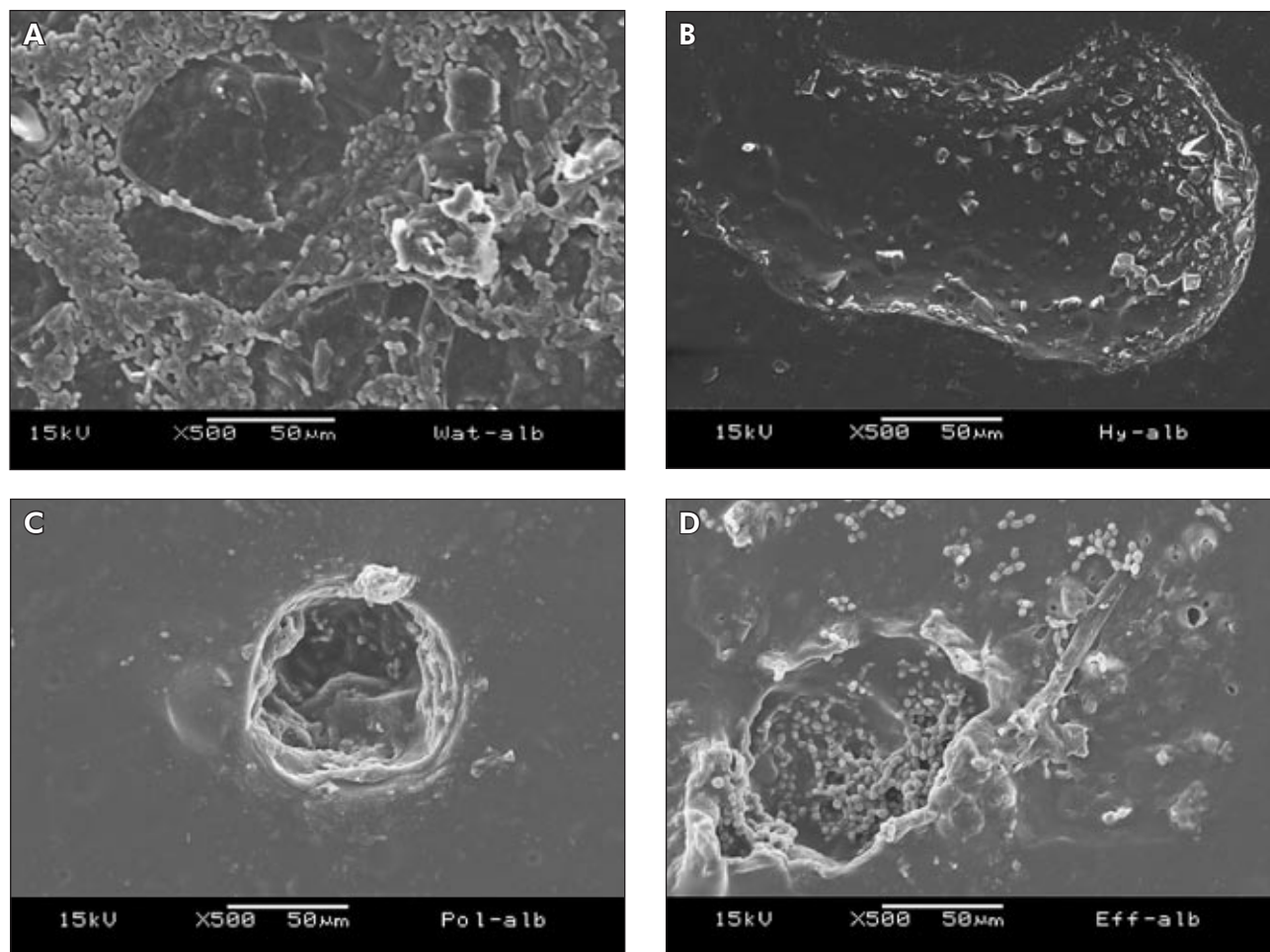
*C. glabrata* showed significantly higher cell counts in comparison to *C. albicans* when treated with both alkaline denture cleansers ( $P < 0.001$ ). However, regarding recolonization, no differences were found between the *Candida* strains after treatment with DW or two alkaline peroxides ( $P > 0.05$ ) (Table 3).

SEM micrographs of specimen surface after the cleansing treatments are presented in Figure 1.

**Table 3** - Cell counts/mL for the *Candida* spp. after the treatments and surface recolonization (Mean  $\pm$  SD; n = 8).

| Treatments | <i>Candida</i>                  |                                  |
|------------|---------------------------------|----------------------------------|
|            | <i>C. albicans</i>              | <i>C. glabrata</i>               |
| DW         | 4.1 $\pm$ 2.1 $\times 10^7$ A,a | 5.2 $\pm$ 4.8 $\times 10^7$ A,a  |
| EFF        | 4.9 $\pm$ 2.7 $\times 10^7$ A,a | 10.0 $\pm$ 4.9 $\times 10^7$ A,c |
| POL        | 8.0 $\pm$ 3.3 $\times 10^7$ A,a | 9.7 $\pm$ 4.0 $\times 10^7$ A,c  |
| HYP        | 0.0                             | 0.0                              |

Different uppercase letters in rows and different lowercase letters in columns indicate statistically significant differences between *Candida* spp., and among denture cleansers, respectively.



**Figure 1** - Representatives SEM micrographs of biofilm developed on denture liner surface after treatment with the different denture cleansers: **(A)** surface after DW treatment; **(B)** surface after HYP treatment; **(C)** surface after POL treatment; **(D)** surface after EFF treatment.

HYP-cleaned specimens showed a cell-free surface (b), while POL (c), EFF (d) and DW (a) showed surfaces with adhered cells.

## Discussion

This study evaluated for the first time the efficacy of denture cleansers on denture liner biofilms immediately after the cleansing treatments and after the treatments followed by surface recolonization. Surface roughness before and after treatments was also evaluated. The biofilm growth model used simulated *in vivo* conditions of static biofilm growth found on the tissue-contacting surface of a denture.<sup>10</sup>

A rougher surface was found after all treatments, which indicated that changes in denture surface probably occur in the mouth, considering that it is

immersed in saliva while it is worn. The increased roughness associated with surface irregularities, such as cracks and pits, found in denture liners, provide a larger surface area and a more sheltered environment for biofilm to develop and protect microorganisms from being removed by cleaning.<sup>19</sup> The results of the present study showed that the specimens treated with denture cleansers POL and EFF presented lower cell counts compared to DW treatment. Nevertheless, these cleansing solutions were not able to remove biofilm completely. When the *Candida* species were compared, the performance of cleansers POL and EFF was the same as that of the control for *C. glabrata*. These results corroborate those found by Ferreira *et al.*,<sup>12</sup> who used the same denture cleansers and *Candida* species, and Sousa *et*

*al.*,<sup>20</sup> who found inefficiency on reduction of *C. albicans* cells after using a similar peroxide cleanser. Another clinical study<sup>21</sup> also found that POL and EFF had similar performances, showing that biofilm growth in the present study model mimicked the *in vivo* environment.

Furthermore, irregularities can serve as reservoirs for fungal species, ready for recolonization of the surface.<sup>8</sup> In the present study, residual biofilm cells are clearly seen on such irregularities in the SEM images. POL and EFF are likely to promote a greater disturbance on biofilm structure, considering the effervescent action (Figures 1C and D), compared to DW, in which biofilm was poorly disturbed (Figure 1A). Results for POL and EFF were not different from each other, even taking in consideration the time elapsed between both treatments.

After 48 h, liner surfaces treated with EFF and POL showed similar counts of viable cells, and it was also observed after the treatments and recolonization. Therefore, cells disturbed by the POL and EFF treatments could develop freely, while DW-treated cells remained on a steady state, which could be a reasonable explanation for the increase in cell counts observed after POL and EFF treatments and recolonization (Table 3).

The results of this study also showed that soaking specimens in 0.5% HYP, which is considered a fungicidal agent, was the only effective treatment against *Candida* species under both conditions, since no viable cells were found after its use for both *Candida* species, which is in accordance with the

findings of a previous study.<sup>19</sup> In addition to its fungicidal effects, sodium hypochlorite acts dissolving mucin and other organic substances, such as extracellular polymeric matrix.<sup>2</sup> Although satisfactory results were found for HYP, the dental literature has shown that this product has the potential to bleach denture-base and may cause surface corrosion,<sup>8</sup> especially of the metal content in partial removable dentures. HYP is thus not indicated for daily use. Nevertheless, these problems seem to have been exaggerated and further studies are required to evaluate different concentrations and immersion times.

The objective of immersing a denture in a disinfectant is to remove biofilm and to decontaminate the surface by destroying the microorganisms, since dentures may function as a reservoir of pathogens.<sup>22</sup> Thus, one of the most important purposes of a denture cleansing protocol is to avoid recolonization of the oral cavity.<sup>12</sup> In the present study, the fungal levels returned to the initial levels within 48 h without significant difference between the *Candida* species. Although this study does not fully reproduce the oral environment, these results may suggest the need for stipulating a routine protocol for denture cleaning.

## Conclusion

Within the limitations of this study, it may be concluded that alkaline peroxide denture cleansers were not effective in removing *Candida* spp. biofilm from denture liner surfaces and biofilm recolonization was not prevented.

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