SÍLVIA CARNEIRO DE LUCENA

"EFFECT OF DENTURE CLEANSER ON MULTISPECIES BIOFILMS: *In vitro* AND *In vivo* EVALUATIONS"

"EFEITO DE LIMPADOR QUÍMICO DE PRÓTESE SOBRE BIOFILMES MULTIESPÉCIES: AVALIAÇÕES In vitro E In vivo"

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UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Orientadora: Profa. Dra. Altair Antoninha Del Bel Cury

"EFEITO DE LIMPADOR QUÍMICO DE PRÓTESE SOBRE BIOFILMES MULTIESPÉCIES: AVALIAÇÕES In vitro e In vivo"

Tese de Doutorado apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do título de Doutora em Clínica Odontológica na área de Prótese Dental.

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Este exemplar corresponde à versão final da tese
defendida pela aluna Sílvia Carneiro de Lucena e
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RESUMO

A adequada higienização das próteses dentais removíveis é fundamental para prevenir o acúmulo de biofilme, que funciona como reservatório de micro-organismos que podem levar a infecções locais e sistêmicas. Apesar de a escovação constituir um método bem estabelecido para tal fim, limitações visual e motora de alguns pacientes reduzem sua eficácia. Assim, o uso de limpadores químicos (LQ) tem sido indicado como método complementar para remoção do biofilme. Frente ao exposto, os objetivos deste trabalho foram (i) avaliar *in vitro* o efeito da imersão diária em LQ sobre biofilmes multiespécies desenvolvidos em resina para base de próteses removíveis e (ii) avaliar *in vivo* o efeito do LQ no controle do biofilme de próteses parciais removíveis (PPR). Para o estudo *in vitro*, foram confeccionados discos de resina acrílica sobre os quais foram desenvolvidos biofilmes multiespécies (*Candida albicans, Veillonella dispar, Streptococcus mutans, Streptococcus oralis, Fusubacterium nucleatum e Actinomyces naeslundii)*. Após 64,5 horas de desenvolvimento do biofilme, os espécimes foram aleatoriamente distribuídos em grupos controle e experimental. Os espécimes do grupo experimental foram submetidos a imersões diárias de 3 minutos na solução de LQ (Polident® 3 minute) por sete dias consecutivos; o biofilme do grupo controle foi desenvolvido durante o mesmo período sem tratamento. O biofilme de ambos os grupos foi coletado após 1, 4 e 7 dias experimentais (n=16) e analisados quanto ao número de células viáveis e concentração de polissacarídeos. Adicionalmente, a topografia superficial do biofilme e a morfologia celular foram analisadas através de microscopia eletrônica de varredura e confocal a laser. Para a avaliação *in vivo*, foram selecionados 25 voluntários usuários de PPR. Estes foram instruídos a complementar a higienização das próteses imergindo-as na solução do LQ (Polident® 3 minute) diariamente, por 3 minutos, durante 15 dias. O biofilme presente nas próteses foi coletado imediatamente antes e após o período experimental e o número de micro-organismos totais, estreptococos totais *e Candida* spp. foi quantificado. No

estudo *in vitro*, foi observado que a imersão diária no LQ reduziu significativamente o número de micro-organismos totais quando comparado ao grupo controle nos três períodos avaliados (Análise de variância a dois fatores, $p<0,05$). Esta redução também foi observada para as bactérias individualmente e *F. nucleatum* e *V. dispar* não foram detectadas nos biofilmes expostos ao LQ. Observou-se um aumento gradativo na contagem de células de *C. albicans* no grupo experimental, comportamento inverso ao grupo controle no qual houve redução do número de células fúngicas viáveis. A concentração de polissacarídeos extracelulares no biofilme foi maior no grupo experimental após sete dias de limpeza quando comparado ao controle (Análise de variância a dois fatores, $p<0,05$). As imagens de microscopia revelaram elevada quantidade de hifas de *C. albicans* no grupo experimental. No estudo *in vivo*, observou-se uma redução significativa do número de micro-organismos totais e estreptococos totais após o período experimental (Teste-t pareado, $p<0,05$). Entretanto, não houve diferença para as contagens de *Candida* spp.. Conclui-se que a exposição diária ao limpador químico reduziu significativamente os micro-organismos totais viáveis em biofilmes multiespécies mas não apresentou eficácia sobre *Candida.*

Palavras-Chave: Prótese Dentária, Higienizadores de Dentadura, *Candida albicans*

ABSTRACT

The maintenance of adequate denture hygiene is of paramount importance to prevent biofilm accumulation which acts as a reservoir of microorganisms that can cause local and systemic infections. Although brushing is a well established method for denture cleaning, visual and manual limitations of some patients can reduce its efficacy. Therefore, the use of denture cleansers (DC) has been indicated as a complementary method for denture hygiene. Considering this, the aims of this study were to (i) evaluate, in an *in vitro* model, the effect of daily immersion in DC on a multispecies biofilm develop on denture-base acrylic resin and (ii) assess the effect of DC on controlling biofilm from removable partial dentures (RPD) surfaces. For the *in vitro* study, specimens were fabricated using acrylic resin on which it was develop a multispecies biofilm (*Candida albicans, Veillonella dispar, Streptococcus mutans, Streptococcus oralis, Fusubacterium nucleatum e Actinomyces naeslundii)*. After 64.5 hours of biofilm growth, the specimens were randomized into control and experimental groups. The specimens of experimental group were submitted to 3-minutes immersions on DC solution daily for seven consecutive days; the biofilms of control group were developed for the same period without any treatment. The biofilms from both groups were collected after 1, 4 and 7 experimental days (n=16) and analyzed for viable cells and polysaccharides concentration. Additionally, the biofilms topography and cellular morphology were analysed by scanning electron microscopy) and confocal microscopy. For *in vivo* evaluation of DC, 25 volunteers that used RPD were selected. They were instructed to complement their dentures hygiene by immersing them on DC solution, for 3 minutes, during 15 days. The biofilm present on RPD was collected immediately before and after the experimental period and the number of total microorganisms, total streptococci and *Candida* spp. were quantified. In the *in vitro* study, it was observed that daily immersion on DC significantly reduced the total microorganisms in comparison to control group, in all periods evaluated. (Two-way Anova, $p<0.05$). This reduction was also observed for individual bacteria and *F. nucleatum* and *V. dispar* were not detected on biofilms

exposed to DC. It was observed a gradual increase of *C. albicans* count in biofilms of experimental group while, in control group, *Candida* counts decreased and it was not observed viable cells after seven experimental days. Additionally, extracellular polysaccharides concentration was significantly higher in experimental group after 7 days of treatment (Two way Anova, $p<0.05$). The microscopy images revealed a remarkable presence of hyphae in experimental group. In clinical study, it was observed a reduction on total microorganisms and total streptococci. However, no difference was observed in *Candida spp.* counts (paired t-test, $p<0.05$). It can be concluded that daily exposure to DC reduced total microorganisms on multispecies biofilms but did no demonstrated efficacy on *Candida.*

Keywords:Dental Prosthesis, Denture Cleansers, *Candida albicans.*

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SUMÁRIO

INTRODUÇÃO

As próteses dentais removíveis constituem uma alternativa de tratamento bastante utilizada na reabilitação de pacientes parcial (Zitzmann *et al.*, 2007) ou totalmente edêntulos (Carlsson & Omar, 2010). Uma vez dentro da cavidade oral, semelhante ao que acontece com os dentes naturais, as próteses estão suscetíveis à formação de uma película de proteínas salivares (Hannig, 1997; Gocke *et al.*, 2002) que servirá de substrato para adesão de micro-organismos. Se uma higiene adequada não for estabelecida, o processo de colonização resultará no desenvolvimento de complexas comunidades microbianas na superfície da prótese conhecidas como biofilme.

O biofilme das próteses removíveis é uma camada densa e complexa de micro-organismos e seus metabólitos, predominantemente bactérias do gênero *Streptococcus* (Theilade *et al.*, 1983; Koopmans *et al.*, 1988; Kulak *et al.*, 1997). O acúmulo de biofilme, além de ser antiestéticoe contribuir para presença de halitose (Goldberg *et al.*, 1997), funciona como reservatório de bactérias e fungos com potencial de causar infecções locais e sistêmicas (Glass *et al.*, 2001; Coulthwaite & Verran, 2007). Dentre as infecções locais, a candidose associada ao uso de prótese é a mais comumente encontrada em usuários de próteses removíveis, afetando cerca de 45,3% desta população (Figueiral *et al.*, 2007). Esta é associada à presença de fungos do gênero *Candida* spp. no biofilme da prótese, tendo como principal espécie envolvida em sua etiologia a *Candida albicans* (Dar-Odeh & Shehabi, 2003; Ramage *et al.*, 2004). Do ponto de vista sistêmico, os micro-organismos encontrados no biofilme das próteses dentais podem ser deglutidos ou aspirados e levar ao desenvolvimento de infecções sistêmicas tais como a pneumonia (Coulthwaite & Verran, 2007).

Diante disto, destaca-se a importância da adequada higiene das próteses não somente para saúde oral como para saúde geral de seus usuários. O método mais popular para remoção do biofilme sobre a prótese é a escovação com pasta abrasiva (Kulak-Ozkan *et al.*, 2002; Dikbas *et al.*, 2006; Baran & Nalcaci, 2009) que é de fácil acesso e baixo custo. Entretanto, apesar de constituir um método

eficaz, estudos têm mostrado que a sua associação com métodos auxiliares de limpeza é superior no controle do biofilme (Paranhos *et al.*, 2007; Nishi *et al.*, 2011). Isto se torna ainda mais relevante em casos de pacientes que, devido à acuidade visual diminuída ou mesmo à perda de habilidade manual, não conseguem realizar a escovação de suas próteses de maneira adequada (Kulak-Ozkan *et al.*, 2002; Padilha *et al.*, 2007).

Dentre os métodos auxiliares, a imersão de próteses em soluções desinfetantes ou limpadores químicos está entre os mais comumente adotados pelos pacientes (Marchini *et al.*, 2004; Dikbas *et al.*, 2006). O hipoclorito de sódio é um exemplo de solução caseira desinfetante amplamente empregada e que é capaz de efetuar uma limpeza eficaz nas próteses. Entretanto, apesar de seus resultados favoráveis, apresenta desvantagens tais como sabor e odor desagradáveis (Shay, 2000) além da possível alteração de cor da resina acrílica quando utilizado em soluções mais concentradas. Adicionalmente, pode promover corrosão de ligas metálicas (Backenstose & Wells, 1977; Felipucci *et al.*, 2011) contra-indicando o seu uso para higienização de próteses parciais removíveis. Neste contexto, o emprego de limpadores químicos comerciais surge como alternativa para a higienização de próteses removíveis.

Os agentes químicos para limpeza de próteses comercialmente disponíveis possuem diferentes mecanismos de ação de acordo com seus principais componentes, sendo os peróxidos alcalinos os mais frequentemente utilizados (Jagger & Harrison, 1995). Sua ação ocorre inicialmente pelo efeito mecânico de limpeza exercido pela efervescência produzida quando o produto é dissolvido em água, resultando em uma solução alcalina de peróxido de hidrogênio. A solução de limpeza contém oxigênio ativo que possui efeito antimicrobiano e de remoção de manchas; alguns produtos possuem ainda enzimas que cuja função é degradar proteínas presentes no biofilme (Budtz-Jorgensen, 1979; Nakamoto et al., 1991).

A eficácia dos limpadores químicos na higienização de próteses removíveis tem sido investigada através de diferentes critérios de avaliação como

a redução do acúmulo de biofilme (Paranhos *et al*., 2007), capacidade de remoção de manchas (Jagger *et al*., 2002) e controle da halitose (de Oliveira *et al*., 2011). Entretanto, parâmetros microbiológicos, tais como a quantificação de micro-organismos viáveis, constituem um dos métodos mais precisos para avaliação do biofilme (Nikawa *et al*., 1999) e diversos trabalhos têm utilizado-o para investigar a eficácia dos limpadores químicos.

Frente à alta prevalência de candidose associada ao uso de prótese, a ação *in vitro* de limpadores químicos sobre biofilmes de *Candida* tem sido amplamente estudada. Os resultados observados demonstraram que os limpadores são capazes de reduzir significantemente os níveis de *Candida* spp. sobre a resina acrílica para base de prótese tanto durante a fase de adesão inicial (Nakamoto *et al*., 1991; Iseri *et al*., 2011) como em biofilmes maduros, mono (Nikawa *et al.,* 1995) e duo-espécie (de Freitas Fernandes *et al*., 2011). Entretanto, apesar da relevância clínica destes estudos, o desenvolvimento de infecções sistêmicas associadas a outros micro-organismos presentes no biofilme das próteses ressalta a importância de investigar a ação dos limpadores químicos sobre biofilmes multiespécies, simulando o que acontece *in vivo*. Adicionalmente, alguns micro-organismos podem ser mais suscetíveis a exposição ao limpador químico (Paranhos *et al*., 2009) o que, em um biofilme complexo, poderia levar à seleção de determinadas espécies.

Neste sentido, alguns estudos foram realizados com o objetivo de avaliar o efeito do limpador químico sobre biofilmes multiespécies, associando a *Candida albicans* a bactérias, tais como *Streptococcus mutans* (Drake et al., 1992), *Escherichia coli* e *Staphylococcus aureus* (Lin *et al*., 1999) ou mesmo em biofilmes mais complexos (Li *et al*., 2010). No entanto, estes trabalhos realizaram uma única imersão dos biofilmes na solução de limpeza expondo assim a necessidade da realização de estudos que simulem o efeito do uso diário do limpador químico sobre um biofilme multiespécies.

Do ponto de vista clínico, a literatura é rica em estudos longitudinais que avaliaram *in vivo* a ação de limpadores químicos sobre biofilme de próteses

totais removíveis (Chan *et al.*, 1991; Gornitsky *et al.*, 2002; Paranhos *et al.*, 2007; de Andrade *et al.*, 2011; de Oliveira *et al.*, 2011); entretanto, seu efeito no controle do biofilme de próteses parciais removíveis é um campo ainda pouco explorado. Porém, a higienização de próteses parciais removíveis não deve ser negligenciada uma vez que estas possuem regiões de difícil acesso durante a escovação que representam sítios adicionais para acúmulo do biofilme, inclusive sobre os dentes suporte (Mine *et al.*, 2009). Adicionalmente, a instalação de próteses parciais tem sido associada a um aumento no número de *Candida* (Gusmao *et al.*, 2011) e *Streptococcus mutans* (Rocha *et al.*, 2003), micro-organismos envolvidos no desenvolvimento da estomatite protética e cárie dental respectivamente. Desta forma, o objetivo do presente trabalho é avaliar o efeito do limpador químico à base de peróxido alcalino sobre biofilmes multiespécies bem como a sua eficácia no controle de biofilme formado sobre próteses parciais removíveis.

CAPÍTULO 1

Daily immersions in denture cleanser influence multispecies biofilm

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ABSTRACT

This study evaluated the effect of daily exposure to a denture cleanser on a multispecies biofilm. Multispecies biofilms (five bacteria and *Candida albicans*) were developed for 64.5 hours on acrylic resin discs and then randomized into control and experimental groups. The biofilms of experimental group were submitted to 3-minutes daily immersions on denture cleanser for seven consecutive days while the biofilms on the control group were developed with no treatment for the same period. The biofilms from both groups were collected after 1, 4 and 7 experimental days (n=16) and analyzed for the number of microorganisms and extracellular polysaccharides concentrations. Biofilms topoghapy and cellular morphology were evaluated by scanning electron microscopy and confocal microscopy. Data were analyzed by two-way anova. The results showed a lower count of total microorganisms and bacterial population in experimental group for all periods evaluated. However, it was observed a continuous increase in *C. albicans* counts in the biofilms exposed to denture cleanser, with a remarkable presence of hyphae. Polysaccharides concentration was significantly higher in biofilms of experimental group. The daily exposure of a multispecies biofilm to a denture cleanser reduces the number of total microorganisms but favored *C. albicans*.

Key words: Oral hygiene, Polident, *Streptococcus mutans*, *Candida albicans*, hyphae, polysaccharides.

INTRODUCTION

Adequate denture hygiene is fundamental for successful rehabilitations with removable dentures. Otherwise, dentures became colonized by a dense layer of complex microbial communities embedded in a polymeric matrix known as biofilm (Spratt, 2003). Beyond aesthetics concerns, biofilm acts as a reservoir for opportunistic microorganisms that can cause local or even systemic diseases

(Glass *et al.*, 2010) emphasizing the important role of appropriate denture hygiene to patient´s health.

Brushing is the most popular method for cleaning dentures (Jagger and Harrison, 1995). However, many individuals fail to perform it correctly as a consequence of poor manual dexterity and/or impaired vision. Besides, acrylic resins present depths and pores that may harbor microorganisms and debris (Glass *et al.*, 2010) that cannot be easily removed by brushing. Therefore, the immersion on cleaning solutions has been recommended to complement denture hygiene (Dills *et al.*, 1988; Jagger and Harrison, 1995).

Alkaline peroxides are the most commonly used denture cleansers. These products, when in contact with water, produce an effervescent alkaline solution of hydrogen peroxide, containing active oxygen. This effervescence exerts a mechanical action for removing debris while the oxygen has antimicrobial and stain removal effects. Some products also add enzymes to break down proteins in biofilm (Budtz-Jorgensen, 1979; Nakamoto *et al.*, 1991; Jagger and Harrison, 1995).

A wide range of *in vitro* studies investigated the efficacy of denture cleansers, however most of them focused on *Candida* spp. (Nakamoto *et al.*, 1991; Nikawa *et al.*, 1995; de Freitas Fernandes *et al.*, 2011; Iseri *et al.*, 2011) or evaluated other microorganisms but in single-species biofilm (Paranhos *et al.*, 2009). Considering that denture biofilm is a community of fungi and bacteria, it is important to investigate the effect of denture cleansers on mixed biofilms. In this direction, some authors assessed the efficacy of these products on bacterial-yeast duo (Drake *et al.*, 1992) or multispecies biofilms (Li *et al.*, 2010) but limited to a single immersion protocol. Thus, the aim of this study was to evaluate the effect of daily immersion in denture cleansers on a multispecies biofilm.

MATERIALS AND METHODS

Specimens Preparations

Cylindrical specimens (10 x 2 mm) of acrylic resin for denture base (QC-20 PMMA – Dentsply Ltd., Weybridge, England) were made according to the manufacturer's instructions. The specimens were then grounded at both sides using progressively smoother aluminum oxide papers (320, 400 and 600 – grit) in a horizontal polisher (model APL-4; Arotec, Sao Paulo, Brazil) to standardize surface roughness. The specimens were washed with sterile distilled water and ultrasonicated to remove residues from the surface (Gomes *et al*., 2011).

Surface roughness was measured using a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan) accurate to 0.01 mm with a measurement length of 3.2 mm at 0.5 mm/s (Gomes *et al*., 2011). Three readings were made at each side of specimens and a mean value was calculated. The average surface roughness obtained was 0.32±0.03 mm. The specimens were placed in 24-well tissue culture plates in vertical position using a disc holder and submitted to sterilization by exposure to ethylene oxide.

Biofilm assay

A multispecies biofilm was developed based on the Zurich model (Guggenheim *et al*., 2001; Guggenheim and Meier, 2011). The biofilm was composed of six microorganisms, five bacteria – *Actinomyces naeslundii* OMZ 745, *Veillonella dispar* OMZ 493, *Fusobacterium nucleatum* OMZ 598, *Streptococcus mutans* OMZ 918 and *Streptococcus oralis* OMZ 607 – and the yeast *Candida albicans* OMZ 110 (Guggenheim and Meier, 2011). The model has been previously detailed (Guggenheim *et al*., 2001) and a brief description will be presented.

Inoculum preparation: all strains were individually cultivated on blood agar and colonies were transferred to 9 mL of a modified fluid universal medium (mFUM) supplemented with 0.3% of glucose. After 15 hours of incubation, aliquots from these pre-cultures were transferred to a new tube containing 5 mL of mFUM

0.3% glucose, incubated at 37°C for 7 hours and then independently adjusted to OD_{550} 1.0±0.02. The six-species inoculum was prepared by mixing equal volumes of each density-adjusted culture.

Biofilm development: the specimens were preincubated in 2 mL of processed whole unstimulated saliva at 37 °C for 4 hours to allow the formation of a salivary pellicle (Guggenheim *et al.*, 2001). The pellicle-coated specimens were transferred to wells containing 0.225 mL of mixed-species inoculum and 1.8 mL of medium (70:30 (v/v) mixture of saliva and mFUM 0.3% glucose). The plates were incubated anaerobically at 37 °C for 64.5 hours. During this period, the biofilms were dipped in saline solution three times a day and the medium was changed at 16.5 and 40.5 hours for a fresh medium (70:30 (v/v) mixture of saliva and mFUM 0.15% glucose and 0.15% sucrose).

Treatment Protocol

After the development of a mature multispecies biofilm (64.5 hours), the specimens were randomly assigned into the groups: control or experimental. The specimens in the experimental group were submitted to 3 minutes daily immersions on an alkaline peroxide enzyme-containing commercial denture cleanser (Polident® 3 Minute; GlaxoSmithKline, Philadelphia, USA) for seven consecutive days. For each immersion, the biofilm was transferred to plastic tubes with 8 mL of denture cleanser solution prepared by dissolving 0.11 g of a powdered tablet of denture cleanser in 8 mL of sterile distilled water at 40 °C. After 3 minutes, the biofilms were washed in saline solution (NaCl 0.9%) and then transferred to fresh medium. The biofilms in the control group were developed for the same period with no treatment. Biofilms of both groups were dipped in saline solution three times a day and culture medium was changed at each 24 hours. Biofilms were analyzed at days 1, 4 and 7 (n=16; Figure 1).

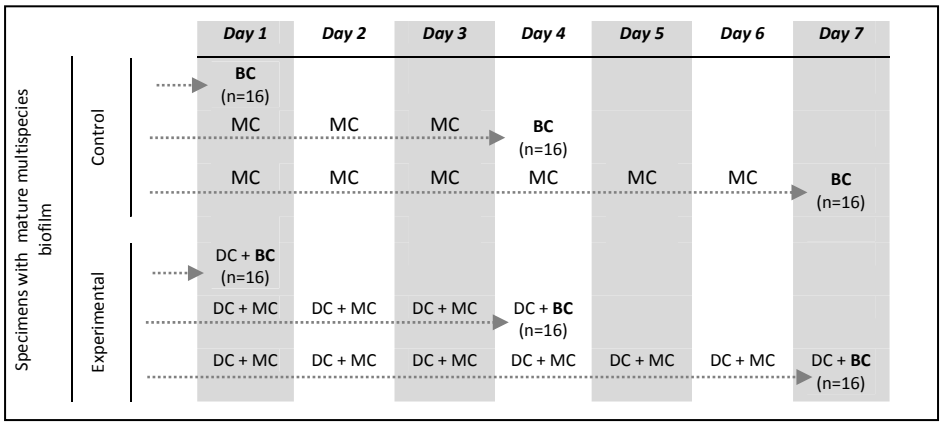

Figure 1. Study design of biofilm treatment and collection in control and experimental groups (BC = biofilm collection; MC = medium change; DC = immersion into denture cleanser).

Biofilm Analysis

The specimens were transferred to cryogenic tubes containing 3 mL of saline solution, sonicated (7 W, 30 seconds) and the resultant suspension was used for biofilm analysis.

Colony forming unit: 0.1 mL of the suspension was 10-fold serially diluted in saline solution and plated in the following culture media: Columbia Blood Agar[§] supplemented with 5% (v/v) defibrinate sheep's blood for total microorganisms count and plus norfloxacin (1 mg/mL), erythromycin (1 mg/mL) and vancomycin (4 mg/mL) for *F. nucleatum*; Mitis Salivarius Agar[§] for *S. mutans* and *S. oralis*; Veillonella Agar (Arif *et al*., 2008) for *V. dispar*; Cadmium Sulfate Fluoride Acridine Trypticase Agar (Zylber and Jordan, 1982) for *A. naeslundii*; and Sabouraud Dextrose Agar[§] with cloramphenicol (0.1 mg/mL) for *C. albicans*. ([§] DIFCO BD Franklin Lakes, USA)

Polysaccharide Quantification: 0.4 mL of the sonicated suspension was used to extract soluble and insoluble extracellular polysaccharides as described by Aires *et al*., 2008. The total carbohydrate was estimated by the phenol sulfuric method (Dubois, 1956) using glucose as standard and the total extracellular polysaccharide was obtained.

Both CFU and Polysaccharide were normalized by biofilm dry weight. All biofilm assays and analysis were performed in quadruplicate in 4 independent experiments on different days.

Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM)

For SEM analyses, two samples of each group were fixed overnight in Karnovsky solution (PBS- pH 7.2). The biofilms were dehydrated in a series of ethanol (60%, 80% and 90% for 5 minutes each and 100% for 10 minutes) and allowed to dry under aseptic conditions. The discs were mounted on stubs, sputter-coated with gold and observed in a Scanning Electron Microscope (LEO 435 VP, Carl Zeiss SMT, Oberkochen, Germany).

For visualization on CLSM (Leica Microsystems CMS, Mannheim, Germany), three samples of each group were submitted to fluorescent *in situ* hybridization using rRNA-specific oligonucleotide probes (Thurnheer *et al.*, 2001; Thurnheer *et al.*, 2004).

In both microscopy analyses, at least five representative randomly optical fields were examined for each specimen.

STATISTICAL ANALYSIS

The data were analyzed by two-way ANOVA followed by Tukey's HSD Test with a significance level fixed at 5% (SAS v. 9.0; SAS Institute, Inc, Cary, NC, USA).

RESULTS

The results showed a significant reduction on total microorganisms of experimental group, irrespective of the period of analysis ($p<0.001$). This significant reduction was also observed for bacteria individually ($p<0.001$) and *F. nucleatum* and *V. dispar* were not detected on experimental group. On the other side, the

biofilms of experimental group presented a higher number of viable *C. albicans* cells than control ($p<0.001$; Table 1).

Regarding the period of biofilm development, total microorganisms and *S. mutans* counts significantly increased after seven experimental days in both groups as well as *A. naeslundii* for experimental group. *F. nucleatum* decreased in control group and no difference was observed for *S. oralis*. For *C. albicans*, while the biofilms exposed to denture cleanser presented a progressive and significant increase in cell counts along the experimental period, in control group the number of viable yeast decreased progressively ($p=0.0014$) and no *Candida* cells were observed after seven days (Table1).

The polysaccharides concentration increased along experimental period for both groups but the biofilms exposed to denture cleanser presented a higher concentration of extracellular polysaccharides than control group at day 7 (Table 1).

SEM images revealed similar superficial characteristics of both biofilms at day 1; however, at days 4 and 7, the biofilms exposed to denture cleanser showed a more porous and irregular aspect when compared to control group (Figure 2). Both SEM and CSLM analyses revealed a remarkable presence of hyphal forms of *C. albicans* in biofilms exposed to denture cleanser at days 4 and 7 as well as the presence of small clusters of *C. albicans* cells covered by dense layers of bacteria after seven experimental days (Figures 2 and 3).

Table 1. Viable microorganisms (CFU/ mg dry weight) and extracellular polysaccharides concentrations (µg/ mg dry weight) for control and experimental groups at days 1, 4 and 7 (mean ± SD).

		*Total Microorganims** ($x10^8$)	*S. mutans** ($x10^7$)	*S. oralis** ($x10^7$)	*A. naeslundii** ($x10^7$)	*F. nucleatum** ($x10^7$)	*V. dispar** ($x10^6$)	*C. albicans** ($x10^4$)	*Extracelullar Polysaccharides **
Control	*D1*	2.9 ±1.4 a	8.8 ± 6.3 a	16.6 ±11.6 a	1.1 ± 0.6 a	2.4 ± 1.3 a	6.9 ± 11.8 a	0.4 ±0.3 a	9.2 ± 4.0 a
	D4	5.6 ± 4.4 a	33.5 ± 30.0 b	15.7 ± 14.3 a	1.9 ± 2.6 a	1.5 ± 1.3 b	2.7 ± 4.3 a	0.04 ± 0.06 b	10.1 ± 3.0 ab
	D7	9.4 ± 3.7 b	78.6 ± 33.1 c	13.4 ± 8.2 a	1.3 ± 0.9 a	1.7 ± 0.7 b	1.9 ± 2.9 a	0.0 c	15.2 ± 3.5 b
Experimental	*D1*	0.1 ± 0.1 a	0.40 ±0.4 a	0.5 ± 1.1 a	0.02 ±0.02 a	0.0 a	0.0 a	0.3 ± 0.3 a	10.1 ± 4.3 a
	D4	0.4 ± 0.4 b	2.7 ± 2.5 b	0.3 ± 0.3 a	0.04 ± 0.03 b	0.0 a	0.0 a	17.0 ± 15.0 b	15.1 ± 9.0 a
	D7	0.9 ± 0.9 b	7.7 ± 9.0 c	0.3 ± 0.2 a	0.06 ± 0.07 b	0.0 a	0.0 a	54.2 ± 33.9 c	35.8 ± 17.4 b

* statistically difference between control and experimental group.

Different lowercase letters indicate statistical difference among days in each group

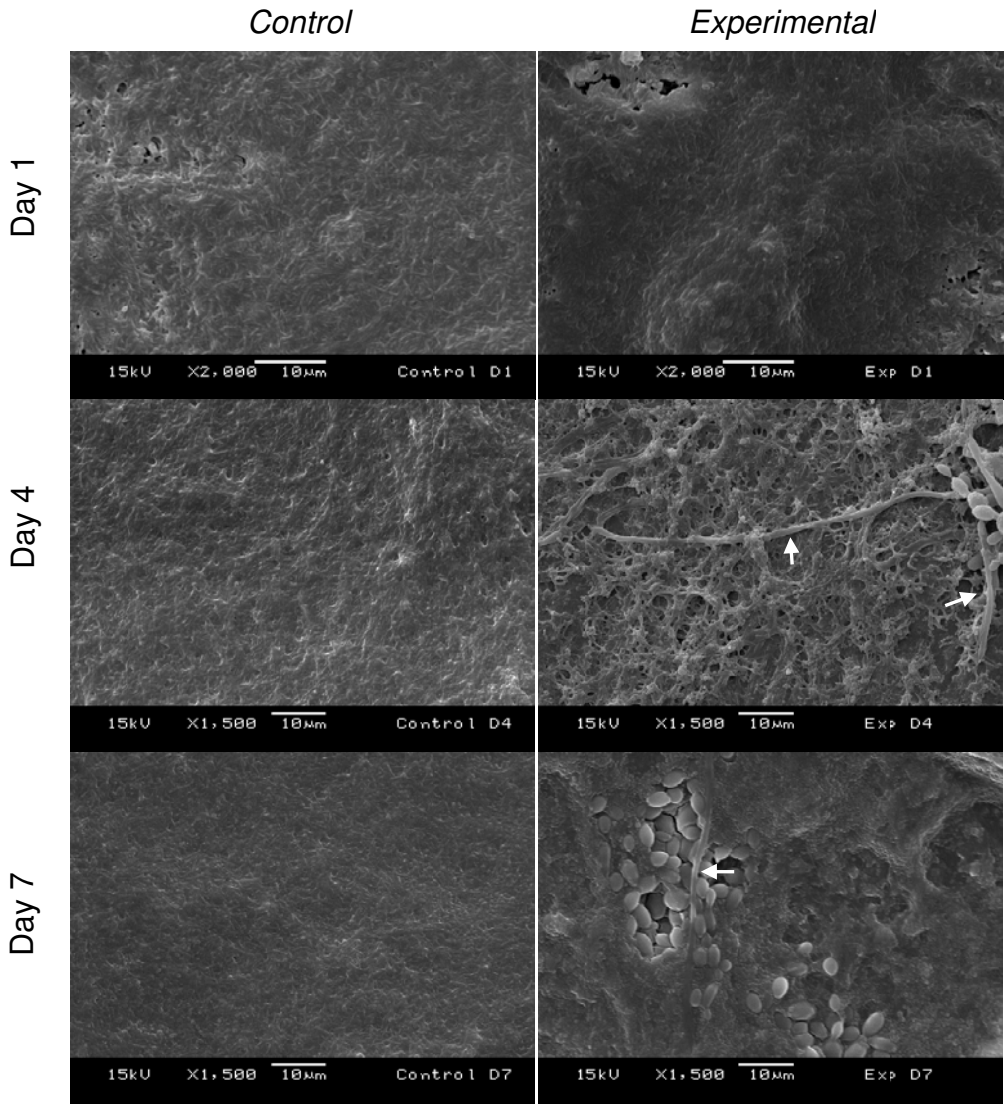

Figure 2. SEM images of control and experimental biofilms at days 1, 4 and 7. Note the presence of *C. albicans* hyphae (arrows) and the cluster of yeast cells covered by bacteria at experimental group, day 7.

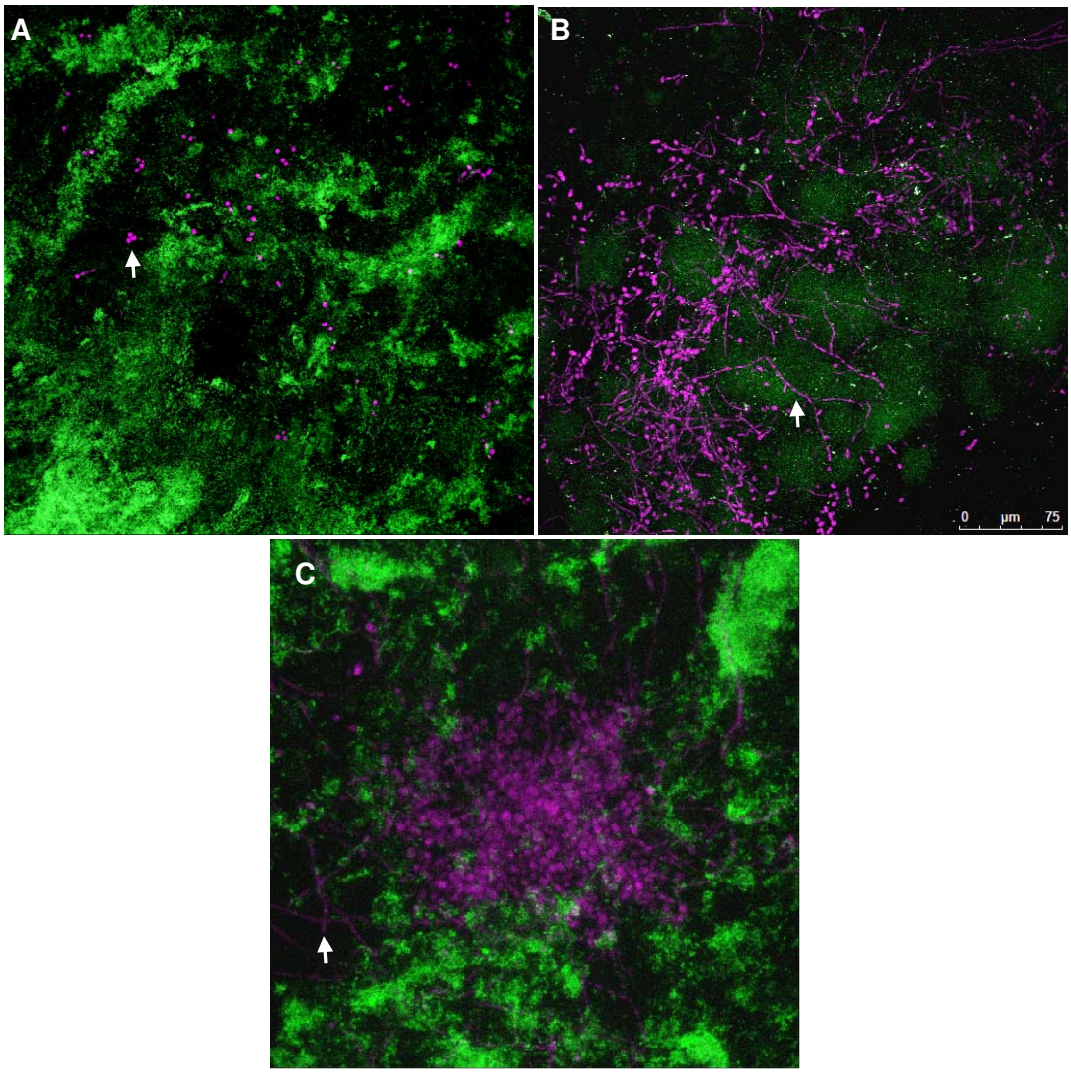

Figure 3 – CLSM images showing biofilm of control group at day 4 (A) and biofilms of experimental group at days 4 (B) and 7 (C). *C. albicans* (purple) was stained with EUK 516; in green, total bacteria (A and C) stained with EUB338 and *S. mutans* (B) stained with MUT 590. Note the remarkable presence of *C. albicans* hyphae in experimental group (arrows in B and C) in comparison with yeast form in control group (arrow in A). In C, a cluster of *C. albicans* cells.

DISCUSSION

The need for assessing the efficacy of denture cleansers on a multispecies biofilm has beeg previously pointed in literature (Paranhos *et al.*, 2009; de Freitas Fernandes *et al.*, 2011). However, to the authors' knowledge, this is the first *in vitro* study that used a daily immersion protocol in a multispecies

biofilm. This model, besides simulating clinical situations, allowed us to observe the ecological changes on an oral biofilm constantly exposed to a denture cleanser.

The general results showed that immersion in a denture cleanser significantly reduced the number of total microorganisms on biofilm, similar to previous reports of *in vivo* studies (Dills *et al.*, 1988; Chan *et al.*, 1991). The complete elimination of *F. nucleatum* and *V. dispar* in experimental group already after the first exposure to denture cleanser could be attributed to the sensitivity of those microorganisms to the active oxygen present in denture cleanser solution since they are the two obligate anaerobic microorganisms in this biofilm model. Dills *et al.* (1988) and Chan *et al.* (1991) had reported this strong effect of denture cleanser against Veillonella and Fusobacteria as well as other anaerobes in denture biofilm. This result may confirm the ability of denture cleansers to eliminate odor-causing bacteria from denture surface pointed by the manufacturer, although this elimination do not seem to have a significant impact on halitosis as observed by de Oliveira *et al.* (2011) in a clinical study

Another important remark was that, in the control group, *C. albicans* was undetected in biofilm at the seventh experimental day. The increasing bacterial population and the disadvantages in the experimental conditions for *C. albicans* (i.e. anaerobic conditions) may have lead to this progressive reduction of yeast cells. On the other hand, in experimental group, *C. albicans* population increased progressively with a growth rate ten times greater than *S. mutans*, suggesting that denture cleanser do not have an effect on *C. albicans* as it does on bacteria. This is an important finding given the clinical relevance of *C. albicans* in denture biofilm as the main pathogen of denture-related stomatitis. Also, in a multispecies biofilm, bacteria may somehow influence the *C. albicans* susceptibility to denture cleanser since the efficacy of cleansing solution observed against pure fungal mature biofilms (Nikawa *et al.*, 1995, de Freitas Fernandes *et al.*, 2011) has not been confirmed when *C. albicans* is present in *in vitro* (Drake *et al.*, 1992) or *in vivo* (Dills *et al.*, 1988; de Andrade *et al.*, 2011) mixed yeast-bacteria mature biofilms. A different result was reported by Li *et al.* (2010) that found a reduction on *C.*

albicans counts in mixed-biofilms exposed to denture cleansers probably associated to the use of a non-mature biofilm that is more sensitive to antimicrobial agents.

It has been suggested that, in a multispecies biofilm, bacteria such as streptococci may be placed in more superficial layers than *C. albicans*, protecting yeast cells from denture cleansers (Drake *et al*., 1992; de Andrade *et al*., 2011). This type of arrangement was found in our SEM images especially at day 7, in which it was observed the presence of clusters of *C. albicans* cells under a dense layer of bacteria. Drake *et al*. (1992) also suggest that *S. mutans*, with its known capacity of secreting polysaccharides, may produce a protective barrier of glucan that limits the exposure of yeast cells to the antimicrobial components of denture cleanser. This hypothesis is also supported by our findings since it was observed a significant increase in polysaccharides concentrations in the biofilms exposed to seven days of cleansing procedures. Although the elevated production of glucans by *S. mutans* in the presence of sucrose is well established, denture cleanser seem to have somehow stimulated matrix production since the experimental group present a much higher polysaccharides concentration. This may have resulted in a more roughed and porous biofilm as observed in SEM images.

Another interesting finding in SEM images was the presence of *C. albicans* in hyphal forms in biofilms exposed to denture cleanser, as confirmed by CSLM images. This yeast-to-hyphal differentiation in *C. albicans* may be triggered by various environmental factors and, although in our experimental conditions some factors could have induced hyphae formation, e.g. anaerobiosis (Thein *et al*., 2007), the role of denture cleanser in this process cannot be discarded. For instance, denture cleanser solutions present hydrogen peroxide, a molecule that has shown to induce hyphal differentiation (Nasution *et al*., 2008).

This multispecies biofilm model was chosen for being well established and composed of common oral microorganism with different metabolisms and roles in biofilm development. This provided important information on how denture cleanser acts in a mixed biofilm. However, as denture biofilm is a much more

complex community with an extremely variable composition, clinical extrapolation of our results is limited and additional studies need to be conducted to confirm the present findings.

CONCLUSION

The daily exposure of multispecies biofilms to denture cleanser reduces the number of total microorganisms and promoted ecological changes, favoring *C. albicans*.

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CAPÍTULO 2

Efficacy of denture cleansers on controlling biofilm of removable partial dentures: a short-term clinical evaluation.

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ABSTRACT

This study investigated the effect of denture cleanser on biofilm from removable partial dentures (RPD). Twenty-five volunteers that used RPD were selected and instructed to complement their RPD hygiene by immersing their prosthesis on a peroxide-based denture cleanser (Polident® 3 minute) once a day, for 15 days. The biofilm was collected immediately before and after the experimental period and analyzed for total microorganisms, total streptococci and C*andida* spp. counts. The results showed a significant reduction on total microorganisms and total streptococci after denture cleanser use but no difference was observed for *Candida* spp. counts.

KeyWords: Removable Partial Denture, Oral Hygiene, Biofilm, Denture Cleanser, *Candida*.

INTRODUCTION

Brushing is a simple and widely used method for removable partial denture (RPD) hygiene [1]. However, the visual and manual limitations presented by some patients [2], associated with the difficulties imposed by RPD design, can impair complete biofilm removal by mechanical methods. Therefore, the use of chemical solutions is usually recommended as complementary method for adequate prosthesis hygiene [1].

Although many studies have investigated the use of denture cleansers for complete denture, little is known about its efficacy in controlling biofilm in RPD. Thus, the aim of this clinical was to evaluate if RPD daily immersion in denture cleansers solutions can reduce microorganisms' counts on biofilm.

MATERIALS AND METHODS

The study population was consisted of patients that were rehabilitated with RPD in the Clinic of Piracicaba Dental School . Subjects were eligible to

participate if they presented good health, did not use any antimicrobial in the last 3 months, had ability to comply with the experimental protocol, were continuously using the RPD and used brushing as the sole method for prosthesis hygiene. The selected volunteers signed the informed consent and denture biofilm collection was performed (baseline). The volunteers received the denture cleansers tablets and were instructed to brush their RPD as usual complementing hygiene by immersing their prosthesis on dentures cleanser (Polident® 3 Minute; GlaxoSmithKline, Philadelphia, PA, USA) once a day for 3 minutes, after the nocturnal brushing. The cleansing solution was prepared by dissolving one tablet of denture cleanser in 200 mL of warm tap water. The volunteers had been previously instructed on how to brush their RPD when they received it and no additional hygiene instructions were provide to avoid any bias. After 15 days, the volunteers returned to the post-treatment biofilm collection.

For both baseline and post-treatment biofilm collections, RPDs were removed, gently washed with sterile distilled water to remove saliva and then a sterile swab was applied on the whole dentures' inner and outer surfaces always in the same way – first clasps followed by teeth and acrylic resin base. The swab was immediately placed in a polypropylene tube containing 3 mL of sterile saline (NaCl 0.9%) solution and then sonicated at 7 W for 30 sec. The resultant suspension containing biofilm was 10-fold diluted and inoculated in triplicate by the drop-counting technique in the following culture media: blood agar, mitis salivarius agar (MSA) and CHROMagar™ for total microorganisms, total streptococci and *Candida* spp. respectively. The plates were incubated for 48 h at 37°C in aerobiosis (CHROMagar™) and 10% carbon dioxide (blood agar and MSA) and the number of colony-forming units (CFU) was counted in the dilutions containing between 6 and 60 CFU. The results from both collections were compared by paired t-test.

RESULTS

The final sample was consisted of 25 volunteers (12 male, mean age: 57±8.6 years) (Figure 1). The mean period of RPD usage was 8,4 months. It was

observed a significant reduction of total microorganisms' counts in RPD biofilms after denture cleanser use (p=0.007; Table 1). This reduction has been previously observed in complete dentures biofilms [3] as well as in RDPs after a single immersion on cleansers solution [4]. Total streptococci also had a significant reduction (p=0.0428; Table 1) in accordance with total microorganism results.

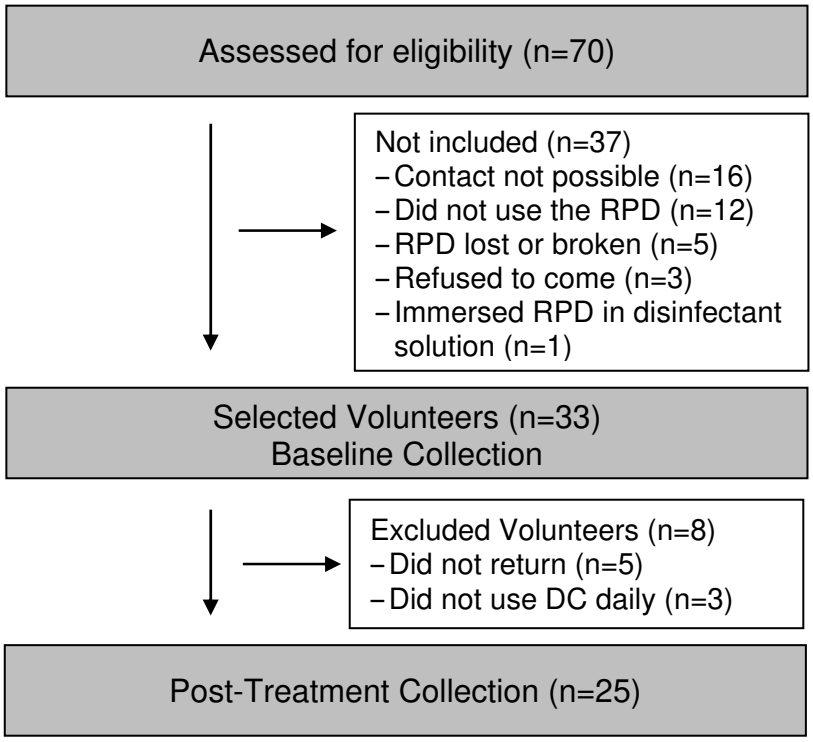

Figure 1. Flow charge of volunteers' selection (DC: denture cleanser)

In the present sample, 11 volunteers had *Candida* at baseline collection and no difference was observed on *Candida* spp. counts after denture cleanser use (p>0.005; Table 1). Actually, some volunteers presented an increase in the number of viable *Candida* cells on biofilm after the experimental period. This lack of action of denture cleanser on *Candida* spp. grown in multispecies biofilms is in agreement

with previously *in vitro* [5] and *in vivo* studies [3, 4]. As suggested by these authors, *Candida* spp. may be protected from denture cleanser action by layers of extracellular matrix and bacterial cells such as *Streptococus mutans*. However, whether these findings for *Candida* spp. have a clinical significance deserves further investigation.

Table 1. Microorganisms count on RDP biofilm at baseline and post-treatment (mean ± sd; n=25)

	Total Microorganisms ($x10^8$)	Total *Streptococci* ($x10^7$)	*Candida* spp. ($x10^4$)
Baseline	2.2 ± 2.4 a	7.3 ± 8.9 a	6.6 ± 12.8 a
Post-treatment	0.8 ± 1.1 b	4.7 ± 6.8 b	9.3 ± 18.2 a

Values followed by different lowercase letters indicate significant difference between baseline and post-treatment data.

CONCLUSION

The use of denture cleanser reduced total microorganisms and total streptococci on RPD biofilm but had no effect on *Candida* spp. counts.

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CONCLUSÃO

Os resultados do presente trabalho demonstraram que a imersão diária em solução de limpador químico para próteses reduz o número de micro-organismos totais em biofilmes multiespécies entretanto não apresenta efeito significativo sobre a *Candida*.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Commitee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos conforme o Medline.

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APÊNDICE 1 – Ilustrações de Materiais e Métodos

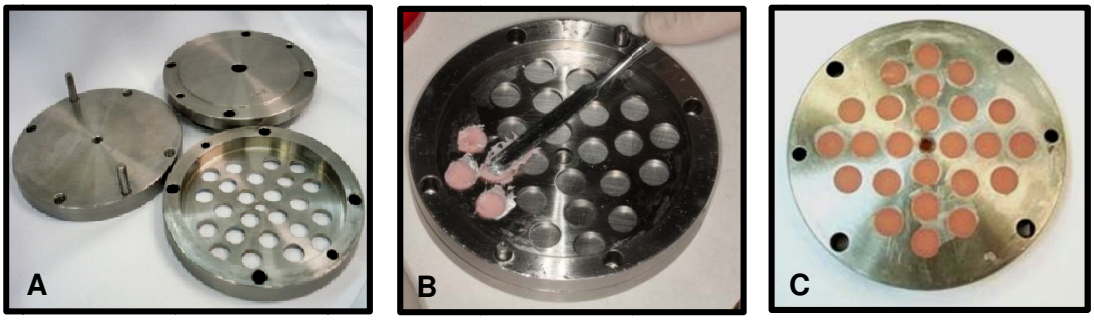

Figura 1. Confecção dos espécimes de resina acrílica: A - Matriz metálica, B - inserção da resina na matriz e C - espécimes após a polimerização.

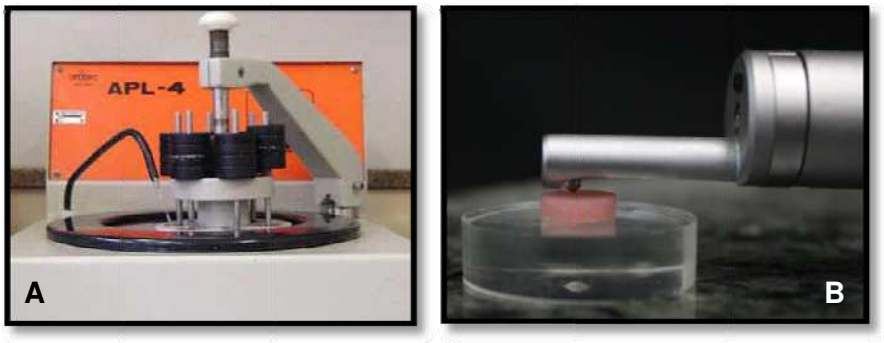

Figura 2. A - Polimento dos espécimes em politriz horizontal (APL-4; Arotec, Sao Paulo, Brazil). B – Leitura da rugosidade de superfície dos espécimes (Rugosímetro Surfcorder SE 1700; Kosaka Laboratory Ltd., Kosaka, Japan).

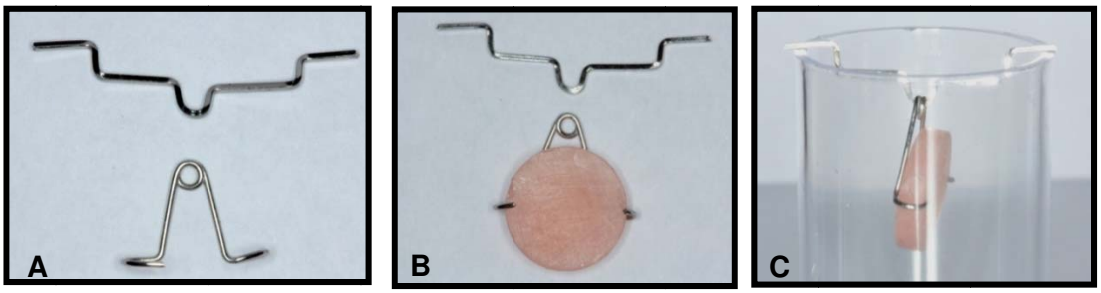

Figura 3. Montagem dos espécimes no suporte metálico: A - Conjunto do suporte metálico, B - fixação do espécime no suporte e C - posicionamento vertical do espécime.

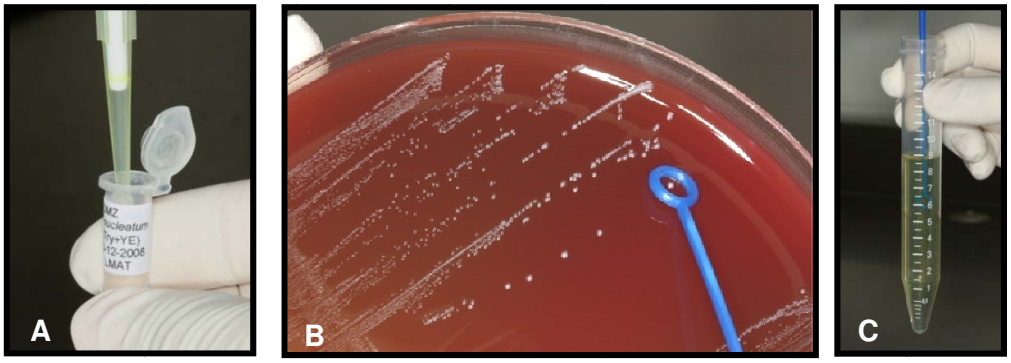

Figura 4. Reativação dos microorganismos para preparo do biofilme. A - Coleta de uma alíquota da cultura de estoque. B - Coleta de colônias em meio sólido (Columbia Blood Agar suplementado com Sangue de carneiro) e C - Transferência das colônias para meio de cultura líquido (FUM 0,3% de glicose).

Figura 5. Posicionamento dos espécimes em placa de 24 poços para formação do biofilme.

Figura 6. Imersão dos espécimes na solução do limpador químico.

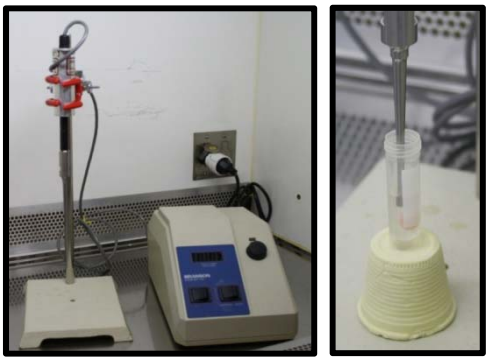

Figura 7. Desagregação do biofilme em sonicador (Sonifier®, Branson Ultrasonics Corporation, Danbury, CT, USA)

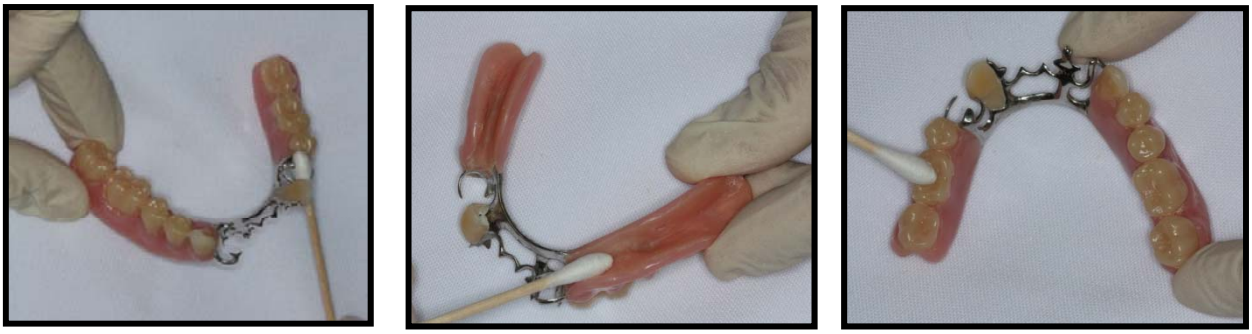

Figura 8. Coleta do biofilme da superfície da prótese parcial removível.

APÊNDICE 2 – Composição do limpador químico testado

Composição do Limpador Químico Polident 3 Minutes (GlaxoSmithKline, Philadelphia, Pa, US)	
▪ Bicarbonato de Sódio ▪ Ácido Cítrico ▪ Carbonato de Sódio ▪ Monopersulfato de Potássio ▪ Perborato de Sódio ▪ Benzoato de Sódio ▪ PEG-180 ▪ TAED ▪ Sodio lauril sulfoacetato	▪ VP/VA ▪ Copolímeros ▪ Aroma ▪ *Subtilisin* - protease ▪ *Blue 1 aluminum lake* ▪ *Blue 2* ▪ *Yellow 5 aluminum lake* ▪ *Yellow 5*

ANEXO 1 - Certificado do Comitê de Ética em Pesquisa – *In vitro*

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Eficácia de limpador químico no controle de biofilmes multiespécie formados sobre a superfície de poli(metil)metacrilato"**, protocolo nº 052/2012, dos pesquisadores Sílvia Carneiro de Lucena e Altair Antoninha Del Bel Cury, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 04/07/2012.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Efficacy of denture cleansers on multispecies biofilm developed on polymethylmethacrylate resin"**, register number 052/2012, of Sílvia Carneiro de Lucena and Altair Antoninha Del Bel Cury, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 07/04/2012.

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2 - Certificado do Comitê de Ética em Pesquisa – *In vivo*

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Eficácia de limpadores químicos no controle do biofilme formado sobre a superfície de próteses removíveis"**, protocolo nº 097/2011, dos pesquisadores Altair Antoninha Del Bel Cury e Sílvia Carneiro de Lucena, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 29/09/2011.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Efficacy of denture cleansers in the control of biofilm formed on removable dentures"**, register number 097/2011, of Altair Antoninha Del Bel Cury and Sílvia Carneiro de Lucena, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 09/29/2011.

Profa. Dra. Lívia Maria Andaló Tenuta
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

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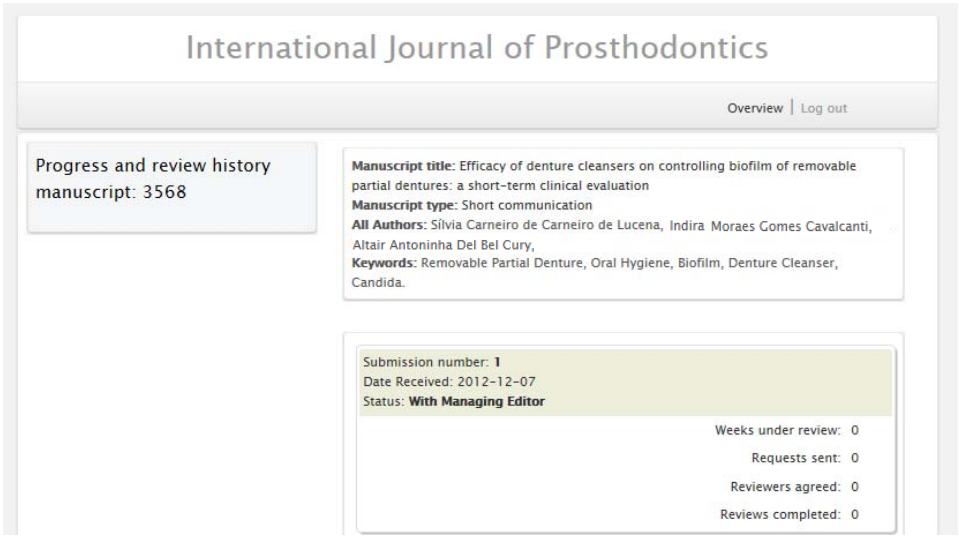
ANEXO 3 – Comprovante de Submissão do artigo



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