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Eficácia de limpadores químicos na remoção e re-colonização de biofilmes de *Candida* spp. formados na superfície de material reembasador

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Orientadora: Profa. Dra. Altair A. Del Bel Cury

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“Disse Deus: Haja luz; e houve luz”

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Resumo

Os reembasadores para base de prótese, após a exposição à cavidade bucal, apresentam alterações de superfície facilitando a adesão e a colonização por micro-organismos. Para a limpeza de superfície desses materiais são indicados os limpadores químicos para evitar os danos mecânicos que podem ser provocados pelas cerdas das escovas dentais. Assim, o objetivo nesta pesquisa foi avaliar a eficácia de limpadores químicos na remoção de biofilme de *Candida* spp. desenvolvido sobre a superfície de um reembasador classificado como permanente à base de poli-metilmetacrilato e na prevenção da re-colonização dessa superfície, especialmente a *Candida spp.*, comumente associada ao desenvolvimento da candidíase. Espécimes (10 mm diâmetro X 3 mm altura) de resina acrílica reembasada com um reembasador mais representativo disponível comercialmente teve sua rugosidade de superfície mensurada antes (baseline) e biofilme de *C. albicans* ATCC 90028 ou *C. glabrata* ATCC 2001 foi desenvolvido sobre os mesmos. Após a formação dos biofilmes os espécimes foram aleatorizados e submetidos aos tratamentos (n=16): AD – Água destilada (Controle), 15 min; POL – Polident 3 minutos, 3 min; EFF – Efferdent, 15 min; HPS - Hipoclorito de sódio a 0,5%, 10 minutos. Metade destes espécimes (n=8) foi utilizada para determinação da eficácia dos limpadores, utilizando contagem de células viáveis, enquanto os espécimes remanescentes (n=8), após os tratamentos, foram novamente colocados em meio de cultura estéril e incubados por mais 48 h a fim de determinar o efeito dos limpadores na prevenção da recolonização. Após os tratamentos os espécimes tiveram a rugosidade de superfície determinada, considerada pós-tratamento. Alguns espécimes de cada uma das espécies de *Candida* tiveram a superfície analisada após os tratamentos, por microscopia eletrônica de varredura (MEV). Os dados foram submetidos à análise de variância e teste de Tukey HSD em nível de significância de 5%. A rugosidade de superfície foi significantemente maior após os tratamentos ($P<0,05$). Quantos aos tratamentos, o HPS mostrou-se efetivo tanto para a

desinfecção quanto na recolonização de ambas as espécies de *Candida,* pois houve ausência total de crescimento. Na avaliação da desinfecção, imediatamente após os tratamentos, quando *C. albicans* foi considerada, não houve diferença significativa entre os peróxidos alcalinos ($p>0,05$) e ambos diminuíram o número de células fúngicas ($p<0,05$) comparado ao tratamento com AD. Entretanto, para *C. glabrata,* os tratamentos com ADD e peróxidos alcalinos não se diferenciaram entre si ($p>0,05$). Na análise dos resultados para a recolonização foi observada que houve inversão no comportamento, pois enquanto, para *C. albicans,* os tratamentos com AD e peróxidos alcalinos não diferiram entre si ($p>0,05$), para *C. glabrata* os tratamentos com peróxidos alcalinos apresentaram valores similares e menores ($p>0,05$),quando comparados com o tratamento com AD ($p<0,05$). Na comparação entre as espécies de *Candida* observou-se que *C. glabrata* apresentou os maiores níveis de células viáveis quando os dados foram avaliados na situação de imediatamente após os tratamentos com os peróxidos alcalinos e foi diferente de *C albicans* ($p<0,05$). Entretanto não houve diferença para a recolonização ($p>0,05$). Os resultados sugerem que os limpadores a base de peróxidos alcalinos não foram efetivos na remoção total dos micro-organismos e também não impediram a recolonização por *Candida* spp.

Palavras chave: *Candida albicans, Candida glabrata,* reembasadores, limpadores, biofilme.

ABSTRACT

The denture liners exhibits surface changes in oral environment by constant loss of its constituent elements, which facilitate microorganisms adherence that leads to biofilm formation. Denture liners surface can be cleaned by brushing or using denture cleaners, which are recommended, in order to avoid mechanic injuries to denture liners by brushing it. Therefore, the aim of this study was to evaluate the long term efficacy of denture cleansers on *Candida spp*. biofilm recolonization on liner surface. Specimens of poly (methylmethacrilate) were lined according to manufacturer instructions (10 mm diameter X 3.0 mm thickness). Surface roughness was measured at baseline and after the treatments. Next, biofilms of *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 were allowed to develop on liner surface for 48 h. Subsequently, the specimens were randomly assigned for the cleaning treatments (n=16): distilled water (DW - control), 15 min; Polident 3 minutes (POL) - 3min; Efferdent (EFF)-15 min; sodium hypochlorite (HYP) - 10 min. After the treatments, specimens (n=8) were sonicated for biofilm disruption and the viable cells were counted (cell/mL). To determine the long term effectiveness of the cleaning process, a set of cleaned specimens (n=8) were submitted to new biofilm growth conditions. After 48 h, biofilm were disrupted by sonication and cell number estimated. Scanning electron microscopy was performed to analyze the specimen topography after denture cleanser treatment. Data were analyzed by ANOVA and Tukey's HSD test was used as post-ANOVA employing a significance level fixed at 5%. The liner surface was rougher after the treatments ($P<0.05$). Results showed significant differences in cleanliness among the treatments ($p<0.05$), however for *Candida* species ($p<0.05$) no significant difference was observed in the recolonization condition ($p>0.05$). Alkaline denture cleansers showed similar cleaning performance and both showed lower cells counts compared with the control ($p<0.05$). Hypochlorite was the only effective treatment as no viable cells were detected even after the recolonization test. Within the limits of this study, it can be concluded that alkaline denture cleansers were not

effective on biofilm removal, once denture liner surface by *Candida* spp biofilm recolonization was not prevented.

Keywords: *Candida albicans*, *Candida glabrata*, denture liners, denture cleansers, biofilm.

SUMÁRIO

INTRODUÇÃO GERAL

Com o aumento da expectativa de vida da população, entre a qual encontram-se usuários de prótese dental removível, a preocupação de manter essas próteses em boas condições no que tange a função e estética é mandatória. Ambas as situações dependem de boa adaptação da prótese dental e remoção do biofilme formado sobre a mesma (1-3).

Em acréscimo, a precisão da adaptação é um fator importante na retenção e estabilidade das próteses removíveis. Entretanto, a reabsorção do osso alveolar é irreversível e pode levar à desadaptação, causando injúrias aos tecidos que revestem o osso alveolar (4) e predispondo essa mucosa inflamada à colonização por microrganismos comensais da cavidade bucal que pode levar ao desenvolvimento de candidíase atrófica cronica (1-3). Nas situações de desadaptações, especialmente quando a fibromucosa de revestimento do remanescente do osso alveolar encontra-se alterada, o uso de reembasadores é o material de escolha, pois possibilita a adaptação precisa da prótese ao rebordo alveolar, permitindo assim melhor distribuição das cargas funcionais e melhor retenção ao rebordo alveolar ao mesmo tempo que auxilia na melhora da mucosa inflamada (4,5).

Os materiais reembasadores resilientes são eficientes em propiciar conforto aos usuários de próteses removíveis, contudo apresentam características de sinérese e embebição que a longo prazo, levam a alteração dimensional e de superfície, aumentando a rugosidade e facilitando o acúmulo de resíduos alimentares e de biofilme microbiano (6-11), especialmente a colonização por *Candida spp*. Autores evidenciam a alta ocorrência de biofilme de C*andida* spp. nos materiais de base de próteses, rígidos ou resilientes, e suas conseqüências para os usuários (12-14), como a candidíase atrófica crônica, a forma mais prevalente da candidose oral, presente em alta porcentagem em usuários de

próteses totais (15, 16). A *C. albicans* está comumente associada a CEC, entretanto outras espécies de *Candida* que não a *C*. *albicans* tem sido isoladas das superfícies das próteses e da mucosa do palato, em particular a *C. glabrata*, um fungo patógeno em evidência em pesquisas recentes devido sua alta virulência. (17, 18)

O desenvolvimento do biofilme sobre a superfície de próteses dentárias removíveis transcorre em diferentes fases. Em um primeiro momento ocorre a interação com a superfície, onde a *Candida spp*. utiliza-se de filamentos extracelulares para sua fixação. Nesse momento inicia-se a fase de colonização, onde ocorre a formação de micro-colônias (16) envoltas por matriz de polissacarídeos extracelular com posterior formação do biofilme. A adesão de células fúngicas sobre a superfície e a formação de microcolonias envolta por matriz extracelular são fases críticas para o desenvolvimento de biofilmes patogênicos. Essa matriz confere ao biofilme sustentação e proteção (19-21), sendo que com o passar do tempo há um aumento dos polissacarídeos extracelulares em torno e entre as microcolonias que aumenta a coesão e a integridade estrutural do biofilme formado, sendo esta a diferença entre biofilmes jovens até 24 horas e biofilmes mais maduros. (20) Essa modificação da distribuição espacial dos polissacarídeos associado às mudanças no padrão de formação, distribuição e morfologia das microcolonias afeta as propriedades de difusão da matriz extracelular (21,22), dificultando a difusão de antifúngicos e nutrientes ao longo do biofilme (23, 24).

Desta maneira, a manutenção da limpeza das próteses não somente contempla a estética como também é de importância fundamental na prevenção da CEC. O método mais comum e rotineiro para limpeza das próteses é a escovação com água e dentifrício (25), contudo para os materiais reembasadores resilientes é contra indicado uma vez que facilita sua remoção da superfície reembasada, além de aumentar a rugosidade de superfície. Outra condição em que a escovação pode não ser eficiente relaciona a pacientes com deficiente

coordenação motora e idosos com limitações de habilidade manual, diminuição da acuidade visual e, portanto não conseguem manter satisfatória higiene da prótese dental, indispensável não só para a qualidade estética, como também para a prevenção dos casos de estomatite protética (26, 27).

Com a finalidade de facilitar a limpeza do biofilme dental, que se forma sobre a superfície de próteses removíveis, estão disponíveis aos usuários destas os limpadores químicos. Estes são reconhecidos por sua eficiência na redução de micro-organismos (28,29), e podem ser classificados em limpadores ou desinfetantes, conforme a função de sua constituição química (30, 31). Os limpadores normalmente contêm enzimas que atuam na degradação e remoção de detritos alimentares e biofilme e como exemplo de desinfetante de superfície, tem-se o hipoclorito de sódio, disponível comercialmente em diferentes concentrações (32, 33). Embora os agentes limpadores tenham sido avaliados quanto à capacidade de remoção de detritos alimentares e redução de biofilme (34, 35), o conhecimento de sua ação sobre a recolonização de biofilme de *Candida* spp sobre a superfície dos materiais reembasadores resilientes foi pouco explorada.

Diante do exposto, o objetivo nesta pesquisa foi avaliar a eficiência de limpadores químicos na remoção de biofilme de *Candida* spp. formado sobre a superfície de reembasador resiliente à base de poli-metilmetacrilato e na prevenção da recolonização dessa superfície.

CAPÍTULO 1

Long term effectiveness of denture cleansers in preventing *Candida spp*. biofilm recolonization on the surface of denture liner

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A**bstract**

Denture cleansers are indicated to remove stains and biofilm that are formed on dentures surface. This study evaluated the long term effectiveness of denture cleansers on *Candida spp*. biofilm recolonization on a liner surface. Specimens were made of poly(methylmethacrilate) - based denture liner in accordance with manufacturer directions and surface roughness was evaluated at baseline and after cleansing treatments. *Candida albicans* or *Candida glabrata* biofilms were formed on liner surface for 48 h. Subsequently, the samples were randomly assigned to one of the cleaning treatments: alkaline peroxides (soaking for 3 or 15 min), 0.5% sodium hypochlorite (10 min) or distilled water as control (15 min). After the treatments, the specimens were sonicated to disrupt the biofilm and viable cells were counted (cell/mL). Long term effectiveness of cleaning processes was determined by submitting a set of cleaned specimens to biofilm growth conditions again. After 48 h, biofilm was disrupted by sonication and cell counts were estimated. Scanning electron microscopy was performed to analyze the specimens topography after cleaning treatments. Data were analyzed by ANOVA and Tukey's HSD test ($\alpha=0.05$). Results showed significant differences in cleanliness among the treatments ($p<0.05$), however for *Candida* species ($p<0.05$) no significant difference was observed in the recolonization condition ($p>0.05$). Alkaline denture cleansers showed similar cleaning performance and both differed from the control ($p<0.05$). Sodium hypochlorite was the only treatment that efficiently removed biofilm, once no viable cells were found after its use. It can be concluded that alkaline peroxide denture cleansers were not effective in removing biofilm from denture liner surfaces, since *Candida* spp. *b*iofilm recolonization was not prevented.

Descriptors: Complete Denture; Biofilms; Candidiasis.

Introduction

Denture liners are important in clinical practice considering their use in patients with compromised alveolar bone support. These materials provide relief for sharp bony undercuts or extreme sensitivity due to submucosal exposure of the inferior alveolar nerve.[1] They are also indicated for post-operative periods, particularly after implant surgery, since they are able to help by absorbing the load on implants.[2] Although denture liners are commonly used, their physical characteristics make them susceptible to sorption, resulting in dimensional changes that favor biofilm formation on their surfaces, leading to easy colonization and infection by *Candida* spp..[3]

Many authors point out the occurrence of *Candida* spp. biofilm on denture base material and its consequence for removable denture wearers,[4,5] such as chronic erythematous candidosis (CEC), the most prevalent form of oral candidosis, affecting more than 65% of denture wearers.[6,7] *C. albicans* is commonly related to CEC, however other non-*albicans* species have been isolated from removable denture surfaces and the palatal mucosa, in particular *C. glabrata*, an emerging fungal pathogen.[8]

Denture cleansers are oral hygiene products developed for cleaning removable dentures, and they are increasingly used by the large consumer base in this specialized healthcare market,[9] mainly due to the increasing 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.[10,11]

Classified into different groups according to their mechanism of action or main components,[12,13] the effervescent tablets are classified as chemical soak type products, and 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.[10,14,15] Although microorganism elimination by denture cleansers has been

evaluated,[10,11,16,17] it is suggested that denture cleansers are not effective in preventing their initial adherence to the denture liners.[18] Another aspect of denture cleaners that is not fully understood is related to recolonization of the host surface by biofilm after using these products. Considering that denture cleansers are applied in an attempt to remove biofilm, it is important that these products are capable of preventing, or at least, delaying surface recolonization.

Therefore, the aim of this study was to evaluate the efficacy of denture cleansers on *Candida* spp. biofilm developed on liner surface as well as to verify their long term effect on biofilm surface recolonization.

Materials and Methods

Experimental Design

The present study was approved by the Local Ethics Committee, and it was a randomized and blinded design. Specimens were fabricated using a water bath poly(methylmethacrylate) acrylic resin discs in accordance with manufacturer's directions relined with a layer of a permanent denture liner. Specimens were decontaminated and randomly divided into two groups according to *Candida* strains (*C. albicans* or *C. glabrata*) and exposed to human whole saliva for acquired pellicle formation. After, specimens were exposed to one of the two strain inoculums (10^7 cells/mL), for biofilm formation for 48 h and submitted to one of the treatments: DW (distilled water) used as control; POL (alkaline peroxide containing enzyme; Polident 3-minutes, GlaxoSmithKline; Philadelphia, PA, USA); EFF (alkaline peroxide; Efferdent, Warner Lambert Co., Morris Plains, NJ, USA); HYP (0.5% sodium hypochloride, Proderma Pharmacy, Piracicaba, Brazil). Afterwards, the specimens were sonicated to disrupt the biofilm and the viable cells were quantified (cell/mL). To determine the long term efficacy of the denture cleansers, another set of specimens covered with biofilm and cleaned by the same treatments were allowed to develop in new fresh culture medium, to obtain a 48h biofilm. Then, the specimens were sonicated to disrupt the biofilm and the cells were quantified (cell/mL). Scanning electronic microscopy (SEM) was used to evaluate the liner surface after cleansing treatment (n=3). Statistical analysis was performed

considering two factors: denture cleanser and *Candida* species and cell counts as the response variable.

PMMA specimens

The water bath poly(methylmethacrylate) PMMA resin used in this study (Lucitone 550, Denstply, Petropolis, RJ), was polymerized at 74 °C for 9 h, according to the manufacturer's recommendations. PMMA was packed in metal flasks and polymerized in a polymerizing unit (Termotron P-100; Termotron Equipamentos Ltd, Piracicaba, Brazil). All flasks were allowed to bench cool and then the samples were removed. The PMMA discs (10.0 x 1.5 mm) were finished and stored in deionized water for 48 h at room temperature (23 ± 1.0°C) for residual monomer release.[19] Next, these discs were relined with PMMA resin liner ,(Kooliner, GC America; Alsip, IL, USA). The uniform 1.5mm liner layer was applied by inserting each disc into a glass mould, pouring in the denture liner, placing glass slides over both ends of the mold are firmly fixing them. The glass slides were separated from the ends of the mold after the material was polymerized, and then the specimens were removed from the moulds. [20,21] These specimens were finished according to manufacturer's recommendation and used immediately in the assay.

Surface roughness

Surface roughness of the relined specimens was measured at baseline and after the treatments, using a profilometer (Surfcorder SE 1700 Kozaka Industry, Kozaka, Japan) with a 0.01 μm resolution, calibrated at sample length of 0.8 mm, 3.2 mm percussion of measure, and 0.5 mm/s. Three readings were taken for each sample and a mean value was calculated.[22] After, the specimens were disinfected by ultrasonic bath for 20 minutes[22] and stored in pre-sterilized, polystyrene, flat-bottomed 24-well microtiter plates for saliva pellicle formation.

Saliva pellicle formation

A healthy volunteer donated saliva by chewing parafilm, which was collected and stored in a sterile plastic tube at 4 °C until processing. The saliva was clarified by centrifugation (10.000 *g*, 10 min, 4°C; MR23i; Jouan Inc.; Winchester, VA) and

sterilized by 0.22 μm membrane filtration (TPP, Trasadingen, Switzerland). Then, 1 mL of saliva was placed in each well of the sterilized flat-bottomed 24-well cell tissue culture plates (TPP, Trasadingen, Switzerland) containing the relined specimens. The plates remained in an orbital shaker (Kline; Novatecnica; Piracicaba, SP, Brazil) for 30 min at 37°C for saliva pellicle formation. After, the specimens were washed with PBS (PH=7.2) twice and placed into another sterile 24-well plate for biofilm formation assay.

Biofilm assay

C. albicans (ATCC 90028) and *C. glabrata* (ATCC 2001) selected for this study had their identity reconfirmed by carbohydrate assimilation tests, using a commercially available API 32C identification system (bioMérieux, Marcy l'Etoile, France). Prior to each experiment, the yeast strains were aerobically cultured at 37°C for 24 h on Sabouraud Dextrose Agar (SDA) and a loopful of growth was inoculated into Yeast Nitrogen Base (YNB) broth (Difco Laboratories, Detroit, MI) supplemented with 50 mM glucose. After 18 to 20 h of incubation, cells were washed with PBS twice and suspended in YNB supplemented with 100 mM glucose. Standard *Candida* spp. suspensions were prepared to a concentration of 10^7 cells/mL.[23]

Biofilm was developed on the liner surfaces placed inside sterilized flat-bottomed 24-well microtiter plates. Aliquots of 2.0 mL of standard cell suspensions of yeasts were transferred into each well containing one disc and incubated for 90 min at 37°C in an orbital shaker (model NT 151; Kline Shaker; Nova Tecnica Laboratory, Sao Paulo, Brazil) at 75 rpm (adhesion phase). After, the cell suspension was gently aspirated and each specimen was washed with PBS twice and 2.0 mL of YNB supplemented with 100 mM glucose was added to each well and the plates were incubated for 48 h at 37°C as described before.

Cleansing Treatment

The specimens covered with biofilm were assigned to one of four cleaning treatments. Each specimen was individually placed in a sterilized beaker

containing 8 mL of one of the treatment solutions. The POL and EFF were prepared in distilled water following the manufacturer's directions. The immersion periods were established as follows: 3 min for POL and 15 min for EFF in accordance with the manufacturer's directions; for HYP the immersion time was 10 min. For DW, the specimens remained in it for15 min as a reference for the longest treatment time used.

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. After this, *Candida* spp. cells were submitted to decimal serial dilution and plated onto SDA. Plates were incubated at 37°C in aerobiose for 48 h, and cell counts were performed and expressed in cell/mL.

Long term efficacy of the treatments (biofilm recolonization)

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 number estimation was performed.

Scanning Electron Microscopy

Surface topography was evaluated using scanning electron microscopy (MEV). Specimens after treatments were rinsed with sterile PBS and placed in 1% osmium tetraoxide for 1 h, subsequently specimens were washed in distilled water, dehydrated in an increasing series of ethanol washes (70% for 10 min, 95% for 10 min and 100% for 20 min) and air dried in a desiccators. After this, the specimens were mounted on aluminum stubs, fixed with copper tape, sputter coated with gold in a low-pressure atmosphere with an ion sputter coater (JEOL JFC1 100: JEOL,

Tokyo, Japan). 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, NC, USA) with the level of significance fixed at 5%. The normality of error distribution and degree of non-constant variance were checked for the response variable and data were transformed as suggested by the software. The cell count values were transformed by logarithm (log10 (χ)). All data were analyzed using two-way ANOVA. Tukey HSD test was used as post- ANOVA comparison.

Results

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

Table 1 - Surface roughness (Ra-μm) at baseline and after the treatments. (Mean ± S.D.)

Treatments	Baseline	After treatments
DW (Control)	3.8 ± 0.5 a	4.0 ± 0.3 b
HYP	3.5 ± 0.9 a	4.0 ± 0.9 b
EFF	3.2 ± 0.4 a	3.8 ± 0.6 b
POL	3.2 ± 0.5 a	3.4 ± 0.4 b

Distinct lower case letters show statistically significant differences between baseline and after treatments ($p<0.013$).

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

Table 2 - Cell counts (mean ± s.d.) for *Candida* spp. (CFU/mL) immediately after cleanser treatments (n=8).

Candida	Treatments			
	DW	EFF	POL	HYP
C. albicans	$5.8 \pm 5.4\times10^{6}$ A,a	$0.17 \pm 0.23\times10^{6}$ A,b	$0.07 \pm 0.1\times10^{6}$ A,b	0.0
C. glabrata	$5.0 \pm 2.3\times10^{6}$ A,a	$4.8 \pm 3.2\times10^{6}$ B,a	$5.7 \pm 7.0\times10^{6}$ B,a	0.0

Distinct upper case letters and distinct lower case letters show statistically significant differences between *Candida* spp, and among denture cleansers respectively. Tukey test, $p<0.05$.

Under the recolonization condition, alkaline peroxides and DW treatments showed similar results in relation to *C albicans* and no statistically significant difference was observed ($p>0.05$). However, for *C. glabrata* the treatments with both alkaline peroxide showed higher counts when compared with the control and a statistically significant difference was found ($p<0.05$) (Table 3).

Table 3 - Cell counts (CFU/mL; mean ± s.d.) for the *Candida* spp. recovery(cell/mL) after treatments and surface recolonization (n=8).

Candida	Treatments			
	DW	EFF	POL	HYP
C. albicans	$4.1 \pm 2.1\times10^{7}$ A,a	$4.9 \pm 2.7\times10^{7}$ A,a	$8.0 \pm 3.3\times10^{7}$ A,a	0.0
C. glabrata	$5.2 \pm 4.8\times10^{7}$ A,a	$10.0 \pm 4.9\times10^{7}$ A,b	$9.7 \pm 4.0\times10^{7}$ A,b	0.0

Distinct upper case letters and distinct lower case letters show statistically significant differences between *Candida* spp, and among denture cleansers respectively. Tukey test, $p<0.05$.

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).

Candida glabrata showed higher cell count values in comparison with *C. albicans* when treated with both alkaline denture cleansers *($p<0.05$*). However, for

recolonization, no differences were found between the *Candida* strains treated with the two alkaline peroxides or DW ($p>0.05$) (Table 3).

Images of specimen surface after cleaning treatments are illustrated by SEM microphotography (Fig.1). HYP cleaned specimens showed a surface free of cells (b), while POL (c), EFF (d) and DW (a) showed surfaces with attached cells.

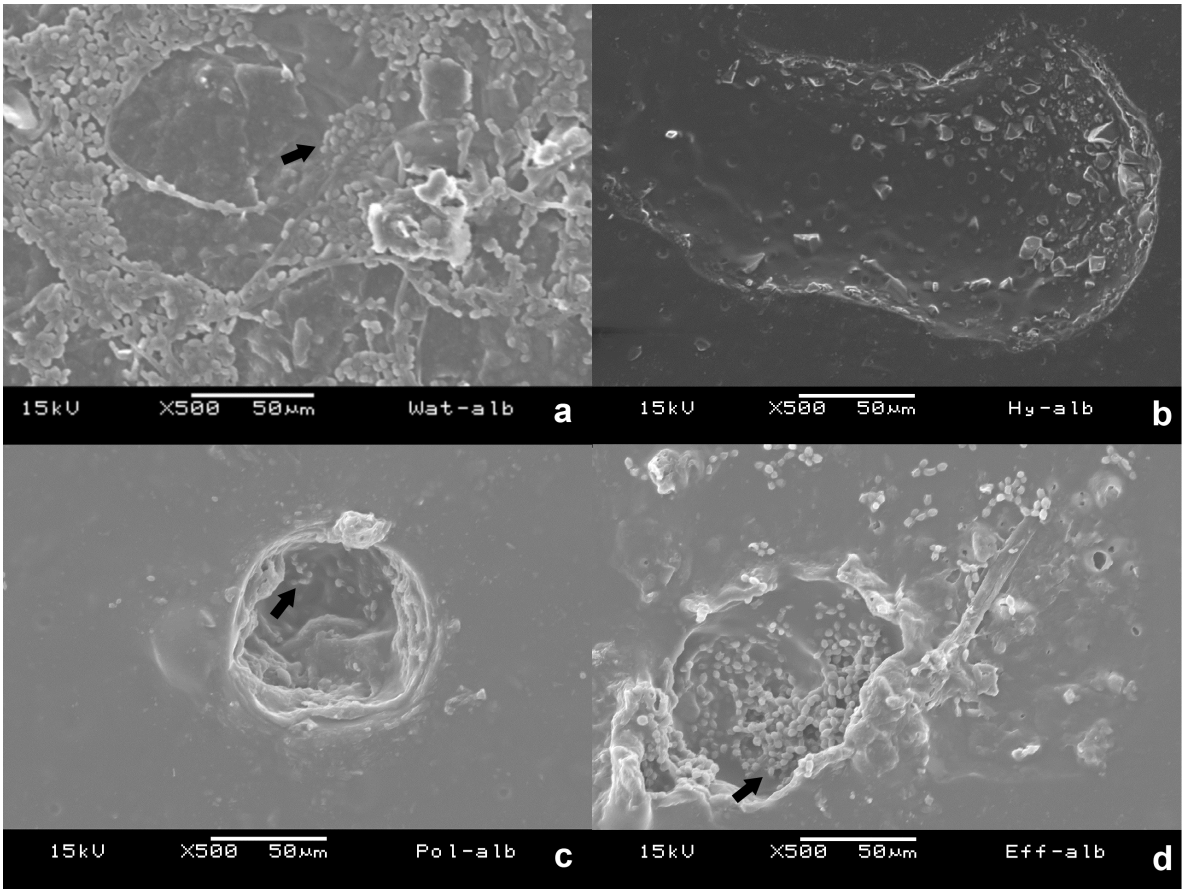

Fig. 1 – Representatives SEM of biofilm developed on denture liner surface after cleanser treatment: **a -** surface after DW treatment; **b -** surface after HYP treatment; **c** - surface after POL treatment; **d** - surface after EFF treatment.

Discussion

In this study it was evaluated for the first time the efficacy of denture cleansers on denture liner biofilms the condition immediately after the treatments and after the treatments followed by a condition of recolonization. The biofim growth model used simulated *in vivo* conditions of static biofilm growth found at the tissue–contacting surface of a denture.[24]

A rougher surface was found after all the treatments was rougher even for the control (DW), which indicated that changes in the denture surface, probably occur in the mouth, considering it is immersed in saliva all the time while is being worn. This 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. Furthermore, irregularities can be claimed as a deposit of fungi, ready for recolonization of the surface[27]. Thus, the increased surface roughness after cleaning is an indicative of changes on the surface occurred, favoring the attachment of cells. SEM images show that residual biofilm cells are clearly seen on such irregularities. POL and EFF probably promote a greater disturb on biofilm structure, considering the effervescent action (fig. 1c and d) when compared with DW treatment, in which biofilm was poorly disturbed (fig. 1a). Results for POL and EFF did not distinguish from each other, even taking in consideration the time lapse between both treatments.

Aqueous denture cleanser solutions are extensively indicated for cleansing and removing biofilm , considering that the effervescent action of the cleanser solutions is capable of penetrating into the irregularities and removing biofilm and debris, and that hydrogen peroxide act as antiseptic and disinfectant.[14,15] The results of the present study showed that denture cleansers POL and EFF presented lower cell counts in comparison with DW treatment. Nevertheless, these cleanser solutions were not able to entirely remove the biofilm, and when *Candida* species were considered, 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*.[18] (2009) who used the same denture cleansers and *Candida species* and Sousa *et al*.[25] (2009), who found inefficiency on reduction of *C. albicans* cells after using a similar peroxide cleanser. Another clinical study,[26] also found that POL and EFF had similar performance, showing that the biofilm growth of the present study model mimicked the "*in vivo"* environment.

After 48 h, liner surfaces treated by EFF and POL showed similar amounts of viable cells both after the treatments and recolonization, disturbed cells from POL and EFF treatment could develop freely, meanwhile DW treated cells still on steady state, what could be a reasonable explanation for the increase in cell counts found in the recolonization condition after POL and EFF treatments (Table 3). Also, it should be considered that adherence of yeasts to surface can also include concentration and viability of yeast cells.[28]

The results also showed that soaking specimens in 0.5% sodium hypochlorite, which is considered fungicidal, was the only effective treatment against *Candida species* under both conditions, since no viable cells were found after its use for both *Candida species,* even *C. glabrata,* which presented higher adherence rates when compared with *C. albicans*, results in accordance with previous study. [29] In addition to its fungicidal effect, sodium hypochlorite acts dissolving mucin and other organic substances such as extracellular polymeric matrix.[15] In spite of its satisfactory results, the dental literature has shown that this product has the potential to bleach denture-base products, in the addition to presenting the risk of surface corrosion,[12] especially of the metal content in partial removable prosthesis, consequently this product is not indicated for daily use. Nevertheless this risk seems to have been exaggerated and further studies are required, and the use of different concentrations and times of immersion should be evaluated.

The objective of immersing a denture in a disinfectant is to remove biofilm and to decontaminate the surface by destroying the microorganism, considering that dentures may function as a reservoir of infection.[30] Thus, the most important

purpose of a denture cleaning protocol is to avoid the recolonization of the oral cavity.[18] In the current study, the fungal levels returned to the initial levels within 48 h without difference between the *Candida* species. Although this study does not fully mimic the oral environment, the results may suggest the need for stipulating a routine protocol for denture cleaning.

Considering the clinical implications of this study, it is possible to state that peroxide cleansers were not effective for removing biofilm. Although HYP was effective to remove biofilm, more studies using longer periods and lower concentrations should be evaluated because of its bleach or corrosive properties.

Conclusion

Within the limitations of this study, it can be concluded that alkaline denture cleansers were not effective for biofilm removal.

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CONCLUSÃO

Dentro das limitações deste estudo, foi possível concluir que limpadores químicos não foram eficazes na remoção de biofilmes de *Candida* spp.formados sobre a superfície de reembasador para prótese.

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* De acordo com a norma da UNICAMP/FOP, baseadas na norma do International Committe of Medical Journal Editors – Grupo Vancouver. Abreviaturas dos periódicos em conformidade com o Medline.

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ANEXOS

Anexo 1: Certificado de aprovação do Comitê de Ética em Pesquisa

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 à base de peróxidos e hipoclorito de sódio na remoção de biofilme de Candida spp. de reembasadores resilientes**", protocolo nº **071/2008**, dos pesquisadores **ALTAIR ANTONINHA DEL BEL CURY, ANA PAULA COELHO VIEIRA e WANDER JOSÉ DA SILVA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 16/07/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Efficiency of chemical cleaners to the peroxides base and sodium hypoclorite in the removal of Candida spp. biofilm developed on the surface of resilient liners**", register number **071/2008**, of **ALTAIR ANTONINHA DEL BEL CURY, ANA PAULA COELHO VIEIRA and WANDER JOSÉ DA SILVA**, 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 16/07/2008.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
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: Materiais utilizados

Tipo de reembasador	*COMPOSIÇÃO*	*Nome Comercial e Fabricante*
Resina acrílica	Pó: Metil Metacrilato, co-polímero de etil-acrilato, di-butil, peróxido de benzoíla *Líquido: metil metacrilato, topanol, etileno glicol di-metil metacrilato*	Lucitone 550, Dentsply, USA
Rembasador *de*finitivo a base de polimetil metacrilato	Pó: cadmio, sílica, quartzo cristalino, peroxide de benzoila, polimetil metacrilato Líquido: 2,4-di-hidroxi benzofenona, isobutil methacrilato	*Kooliner, GC America, Alsip, IL, USA*
Limpador químico com enzimas	Perborato de sódio, Monopersulfato de potássio, Enzima proteolítica, *Detergente, Base efervescente.*	*Polident 3 minutes GlaxoSmithKline, Philadelphia, PA, USA.*
Limpador quimico	Monopersulfato de potássio, Perborato de sódio monohidratado, Latanol, Ácido cítrico, Bicarbonato de sódio, EDTA, Sulfato de potássio, Carbonato de sódio, Estereato de magnésio, Essência, Corante.	*Efferdent* *Warner-Lambert Co.,Morris Plains,Jersey City, U.S.A.*
Hipoclorito de sódio 0,5%	*Hipoclorito de sódio (NaOCl)*	*Proderma Farmácia de Manipulação, Piracicaba, Brasil*

Anexo 3: Resultados estatísticos

```
---------------------- tratamento=limpeza cleaner=efferden strain=albicans ----------------------

         Variable      N            Mean         Std Dev         Minimum         Maximum
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff
         cell          8       237828.75       479238.98         9330.00      1400000.00
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff

---------------------- tratamento=limpeza cleaner=efferden strain=glabrata ----------------------

         Variable      N            Mean         Std Dev         Minimum         Maximum
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff
         cell          8      5738750.00      6958519.72        73300.00     17500000.00
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff

---------------------- tratamento=limpeza cleaner=hipoclor strain=albicans ----------------------

         Variable      N            Mean         Std Dev         Minimum         Maximum
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff
         cell          8               0               0               0               0
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff

---------------------- tratamento=limpeza cleaner=hipoclor strain=glabrata ----------------------

         Variable      N            Mean         Std Dev         Minimum         Maximum
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff
         cell          8               0               0               0               0
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff

---------------------- tratamento=limpeza cleaner=polident strain=albicans ----------------------

         Variable      N            Mean         Std Dev         Minimum         Maximum
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff
         cell          8       172175.00       231558.55        12700.00       687000.00
         ffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffffff
```

---------- tratamento=limpeza cleaner=polident strain=glabrata ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	4797875.00	3182521.75	193000.00	10000000.00

---------- tratamento=limpeza cleaner=water strain=albicans ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	5797875.00	5427483.41	513000.00	13300000.00

---------- tratamento=limpeza cleaner=water strain=glabrata ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	4998750.00	2336367.37	2730000.00	9730000.00

---------- tratamento=recupera cleaner=efferden strain=albicans ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	77825000.00	32703724.12	37300000.00	120000000

---------- tratamento=recupera cleaner=efferden strain=glabrata ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	96900000.00	40116758.17	45300000.00	160000000

---------- tratamento=recupera cleaner=hipoclor strain=albicans ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	48925000.00	27351455.95	14000000.00	91300000.00

---------- tratamento=recupera cleaner=hipoclor strain=glabrata ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	104287500	49416898.14	43300000.00	207000000

---------- tratamento=recupera cleaner=polident strain=albicans ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	1516250.00	3388315.80	0	9800000.00

---------- tratamento=recupera cleaner=polident strain=glabrata ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	0	0	0	0

---------- tratamento=recupera cleaner=water strain=albicans ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	41396250.00	20818366.85	3870000.00	67300000.00

---------- tratamento=recupera cleaner=water strain=glabrata ----------

Variable	N	Mean	Std Dev	Minimum	Maximum
cell	8	52116250.00	47638023.21	2130000.00	127000000

The ANOVA Procedure

Class Level Information

Class	Levels	Values
tratamento	3	limpeza recupera tratamen

Number of observations 129

The ANOVA Procedure

Dependent Variable: cell_log

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	90.970146	90.970146	10.58	0.0015
Error	126	1083.662944	8.600500		
Corrected Total	127	1174.633090			

R-Square	Coeff Var	Root MSE	cell_log Mean
0.077446	55.83769	2.932661	5.252117

Source	DF	Anova SS	Mean Square	F Value	Pr > F
tratamento	1	90.97014610	90.97014610	10.58	0.0015

Tukey's Studentized Range (HSD) Test for cell_log

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	126
Error Mean Square	8.6005
Critical Value of Studentized Range	2.79869
Minimum Significant Difference	1.026

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	tratamento
A	6.0951	64	recupera
B	4.4091	64	limpeza

---------- tratamento=limpeza ----------

The GLM Procedure

Class Level Information

Class	Levels	Values
cleaner	4	efferden hipoclor polident water
strain	2	albicans glabrata

Number of observations 64

---------- tratamento=limpeza ----------

The GLM Procedure

Dependent Variable: cell_log

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	445.0242866	63.5748981	181.08	<.0001
Error	56	19.6609754	0.3510888		
Corrected Total	63	464.6852620			

R-Square	Coeff Var	Root MSE	cell_log Mean
0.957690	13.43879	0.592528	4.409084

Source	DF	Type III SS	Mean Square	F Value	Pr > F
cleaner	3	427.3948709	142.4649570	405.78	<.0001
strain	1	9.5740792	9.5740792	27.27	<.0001
cleaner*strain	3	8.0553365	2.6851122	7.65	0.0002

---------- tratamento=limpeza ----------

The GLM Procedure

Least Squares Means

cleaner	cell_log LSMEAN	LSMEAN Number
efferden	5.37357423	1
hipoclor	-0.00000000	2
polident	5.67899588	3
water	6.58376561	4

Least Squares Means for effect cleaner

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: cell_log

i/j	1	2	3	4
1		<.0001	0.1504	<.0001
2	<.0001		<.0001	<.0001
3	0.1504	<.0001		<.0001
4	<.0001	<.0001	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

---------- tratamento=limpeza ----------

The GLM Procedure

Least Squares Means

strain	cell_log LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
albicans	4.02230882	<.0001
glabrata	4.79585904	

---------- tratamento=limpeza ----------

The GLM Procedure

Least Squares Means

cleaner	strain	cell_log LSMEAN	LSMEAN Number
efferden	albicans	4.73104722	1
efferden	glabrata	6.01610124	2
hipoclor	albicans	-0.00000000	3
hipoclor	glabrata	-0.00000000	4
polident	albicans	4.85262965	5
polident	glabrata	6.50536212	6
water	albicans	6.50555841	7
water	glabrata	6.66197281	8

Least Squares Means for effect cleaner*strain

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: cell_log

i/j	1	2	3	4	5	6	7	8
1		<.0001	<.0001	<.0001	0.6831	<.0001	<.0001	<.0001
2	<.0001		<.0001	<.0001	0.0002	0.1042	0.1041	0.0335
3	<.0001	<.0001		1.0000	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	1.0000		<.0001	<.0001	<.0001	<.0001
5	0.6831	0.0002	<.0001	<.0001		<.0001	<.0001	<.0001
6	<.0001	0.1042	<.0001	<.0001	<.0001		0.9995	0.5992
7	<.0001	0.1041	<.0001	<.0001	<.0001	0.9995		0.5996
8	<.0001	0.0335	<.0001	<.0001	<.0001	0.5992	0.5996	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

---------- tratamento=limpeza ----------

The GLM Procedure

Tukey's Studentized Range (HSD) Test for cell_log

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	56
Error Mean Square	0.351089
Critical Value of Studentized Range	2.83308
Minimum Significant Difference	0.2968

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	strain
A	4.7959	32	glabrata
B	4.0223	32	albicans

---------- tratamento=limpeza ----------

The GLM Procedure

Tukey's Studentized Range (HSD) Test for cell_log

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	56
Error Mean Square	0.351089
Critical Value of Studentized Range	3.74475
Minimum Significant Difference	0.5547

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	cleaner
A	6.5838	16	water
B	5.6790	16	polident
B			
B	5.3736	16	efferden
C	0.0000	16	hipoclor

---------- tratamento=recupera ----------

The GLM Procedure

Class Level Information

Class	Levels	Values

cleaner	4	efferden hipoclor polident water
strain	2	albicans glabrata

Number of observations 64

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---------- tratamento=recupera ----------

The GLM Procedure

Dependent Variable: cell_log

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	537.1722581	76.7388940	52.53	<.0001
Error	56	81.8054235	1.4608111		
Corrected Total	63	618.9776817			

R-Square	Coeff Var	Root MSE	cell_log Mean
0.867838	19.82954	1.208640	6.095149

Source	DF	Type III SS	Mean Square	F Value	Pr > F
cleaner	3	513.7510541	171.2503514	117.23	<.0001
strain	1	4.0154349	4.0154349	2.75	0.1029
cleaner*strain	3	19.4057691	6.4685897	4.43	0.0073

---------- tratamento=recupera ----------

The GLM Procedure

Least Squares Means

cell_log	LSMEAN

cleaner	LSMEAN	Number
efferden	7.90381900	1
hipoclor	7.80027255	2
polident	1.19521791	3
water	7.48128850	4

Least Squares Means for effect cleaner
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: cell_log

i/j	1	2	3	4
1		0.8094	<.0001	0.3270
2	0.8094		<.0001	0.4585
3	<.0001	<.0001		<.0001
4	0.3270	0.4585	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

---------- tratamento=recupera ----------

The GLM Procedure
Least Squares Means

strain	cell_log LSMEAN	H0:LSMean1= LSMean2 Pr > |t|
albicans	6.34563136	0.1029
glabrata	5.84466761	

---------- tratamento=recupera ----------

The GLM Procedure
Least Squares Means

cell_log	LSMEAN

cleaner	strain	LSMEAN	Number
efferden	albicans	7.85499220	1
efferden	glabrata	7.95264580	2
hipoclor	albicans	7.62206793	3
hipoclor	glabrata	7.97847717	4
polident	albicans	2.39043582	5
polident	glabrata	-0.00000000	6
water	albicans	7.51502951	7
water	glabrata	7.44754749	8

Least Squares Means for effect cleaner*strain

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: cell_log

i/j	1	2	3	4	5	6	7	8
1		0.8722	0.7014	0.8388	<.0001	<.0001	0.5760	0.5029
2	0.8722		0.5865	0.9661	<.0001	<.0001	0.4720	0.4068
3	0.7014	0.5865		0.5577	<.0001	<.0001	0.8601	0.7738
4	0.8388	0.9661	0.5577		<.0001	<.0001	0.4464	0.3834
5	<.0001	<.0001	<.0001	<.0001		0.0002	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	0.0002		<.0001	<.0001
7	0.5760	0.4720	0.8601	0.4464	<.0001	<.0001		0.9115
8	0.5029	0.4068	0.7738	0.3834	<.0001	<.0001	0.9115	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

---------- tratamento=recupera ----------

The GLM Procedure

Tukey's Studentized Range (HSD) Test for cell_log

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	56
Error Mean Square	1.460811
Critical Value of Studentized Range	2.83308
Minimum Significant Difference	0.6053

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	strain
A	6.3456	32	albicans
A			
A	5.8447	32	glabrata

---------- tratamento=recupera ----------

The GLM Procedure

Tukey's Studentized Range (HSD) Test for cell_log

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	56
Error Mean Square	1.460811
Critical Value of Studentized Range	3.74475
Minimum Significant Difference	1.1315

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	cleaner
A	7.9038	16	efferden
A			
A	7.8003	16	hipoclor

```
A
A        7.4813     16    water

B        1.1952     16    polident
```