FREDERICO SILVA DE FREITAS FERNANDES

EFEITO DE LIMPADORES QUÍMICOS SOBRE BIOFILMES DE *CANDIDA* FORMADOS SOBRE A SUPERFÍCIE DE MATERIAIS PARA BASE DE PRÓTESES REMOVÍVEIS

Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do título de Mestre em Clínica Odontológica – Área de Prótese Dental.

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RESUMO

Biofilme de *Candida spp* formado na superfície de próteses removíveis é considerado o principal fator etiológico da candidose, a qual é a infecção oral fúngica mais prevalente em humanos. Em pacientes com comprometimento motor, o uso de limpadores químicos é indicado para o controle desse biofilme, entretanto, pouco se conhece sobre o efeito desses agentes sobre o biofilme de *Candidas* não-*albicans*. Adicionalmente, a literatura é escassa de estudos avaliando a formação de biofilme de *Candida* sobre novos materiais para base de próteses. Assim, o objetivo desse estudo foi avaliar o efeito de limpadores químicos sobre o biofilme mono e multi-espécie de *Candida* formado sobre a superfície de materiais para confecção de próteses removíveis. Foram confeccionados espécimes de resina de polimetilmetacrilato (PMMA) e resina poliamida, os quais, após a padronização da rugosidade de superfície (0,34 ± 0,02 µm), foram submetidos à avaliação da energia livre de superfície (ELS) ou à formação de biofilme. Biofilme de *Candida albicans* e/ou *Candida glabrata* foi formado por 72 h, sendo os espécimes, previamente, submetidos à formação da película adquirida. Após o período de formação do biofilme, os espécimes foram submetidos aos tratamentos, segundo o tempo recomendado por cada fabricante: limpador químico enzimático (3 min); limpador químico sem enzimas (5 min); e hipoclorito de sódio (NaOCl) a 0,5% (10 min). A água destilada e deionizada foi utilizada como controle. Após os tratamentos, os espécimes foram sonicados (7W por 30s) em solução salina, para remoção das células aderidas. Essa solução foi serialmente diluída em solução salina e semeada em CHROMagar® *Candida*. O número de células viáveis de *Candida* foi expresso em unidades formadoras de colônia (UFC)/mm^2. Os dados da ELS e ângulo de contato foram submetidos a ANOVA um fator, enquanto que os dados de células viáveis de *Candida* foram submetidos a ANOVA três fatores, seguido do teste de Tukey-Kramer. Todos os biofilmes avaliados apresentaram maior crescimento na resina de poliamida ($p<0,0001$), entretanto, essa resina apresentou um menor valor de ELS quando

comparada à resina de PMMA. Os limpadores químicos, contendo ou não enzimas, reduziram significantemente os níveis de *Candida*, sem haver diferença estatística entre eles (p=0,9999). Entretanto, o NaOCl a 0,5% foi mais eficaz, na medida em que resultou na ausência de células viáveis. Em todas as situações avaliadas, a *C. glabrata* apresentou maiores valores de células viáveis do que a *C. albicans* (p=0,0002). Nas condições desse estudo, conclui-se que a resina de poliamida possibilitou uma maior proliferação de *Candida*; e os limpadores químicos comerciais foram eficazes na redução dos níveis de *Candida* spp, mas apenas a solução de hipoclorito de sódio a 0,5% resultou na ausência de células viáveis na superfícies dos materiais testados.

Palavras-chave: Resina de poliamida, *Candida glabrata*, *Candida albicans*, biofilme, limpadores químicos de prótese

ABSTRACT

Candida denture biofilm is considered the the primary aetiological agent for the development of oral candidosis, which is the most common fungal oral infection in humans. Although, for patients with limited motor capacity, chemical cleansing with immersion in denture cleansers has been shown to be effective in controlling *Candida* biofilm accumulation, limited data is available on the effect of those cleansing agents on other *Candida* species biofilms. Additionally, few studies have examined the development of *Candida* biofims on novel denture materials. This study evaluated the efficacy of denture cleansers on *C. albicans* and *C. glabrata* single and dual-species biofilms formed on novel denture base materials. Specimens of polymethylmetacrylate resin (PMMA) and polyamide resin were prepared and had their surface roughness standardized (0.34 ± 0.02 µm). Part of the specimens had their surface free energy measured and the other specimens were submitted to the biofilm assays. *C. albicans* and/or *C. glabrata* biofilm was formed for 72 hours on saliva-coated specimens. On the 3rd day, specimens were treated with an enzymatic cleanser, denture cleanser or 0.5% sodium hypochlorite (NaOCl) solution by soaking for, 3, 5 and 10 min, respectively. Water was used as negative control. After treatment, adhered cells were detached from the acrylic resin surface by ultrassonic waves at 7 watts for 30 seconds in phosphate buffered saline solution (PBS). This solution was serially diluted in PBS and plated on CHROMagar® *Candida*. *Candida* viable cell were expressed in colony forming units per surface area (CFU/mm2). Data of surface free energy and contact angle were analyzed by one-way ANOVA, and data of *Candida* species were analyzed by three way-ANOVA followed by Tukey-Kramer test. All tested biofilms displayed significantly higher growth on polyamide thermoplastic resin ($p<0.0001$), which presented the lowest SFE. Denture cleansers significantly decreased *Candida* spp levels, with no statistical difference between them ($p=0.9999$); however, 0.5% NaOCl solution was more effective, since, after treatment, no viable cell was observed. *Candida glabrata* revealed significantly higher CFU counts when

compared to *Candida albicans* under all experimental conditions (p=0.0002). Our study has shown that polyamide resins may present a convenient substratum for microbial colonization. Although denture cleansers reduced *Candida* levels, sodium hypochlorite should be preferred as it was efficient to eliminate *Candida* cells from the tested materials.

Key Words: Polyamide resin, *Candida glabrata*, *Candida albicans*, biofilm, denture cleansers

SUMÁRIO

INTRODUÇÃO

Nos últimos vinte anos, tem-se observado um aumento na incidência de infecções oportunistas, dentre elas, destacam-se as infecções causadas por fungos (Muzyka, 2005). Esse aumento se deve à presença, cada vez maior, de determinadas condições de saúde geral predisponentes ao desenvolvimento dessas infecções. Dentre elas, destacam-se os tratamentos imunossupressores em transplantados e na terapia do câncer, nutrição parenteral, uso indiscriminado de antibióticos, idade avançada e a pandemia de AIDS (Nucci e Marr, 2005; Cheng *et al.*, 2005). Secundária a outros fatores locais ou sistêmicos, a candidose é a infecção oral fúngica mais comumente diagnosticada em humanos (Muzyka, 2005), estando presente em 67% dos usuários de próteses removíveis (Akpan e Morgan, 2002).

Também denominada estomatite induzida por prótese, a candidose apresenta-se como uma inflamação dos tecidos moles orais, geralmente em contato com superfícies de próteses mal adaptadas e/ou precariamente higienizadas (Wilkieson *et al.*, 1991; Coulthwaite e Verran, 2007). A resina da base das próteses funciona como um nicho bastante favorável à proliferação de microorganismos, onde estes, organizados em biofilme, estão mais protegidos da ação antimicrobiana e física da saliva e de outros agentes químicos (Rocha *et al.*, 2002; Li *et al.*, 2007; Tanaka *et al.*, 2009). Por sua capacidade de penetrar e colonizar facilmente a base das próteses e a mucosa subjacente, a *Candida albicans* é considerada o principal agente etiológico da estomatite induzida por prótese (Cannon e Chaffin, 1999; Akpan e Morgan, 2002; Barbeau *et al.*, 2003).

Apesar da *Candida albicans* ser o fungo mais prevalente em pacientes com estomatite induzida por prótese, outras espécies de *Candida* têm sido freqüentemente isoladas desses pacientes, sendo responsáveis por mais 50% dos casos de infecção (Li *et al.*, 2007; Pereira-Cenci *et al.*, 2008; ten Cate *et al.*, 2009). Os motivos dessa mudança ainda não estão completamente esclarecidos, sendo em muitas circunstâncias relacionados à repetidas profilaxias antifúngicas, as

quais têm maior efeito sobre as espécies de *Candida albicans* (Procop e Roberts, 2004). Adicionalmente, é sabido que técnicas mais precisas de identificação celular e molecular tornaram possível a identificação de outras espécies que outrora eram desconhecidas (Li *et al.*, 2007). O aumento na prevalência de infecções causadas por *Candidas* não-*albicans* é preocupante, na medida em que essas espécies são mais resistentes aos agentes antifúngicos comumente utilizados (Bagg *et al.*, 2003; Li *et al.*, 2007). Sendo assim, candidoses associadas a altos níveis dessas espécies de *Candida* são mais difíceis de serem tratadas (Willocks *et al.*, 1991; Hitchcock *et al.*, 1993), em especial as infecções ocasionadas pela *Candida glabrata*, a qual está fortemente associada a infecções sistêmicas generalizadas com alta taxa de mortalidade (Li *et al.*, 2007; Pfaller *et al.*, 2007).

Responsável por 15% das candidoses ocorridas na mucosa e sistemicamente (Cormack *et al.*, 1999), a *Candida glabrata* vem sendo considerada a espécie de *Candida* não-*albicans* com maior potencial de virulência no desenvolvimento da estomatite induzida por prótese, o que pode estar relacionado, dentre outros fatores, à sua alta capacidade de aderência às superfícies acrílicas (Luo e Samaranayake, 2002; He *et al.*, 2006; Pereira-Cenci *et al.*, 2007). Estudos recentes observaram uma alta prevalência da *Candida glabrata* em casos severos de candidose, especialmente quando em associação com a *Candida albicans* (Barbeau *et al.*, 2003; Coco *et al.*, 2008). De acordo com alguns autores, existiria um sinergismo entre essas duas espécies (Li *et al.*, 2007; Pereira-Cenci *et al.*, 2008), tornando o biofilme misto de *Candida albicans* e *Candida glabrata* menos suscetível à ação da saliva e de agentes químicos e, portanto, mais patogênico. Apesar da alta prevalência desse biofilme misto em pacientes com estomatite induzida por prótese (Barbeau *et al.*, 2003; Coco *et al.*, 2008), a literatura é escassa de estudos avaliando o efeito dos métodos de higienização, dentre eles o uso de limpadores químicos, no controle do biofilme misto de *Candida albicans* e *Candida glabrata* formado sobre a superfície de próteses removíveis.

A escovação diária das próteses removíveis é considerada um método de higienização eficaz no controle de biofilme (Baysan *et al.*, 1998; Barnabe *et al.*, 2004), entretanto não é o método mais indicado para pacientes com limitação motora, como os idosos e os pacientes portadores de necessidades especiais (Odman, 1992; Kulak-Ozkan *et al.*, 2002; de Castellucci Barbosa *et al.*, 2008). Para esses pacientes, a higienização da superfície de próteses removíveis deve ser realizada por meio dos limpadores químicos, os quais, além de interferirem com o processo de adesão inicial da *Candida* (Nakamoto *et al.*, 1991), são capazes de desorganizar o biofilme formado sobre a resina de polimetilmetracrilato (PMMA) (Nikawa *et al.*, 1995). Apesar de inúmeros estudos comprovarem a eficácia dos limpadores químicos de prótese frente à colonização da *Candida albicans* em resinas de PMMA (Nikawa *et al.*, 1999), a literatura é escassa de trabalhos avaliando o efeito desses limpadores sobre espécies de *Candida* não-*albicans* (Ferreira *et al.*, 2009) e sobre novos materiais que vêm sendo utilizados para confecção de próteses removíveis. Somado a isso, não foram encontrados trabalhos na literatura avaliando o comportamento desses limpadores frente a biofilmes multiespécie, como o misto de *Candida glabrata* e *Candida albicans*.

Nos últimos anos, novos materiais surgiram como opção para confecção de próteses removíveis. Entretanto, poucos estudos têm avaliado a formação de biofilme de *Candida* sobre esses materiais, assim como a influência da energia livre de superfície (ELS) dos novos materiais na adesão, colonização e formação desse biofilme (Pereira-Cenci *et al.*, 2008; ten Cate *et al.*, 2009). Dentre esses materiais, destaca-se a resina termoplástica de poliamida, a qual, apesar de recentemente desenvolvida para uso odontológico, já vem sendo amplamente utilizada em reabilitações orais. Esse material apresenta a vantagem de ser mais flexível que as resinas de PMMA, o que proporciona mais conforto ao paciente reabilitado, sendo, portanto, bastante indicada para pacientes idosos e portadores de necessidades especiais (Negrutiu *et al.*, 2005). Tendo em vista que a limitação motora apresentada por esses pacientes dificulta a higienização adequada da

prótese por meio da escovação, o uso de limpadores químicos tem sua indicação precisa para controle do biofilme de *Candida* formado sobre a superfície de resinas para base de próteses removíveis.

Dessa forma, considerando o importante papel desempenhado pelo biofilme de *Candida*, pelas propriedades de superfície do material utilizado na confecção de próteses removíveis e pela higienização no desenvolvimento e severidade da estomatite induzida por prótese, o objetivo deste estudo foi avaliar o efeito de limpadores químicos sobre o biofilme mono e multi-espécie de *Candida* spp formado sobre a superfície de resina de polimetilmetracrilato e de resina de poliamida, assim como verificar a influência da ELS desses materiais sobre a colonização de *Candida*.

CAPÍTULO

Efficacy of denture cleansers on *Candida* spp biofilm formed on polyamide resin

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ABSTRACT

Statement of problems. There is limited information on the effect of cleansing agents on non-*albicans* species biofilms. Additionally, few studies have examined the development of *Candida* biofims on novel denture materials.

Purpose. The purpose of this study was to evaluate the efficacy of denture cleansers on *Candida* single and dual-species biofilms formed on polyamide resin.

Material and methods. Specimens of polymethylmetacrylate resin and polyamide resin were prepared and had their surface roughness standardized (0.34 ± 0.02 µm). Part of the specimens had their surface free energy measured (n=20 per resin), while the other part was randomly divided into 24 groups (n=8) for biofilm assay. *C. albicans* and/or *C. glabrata* biofilm was formed for 72 hours. After, specimens were treated with an enzymatic cleanser solution, a cleanser solution or a 0.5% sodium hypochlorite (NaOCl) solution. Water was used as negative control. Remaining adherent micro-organisms were removed from the treated specimens by ultrasonic waves and colony forming units (CFU) of each micro-organism were calculated. Data of SFE were analyzed by one-way ANOVA, and data of *Candida* species were analyzed by three way-ANOVA followed by Tukey-Kramer test.

Results. All tested biofilms displayed significantly higher growth on polyamide resin ($p<0.0001$), which presented the lowest SFE. Denture cleansers significantly decreased *Candida* levels; however, 0.5% NaOCl solution was the most effective. *C. glabrata* revealed significantly higher CFU counts under all experimental conditions ($p=0.0002$).

Conclusions. The chemical cleansers tested were effective in controlling *Candida spp* biofilms formed on both studied resins.

Clinical Implications. The results of this in vitro study suggest that polyamide resin, similarly to polymethylmetacrylate resin, is a suitable substratum for microbial colonization; and the denture cleansers tested were able to reduced *Candida* spp levels.

INTRODUCTION

Oral candidosis is a common opportunistic infection in denture wearers,[1] with *Candida* species being the primary aetiological agent.[2,3] Denture surfaces usually act as a reservoir of yeasts and the disease is aggravated by poorly fitting and inadequate oral and denture hygiene.[4] Although *Candida albicans* is considered the predominant isolate in this infection,[5,6] a shift towards non-*albicans* species, such as *Candida glabrata,* was observed.[7,8]

C. glabrata, an emerging fungal pathogen, exhibits superior adhesion to acrylic resins surfaces compared with *Candida albicans*,[9-12] while it is responsible for 15% of mucosal and systemic candidosis.[13] *C. glabrata* is highly associated with severe inflammation in denture wearers,[14] particularly in combination with *C. albicans*, suggesting a synergistic relationship between these two species.[8,12] Considering the pathogenicity and the high prevalence of mixed *C. albicans* and *C. glabrata* biofilms in patients with denture stomatitis,[5,14] it is crucial to assess the effectiveness of methods that can effectively remove both *C. albicans* and *C. glabrata* from colonized dentures and oral mucosa.

Apparently, mechanical cleansing of the dentures is an effective measure for routine biofilm control;[15] however, some denture wearers usually present difficulty in keeping their dentures clean, especially geriatric patients and those with limited motor capacity.[16,17] For those patients, an association of mechanical and chemical cleansing with immersion in denture cleansers is mandatory for reducing microbial biofilm accumulation on removable prostheses.[18,19] Despite the fact that soaking dentures in disinfectant solutions[20-22] or denture cleansers[23-26] has been shown to be an effective method to control *Candida albicans* colonization, there is a lack of evidence about the comparative effectiveness of those cleansing agents on other *Candida* biofilms as *C. glabrata*[27] and mixed *C. albicans* and *C. glabrata* biofilms, which play an important role in the pathogenesis of denture stomatitis.

Additionally, in the last few years, new materials have been developed to fabricate removable prostheses such as polyamide thermoplastic resin, which is

largely employed in oral rehabilitation. This novel material is more flexible, providing long-term comfortable use for the patient, therefore, being recommended for geriatric and handicapped denture wearers.[28] Considering the poor denture hygiene of these patients due to their limited motor capacity, it is important to access the effectiveness of denture cleansers on *Candida* biofilms formed on polyamide resins. Also, few studies have examined the development of *Candida* biofims and how surface properties e.g. surface free energy (SFE) relate to fungi colonization. SFE is considered an important property, as it may alter the denture pellicle composition and initial adherence of microorganisms to denture surfaces.[29-31]

Since *Candida* species biofilms, denture base materials and oral hygiene plays an important role on the onset and severity of denture stomatitis, the purpose of this study was to compare *C. albicans* and *C. glabrata* single and dual-species biofilm development on polyamide resin, and to evaluate the efficacy of denture cleansers on *Candida* single and dual-species biofilms formed on denture resins. The null hypothesis assumed that: (1) the substratum type would not interfere with biofilm formation, (2) the type of biofilm would not interfere with denture cleansers efficacy, and (3) there would be no difference among the chemical cleansers in decreasing *Candida* levels.

MATERIAL AND METHODS

Experimental design

This *in vitro* study had a completely randomized and blinded design (regarding CFU counts), with substratum type (microwave-polymerized polymethylmetacrylate resin and polyamide thermoplastic resin), biofilm type (single-species biofilms: *C. albicans* and *C. glabrata*, and dual-species biofilms: *C. albicans* plus *C. glabrata*) and treatment with chemical cleansers (enzymatic cleanser solution, cleanser solution or 0.5% sodium hypochlorite – NaOCl) as factors. Contact angle, surface free energy and colony-forming units (CFU) counts

of *C. albicans* and *C. glabrata* were the variables. Surface roughness was standardized for both studied resins.

Specimens were fabricated according to the manufacturer's instructions. After the standardization of the surface roughness, part of the specimens had their surface free energy measured (n=20 per resin), while the other part was randomly divided into 24 groups (n=8) for biofilm assay. Single and dual-species biofilms were formed for 72 h and assigned to one of the four treatments with denture cleansers. Remaining adherent micro-organisms were removed from the treated specimens by sonication and CFU counts of each micro-organism were calculated.

Preparation of specimens

All materials were prepared according to the manufacturers' instructions at room temperature (25 ± 1 ºC and 50 ± 5% relative humidity), under aseptic conditions. Initially, cylindrical wax patterns discs (10 mm in diameter and 2 mm in thickness) were prepared using an aluminum matrix. Discs were invested in plastic or injection flasks for microwave-polymerized polymethylmetacrylate (PMMA) (Acron MC, GC America, Alsip, IL, USA) or polyamide thermoplastic resin (Flexite, Rapid Injection Systems Corp., Minneola, NY), respectively, and subsequently boiled out to soften and eliminate the wax. The PMMA resin was then packed and the plastic flasks were placed in a microwave oven for polymerization, while the injection flasks were sprued, closed and injected with the polyamide resin. Once processed, all flasks were allowed to bench cool for 2 h, when the specimens were removed and immersed in distilled water at 37ºC for 12 h for residual monomer release.[32] Specimens were ground using progressively smoother aluminum oxide papers (320-, 400-, and 600-grit) in a horizontal polisher (model APL-4; Arotec, Sao Paulo, Brazil).

Subsequently, surface roughness (Ra) of the specimens was measured using a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd, Kosaka, Japan) with a 0.01-mm resolution, calibrated at a specimen length of 0.8 mm, 2.4-mm percussion of measure, and 0.5 mm/s. Three readings were made for each

specimen, and a mean value was calculated.[33] For both studied resins, Ra was standardized in 0.34 ± 0.02 µm.

After surface roughness measurements were completed, the specimens were ultrasonically cleansed (Thornton T 740; Thornton-Inpec Eletronica Ltda, Vinhedo, SP, Brazil) in sterilized distilled water for 20 min previously to the surface free energy (SFE) measurements or biofilm formation, to remove any contaminants and artifacts from the surfaces.[9] All procedures were carried out by a single operator.

Contact angles and surface free energy measurements

Three liquids were chosen for contact angle measurement: distilled water, formamide and 1-bromonaphthalene. Contact angle (degrees) was measured by dispensing a droplet (10 µL) of each liquid on the specimen surface. The images of the droplets were taken immediately and contact angles were measured (AutoCAD R14; Autodesk, Inc, San Rafael, CA, USA) from the left boundaries of the magnified picture to the point of air-water-specimen intersection. Each specimen was measured three times for each liquid at 25 ± 1.0 ºC and a mean was determined. The surface free energy ($mN.m^{-1}$) was calculated using cosine of contact angles values obtained previously.[34]

Inoculum and growth conditions

A loopful of stock yeast cultures of *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 2001) were reactivated from their original cultures at -70°C and incubated for 24h at 37°C. Cells were harvested, suspended in Yeast Nitrogen Base (YNB) broth (Difco Laboratories, Detroit, MI, USA) supplemented with 100 mM glucose, and standardized to 1 to 5 x 10^6 $cells.mL^{-1}$, ascertained spectrophotometrically (Bausch & Lomb Spectronic 20, San Pablo, CA, USA) to a concentration of 0.38 at 520 nm.[32]

Biofilm assays

Biofilm assays were performed with single-species biofilms of *C. albicans* or *C. glabrata*, and dual-species biofilms of *C. albicans* plus *C. glabrata*. Discs of the two materials were placed vertically on 24-well (15 mm diameter each well) polystyrene tissue culture plates (bio-one; Greiner, Frickenhausen, Germany). Subsequently, 2 ml of each cell suspension (10^6 CFUs *C. albicans* and/ or *C. glabrata* in YNB) was added to each well.

Biofilms were formed on saliva-coated PMMA and polyamide resin discs. Disc surface area was 219.8 mm^2. The discs were prepared by incubation with clarified human whole saliva for 30 minutes at 37°C. Human whole saliva was collected during masticatory stimulation with Parafilm M (American Can Co., Greenwich, CT, USA) in an ice-chilled polypropylene tube and clarified by centrifugation at 10000g for 10 min at 4°C. The volunteer provided written informed consent previously approved by the Local Ethics Committee. For every experiment the saliva sample was collected at the same time of day and the volume limited to 50ml per collection period, such as to account for the circadian rhythm in saliva composition.[35] The supernatant was removed and immediately used.

All biofilm assays were performed in duplicate in at least four independent experiments on different days. The organisms were grown at 37ºC at 75 rpm in an orbital shaker (model NT 151; Kline Shaker; Nova Tecnica Laboratory, Sao Paulo, Brazil) during 72 hours to allow biofilm formation.

Treatment protocols

After the biofilm development phase (72 h), specimens were randomly assigned to four groups of separate treatments: group 1: distilled water - negative control; group 2: enzymatic cleanser solution - Polident 3 min (GlaxoSmithKline, Philadelphia, PA, USA); group 3: cleanser solution – Corega Tabs (Block Drug Company, Jersey City, NJ, USA); or group 4: 0.5% sodium hypochlorite solution (NaOCl) – positive control (Proderma Pharmacy, Piracicaba, SP, Brazil). Cleaning tablets were placed into 8mL (40°C) deionised distilled water.[27] Exposure to the

immersion effervescent denture cleansers was controlled to allow all surfaces of the specimen to be in contact with the cleanser. Denture cleansers solutions were prepared according to manufacturers' instructions. In group 2 specimens were treated for 3 min, in group 3 specimens were treated for 5 min. and in group 4, specimens were treated for 10 min. The negative control group was not subjected to any treatment as it would be impossible to prepare a common placebo for the two denture cleansers tested. In this group, specimens remained 10 min in deionised distilled water as a reference for the higher time used (group 4).

Before and after the treatment each specimen was subsequently removed and gently washed twice in a new well containing 2ml of sterilized phosphate-buffered saline solution (PBS) for 2s.

Biofilm analysis

After treatment, discs were subsequently placed inside a polypropylene tube containing 3ml of sterilized PBS. Adherent micro-organisms were removed from the specimens by sonication at 7W for 30s.[36] The sonicated solutions were serially diluted in PBS and 20 µl samples were plated in triplicate on CHROMagar™ *Candida* and blood agar. The latter was used to verify possible contamination. The plates were incubated at 37 °C, under aerobic conditions for 24–72 h. CFU were counted using a stereomicroscope, and the results were expressed in colony-forming units per area.

Statistical analysis

Statistical analyses were done using SAS software (SAS Institute Inc., version 9.0, Cary, N.C., USA) employing a significance level fixed at 5%. The assumptions of equality of variances and normal distribution of errors were checked with Shapiro-Wilk test for the response variables data. Data of surface free energy and contact angle were analyzed by one-way ANOVA, while data of viable cells of *Candida* species were analyzed by three way-ANOVA followed by Tukey-Kramer test.

RESULTS

One-way ANOVA revealed statistically significant differences in surface free energy (p=0.0436). PMMA resin exhibited significantly higher SFE compared to the polyamide resin (Table I).

Table I: Contact angle (degrees) of each liquid used, and surface free energy (mN/m) of the resins (Mean ± SD; n=20).

Material	Water	Formamide	1-bromonaphthalene	SFE
PMMA	85.5 ± 3.2a	62.4 ± 5.3a	34.0 ± 2.8a	37.16 ± 1.06a
Polyamide	94.6 ± 4.6b	65.0 ± 3.0a	36.3 ± 3.3a	36.35 ± 1.36b

Distinct lower case letter represents statistically significant differences between materials. (One-Way ANOVA; $p<0.05$).

All tested biofilms (single and dual-species) displayed significantly higher growth on polyamide resin (Table II, $p<0.0001$). *Candida glabrata* single-species biofilm revealed significantly higher CFU counts in all treatments compared to *Candida albicans* single-species biofilm (Table II, p=0.0002), under all experimental conditions; however, dual-species biofilm did not differ from both single-species biofilms, with respect to *Candida* counts (Table II, $p>0.05$). Denture cleansers significantly decreased Candida species levels compared with negative control; however, 0.5% NaOCl solution was more effective as, after treatment, no viable cell was observed. There was no statistical difference between the denture cleansers solutions in decreasing *Candida* species levels (Table II, p=0.9999), even though both chemical agents were more effective in diminishing *C. albicans* than *C. glabrata* from the biofilms tested (Table III).

Table II: *C. albicans* and *C. glabrata* viable cells (CFU/mm^2) after treatment (Mean ± SD).

Type of biofilm	Treatment	Material	
		PMMA	Polyamide
C. albicans	H_2O *	1075 ± 712 Aa	1826 ± 1596 Ba
C. glabrata		1209 ± 767 Ab	2235 ± 1528 Bb
C. albicans + C. glabrata		1189 ± 1047 Aa,b	1596 ± 1213 Ba,b
C. albicans	Polident §	401 ± 274 Aa	489 ± 280 Ba
C. glabrata		600 ± 519 Ab	1698 ± 1352 Bb
C. albicans + C. glabrata		412 ± 243 Aa,b	850 ± 491 Ba,b
C. albicans	Corega Tabs §	244 ± 284 Aa	535 ± 307 Ba
C. glabrata		542 ± 508 Ab	1725 ± 1135 Bb
C. albicans + C. glabrata		562 ± 358 Aa,b	837 ± 487 Ba,b
C. albicans	0.5% NaOCl #	0 ± 0 Aa	0 ± 0 Aa
C. glabrata		0 ± 0 Aa	0 ± 0 Aa
C. albicans + C. glabrata		0 ± 0 Aa	0 ± 0 Aa

Distinct upper case letters represent statistically significant differences between materials. Distinct lower case letters represent differences among types of *Candida* biofilm within materials. Different symbols represent statistical differences among treatments. (Tukey-Kramer test; $p<0.05$).

Table III: Reduction of *C. albicans* and *C. glabrata* after treatment compared with negative control.

		PMMA		Polyamide	
Type of biofilm	**Treatment**	***C. albicans***	***C. glabrata***	***C. albicans***	***C. glabrata***
C. albicans		-	-	-	-
C. glabrata	H_2O	-	-	-	-
C. albicans + C. Glabrata		-	-	-	-
C. albicans		62,7%	-	73,2%	-
C. glabrata	Polident	-	50,4%	-	24%
C. albicans + C. glabrata		74,9%	63,5%	71,4%	39,3%
C. albicans		77,3%	-	70,7%	-
C. glabrata	Corega Tabs	-	55,2%	-	22,8%
C. albicans + C. glabrata		73,4%	48,5%	82,2%	37,1%
C. albicans		100%	-	100%	-
C. glabrata	0.5% NaOCl	-	100%	-	100%
C. albicans + C. glabrata		100%	100%	100%	100%

DISCUSSION

This study has shown that the novel denture material studied is easily colonized by *Candida* species, suggesting that polyamide resins may present a convenient substratum for microbial colonization, as usually occurs on polymethylmetacrylate resin surfaces. *Candida* growth on those materials was even higher than that observed on the PMMA material. Although a linear relationship between SFE and *Candida* adherence has been demonstrated,[29,30] in the present study, this surface property seemed to have no direct influence on *Candida* biofilm development, in which the polyamide resin presented the highest *Candida* colonization and the lowest SFE values compared to the PMMA resin. These results corroborate recent studies that found no correlation between SFE values and *Candida* colonization, suggesting that other factors may be involved in *Candida* initial adherence and biofilm formation on denture materials.[11,37,38] Previous studies have suggested that the residual monomer found in the PMMA resin produces differences in the resin surface charge that can reduce the adhesion and inhibit the growth of *Candida*.[37,39] In the present study, the immersion in distilled water for 12 h for residual monomer release[32] may has not been sufficient to completely eliminate the residual monomer from the PMMA specimens,[40] what may explain why all tested biofilms displayed significantly lower growth on PMMA resin compared to the polyamide resin. Additionally, it has been shown that saliva immersion reduce differences in SFE of denture resins,[31] therefore, further studies evaluating the SFE of saliva coated specimens are needed to further increase the understanding on the role of this substrate surface properties in controlling *Candida* colonization.

Although many studies evaluated the effect of denture cleansers and disinfection solutions against initial *Candida* adherence on denture base materials,[25-27] little attention has been paid to the effect of those denture-cleansing agents on *Candida* mature biofilm,[19] in which cells are known to be more resistant to antimicrobials compounds and chemical cleansing.[23] This is why this study have used 72 h biofilm to gain understanding of (dual-species) biofilms formed on the

different surfaces and its relation to denture cleansing. The results have shown that the denture cleanser solutions, with or without enzyme, were effective to control *Candida* spp biofilms, especially reducing *C. albicans* levels; however they were not able to completely eliminate *Candida* cells from the dental materials, as did the sodium hypochlorite solution.

Previous studies have shown that soaking dentures in 5.25% sodium hypochlorite solution is an effective method for killing adherent *Candida albicans*;[21,22] on the other hand, this disinfection solution in high concentrations may damage the denture materials.[18] In this study, hypochlorite solution in a lower percentage was able to completely eliminate the *Candida* cells from the specimens. The effectiveness 0.5% sodium hypochlorite is already proven regarding *C. albicans*[20] and *C. glabrata*[27] initial adherence, regardless of the fact that the cited reports did not assess the effect of this disinfection solution on a mature and mixed *Candida* species biofilm as did the present study.

The ability of *Candida* species to form biofilm on the surfaces of denture materials was proposed to be of upmost importance in the virulence of *Candida*.[8] This study demonstrates a significantly higher *Candida glabrata* colonization to the resins surfaces studied compared with *C. albicans*. These results are in accordance with previous findings,[9-12] although these studies did not assess the effect of denture cleansers. The different colonization results may be explained by the complexity and phenotypic heterogeneity of the *Candida* species population. This heterogeneity is displayed by a variable surface hydrophobicity, the absence or presence of secreted extracellular proteinases, hyphae formation and/or thigmotropism, all directly influencing *Candida* adherence to plastic surfaces.[8,9]

In light of the fact that recent studies have found that *C. glabrata* is frequently co-isolated with *C. albicans* from severe inflammation in denture wearers,[5,14] it is intriguing to speculate that a synergistic relationship may be involved in the enhanced pathogenic potential of this combination.[12] The present study was the first to evaluate the effect of chemical cleansers on this pathogenic mixed *C. albicans* and *C. glabrata* biofilm. It was observed that the denture

cleansers tested were more effective in diminishing *C. albicans* than *C. glabrata* from *Candida* mixed biofilm. These new results are important as the daily use of cleansers solutions regularly used in clinical practice may promote a population shift towards non-*albicans* species, such as *C. glabrata*, which is increasingly implicated in human infection and associated with systemic infections having a high mortality rate.[8]

Although the results of this study should be interpreted with care, since the nutrient-rich environment of the oral cavity does not fully match the *in vitro* nature of the present study, these results are an important clue on how different *Candida* species, in a single or duo-species biofilm, behave in the presence of novel denture base materials, treated with dentures cleansers regularly used in clinical practice. Further studies on multi-species biofilms with a larger number of yeast strains and oral bacterial species are needed to further increase the understanding of the oral ecosystem and the effect of denture cleanser solutions on those biofilms formed on contemporary denture base materials.

CONCLUSION

Within the limitations of this study it can be concluded that polyamide resin, similarly to polymethylmetacrylate resin, is a suitable substratum for microbial colonization; and the denture cleansers tested were effective to reduce *Candida* biofilms levels on both studied resins.

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CONCLUSÃO

Os resultados deste estudo indicam que os biofilmes mono e multi-espécie de *Candida* apresentaram um maior crescimento na resina de poliamida, não sendo influenciados pela ELS do material. Somado a isso, os resultados suportam que os limpadores químicos, contendo ou não enzimas, foram eficazes na redução dos níveis de *Candida* dos biofilmes avaliados, entretanto somente o tratamento com NaOCl a 0,5% resultou na ausência de células viáveis na superfície dos materiais testados.

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ANEXO 1 – Certificado de Aprovação do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Piracicaba

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 **"Efeito de limpadores químicos sobre o biofilme de *Candida* spp. formado sobre a superfície de diferentes materiais para base de próteses"**, protocolo nº **035/2008**, dos pesquisadores **ALTAIR ANTONINHA DEL BEL CURY, FREDERICO SILVA DE FREITAS FERNANDES e TATIANA PEREIRA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 07/05/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Effect of denture cleansers on *Candida* species biofilm formed on the surface of different materials used in dentures base"**, register number **035/2008**, of **ALTAIR ANTONINHA DEL BEL CURY, FREDERICO SILVA DE FREITAS FERNANDES and TATIANA PEREIRA**, 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 07/05/2008.

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 – Confirmação de submissão ao periódico *The Journal of Prosthetic Dentistry*

Dear Dr. Del Bel Cury:

The manuscript entitled "Efficacy of denture cleansers on Candida spp biofilm formed on polyamide resin," which you recently submitted to the Journal, has been received and assigned number 18641. Please refer to this number in all correspondence relating to your article. All articles are considered in the order in which they were received.

When it is convenient, please address the question(s) contained in the attached title page, then return this document to us as an email attachment. Other than to provide the requested information, please do not change the formatting of this document. (You may wish to keep this title page in your files as an example of our required title page format.)

Thank you for your interest in The Jo urnal of Prosthetic Dentistry.

Best regards,
Dora Lockhart
Manuscript Editor