FREDERICO SILVA DE FREITAS FERNANDES

EFEITO DO USO DIÁRIO DE UM LIMPADOR QUÍMICO ENZIMÁTICO SOBRE O BIOFILME DE *Candida albicans* FORMADO SOBRE MATERIAIS PARA BASE DE PRÓTESES REMOVÍVEIS

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

Orientadora: Profa. Dra. Altair Antoninha Del Bel Cury

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RESUMO

Os limpadores químicos de prótese têm sido bastante indicados para o controle do biofilme formado sobre próteses removíveis de pacientes com comprometimento motor. Apesar de estudos prévios terem mostrado que uma única imersão nesses agentes é capaz de reduzir os níveis de *Candida albicans* do biofilme formado sobre próteses removíveis, pouco se sabe sobre o efeito do uso diário desses limpadores sobre o biofilme residual de *Candida*. Assim, o objetivo desse estudo foi avaliar a eficácia do uso diário de um limpador químico enzimático sobre o biofilme de *C. albicans* formado sobre a superfície de materiais para confecção de próteses removíveis; bem como a atividade enzimática das células de *Candida* desse biofilme após exposições diárias a esse limpador de prótese. Foram confeccionados espécimes de resina de polimetilmetacrilato (PMMA) e resina de poliamida, nos quais foi realizada, inicialmente, a padronização da rugosidade de superfície (0,34 ± 0,02 µm). Após a formação da película adquirida, os espécimes foram divididos aleatoriamente em 12 grupos (n=9) para formação do biofilme de *C. albicans* por 72 horas. Após esse período, os espécimes foram tratados por 1, 4 ou 7 dias, sendo realizado um tratamento por dia, com um limpador químico enzimático (Polident 3 Minutes) ou com água destilada (controle negativo). Após os respectivos períodos de tratamento, os microrganismos remanescentes foram removidos da superfície dos espécimes por meio de ondas ultra-sônicas (7W por 30s). Em seguida, as unidades formadoras de colônia (UFC) foram calculadas e a atividade enzimática das células remanescentes foi avaliada. Os dados foram submetidos à ANOVA um fator ou dois fatores, seguido do teste de Tukey-Kramer. O biofilme de *Candida albicans* formado sobre a resina de poliamida apresentou maiores níveis de *Candida* e uma maior atividade fosfolipásica que o biofilme formado sobre a resina de PMMA ($p<0,001$). O limpador químico enzimático reduziu significantemente os níveis de *Candida albicans* em todos os períodos avaliados ($p<0,001$), entretanto os níveis desse microrganismo aumentaram com o tempo, sendo observada diferença estatisticamente significante entre os períodos avaliados ($p<0,001$). As exposições diárias a esse limpador químico aumentaram

a virulência das células de *Candida*, no que diz respeito à atividade fosfolipásica. Nas condições desse estudo, conclui-se que o uso diário do limpador químico enzimático não foi capaz de impedir a proliferação de *Candida albicans* no biofilme residual, apesar de ter interferido no crescimento desse biofilme.

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

ABSTRACT

Chemical cleansing with immersion in denture cleansers has been indicated for denture biofilm control in patients with limited motor capacity. Although previous studies have shown that a single immersion in those agents is able to substantially reduce *Candida albicans* biofilm levels, the effect of the routine use of denture cleansers on the *Candida* residual biofilm is poorly understood. This study evaluated the efficacy of daily use of an enzymatic denture cleanser on *C. albicans* biofilm formed on denture base materials; and the enzymatic activities of *Candida* biofilm cells after daily exposure to this cleanser agent. Polymethyl methacrylate (PMMA) and polyamide resins specimens were prepared (n=54), and their surface roughness was standardized (0.34 ±0.02 µm). Saliva-coated specimens were randomly divided by lottery into 12 groups (n=9) for biofilm assay. *C. albicans* biofilm was formed for 72 hours, and then specimens were treated for 1, 4 or 7 days, once a day, with an enzymatic cleanser (Polident 3 Minutes), or distilled water (negative control). Remaining adherent microorganisms were removed from the treated specimens by ultrasonic waves at 7 watts for 30 seconds, and then colony-forming units (CFU) were calculated and remaining cells enzymatic activities were determined. Data were analyzed by 1-way or 2-way ANOVA followed by the Tukey-Kramer test. *C. albicans* biofilm formed on polyamide resin showed significantly higher *Candida* levels and phospholipase activity ($p<0.001$) than biofilm formed on PMMA resin. The enzymatic cleanser significantly reduced *C. albicans* levels in all evaluated periods ($p<0.001$); however, the number of this microorganism increased with time, showing statistical difference among the treatment periods ($p<0.001$). The daily exposure to the denture cleanser increased *Candida* cells virulence, with regard to phospholipase activity. Our study has shown that the enzymatic cleanser daily use did not prevent C. albicans proliferation in residual biofilm; however, this agent reduced this fungus rate of growth.

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

SUMÁRIO

INTRODUÇÃO

A candidose é a infecção oral fúngica mais comumente diagnosticada em humanos (Muzyka, 2005), sendo a *Candida albicans* considerada o principal agente etiológico (Coco *et al.*, 2008, ten Cate *et al.*, 2009). Embora seja um microrganismo comensal que habita a cavidade oral de indivíduos sadios (Perezous *et al.*, 2005, Pereira-Cenci *et al.*, 2008), a ocorrência de fatores predisponentes pode aumentar os níveis orais desse fungo e, desencadear o aparecimento da candidose. Dentre esses fatores, destacam-se os tratamentos imunossupressores em transplantados e na terapia do câncer, o uso indiscriminado de antibióticos e a presença na cavidade oral de próteses removíveis com higienização deficiente (Coulthwaite & Verran, 2007). Quando associada ao uso de próteses, a candidose é também denominada de estomatite induzida por prótese, estando presente em até 65% dos usuários de próteses removíveis (Akpan & Morgan, 2002).

Candida albicans apresenta alta hidrofobicidade de superfície celular, aderindo facilmente à base das próteses removíveis (Webb *et al.*, 1998), dando início ao processo de colonização dessa superfície pela formação de micro-colônias. O desenvolvimento celular e a maturação dessas colônias levam à formação de uma comunidade microbiológica altamente especializada, denominada biofilme que encontra-se envolta por uma matriz de polissacarídeos extracelulares que lhe confere sustentação e proteção contra a ação antimicrobiana e física da saliva e de outros agentes químicos (Radford *et al.*, 1999; Mukherjee *et al.*, 2005, da Silva *et al.*, 2010). Uma vez em contato com a mucosa oral, as células de *Candida* do biofilme formado sobre a prótese invadem e colonizam esse tecido pela liberação de enzimas hidrolíticas (Schaller et al., 2005). Essas enzimas promovem a ruptura da membrana plásmatica das células epiteliais (fosfolipase e lipase), alteram a permeabilidade do epitélio nos espaços intercelulares (condroitinase), provocam a lise de eritrócitos e linfócitos (hemolisina) e a degradação das proteínas do hospedeiro (proteinase), sendo,

portanto, consideradas um dos principais fatores de virulência para o desenvolvimento da candidose (Ghannoum & Abu-Elteen, 1990). A produção dessas enzimas pode ser estimulada por alguns fatores, como os níveis de *Candida* no biofilme da base da prótese, na medida em que quanto maior a população celular, maior o estresse das células de *Candida*, o que pode levar a uma maior secreção de enzimas hidrolíticas (Thiele *et al.*, 2008). Por outro lado, o acúmulo de biofilme na base da prótese pode ser influenciado pela natureza do material empregado na confecção da prótese removível e pela higienização da prótese pelo paciente (Mukherjee *et al.*, 2005; Pereira-Cenci *et al.*, 2008).

A resina de polimetilmetacrilato (PMMA) tem sido o material de escolha para a confecção de próteses removíveis, tendo como vantagens a estética e a estabilidade dimensional, além de propriedades físicas satisfatórias (Straioto *et al.*, 2010). Entretanto, o uso desse material na reabilitação de pacientes parcialmente desdentados apresenta a desvantagem da necessidade de uma estrutura metálica, a qual pode comprometer a estética por meio dos grampos de retenção. Somado a isso, a presença de monômero residual em sua superfície pode causar reações alérgicas (Sadamori *et al.*, 1992), inviabilizando o uso desse material por alguns pacientes. Com o objetivo de suprir as deficiências da resina de PMMA, nos últimos anos, novos materiais surgiram como opção para confecção de próteses removíveis, dentre eles destaca-se a resina termoplástica de poliamida. Essa resina não apresenta monômero residual e ganhou popularidade por sua flexibilidade e por sua natureza altamente elástica (Takabayashi, 2010), características que possibilitam a substituição dos grampos métalicos anti-estéticos por grampos confeccionados com o próprio material, além de proporcionarem um maior conforto ao paciente reabilitado, sendo bastante indicada para pacientes idosos e portadores de necessidades especiais (Negrutiu *et al.*, 2005). Entretanto, deve ser destacado que a resina de poliamida apresenta a desvantagem de ser mais suscetível à formação de biofilme de *Candida* do que a resina de PMMA (Fernandes *et al.*, 2011). Dessa forma, quando essa resina é utilizada para a reabilitação de pacientes idosos, cuja acuidade visual ou alguma

limitação motora esteja presente, impedindo a higienização adequada das próteses por meio da escovação, a higienização das próteses deve ser realizada com auxílio de agentes químicos (Odman, 1992, de Castellucci Barbosa *et al.*, 2008).

Dentre os agentes químicos para limpeza de próteses disponíveis no mercado, os limpadores à base de peróxidos são os mais utilizados, tendo em vista serem simples de usar, apresentarem odor e sabor agradável, serem compatíveis com os materiais da prótese e terem efeito bactericida e fungicida (Shay, 2000, de Souza *et al.*, 2009). O produto pode ser apresentado na forma de pó ou tablete, que quando dissolvidos em água geram uma efervescência criada pela liberação de bolhas de oxigênio, que promovem além da limpeza química, uma limpeza mecânica adicional na prótese (Abelson, 1981). A adição de enzimas proteolíticas aos peróxidos tem, como principal função, a remoção da película adquirida, que constitui a primeira etapa no processo de formação do biofilme (Rodrigues Garcia *et al.*, 2004, Lima *et al.*, 2006). Entretanto, para que esses limpadores possam efetivamente contribuir para o tratamento da estomatite protética, é de fundamental importância que esses agentes interfiram não só com o processo de adesão inicial da *Candida albicans* (Tamamoto *et al.*, 1985, Nakamoto *et al.*, 1991, Buergers *et al.*, 2008), mas também sejam capazes de desorganizar o biofilme de *Candida* formado sobre as resinas utilizadas na confecção de próteses removíveis, diminuindo a virulência desse biofilme (Nikawa *et al.*, 1999). Apesar de estudos recentes (Fernandes *et al.*, 2011; Jose *et al.*, 2010) terem verificado que uma única imersão nos limpadores químicos enzimáticos é capaz de reduzir significativamente os níveis de *Candida* do biofilme formado sobre as resinas de PMMA e poliamida, não foram encontrados trabalhos na literatura avaliando se limpezas diárias com esses agentes seriam capazes de manter baixos os níveis de *Candida albicans* no biofilme residual, e se o estresse causado às células de *Candida,* pela exposição diária a esses agentes, pode interferir com a secreção de enzimas hidrolíticas e, consequentemente, com a virulência desse biofilme.

Dessa forma, o objetivo desse estudo foi avaliar a eficácia do uso diário de um limpador químico enzimático sobre o biofilme de *Candida albicans* formado sobre a superfície de resinas de PMMA e poliamida, bem como a atividade enzimática desse biofilme após exposições diárias a esse limpador de prótese.

CAPÍTULO

Effect of the daily use of an enzymatic denture cleanser on *Candida albicans* biofilm formed on polyamide and polymethyl methacrylate resins

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ABSTRACT

Statement of problem. Although a single immersion in denture cleansers is able to substantially reduce *Candida albicans* biofilm levels, the effect of the routine use of this agent on the residual biofilm is poorly understood.

Purpose. The purpose of this study was to evaluate the effect of daily use of an enzymatic cleanser on *C. albican*s biofilm formed on denture base materials.

Material and methods. Polymethyl methacrylate (PMMA) resin (Onda Cryl) and polyamide resin (Flexite M.P.) specimens (n=54) were prepared, and their surface roughness was standardized (0.34 ±0.02 μm). Specimens were randomly divided by lottery into 12 groups (n=9) for biofilm assay. *C. albicans* biofilm was formed for 72 hours, and then specimens were treated for 1, 4 or 7 days, once a day, with an enzymatic cleanser (Polident 3 Minutes), or water (negative control). Remaining adherent microorganisms were removed from the treated specimens by ultrasonic waves, then colony-forming units (CFU) were calculated and biofilm cells enzymatic activities were determinate. One-way ANOVA was used to compare the materials and 2-way ANOVA, followed by the Tukey-Kramer test, to compare the types and periods of treatment.

Results. *C. albicans* biofilm formed on polyamide resin showed significantly higher *Candida* levels and phospholipase activity ($P<.001$). The enzymatic cleanser significantly reduced *C. albicans* levels in all evaluated periods ($P<.001$). The number of this microorganism increased with time, showing statistical difference among the treatment periods ($P<.001$). The denture cleanser increased *C. albicans* phospholipase secretion.

Conclusions. Enzymatic cleanser daily use did not prevent *C. albicans* proliferation in residual biofilm; however, this agent reduced this fungus rate of growth.

Clinical Implications. Based on the results of this study, enzymatic denture cleanser should not be employed as a sole means for routine biofilm control.

INTRODUCTION

Denture stomatitis is an opportunistic disease affecting 65% of denture wearers,[1] with *Candida albicans* being the primary etiological agent.[2] A major virulent attribute of *C. albicans* is its ability to adhere to denture surfaces, and form surface-attached microbial communities known as biofilms,[3] which protect these organisms from detachment by shear forces and chemical agents.[4] Once in contact with oral mucosa, *C. albicans* biofilm cells may colonise and penetrate in the oral epithelium by the release of hydrolytic enzymes,[5-7] triggering the inflammatory process known as denture stomatitis. There are many factors that influence the onset and severity of this disease, such as the nature of the denture base material and denture cleanliness.[4,8]

Polyamide thermoplastic resin has been considered an alternative to polymethyl methacrylate (PMMA) resin in cases that the esthetics is compromised by visible metal clasps, and for patients with allergy to monomer.[9,10] Additionally, polyamide resin highly elastic nature and flexibility[11] provide patients with more comfortable long-term use than acrylic resins. Therefore, it is often recommended for geriatric and disabled denture wearers.[12] However, this denture base material has the disadvantage of being more susceptible to *Candida* biofilm growth than PMMA resin.[13] Considering the poor denture hygiene of those patients as a result of their limited motor capacity,[14] the use of a simple and effective method to promote the daily removal of *Candida* biofilm is indispensable for clinical use of the polyamide resin.

Denture cleansers have been considered the first option for denture wearers that present difficulty in keeping their dentures clean by mechanical cleansing method.[15-17] Among these cleansers, enzymatic cleansers are considered the best choice, since they are able to interfere with biofilm development[18] without changing the resins surface properties.[19] Previous studies have shown that a single immersion in a cleanser solution is an effective method to control *C. albicans* initial adherence[20-22] and to significantly reduce this microorganism levels in a mature biofilm formed on PMMA and polyamide

resins.[13,23,24] However, these studies did not evaluate whether daily use of these agents could prevent *Candida albicans* proliferation in residual biofilm, and if the daily exposure to these agents may interfere with *C. albicans* virulence with regard to its enzymatic activity.

Therefore, the purpose of this study was to evaluate the efficacy of daily use of an enzymatic denture cleanser on *Candida albican*s biofilm formed on PMMA and polyamide resins; and the enzymatic activities of *Candida* cells after daily exposure to this cleanser agent. The null hypotheses assumed that substratum type, periods or type of treatment would not interfere with biofilm growth and *C. albicans* enzymatic activities.

MATERIAL AND METHODS

Experimental design

This in vitro study had a randomized and blinded design (with regard to CFU counts and enzymatic activities), with substratum type (microwave-polymerized polymethyl methacrylate resin and polyamide thermoplastic resin), periods of treatment (one, four, or seven days), and type of treatment (enzymatic cleanser or water) as factors. Colony-forming unit (CFU) counts of *Candida albicans* and biofilm cells enzymatic activities were the response variables. Surface roughness was standardized for both resins. Specimens were fabricated according to the manufacturer's instructions, and the number of specimens in each test group was determined by preliminary tests, which demonstrated that the sample size yielded an adequate power (80%) for detecting statistically significant differences (n=54). After surface roughness standardization, the specimens were randomly divided by lottery into 12 groups for biofilm assay (n=9). *C. albicans* biofilm was formed for 72 hours, and specimens were, then, treated for 1, 4 or 7 days, once a day, with an enzymatic cleanser solution or water. After the period of treatment, remaining adherent microorganisms were removed from the treated specimens by ultrasonic waves, then CFU counts were calculated and biofilm cells enzymatic activities were determined.

Specimen preparation

All materials were prepared according to the manufacturers' instructions at room temperature (25 ±1°C and 50 ±5% relative humidity), under aseptic conditions. Initially, cylindrical wax pattern discs (10 mm in diameter and 2 mm thick) were prepared using an aluminum matrix. Discs were invested in plastic or injection flasks for microwave-polymerized polymethyl methacrylate (PMMA) (Onda Cryl; Artigos Odontológicos Clássico Ltd, São Paulo, Brazil) or polyamide thermoplastic resin (Flexite M.P.; Rapid Injection Systems Corp, Mineola, NY), respectively, and the wax was softened and eliminated with boiling water. 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 hours. Afterwards, the specimens were removed and immersed in distilled water at 37°C for 48 hours for residual monomer release.[25] Specimens were ground using progressively smoother aluminum oxide papers (320-, 400-, and 600-grit) in a horizontal polisher (model APL-4; Arotec, São Paulo, Brazil) to standardize surface roughness.

Subsequently, surface roughness (Ra) of the specimens was measured using a profilometer (Surfcorder SE1700; Kosaka Laboratory Ltd, Tokyo, Japan) with a resolution of 0.01 mm, calibrated at a specimen length of 0.8 mm, 2.4-mm percussion of measure, and 0.5 mm/s. Three readings were made on each side of the specimen, and a mean value was calculated.[26] For both resins, Ra was standardized at 0.34 ±0.02 μm.[13]

After Ra measurements were completed, the specimens were ultrasonically cleansed (Thornton T 740; Thornton-Inpec Eletrônica Ltda, Vinhedo, Brazil) in sterilized, distilled water for 20 minutes prior to biofilm formation, to remove any contaminants and artifacts from the surfaces (Luo and Samaranayake, 2002). All procedures were performed by a single operator.

Inoculum and growth conditions

A loopful of stock yeast culture of *C. albicans* (ATCC 90028) was reactivated from its original culture stored at -70°C and incubated for 24 hours at 37°C. Cells were harvested, suspended in Yeast Nitrogen Base (YNB) broth (Becton Dickinson, Franklin Lakes, NJ) supplemented with 100-mM glucose, and standardized to 1 to 5 x 10^6 cells.mL^{-1}, ascertained spectrophotometrically (Spectronic 20; Bausch & Lomb, Rochester, NY) at an absorbance of 0.38 at 520 nm.[27]

Biofilm assays

Biofilm assays were performed with *C. albicans* biofilm. Discs of the 2 materials were placed vertically in 24-well (each well was 15 mm in diameter) polystyrene cell culture plates (Cellstar 24 Well Cell Culture Plates; Greiner Bio-One GmbH, Frickenhausen, Germany). Subsequently, 2 ml of the cell suspension (10^6 CFUs *C. albicans* in YNB) was added to each well.

Biofilm was formed on saliva coated PMMA and polyamide resin discs. The 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 flexible film (Parafilm M; American Can Co, Neenah, Wis) in an ice-chilled polypropylene tube and clarified by centrifugation at 10,000 g for 10 minutes at 4°C. The volunteer provided written informed consent, and the study was previously approved by the Research and Ethics Committee of Piracicaba Dental School, State University of Campinas. For each experiment, the saliva sample was collected at the same time of the day and the volume was limited to 50 ml per collection period, so as to account for the circadian rhythm in saliva composition.[28] The supernatant was removed and immediately used.

Biofilm assays were performed in triplicate in at least 3 independent experiments on different days. The organisms were grown at 37°C at 75 rpm in an

orbital shaker (Kline agitator NT 151; Nova Técnica, São Paulo, Brazil) for 72 hours to allow biofilm formation. The medium was changed every 24 hours.

Treatment protocols

After the biofilm development phase (72 hours), specimens were randomly divided by lottery into 3 groups of separate periods of treatment: 1, 4 or 7 days. Treatments were performed for 3 minutes per day with distilled water (DDW), negative control; or enzymatic cleanser solution (PD) (Polident 3 Minute; GlaxoSmithKline, Philadelphia, Pa). Denture cleanser solutions were prepared according to the manufacturers' instructions. Cleaning tablets were placed into 200 ml (40°C) of deionised distilled water. Then, each specimen was individually placed in a sterile beaker containing 8 ml of the treatment solution.[13] Exposure to the immersion effervescent denture cleansers was controlled to allow all surfaces of the specimen to be in contact with the cleanser.

After the treatment, each specimen was subsequently removed and gently washed twice in a new well of a new polystyrene tissue culture plate (TPP AG, Trasadingen, Switzerland) containing 2 ml of sterilized phosphate-buffered saline solution (PBS; Sigma-Aldrich Corp, St. Louis, Mo), at pH 7.4 for 2 seconds. Then, specimens treated only once (1 day period) were placed into a polypropylene tube containing 3 ml of sterilized PBS for biofilm analysis, while the specimens treated for 4 or 7 days were transferred to a new sterile 24-well polystyrene cell culture plate containing 2 ml YNB supplemented with 100-mM glucose and residual biofilm was allowed to develop for 24 hours, when a new treatment was performed. This procedure was repeated each day until the end of these periods when the specimens were subjected to biofilm analysis.

Biofilm analysis

Adherent microorganisms were removed from the specimens by sonication at 7 W for 30 seconds.[29] The sonicated solutions were serially diluted in PBS, and 20-µL aliquots were plated in duplicate on agar (Sabouraud agar; Difco,

Sparks, Md) and blood agar (Blood Agar Base; Becton Dickinson). The latter was used to verify possible contamination. The plates were incubated at 37°C under aerobic conditions for 24-72 hours. CFU were counted using a stereomicroscope (Coleman Equipamentos para Laboratórios, Santo Andre, Brazil), and the results were expressed as colony-forming units per area.

Assessments of enzymatic activity were made by the agar plate method. The phospholipase, aspartyl-protease, hemolisyn, lipase and chondroitinase activities were performed using egg yolk medium,[30] BSA (bovine serum albumin) medium,[31] blood agar,[32] Tween 80 opacity test medium,[33] and chondroitin sulphate medium,[34] respectively. The sonicated solutions were centrifuged at 3,000 g for 6 minutes. Cells were washed twice with PBS and suspended in 100-µL of PBS. Then, 5-µL of this suspension was plated on each one of the test medium. Plates were incubated at 37°C under aerobic conditions for 48 hours (phospholipase), 7 days (hemolisyn, lipase and chondroitinase) or 20 days (aspartyl-protease) period. After, the enzymatic activity was determined by the formation of a halo around the yeast colony, and measured in terms of the ratio of the diameter of the colony to the total diameter of colony plus zone of precipitation (Pz). Pz=1.00 indicates absence of enzymatic activity, and the lower the Pz the higher the enzymatic activity.[30]

Statistical analysis

The statistical analysis was performed using statistical software (SAS v. 9.0; SAS Institute, Inc, Cary, NC) with a significance level fixed at 5%. The assumptions of equality of variances and normal distribution of errors were evaluated for each variable, and, when violated, the data were transformed as suggested by the software.[35] The data of viable cells of *C. albicans* were transformed by logarithmic [$\log_{10}$ (y)]. One-way ANOVA was used to compare the materials and 2-way ANOVA to compare the types and periods of treatment; regarding the data of *C. albicans* biofilm viable cells and biofilm enzymatic activities. Post hoc comparisons were performed using the Tukey-Kramer test.

RESULTS

One-way ANOVA revealed a statistically significant difference between the denture materials; biofilm formed on polyamide resin showed significantly higher *Candida* levels (*df*=1; F=33.6, *P*<.001) and phospholipase activity (*df*=1; F=4.6, *P*=.03). Two-way ANOVA showed an interaction between the type and periods of treatment only for the phospholipase activity of polyamide biofilm (*P*=.03) (Table I and II).

Table I: Two-way ANOVA for PMMA resin, considering type and period of treatment on cell counts and enzymatic activities.

Depedent Variable	**Source of variation**	***Df***	**Sum of Squares**	**Mean Square**	**F**	***P***
Cell counts	Type of treatment	1	4.755	4.755	167.73	<.0001
	Period of treatment	2	3.900	1.950	68.78	<.0001
	Type × Period of treatment	2	0.067	0.033	1.18	.316
Phospholipase activity	Type of treatment	1	0.006	0.006	12.17	.001
	Period of treatment	2	0.229	0.114	216.11	<.0001
	Type × Period of treatment	2	0.003	0.002	2.96	.061

Table II: Two-way ANOVA for Polyamide resin, considering type and period of treatment on cell counts and enzymatic activities.

Depedent Variable	**Source of variation**	***Df***	**Sum of Squares**	**Mean Square**	**F**	***P***
Cell counts	Type of treatment	1	1.730	1.730	38.39	<.0001
	Period of treatment	2	8.076	4.038	89.60	<.0001
	Type × Period of treatment	2	0.160	0.080	1.78	.180
Phospholipase activity	Type of treatment	1	0.015	0.015	37.03	<.0001
	Period of treatment	2	0.156	0.078	192.91	<.0001
	Type × Period of treatment	2	0.003	0.002	3.88	.027

The denture cleanser significantly decreased *C. albicans* levels and biofilm rate of growth, since, in all evaluated periods, this microorganism showed significantly higher cell counts in the negative control group ($P<.001$) (Table III). C. albicans levels increase with time, showing statistical difference among the treatment periods ($P<.001$) (Table III).

Table III: *C. albicans* viable cells (CFU/mm^2) after treatments and periods (Mean ±SD) and reduction (%) of *C. albicans* after treatment with enzymatic cleanser compared to negative control.

	Period of	Treatment		
Material	**treatment**	**H_2O**	**Polident**	**Reduction**
PMMA	1 day	1062 ± 423^{Aa}	242 ± 92^{Ba}	77.2%
	4 days	2624 ± 697^{Ab}	869 ± 379^{Bb}	66.9%
	7 days	4485 ± 1711^{Ac}	997 ± 334^{Bc}	77.8%
Polyamide	1 day	2252 ± 1113^{Aa}	761 ± 510^{Ba}	66.2%
	4 days	6460 ± 2113^{Ab}	3268 ± 1118^{Bb}	49.4%
	7 days	14028 ± 6228^{Ac}	7442 ± 2626^{Bc}	47.0%

Different uppercase letters represent statistically significant differences between treatments within periods. Different lowercase letters represent differences among periods within treatments. (Tukey-Kramer test; $P<.05$).

Phospholipase activity was substantially influenced by the factors under study; a statistically significant difference among periods and treatment was observed for the Pz value ($P<.001$) (Table IV). The type and period of treatment did not influence *Candida albicans* aspartyl-protease and hemolisyn activity for both resins ($P>.05$). *Candida* cells were negative for lipase and chondroitinase (Pz=1.00).

Table IV: Phospholipase activity (Pz) of *C. albicans* after treatments and periods (Mean ±SD)

Material	Period of treatment	Treatment	
		H_2O	Polident
PMMA	1 day	0.68 ± 0.02^{Aa}	0.69 ± 0.02^{Ba}
	4 days	0.59 ± 0.02^{Ab}	0.63 ± 0.03^{Bb}
	7 days	0.52 ± 0.01^{Ac}	0.53 ± 0.03^{Bc}
Polyamide	1 day	0.63 ± 0.02^{Aa}	0.65 ± 0.02^{Ba}
	4 days	0.55 ± 0.02^{Ab}	0.61 ± 0.02^{Bb}
	7 days	0.50 ± 0.01^{Ac}	0.52 ± 0.02^{Bc}

Different uppercase letters represent statistically significant differences between treatments within periods. Different lowercase letters represent differences among periods within treatments. (Tukey-Kramer test; $P<.05$).

DISCUSSION

The null hypotheses that substratum type, periods or type of treatment would not interfere with biofilm growth and *C. albicans* enzymatic activities were rejected. Denture cleansers have been considered a simple and effective measure for routine biofilm control in elderly or disabled patients lacking motor coordination.[15] Previous studies have shown that those agents are able to substantially reduce *C. albicans* initial adherence[20-22] and the levels of this microorganism in a mature biofilm.[13,23,24] Although these studies have related the presence of a resultant biofilm which cannot be completely removed by a single immersion in chemical agents, the effect of the routine use of denture cleansers on this residual biofilm had not been yet studied. This study was the first to evaluate the efficacy of daily use of an enzymatic denture cleanser on *C. albicans* mature biofilm. The results of the present study are important as the routine use of this agent was not able to prevent *C. albicans* proliferation in residual biofilm, which significantly increased with time. This fact could be due to the complexity structure of biofilms associated with the denture resin surface properties which does not allow denture cleansers to completely eliminate *Candida* cells from the denture materials.[4,13,23] Once protected by the tenacious resultant biofilm, *Candida* cells

proliferate in the period between treatments, considering the absence of a residual effect of the enzymatic product.

Even though in the present study the daily use of the denture cleanser did not prevent *Candida albicans* proliferation in residual biofilm, the continuous use of this product decreased the rate of growth of this fungus. This is confirmed by the fact that *C. albicans* levels were lower in the group treated with the denture cleanser compared with control group in all evaluated periods, for both studied resins. Regardless the use of denture cleansers may contribute for routine *C. albicans* biofilm control, it should not be employed as a sole means of denture hygiene, as it is not able to promote a complete biofilm eradication. On the other hand, denture cleansers can be indicated as an auxiliary denture care method, especially when used in combination with mechanical cleansing method, since these cleansers have shown to improve the efficacy of this physical method in removing denture biofilm.[16-18]

Polyamide resin showed higher *Candida* biofilm colonization, results which are in accordance with previous study conducted by Fernandes et al. (2011).[13] However, in that study, *C. albicans* biofilm virulence with regard to its enzymatic activity was not evaluated. The higher phospholipase activity found for biofilm formed on polyamide resin surface could be related to the large accumulation of biofilm, since the greater the number of cells in the biofilm, the higher the cell-population stress, which has been considered an important factor for the selection of more secretory strains.[7] In the same way, the increased amount of biofilm that occurred with time may have contributed to increased phospholipase production of *Candida* cells observed over the evaluated periods. Another reason for this increase in phospholipase activity with time is the maturation of biofilm, which is associated with a greater amount of hyphae, and consequently, with a higher enzymatic activity.[6] *C. albicans* cells levels substantially decrease after treatment with the enzymatic denture cleanser; however, their phospholipase activity not reduced in proportion. This increased phospholipase secretion of the remaining cells may have been stimulated by the stress caused by the daily

exposure to the denture cleanser. These results are important since phospholipases play an active role in the invasion of lesions in the host tissue, causing the rupture of the epithelial cell membrane and permitting the penetration of the *Candida* cells into the cytoplasm.[30] On the other hand, the secretion of other enzymes, such as aspartyl-protease, hemolisyn, lipase and chondroitinase, also involved in the mucosal colonization was not influenced by the factors under study.

The results of the present study should be interpreted with care, since the in vitro nature of the present study does not fully match the environment of the oral cavity. However, these results provide important data on how *C. albicans* biofilm behave with the daily use of an enzymatic denture cleanser regularly used in clinical practice. In the present study, *Candida albicans* biofilms were used since this microorganism is the primary pathogens responsible for the development of denture stomatitis. However, further studies are needed on other *Candida* species in a single or mixed biofilm, which are increasingly implicated in denture stomatitis.

CONCLUSION

Within the limitations of this study, it can be concluded that the daily use of an enzymatic denture cleanser did not prevent *C. albicans* proliferation in residual biofilm; however, this agent reduced this fungus rate of growth.

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CONCLUSÃO

Os resultados deste estudo indicam que o uso diário do limpador químico enzimático não foi capaz de impedir a proliferação de *Candida albicans* no biofilme residual, apesar de ter interferido no crescimento desse biofilme. Somado a isso, as exposições diárias a esse limpador químico aumentaram a virulência das células de *Candida*, no que diz respeito à atividade fosfolipásica.

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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. Abreviaturas dos periódicos em conformidade com o Medline.

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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 daily use of an enzymatic denture cleanser on Candida albicans biofilm formed on polyamide and polymethyl methacrylate resins," which you recently submitted to the Journal, has been received and assigned number 19018. Please refer to this number in all correspondence (including subject line of your e-mails). All articles are considered in the order in which they were received.

Thank you for your interest in The Journal of Prosthetic Dentistry.

Drew Landrum
Editorial Assistant

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