UNIVERSIDADE DE SÃO PAULO

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Synthetic and Biological adhesives influence on the ability of
Candida albicans **in forming biofilms on different PMMA surfaces**

Influência de adesivos sintéticos e biológicos na capacidade de
formação de biofilme de *Candida albicans* **em diferentes superfícies de PMMA**

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Tese constituída por artigos apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutora em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Reabilitação Oral
Orientador: Prof. Dr. Vinicius Carvalho Porto

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DEDICATÓRIA

Dedico esta tese a todos aqueles que buscam o seu melhor diariamente. A nossa capacidade é infinita, a limitação quem impõe somos nós.

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*"Talvez não tenha conseguido fazer o melhor,
mas lutei para que o melhor fosse feito. Não sou
o que deveria ser, mas Graças a Deus, não sou o
que era antes".*

Martin Luther King

RESUMO

A aderência de microrganismos aos materiais que constituem as próteses e aos tecidos bucais é um fator determinante para a colonização microbiana, o que pode desencadear, em principal, infecções como a estomatite protética (EP). A aderência de microorganismos a superfície da prótese esta relacionada as características de superfície da resina acrílica, assim, a fim de minimizar esta aderência o objetivo do presente estudo foi avaliar se adesivos a base ethil cianocrilato possui efeito sobre a adesão de *C. albicans* por 7, 14 e 30 dias e se a incorporação de *Punica Granatum* e digluconato de clorexidina no biopolimero de fibrina a base de veneno de cobra possui efeito sobre a formação de biofilme de *C. albicans* após 24, 48 e 72 horas sobre superfície de resina termo e pré polimerizada (Cad-Cam). Amostras circulares (10×2 mm), com uma rugosidade superficial de 2-3 µm, foram produzidas. Para ensaios com ethil cianoacrilato, os espécimes foram divididos em 3 grupos: controle (sem tratamento prévio), ethil cianoacrilato convencional e ethil cianoacrilato gel. Foram aplicadas duas camadas contendo 50 µL de cada material. O ensaio de unidades formadoras de colônias (UFC) foi utilizados para quantificar as células vivas do biofilme em resina acrílica termopolimerizável pré-tratada, após 7, 14 e 30 dias de desenvolvimento de biofilme. Os dados foram avaliados usando ANOVA e teste de Tukey para análise post hoc ($P = 0,05$). Para ensaios com o biopolimero de fibrina, os espécimes foram divididos em 4 grupos: CT (grupo controle), sem tratamento de superfície; revestimento de biopolímero de fibrina (FB); biopolímero de fibrina com *Punica Granatum* (FBPg); biopolímero de fibrina com digluconato de clorexidina (FBCh). As amostras foram inoculadas com *C. albicans* SC5314 ($1x10^7$ células / mL) e incubadas por 24, 48 e 72 horas. Para quantificar a biomassa total do biofilme e as células vivas do biofilme em todas as amostras foram realizados ensaios de UFS

e cristal violeta (CV). Uma análise qualitativa foi realizada utilizando microscopia confocal de varredura a laser. Os dados foram analisados estatisticamente por ANOVA 3 critérios (p <0,05). Ambos os adesivos de ethil cianoacrilato reduziram significativamente a formação de biofilme (P <0,05) em comparação com o controle em todos os períodos avaliados (24, 48e 72 horas). Os grupos FBPg e FBCh apresentaram capacidade inibitória nos biofilmes de *Candida albicans* em todos os períodos e em ambos os materiais avaliados, destacando o FBCh que apresentou melhor desempenho (p <0,05). Todos os grupos apresentaram um aumento no desenvolvimento de biofilme após 72 horas de desenvolvimento de biofilme (p <0,05). Não foi encontrada diferença estatística entre os diferentes materiais a base de PMMA, exceto no grupo FB. Observou-se um potencial para prevenir e controlar a formação de biofilme fúngico sobre PMMA com o revestimento com adesivos a base de ethil cianocrilato e com biopolímero de fibrina incorporado com digluconato de clorexidina e *P. granatum.*

ABSTRACT

The adherence of microorganisms to the base materials of dentures and on oral tissues is a determining factor for microbial colonization. This can trigger infections such as denture stomatitis (DS). The adhesion of microorganisms to the surface of the prosthesis is related to the surface characteristics of the acrylic resin. Therefore, in order to minimize this adhesion, the present study aimed to evaluate whether adhesives based on ethyl cyanoacrylate had an effect on the adhesion of *Candida. albicans* after 7, 14, and 30 days, and whether the incorporation of *Punica Granatum* and chlorhexidine in a fibrin biopolymer derived from snake venom had an effect on the formation of *C. albicans* biofilm after 24, 48, and 72 h on a heat-polymerized and pre-polymerized resin (computer-assisted design/computer assisted manufacturing/Cad-Cam). Circular samples (10 × 2 mm) with a surface roughness of 2-3 μm were produced. For tests with ethyl cyanoacrylate, the specimens were divided into three groups: control (without previous treatment), conventional ethyl cyanoacrylate, and ethyl cyanoacrylate gel. Two layers containing 50 μL of each material were used. Colony-forming units assay was used to quantify the living cells of the biofilm in pre-treated thermopolymerized acrylic resin after 7, 14, and 30 days of biofilm development. The data were evaluated using one-way analysis of variance (ANOVA) and Tukey's post-hoc test for post hoc analysis (P = 0.05). For tests with the fibrin biopolymer, the specimens were divided into four groups: CT (control) without surface treatment, fibrin biopolymer (FB) coating, fibrin biopolymer with *Punica Granatum* (FBPg), and fibrin biopolymer with chlorhexidine digluconate (FBCh). The samples were inoculated with *C. albicans* SC5314 (1 × 107 cells/mL) and incubated for 24, 48, and 72 h. The tests of colony-forming units and violet crystals were used to quantify the total biomass of the biofilm and the living cells of the biofilm in all the samples. Qualitative analysis was performed using confocal laser-scanning

microscopy. The data were analyzed statistically using ANOVA with three factors (p <0.05). Ethyl cyanoacrylate adhesives significantly reduced biofilm formation (P <0.05) compared to the control in all the evaluated periods (24, 48, and 72 h). The FBPg and FBCh groups showed an inhibitory capacity to the biofilms of *C. albicans* in all the periods. Both materials evaluated highlighting the FBCh showed the best performance (p <0.05). All groups showed an increase in biofilm development after 72 h (p <0.05). No statistical difference was found between the different materials based on polymethylmethacrylate (PMMA), except in the FB group. There was a potential to prevent and control the formation of fungal biofilms on PMMA coated with adhesives based on ethil cyanocrilate and fibrin biopolymer incorporated with chlorhexidine digluconate and *P. granatum.*

LISTA DE TABELAS

LISTA DE ABREVIATURA E SIGLAS

FB	Fibrin Biopolymer
FBCh	Fibrin biopolymer with chlorhexidine digluconate
FBPg	Fibrin biopolymer with *Punica Granatum*
CAD	Computer-aided design
CAM	Computer-aided manufacturing
CD	Complete Denture
céls/mL	Cells per milimiliter
°C	Celsius Degrees
CT	Control
DMSO	Dimethylfulfoxide
DS	Denture stomatitis
EtOH 70%	Alcohol 70 percent (Crude Extract)
g	Grams
h	Hours
Kg	Kilograms
kgf	Kilogram force
L	Liters
MeOH	Methanol
mg	Milligrams
mg/mL	Milligrams per milliliter
min	Minutes
mL	Milliliters
mm	Millimeters
PMMA	Poly Methyl Methacrylate

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1 INTRODUCTION

Edentulism is an important public health problem that affects the elderly population (CUNHA et al., 2013; DELLA et al., 2013). Despite the great advances in dentistry, especially in the area of prevention, there are still a large number of edentulous people in Brazil (SILVA; SOUSA, 2006). According to the Ministry of Health, 92% of elderly Brazilians are edentulous, and for adults (35 to 44 years old), this rate accounts for 68% of the general population. Thus, it is estimated that 16 million people are users or require dental prostheses in Brazil (BRASIL, 2010). In addition, more than half of this edentulous population seek complete or partial removable prostheses as their treatment options (FONSECA et al., 2013).

Aging, despite being a natural process, submits the body to several changes that require special attention for the maintenance of general and oral health. Denture stomatitis (DS) is a chronic disease that is the most prevalent oral condition among prosthetic patients. It is frequently associated with the elderly (MUJICA et al., 2008; LANGLAIS et al., 2002; TAIWO et al., 2009), defined by a chronic localized or generalized inflammatory lesion in the oral mucosa. It is characterized by the presence of an erythematous area and is usually asymptomatic (BUDTZ-JORGENSEN, 1974; ALTARAWNEH et al., 2013; PEREIRA et al., 2013). This pathology is related to several etiological factors, such as poor oral and prosthetic hygiene, trauma caused by complete dentures, continuous use of a prosthesis, allergy to residual monomer, use of maladaptive prostheses, systemic factors such as diabetes, xerostomia, immunosuppression, and mainly, the presence of *Candida albicans* in the form of a microbial biofilm, adhered and retained on the internal surface of the denture base

(BUDTZ-JORGENSEN, 1978; KOREG, 1991; IACOPINO; WATHEN, 1992; ALLEGRA; GENNARI, 2000; ESPINOZA et al., 2003; HUANG; WU, 2005; PETERSON et al., 2005).

The adhesion of *C. albicans* is essential for the initiation of inflammatory processes and the formation of biofilms (SPRATT et al., 2003; RAMAGE et al., 2004). The adhesion process involves a complex and varied mechanism. This is influenced by the exposed medium, virulence of the pathogen, and the physico-chemical characteristics of the prosthetic surface (ALI; ALHARBI; SURESH, 2013; AL-DWAIRI; AL QUIRAN; AL OMARO, 2012; BOURLIDI et al., 2016; CALDERONE; FONZI et al., 2001; PEREIRA-CENCI et al., 2007; HAHNEL et al., 2010; RODRIGUEZ ACOSTA et al., 2015). The relationship between microorganisms and denture bases has been widely studied. It was determined that the surface roughness and hydrophobicity of the substrate directly influence the adhesion of *C. albicans* (BULAD et al., 2004; NIKAWA et al., 1992).

Despite the development of dental materials, polymethylmethacrylate (PMMA) is used extensively in the manufacture of denture bases because of its low cost, acceptable esthetics, ease of handling, and low weight (RODRIGUEZ et al., 2013). However, one of its main limitations is associated with its surface characteristics, which are porous and rough because the internal surface is not usually subjected to the finishing and polishing processes. This is an important factor because polishing can influence the adaptation, settlement, and retention of the complete denture. This allows the adhesion and penetration of pathogens inside the prosthesis (RAHAL et al., 2004; PEREIRA CENCI et al., 2007). In addition, these pathogens have the ability to use these irregularities as niches to protect themselves against the shear forces produced during swallowing, the self-cleaning effect of saliva, and the cleaning process of the prostheses. This makes their removal even more difficult (RAMAGE et al.,

2004). Furthermore, the acidic and anaerobic environment found between the base of the prosthesis and the mucosa provides an ideal reservoir for the growth and dispersion of the *C. albicans* biofilm (BUDTZ-JORGENSEN, 1974; MAHONEN; VIRTANEN; LARMAS, 1998).

Several methods for the treatment of DS have been proposed in the literature. Traditionally, topical and/or systemic antifungal therapies have been indicated. However, despite being able to relieve the signs and symptoms of DS (BANTING; HILL, 2001), these drugs do not reach the internal surface of the contaminated prosthesis properly (BARBEAU et al., 2003). Topical antifungals, in contrast, might cause allergic reactions and nausea due to their extremely unpleasant taste (NEVILLE et al., 2009). Therefore, this can lead to treatment interruption by prosthesis users. Both therapies are associated with recurrence (QUIRYNEN; BOLLEN 1995; BABEAU et al., 2003).

The use of chemical agents and microwave irradiation for cleaning dentures has been highlighted as an effective means of cleaning. These act as adjuncts to brushing to control bacterial plaque and prevent DS associated with *Candida* colonization (SESMA et al., 1999; NEPPELENBROEK et al., 2008). However, these cleaning mechanisms can cause deleterious effects on the base material of the prosthesis, thus, causing damage to the physical and mechanical properties and leading to a decreased durability (POLYCHRONAKIS et al., 2018; POLYCHRONAKIS et al., 2014).

The development of DS is directly related to the adhesion of microorganisms to the internal surface of the prosthesis. Thus, several alternative treatments have been directed toward the modification of the surface characteristics of biomaterials in order to reduce colonization by biofilms. The incorporation of antimicrobial agents in the acrylic resin used as the base of the

prosthesis have been evaluated and demonstrated to have a promising antifungal effect (SRIVATSTAVA et al., 2013; AMORNVIT et al., 2014; SHARMA AND HEDGE, 2014). However, this incorporation influenced the mechanical properties and a color change in the acrylic resin (NAWASHI; GAD; EL ZAYAT, 2018; GAD et al., 2018). In addition, the application of coatings on the base of the prosthesis have been evaluated. It provided biocompatibility through a very thin and smooth coating layer, such as silica (AZUMA et al., 2012) and silane-SiO2 nanocomposite (YODMONGKOL et al., 2014) and cyanoacrylates (ALI; ALHARBI; SURESH, 2013; TAVORA et al., 2018), showing promising results and significantly reduced the adhesion of *C. albicans.*

Given the promising results previously mentioned, this study investigated the longitudinal effect of conventional ethyl cyanoacrylate and gel on *C. albicans* biofilms and the effect of the incorporation of antimicrobials into the fibrin biopolymer on the *C. albicans* biofilm on a heat-cured and pre-cured (computer-assisted design/computer assisted manufacturing/CAD/CAM) resin surface.

2. ARTICLES

The present research allowed the drafting, discussion and production of two manuscripts,

which have been submitted to the journal detailed:

1. **Longitudinal Evaluation of Ethyl Cyanoacrylate Adhesives on *Candida albicans* Biofilm**. Inidan Journal of Dental Research. N 1998-3603 Article code number and Journals´s Index in Annex 1.

2. **Fibrin biopolymer incorporated with antimicrobial agents: A proposal for coating denture bases.** Materials. N 1996-1944. Article code number and Journals´s Index document in Annex 2.

Article 1

Title of the article: **Longitudinal Evaluation of Ethyl Cyanoacrylate Adhesives on**

Candida albicans **Biofilm**

Abstract:

Context: The adhesion and development of *Candida albicans* biofilm is strongly related to the surface characteristics of the material base. Applying cyanoacrylate adhesives on the fitting surface of the denture could potentially change its surface properties and be an effective alternative for preventing and control denture stomatitis.

Aims: To evaluate the effect of two ethyl cyanoacrylate-based adhesives on the growth of *Candida albicans* biofilms on a heat-polymerized resin, after 7, 14, and 30 days of exposure.

Settings and Design: Laboratory setting and experimental design.

Methods and Material: Ninety circular (10×2 mm) heat-polymerized resin specimens were equally divided into three groups: control, conventional ethyl cyanoacrylate (ECAc), and ethyl cyanoacrylate gel (ECAg). Two layers of 50 µL of each material were applied to the respective groups. *C. albicans* SC5314 strain was activated and standardised to 10^7 cells/mL^{-1}. Specimens were immersed in 1 mL of artificial saliva and deposited in 1 mL fungal suspension, washed, and immersed in 1 mL of RPMI for 7, 14, and 30 days. The medium was changed at 48-hour intervals. The final suspension was diluted (10^{-1} to 10^{-4}) and deposited on Sabouraud dextrose agar for 48 h at 37 °C. After this period, the colonies were quantified using the CFU/mL calculation.

Statistical analysis used: Data were evaluated using one-way ANOVA and Tukey's test for post-hoc analysis ($P=0.05$).

Results: Both adhesives significantly reduced ($P<0.05$) biofilm formation compared to the control at all evaluated periods.

Conclusions: An immediate and long-term inhibitory effect on *C. albicans* biofilm formation was observed.

Key Messages: A commercially available low-cost cyanoacrylate-based adhesives showed long-term inhibitory effects on *C. albicans* biofilm formation and can be an alternative on therapy for denture stomatitis.

Introduction:

Polymethylmethacrylate (PMMA) has been used for the fabrication of complete denture bases for many decades. It has advantages such as ease of handling and repair, low cost, low weight, and acceptable aesthetics[1,2]. However, one of the major disadvantages of PMMA is the material porosity and surface roughness, which combined with the oral environment, allows for pathogenic biofilm attachment to the denture base. Thus, an acidic environment favourable to the growth and development of yeasts such as *Candida albicans* is created, leading to oral infections such as denture stomatitis (DS)[3,4].

The treatment of DS commonly involves systemic and topical medication, repackaging, hygiene, and disinfection instructions[5]. However, systemic drugs can cause nephrotoxic and hepatotoxic effects[6]. Topical agents such as nystatin and miconazole are widely used for the treatment of DS. However, they have an unpleasant taste that prevents the daily use of the complete denture. Moreover, their use prolongs treatment as the drug must be in direct contact with the mucosa[7,8]. Prolonged use of disinfectants such as sodium hypochlorite 1% and chlorhexidine digluconate 2% can cause damage to the physical properties of acrylic resin, thus decreasing its longevity[9,10]. Hence, studies are now focussed on new approaches to improve the denture base surface in order to control and/or prevent biofilm formation without compromising oral health and material properties.

Authors have reported the coating of PMMA surfaces[11,12] with cyanoacrylate-based adhesives as beneficial against *C. albicans* development. Ali et al.[12] studied the effects of octyl-cyanoacrylate adhesives coated on plates of heat-cured acrylic resin and reported complete inhibition of fungal adhesion. Among cyanoacrylate adhesives, ethyl cyanoacrylate has shown promising results against *C. albicans*. It stands out because of its low cost and bactericidal effect. Távora et al.[11] studied *C. albicans* growth on relining acrylic resin coated

with ethyl-cyanoacrylate (Superbonder) and verified that this adhesive reduced the initial fungal development.

In this context, the possibility of applying cyanoacrylate-based adhesives as coatings on acrylic resins was considered, thus modifying its surface properties, reducing roughness, and increasing hydrophobicity[11]. However, no study has assessed its long-term efficacy. Therefore, the inhibitory effect of two ethyl cyanoacrylate adhesives on *C. albicans* biofilm was evaluated after 7, 14, and 30 days in this study.

Subjects and Methods:

Fabrication of specimens

A total of 90 circular specimens (10 × 2 mm) were fabricated using a heat-polymerized acrylic resin (Vipi Cril Plus, Vipi Indústria, Comércio, Exportação e Importação de Produtos Odontológicos, Pirassununga, SP, Brazil) following the manufacturer's instructions. Subsequently, one surface of each specimen was roughened using a polishing machine (PFL, Fortel Indústria e Comércio, São Paulo, SP, Brazil) with 120-grit sandpaper (Norton Abrasivos, São Paulo, SP, Brazil) for 15 s. The surface roughness of all specimens was measured using a roughness tester (Surftest SJ-301, Mitutoyo Corporation, Kanagawa, Japan). Readings were taken at four different positions on the roughened surface[13] and standardised to 2-3 µm to simulate the inner surface of a complete denture[14]. Subsequently, the specimens were immersed in 2 mL of distilled water at 37 °C for 48 h to allow the release of the residual monomer (ISO 1567, International Organization for Standardization, 1988) and were further sterilised using ethylene oxide (Acecil, Central de Esterilização de Comércio e Indústria Ltda., Campinas, São Paulo, Brazil)[15]. Finally, the specimens were randomly distributed into three groups: control or without coating (GC), coated with conventional ethyl cyanoacrylate (ECAc, G1), and coated with ethyl cyanoacrylate gel (ECAg, G2) (Table 1).

Surface treatment of specimens

The experimental specimens were coated with 50 µL of adhesive (ECAc or ECAg), which was equally distributed across the surface using a disposable brush tip (Disposable Brush Tips/60, 3M ESPE, St Paul, Minnesota, USA). Afterwards, the specimens were dried at room temperature for 40 min. This procedure was repeated to obtain a second layer of coating[11].

Yeast strain and growth conditions

Initially, *C. albicans* (strain SC5413) were incubated in tryptic soy broth (TSB) (Accumidia manufactures Inc., Lansing, MI, USA) with 1% chloramphenicol (Quemicetina Succinato, Carlo ErbaR, Milano, Mi, Italy) at 30 ºC for 24 h under aerobic conditions. Afterwards, the suspension was centrifuged at 5000 rpm for 10 min at 22 °C. Cells were harvested and washed with phosphate-buffered saline (PBS, pH 7.2) and standardised to 1×10^7 cell/mL^{-1} [15].

Biofilm development

Specimens were exposed to 1 mL of artificial saliva for 2 h at 37 °C using a 24-well tissue culture plate (Cell Culture Plate, Nest Biotech Co., Ltd., China)[14]. Subsequently, the specimens were immersed in 1 mL of the previously standardised *C. albicans* cell suspension and incubated for 90 min at 37 °C at 75 rpm. The non-adherent cells were removed from the specimens by washing with 1 mL of PBS. Finally, the specimens were immersed in 1 mL of Roswell Park Memorial Institute solution (RPMI-1640, GibcoR, Grand Island, NY, USA) for 7, 14, and 30 days at 37 °C at 75 rpm. During these periods, the medium was changed at 48-hour intervals[15].

Viable cell count (CFU/mL)

For all groups, three independent experiments were performed to measure the number of viable *C. albicans* cells adhering to the specimens. After each period (7, 14, and 30 days), the specimens were removed from the culture plate and washed with PBS in another 24-well tissue culture plate. Afterwards, the biofilm was removed from the surface of the specimens with a sterilised cell scraper and the cells were stored in 1 mL of PBS, and the suspension was serially diluted (10^{-1} to 10^{-4}). Subsequently, 50 µL of these dilutions were inoculated into

a Sabouraud agar plate and incubated for 48 h at 37 °C. After this period, the mean values obtained indicated the growth of biofilms in CFU/mL and all colonies were counted and expressed as CFU/mL[15].

Statistical methods

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 23. Additionally, to compare biofilm formation between groups and their correlation with coating material performance, a one-way ANOVA test was used, followed by the Tukey's correction test for multiple comparisons *(P=0.05)*.

Results:

According to the results, cyanoacrylate adhesives showed a marked inhibitory effect on microbial biofilm formation with statistically significantly lower values compared to the control group: ECAc *(P=0,00)* and ECAg *(P=0,00)* (Table 1).

Considering the evaluated periods, the cyanoacrylate groups did not show statistically significant differences. However, the control group showed a significant increase in the colony count during the 14- and 30-day periods *(P<0,05)* (Table 1).

Table:

Table 1. Mean values and Standard Deviation (SD) in number of Colony-Forming Units (CFU) of *Candida albicans* over the evaluation periods of 7, 14 and 30 days.

Groups	Periods	CFU/mL
GC	7	$1445,71 \pm 452,65 \ (10^3)$ aA
	14	$4794,29 \pm 997,78 \ (10^3)$ bA
	30	$4854,29 \pm 1134,53 \ (10^3)$ bA
ECAc	7	$32,86 \pm 17,28 \ (10^3)$ B
	14	$38,29 \pm 18,49 \ (10^3)$ B
	30	$290 \pm 129,31 \ (10^3)$ B
ECAg	7	$22,57 \pm 18,17 \ (10^3)$ B
	14	$180,29 \pm 97,33 \ (10^3)$ B
	30	$466 \pm 383,21 \ (10^3)$ B

GC (control group), ECAc (ethyl-cyanoacrylate), and ECAg (ethyl-cyanoacrylate formulation gel). Different lowercase letters at the end of the lines indicate a statistical difference between the experimental periods (P<0.05). Different capital letters at the end of the lines indicate a statistical difference between the groups (P<0.05).

Discussion:

The cyanoacrylate adhesives evaluated in this study have been successfully used in surgical procedures, both in medicine and dentistry[16;17] for the control of hemorrhages[18]. Recently, cyanoacrylates and fibrin biopolymers have also been studied for use as denture coating adhesives[11,12,19].

This study established that ECAc and ECAg showed promising long-term results in inhibiting *C. albicans* biofilm formation. These findings agree with other studies reported in the literature. Távora et al.[11] verified the lower adhesion of *C. albicans* to acrylic resin treated with different cyanoacrylate adhesives, including conventional ethyl cyanoacrylate and formulation gel. Similarly, Ali et al.[12] highlighted the inhibitory effects of octyl cyanoacrylate coated prostheses on *C. albicans* biofilms after 24 h of exposure.. This may be associated with the changes in surface energy and roughness (*the main factors for the development of C. albicans* biofilm on dentures) of acrylic resins that represent niches for *C. albicans* biofilm adhesion. Moreover, the cyanoacrylate adhesives tested in this study contained the compound methyl 2-cyanoacrylate ($C_5H_5NO_2$), which could be another important factor in inhibiting *C. albicans* biofilm development[19].

In addition, some authors attribute this microbial reduction to the release of formaldehyde and cyanoacetate compounds during its degradation. These by-products could trigger antifungal activity because of their cytopathic effect[20,21] that tends to decrease after 14 days and remains low for 28 days[19].

The degradation of cyanoacrylates is related to the length of the side chain; the smaller the side chain, the faster the degradation [22-24]. In a previous study, the inhibitory effect of two short-chain adhesives was evaluated, with evidence indicating that they degrade rapidly. Thus, it was suggested that with the decrease in the cytopathic reaction, *C. albicans* could adapt or colonise the surface as the coating gradually degraded. However, this was not

observed in the present study, as the development of the *C. albicans* biofilm significantly reduced for up to 30 days after the application of ethyl cyanoacrylate coating.

The cyanoacrylate groups presented no statistically significant difference at all evaluated periods, although the ECAc presented a slightly better performance. This may be associated with the low viscosity of the ECAc liquid, which seeps in filling up the porosities and cracks, thus providing a smooth and regular surface.

The results obtained in this study are promising as cyanoacrylate adhesives showed long-term inhibitory effects on *C. albicans* biofilm formation; however, the lack of information regarding their resistance to biological changes and sustainability against chemical and mechanical challenges (commonly caused during the disinfection of a complete denture) warrants further investigation.

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Article 2

Fibrin biopolymer incorporated with antimicrobial agents: A proposal for coating denture bases

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Abstract: The characteristics of the denture base surface in combination with the oral environment, promote the colonization and development of *Candida albicans* biofilm, which is the main cause of denture stomatitis, thus this study evaluates the effectiveness of fibrin biopolymer with digluconate chlorhexidine or *Punica granatum* alcoholic extract to prevent *C. albicans* biofilm. Conventional heat polymerized and pre-polymerized PMMA circular specimens (10×2 mm) were divided into groups: no treatment (control - CT), fibrin biopolymer coating (FB), fibrin biopolymer with *P. granatum* (FBPg), or digluconate of chlorhexidine (FBCh) coating. The specimens were inoculated with *C. albicans* SC5314 (1×10^7 cells/mL) and incubated for 24, 48, and 72 h. Crystal violet and colony-forming unit assays were used to quantify the total biofilm biomass and biofilm-living cells. A qualitative analysis was performed using confocal laser scanning microscopy. The FBPg and FBCh groups inhibited the growth of *C. albicans* biofilm at all periods and in both PMMA materials, with FBCh performing better ($P<.05$). All groups showed an enhancement in biofilm development up to 72 h ($P<.05$). No statistical difference was found between the PMMA base materials ($P>.05$), except in FB group. Coating using fibrin biopolymer and digluconate chlorhexidine or *P. granatum* could be used to prevent and control the formation of *C. albicans* biofilm on denture bases.

Keywords: *Candida albicans*; Denture stomatitis; Complete denture; Acrylic resin; CAD-CAM.

1. Introduction

Denture stomatitis (DS) is a chronic inflammatory condition that affects 15 to 70% of denture wearers and is mainly related to inflammation caused by *Candida albicans* of the palatal mucosa supporting the denture [1,2,3]. *C. albicans* can colonize and develop a biofilm on the inner surface of the dentures, a process influenced by the hydrophobicity, surface free energy, and surface roughness of the poly(methyl methacrylate) (PMMA) resins[4,5].

The adhesion of *C. albicans* to the denture base is a critical factor in the development of DS [6,7]. This adhesion occurs through the colonization of microorganisms to the denture surface. These act as protective reservoirs that inhibit Candida from being removed by the self-cleaning effect of saliva, mechanical cleaning, or dislodgment forces [8]. This yeast has significant proliferation capacity, especially for immunocompromised patients [9].

Conventional heat-cured PMMA resin is the most widely used material for denture bases, and it has been proven that the processing method used increases the porosity of the acrylic surface [10]. In addition to conventional PMMA, newer processing systems for obtaining denture bases have been reported, such as computer-aided design and computer-aided manufacturing (CAD-CAM) PMMA-based polymers[11-13]. CAD-CAM-fabricated complete dentures have several promising advantages, including a decrease in porosity as the polymer base is formed from a pre-polymerized block of acrylic resin industrially polymerized under protocol conditions at high heat and pressure [13-15]. Moreover, denture bases fabricated using CAD-CAM release a small amount of monomer, which may affect microbial adhesion [16]. The internal surface of the denture bases in both PMMA processing methods are not polished, which may affect the roughness threshold, favoring *Candida* biofilm adhesion [17,18].

DS treatment usually involves reinforcing oral hygiene, the administration of local and systemic antifungals, and chemical solutions [2,19,20]. However, these approaches may have certain setbacks such as local and systemic side effects, antifungal resistance, and deleterious effects on the physicomechanical properties of PMMA, which can lead to a recurrence of DS [2,20]. Thus, it is necessary to develop new alternatives to promote anti-adherent and antimicrobial activity on PMMA substrates.

In this context, a growing interest in medicinal plants has been recently observed, and their therapeutic and preventive effects have been reported, including for DS [2,21,22]. The pomegranate (*Punica granatum*) is a fruit that is commonly consumed fresh or in beverages and has high antioxidant activity [22]. Furthermore, its alcoholic extract has already been proven to possess antifungal activity against *Candida* species [2,19,22-24]. Almeida et al. [2] incorporated *in vitro* anti-*C. albicans*, an alcoholic extract of *P. granatum,* into a denture adhesive. Furthermore, a study by Esawy et al. [25] showed that mouthwashes with fractions of *P. granatum* extract were effective as anti-calculus and anti-hemorrhagic agents.

In addition, coating materials have also been suggested as an alternative to promote changes in the topography and physicochemical characteristics of the PMMA, resulting in it being less prone to the adhesion and growth of *C. albicans*, such as synthetic tissue adhesives, specifically cyanoacrylates (CA) [1,4,26,27]. However, the high cost and controversial biocompatibility of CA continue to be issues [28].

More recently, a new fibrin sealant composed of fibrinogen-rich cryoprecipitate, extracted from *Bubalus bubalis* buffalo blood, and a thrombin-like enzyme, purified from the venom of *Crotalus durissus terrificus,* has been considered as a promising material in medicine and dentistry [29-33]. This biopolymer has biocompatibility, low-cost to produce in addition to hemostatic, sealant, adhesive, scaffold, and drug delivery properties [29-36]. In the presence of calcium, the trombin-like enzyme acts on fibrinogen molecules, turning them into fibrin monomers, resulting in a stable clot [33-35]. In dentistry, Barbosa et al. [32,37] and Chiquito et al. [38] reported that using FB (fibrin biopolymer) to immobilize free gingival grafts is as effective as conventional sutures. In addition, it promoted advanced healing and reduced inflammatory cell density. However, its application as a coating material for complete dentures has not yet been elucidated.

According to literature the incorporation of antimicrobial agents into coating materials has demonstrated promising results, providing clinical advantages as a useful strategy to avoid microbial colonization [39-41]. Redding et al. [39] evaluated the effectiveness of incorporating chlorhexidine diacetate, amphotericin B, and nystatin into a thin-film polymer. Although all antifungals promoted satisfactory biofilm inhibition, the chlorhexidine diacetate group presented significantly better results. Therefore, considering the lack of studies and the necessity of new alternatives to treat and prevent DS, the aim of this study was to evaluate the effectiveness of a new fibrin sealant incorporated with digluconate chlorhexidine or *P. granatum* alcoholic extract against *C.*

albicans biofilm using two different PMMA base materials. The null hypothesis was that the different coating conditions would not affect the formation of *C albicans* biofilm.

2. Materials and Methods

2.1. Plant material and extract preparation

P. granatum fruit were purchased at the 'Boa Fruta' fruit and seedling distributor supermarket. The fruit was cultivated in Petrolina, Pernambuco, Brazil (9°4630'S, 24°2130′W). For this study, crude powder extract produced from the peel of the *P. granatum* fruit was used. The peels were dried in an air circulation oven at 45 °C (Drying Kiln with Renovation/Air Circulation Marconi N480 Novus; Marconi Ltda) and crushed in a knife mill (MA340 - Macro Moinho de Willey Knives; MarconiLtda) [2]. For the extraction of its compounds, percolation was performed at room temperature. This produced a hydroalcoholic extract [2]. The concentrated solution was subjected to a rotary evaporator (Bath Model Hei Vap Precision G3; Heidolph) under reduced pressure and at temperatures below 50 °C to remove the organic solvent. Finally, the crude extract was lyophilized in its powder form [22,42].

2.2. Specimen preparation

A total of 468 circular specimens (10×2 mm) using a pre-polymerized resin (Vipi block gum; Vipi) and heat polymerized acrylic resin (Vipi Cril Plus; Vipi) were prepared in accordance with the manufacturer's instructions. To obtain a highly rough surface compatible with the growth of microorganisms (2-3 μm range), each specimen had one surface sanded using a polishing machine (PFL; Fortel) with 120-grit sandpaper (Norton Abrasivos) for 15s [27,43]. Surface roughness was measured using a roughness tester (Hommel Tester T 1000 basic; Hommelwerke GmbH), and four readings were taken at different positions on the roughened surface of each specimen [44]. Subsequently, the specimens were immersed in 2 mL of distilled water at 37 °C for 48 h to allow for the release of residual monomer (ISO 10139-2, International Organization for Standardization, 1999) and were sterilized using ethylene oxide (Acecil Ltda) [44,47]. Finally, the specimens were randomly distributed into four groups: without coating or control group (CT), coated with FB only, coated with biopolymer fibrin incorporated with chlorhexidine (FBCh) or *P. granatum* (FBPg).

2.3. Surface treatment of the specimens

The fibrin biopolymer was supplied by the Center for the Study of Venoms and Venomous Animals from São Paulo State University, Botucatu, São Paulo, Brazil. The components and formula of the product are covered by patents (registry numbers: BR1020140114327 and BR1020140114360) and in these reviews [31,34,35].

The concentrations of the tested antimicrobials that were incorporated into the fibrin biopolymer were established according to a pilot study and corresponded to 20 mg/mL of *P. granatum* and 4 mg/mL of chlorhexidine (data not shown). The biopolymer is available in three solutions: fraction 1 (0.400 mL) - Crotalus durissus terrificus thrombin-like enzyme, fraction 2 (1 mL)-, buffalo cryoprecipitate solution, and diluent (0.600 mL) - containing calcium chloride. These compounds were kept frozen and thawed prior to use. Then, the powdered medicines were incorporated into the biopolymer until a homogeneous mixture was formed.

The experimental specimens were coated with 50 μL of each experimental product, which was equally distributed across the surface using a disposable brush tip (Disposable Brush Tips/60; 3M ESPE). Afterward, the specimens were dried at room temperature for 40 min.

2.4. Yeast strain, growth conditions and biofilm development

C. albicans (strain SC5413) frozen culture stocks (-80 °C) were incubated in tryptic soy broth (Accumidia manufactures Inc) with 1% chloramphenicol (Quemicetina Succinato) at 30 °C for 24 h under aerobic conditions. Subsequently, the suspension was centrifuged at 5000 rpm for 10 min at 22 °C and the cells were harvested and washed with phosphate-buffered saline (PBS, pH 7.2) and standardized to 1×10^7 cell/mL^{-1} in PBS using a hemocytometer [43,46].

To form the biofilm, all acrylic resin specimens were carefully washed with PBS and immersed in 1 mL of the previously standardized cell suspension and incubated for 90 min at 37 ℃, at 75 rpm. Then, the specimens were washed in 1 mL of PBS to remove non-adherent organisms and immersed in 1 mL of Roswell Park Memorial Institute solution (RPMI-1640; GibcoR) for 24, 48, and 72 h at 37 °C (75 rpm). The medium was changed at 24-h intervals [2,43].

2.5. Viable cell count (colony-forming units (CFU)/mL)

After each evaluation period, the specimens were gently washed in 1 mL of PBS. The biofilm was removed from the specimen surface with the aid of a cell scraper (Costar® 3010; Corning) and stored in 1 mL of PBS [2,47]. These suspensions were serially diluted (10^{-1} to 10^{-4}), and aliquots (50 μL) of each dilution were plated in triplicate on

Sabouraud Dextrose agar (Accumedia Manufacturers) and incubated for 48 h at 37°C [48]. After this period, the colonies were counted and expressed as mean CFU/mL values.

2.6. Total biomass of the C. albicans biofilm

After the formation of the biofilm, the non-adherent cells were removed by washing the specimens in 1 ml of PBS in each well. Then, the biofilm formed on the rough surface was fixed with 1 mL of 99% methanol (Merck Millipore) for 15 min, dried at room temperature, and immersed in 2 mL of 0.1% violet crystal (VC) solution (Sigma-Aldrich) for 20 min. The specimens were then washed with distilled water to remove the excess VC [49]. To dissolve the stain, the specimens were immersed in 2 mL of 95% ethanol (Synth) [50], and an aliquot of 100 μL was transferred to a 96 wells microtiter plate for spectrophotometer reading, programmed with a wavelength of 570 nm [43, 51].

2.7. Confocal laser scanning microscopy

After 24, 48, and 72 h of incubation, 36 specimens were transferred to a sterile 24-well plate and carefully washed with PBS. In sequence, in the absence of light, the specimens were stained with LIVE/DEAD® BaclightTM L7007 Kit (Molecular Probes; Invitrogen Ltd) at 1% for 20 min at 37 °C. The stained *C. albicans* biofilm remaining on the acrylic resin surface was qualitatively analyzed using confocal laser scanning microscopy (CLSM) (TCS-SPE; Leica Mycrosystems) [1].

2.8. Statistical analysis

Data obtained from the CFU and VC assays are presented as means and standard deviations and were statistically analyzed using a 3-way analysis of variance (α=.05).

3. Results

3.1. CFU assay

Data from the CFU assay is shown in Tables 1 and 2. The chlorhexidine group incorporated in the fibrin biopolymer (FBCh) exhibited high inhibitory values in all of the materials studied, showing statistically significant differences when compared with the CT, FB, and FBPg groups (P =.00). In addition, it can be confirmed that this material has an inhibitory capacity of up to 72 h of exposure in microbial biofilm, with no significant differences between the periods evaluated (P=.275) in all of the materials studied (Tables 1 and 2).

At 24 h, it was evident that the FBPg group was not statistically different (P<.05) than the control group in all of the materials studied (P>.05). However, at 48 and 72 h, this group showed inhibitory capacity when compared to the control group (P<.05) in all of the materials studied. FB favored *C. albicans* biofilm growth compared to the control group at 24 and 48 h in all of the materials evaluated (P<.05). However, at 72 h, these differences were not significant (P>.05; Tables 1 and 2).

Comparison among materials in the evaluation periods revealed significant differences between heat polymerized and CAD-CAM resins when BF coating was applied (P=.01).

Table 1. Mean values of CFU/mL (10^3) ±standard deviations in heat polymerized resin

Heat polymerized resin	Experimental periods			Mean ± DP
	24h	**48h**	**72h**	
CT	1304.44 ±500.07 Aa	1726.44 ±386.45 Aa	3144.44 ±474.11Ba	**2058.44 ±453.55**
FB	2297.78 ± 613.55 Ab	2557.78 ±758.19 Ab	3220 ±424.15 Ba	**2691.85 ±598.63**
FBPg	891.11 ±178.36 Aa	1277.78 ±196.58 Ac	2186.67 ±220.45 Bb	**1451.85 ±198.46**
FBCh	0.0 ±0.0 c	0.07 ±0.2d	0.02 ±0.07c	**0.03 ±0.09**
				Groups
Mean ± DP	**1123.33 ±322.99 (10^3)**	**1390.52 ±335.35(10^3)**	**2137.78 ±279.69(10^3)**	
				Periods

CT: Control group; FB: Fibrin biopolymer; BFPg: Punica granatum incorporated in fibrin biopolymer; BFCh: chlorhexidine incorporated in fibrin biopolymer. Different capital letters indicate a statistical difference between the experimental periods (P<.05). Different lowercase letters indicate a statistical difference between the groups (P <.05).

Table 2. Mean values of CFU / mL (10^3) ±standard deviation in CAD / CAM resin

Pre-cured resin (CAD/CAM)	Experimental periods			Mean ± DP
	24h	**48h**	**72h**	
CT	1271.11 ±315.73 Aa	1504.44 ±458.89 Aa	2664.44 ±402.71 Ba	**1813.33 ±392.44**
FB	1842.22 ±410.18 Ab	1753.33 ±597.99 Ab	3044,44 ±434.14 Ba	**2213.33 ±480.77**

	24h	48h	72h	Mean ± DP
FBPg	888.89 +251.62 Aa	1053.33 +190.79 Ac	1753.33 +410.97 Bb	**1231.85 +284.46**
FBCh	0.02 +0.07 c	0.02 +0.07 d	0.09 +0.2 c	**0.04 +0.11**
Mean ± DP	**1000.56 +244.39 (10^3)**	**1077.78 +311.94 (10^3)**	**1865.58 +312.01(10^3)**	Groups
				Periods

CT: Control group; FB: Fibrin biopolymer; FBPg: Punic granatum incorporated in fibrin biopolymer; FBCh: chlorhexidine incorporated in fibrin biopolymer. Different capital letters indicate a statistical difference between the experimental periods ($P < .05$). Different lowercase letters indicate statistical difference between the groups evaluated ($P < .05$).

3.2. Metabolic activity test (VC)

Cellular accounting, expressed in absorbance values, is described in Tables 3 and 4. Significant differences were observed for both the factors "groups" and "periods" ($P < .00$). However, no statistical significance was found for the factor "material" ($P > .83$). Inhibitory effects were observed for the FBPg and FBCh groups when compared to the control group ($P < .05$), with FBCh having the lowest values. However, there were no significant differences between the CT and FB groups ($P > .05$)

Regarding the factor "period," a significant increase in the biofilm adhered to the surface was observed at 72 h in all the groups evaluated ($P < .00$).

Table 3. Mean absorbance values ± standard deviations (nm) of the metabolic activity of the *C. albicans* biofilm for the adhesives tested on specimens made with conventional heat polymerized resin

Heat polymerized resin	Experimental periods			Mean ± DP
	24h	48h	72h	
CT	1576.56 +850. 56	1897.67 +702.62	2763.444 +387.19	**2079.23 +646.79 a**
FB	1879.78 +702.88	2003.44 +776	2102.333 +1179.4	**1995.18 +886.09 a**
FBPg	1153.44 +647.59	1470.56 +591.57	1598.333 +672.35	**1407.44 +637.17 b**
FBCh	110 +2.74	120.11 +4.76	127.889 +5.71	**119.33 +4.4 c**
Mean ± DP	**1179.95 +550.94 A**	**1372.95 +518.74 A**	**1647.99 +561.16 B**	Groups
				Periods

CT: Control group; FB: Fibrin biopolymer; FBPg: Punic granatum incorporated in fibrin biopolymer; FBCh: chlorhexidine incorporated in fibrin biopolymer. Different capital letters indicate a statistical difference between the experimental periods ($P < .05$). Different lowercase letters indicate statistical difference between the groups evaluated ($P < .05$).

Table 4..Average absorbance values ± standard deviations (nm) of the metabolic activity of the *C. albicans* biofilm for the adhesives tested on specimens made with CAD / CAM resin

Pre-cured resin (CAD/CAM)	Experimental periods			Mean ± DP
	24h	48h	72h	
CT	1591.778 +583.75	1475.667+612.7	2126.556 +1204.09	**1731.33 +800.18 a**
FB	1925.889 +438.85	1777.667 +685.53	2643.333 +340	**2115.63 +488.13 a**
FBPg	1500.778 +177.55	1330.222 +520.12	1807.111 +976.89	**1546.04 +558.19 b**
FBCh	136.222 +2.28	126 +14.16	141.333 +12.37	**134.52 +9.6 c**
Média ± DP	**1288.67 +300.61 A**	**1177.39 +458.13 A**	**1679.58 +633.34 B**	Groups
				Periods

CT: Control group; FB: Fibrin biopolymer; FBPg: Punic granatum incorporated in fibrin biopolymer; FBCh: chlorhexidine incorporated in fibrin biopolymer. Different capital letters indicate a statistical difference between the experimental periods ($P < .05$). Different lowercase letters indicate statistical difference between the groups evaluated ($P < .05$).

3.3. Qualitative confocal analysis

After 24 h of incubation, the control counterpart showed a higher proportion of uniform and elongated *C. albicans* yeast arranged in clusters (Figure 1 (a), (e)). Similarly, at the same time point, the FB group was densely populated with uniform yeast (Figure 1 (b), (f)). In contrast, growth suppression of *C. albicans* by FBCh (Figure 1(d), (h)) and FBPg (Figure 1 (c), (g)) was seen with LIVE/DEAD staining, confirming the CFU and VC assay data.
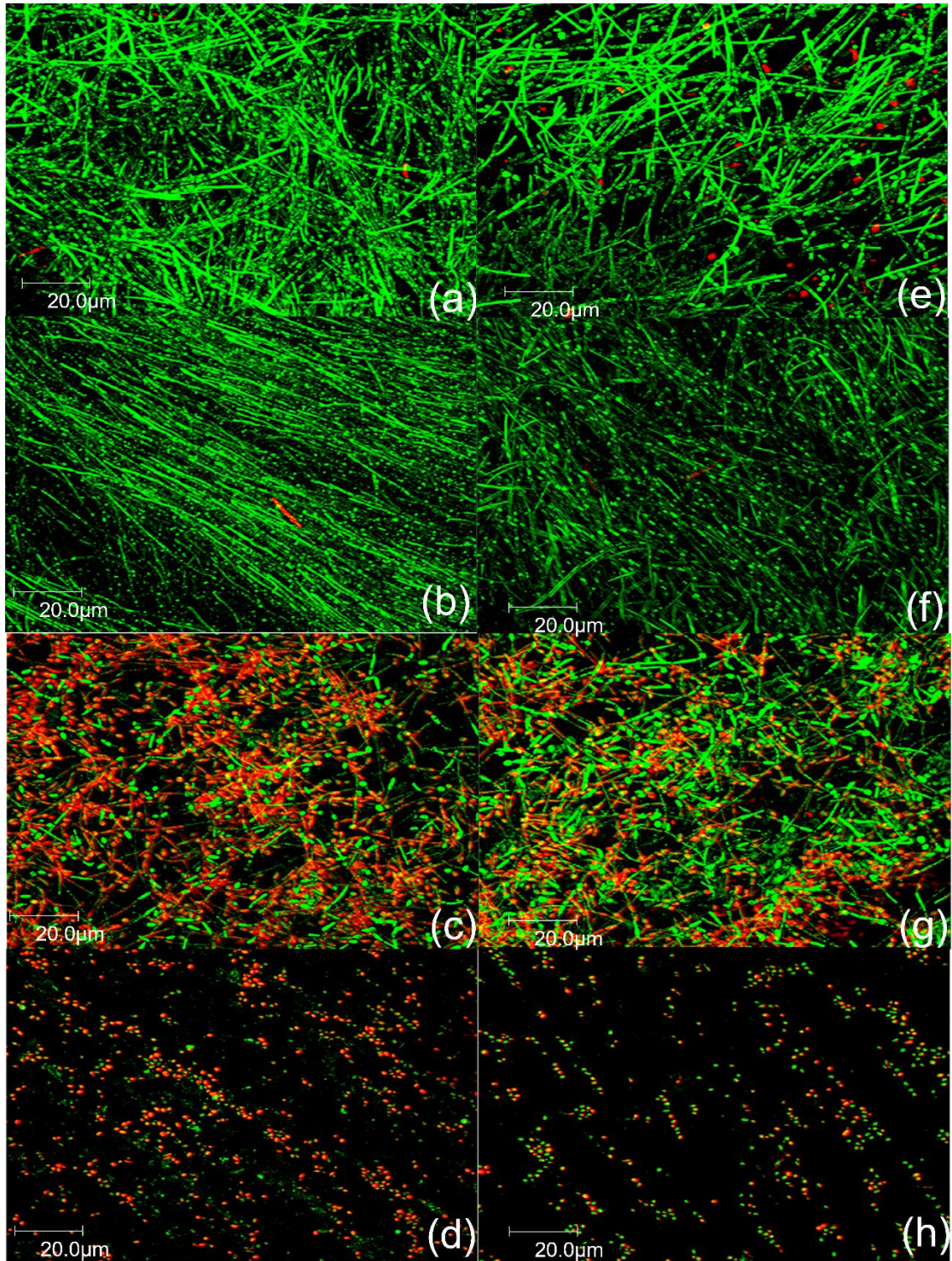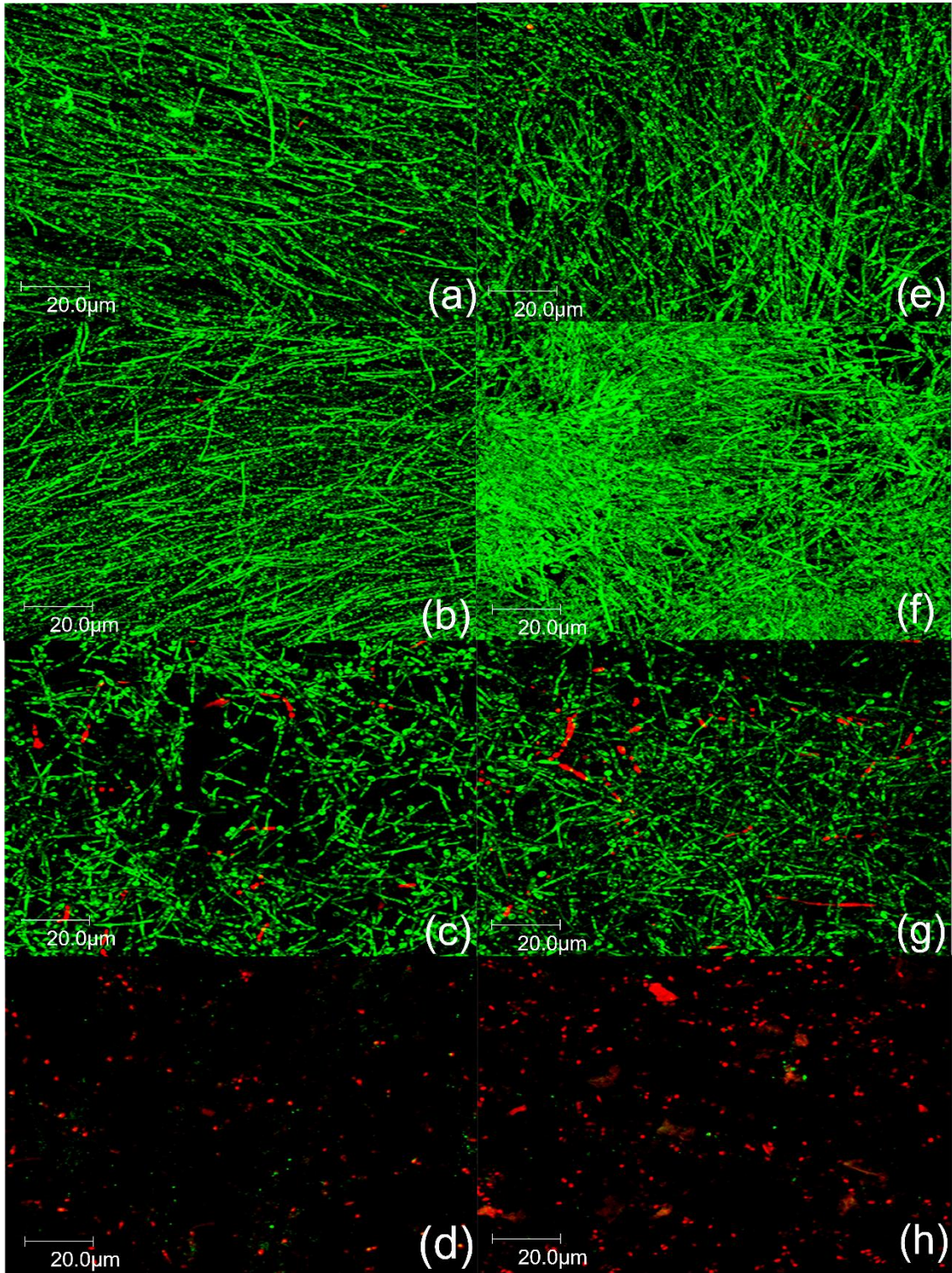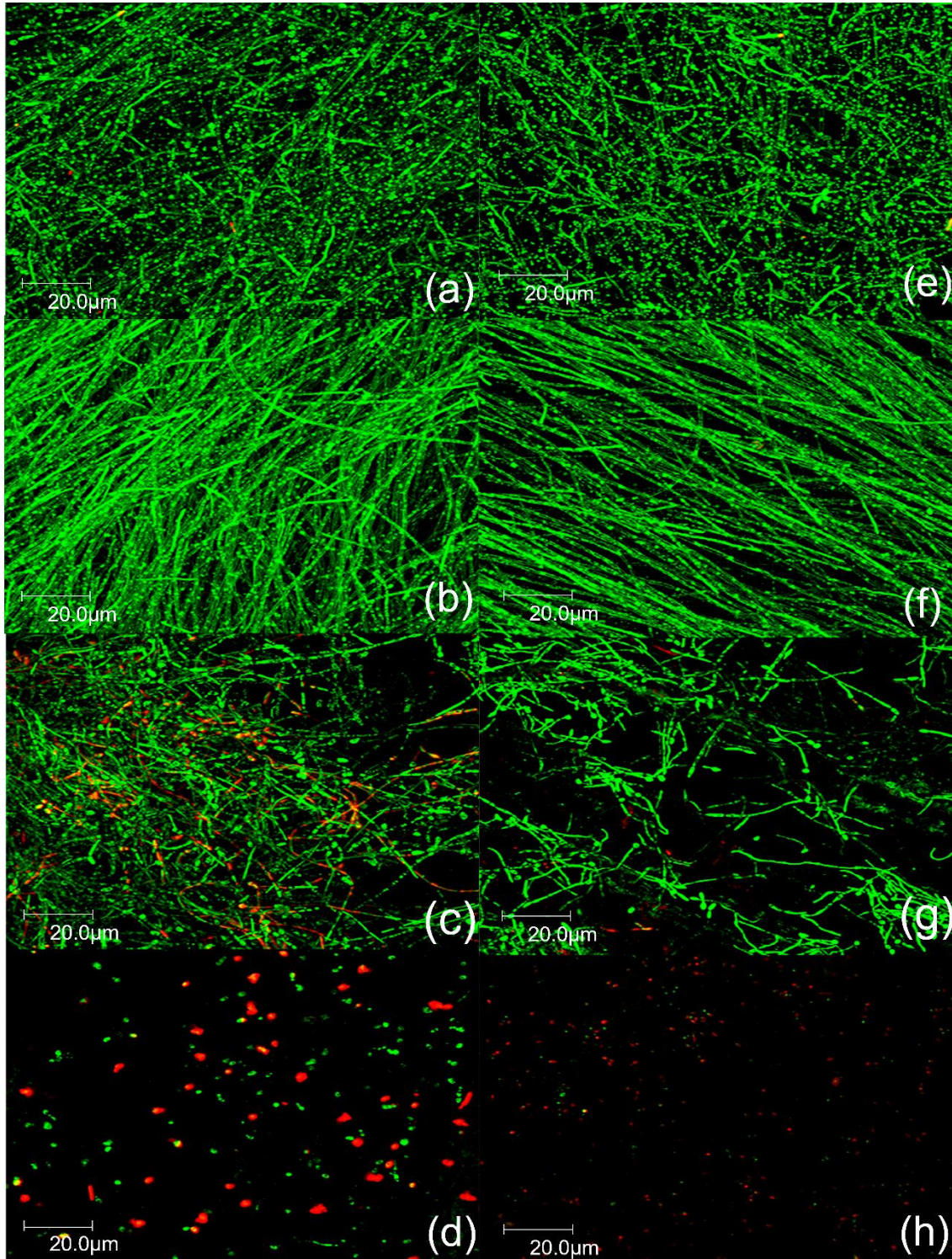
Figure 1. Confocal images of *Candida albicans* biofilms develop on heat-polymerized (HP) and pre-polymerized (PP) surface after 24 h of incubation: HP - (a): control; (b): Fibrin biopolymer; (c): Punic granatum incorporated in fibrin biopolymer; (d): Chlorhexidine incorporated in fibrin biopolymer; PP - (e): control; (f): Fibrin biopolymer; (g): Punic granatum incorporated in fibrin biopolymer; (h): Chlorhexidine incorporated in fibrin biopolymer.

After 48 h of incubation, In the control (Figure 2 (a),(e)) and BF groups (Figure 2 (b), (f)), a large amount of yeast and hyphae were evident, ratifying the quantitative CFU and VC findings. Some *C. albicans* blastopores and yeasts were noted in biofilms treated with

FBPg (Figure 2 (c), (g)), while sparsely distributed *C. albicans* blastopores and dead yeast were detected in the FBCh group (Figure 2 (d), (h)).

Figure 2. Confocal images of *Candida albicans* biofilms develop on heat-polymerized (HP) and pre-polymerized (PP) surface after 48 h of incubation: HP - (a): control; (b): Fibrin biopolymer; (c): Punic granatum incorporated in fibrin biopolymer; (d): Chlorhexidine incorporated in fibrin biopolymer; PP - (e): control; (f): Fibrin biopolymer; (g): Punic granatum incorporated in fibrin biopolymer; (h): Chlorhexidine incorporated in fibrin biopolymer.

After 72 h of incubation, the FB (Figure 3 (f), (b)) and control groups (Figure 3 (a), (e)) were highly densely populated with uniform yeast, pseudohyphae, and hyphae, similar to the results found in the CFU and VC assay. At the same time point, sparsely distributed *C. albicans* blastopores and a considerable amount of dead yeast were visible among scattered *C. albicans* in the FBCh group (Figure 3 (d), (h)). Yeast and some hyphae of diffuse formats were evident in the group treated with FBPg (Figure 3 (c), (g)).

Figure 3. Confocal images of *Candida albicans* biofilms develop on heat-polymerized (HP) and pre-polymerized (PP) surface after 72 h of incubation: HP - (a): control; (b): Fibrin biopolymer; (c): Punic granatum incorporated in fibrin biopolymer; (d): Chlorhexidine incorporated in fibrin biopolymer; PP - (e): control; (f): Fibrin biopolymer;

(g): Punic granatum incorporated in fibrin biopolymer; (h): Chlorhexidine incorporated in fibrin biopolymer.

4. Discussion

DS, the most common pathology involving denture wearers, is mainly caused by the adherence of *C. albicans* to the porous and rough surfaces of denture base materials [7,8]. Coating the denture surface may alter these rough characteristics and prevent the colonization of this yeast. Strategies such as the incorporation of antimicrobials into the composition of coating materials can provide additional benefits in preventing the development of biofilms on the inner surface of removable dentures [39,40]. Thus, in the present study, we assessed whether the coating of fibrin biopolymer adhesive with *P. granatum* and digluconate chlorhexidine could affect the adhesion of *C. albicans* on the surface of two different PMMA resins.

The results showed that heat-treated and pre-polymerized resin specimens coated with fibrin biopolymer incorporated with chlorhexidine (FBCh) significantly reduced *C. albicans* biofilm formation and growth in all of the evaluated assays and periods. These findings are supported by Redding et al. [39] who evaluated chlorhexidine incorporated thin-film polymer on acrylic resin specimens after 24 h of incubation. In addition, Garaicoa et al. [41] showed the antifungal capacity of chlorhexidine incorporated in denture adhesives. Ellepola and Samaranayake [52] found that the chlorhexidine digluconate compound provoked rupture in the cell membrane yeast, even at low concentrations, and was a potent antifungal. Moreover, the antimicrobial efficacy of chlorhexidine is also associated with its substantivity, assuring its gradual release, and the promotion of its efficacy over a long period [53].

Although not as efficient as chlorhexidine, the incorporation of *P. granatum* in fibrin biopolymer (FBPg) also showed inhibitory capacity on *C. albicans* biofilm at all periods and in both evaluated PMMA materials. Almeida et al. [2] also reported reduced biofilm development up to 12 h after biofilm induction in specimens treated with prosthetic adhesive with *P. granatum* incorporation. The antifungal effect of crude hydroalcoholic extract from *P. granatum* peel has been attributed to several structural compounds of the peel, specifically the punicalagin and ellagitannin derivatives [22,23,54]. These components are found in abundance in the crude hydroalcoholic extract of *P. granatum* peels, causing serious damage to the cellular structure of *C. albicans* yeast [23,24], possibly related to their molecular structure and toxicity, and astringent properties of tannins [42].

In contrast, FB favored the development of biofilm for up to 72 h in both quantitative assays. To the best of our knowledge, no study was found to confirm this finding; thus, only indirect comparisons can be made with previous investigations. Biofilm overgrowth could be related to the presence of abundant fibrin net - a special biological material - acting as a nutrient reservoir for C. albicans development on the resin surface [55]. However, it is noteworthy that this study proved the wet tolerance of BF and its efficiency as a drug delivery system for antimicrobials or antifungals. In this way, FBs incorporated with antimicrobials could be a sustainable alternative for the local prevention and management of DS. Moreover, previous studies have demonstrated the biocompatibility or absence of cytotoxicity of FB in human and animal cells [29-31]. It should be noted that for the first time a biodegradable biological material (fibrin biopolymer) was applied together with antimicrobial-yeast candidate agents to prevent DS.

Among the two different PMMA materials, the CAD/CAM pre-polymerized resin presented the best inhibitory effect to the development of *C. albicans* biofilm. Recent studies have demonstrated that the surface of CAD/CAM specimens exhibit significantly less adherence to *C. albicans* than heat-cured specimens [11,14]. These results could be attributed to the surface roughness of the materials once the pre-polymerized PMMA presents a smoother surface compared to heat-cured resin [14,15].

Considering the time exposure evaluated in this investigation, all groups presented a significant overgrowth of *C. albicans* biofilm after 72 h, as observed in other *Candida* species-related studies [41]. The development of *C. albicans* biofilm proceeds in three developmental phases: early (0-11 h), intermediate (12-30 h), and maturation (38-72 h). In the present study, overgrowth of *C. albicans* biofilm was detected in the mature phase of biofilm development, which is probably associated with its highly heterogeneous architecture and extracellular material [46], besides to greater drug tolerance since mature biofilm starts to express resistance genes [3,49]. In addition, 'persister' necrotic fungal cells in mature biofilm subjected to antifungal agents, which are subpopulations of cells highly tolerant of stress conditions, protected by the cell-matrix can repopulate the biofilm and interact and co-aggregate with other microorganisms present in the oral environment. This represents an important factor in its virulence [56,57].

In summary, the present study suggests that the incorporation of *P. granatum* or digluconate chlorhexidine in a vehicle tolerant to a wet environment has the potential to prevent and treat DS by acting on the main etiological factor, *C. albicans*. However, it is worth mentioning that the findings of this in vitro study must be carefully applied to clinical conditions, since the adhesive will be subjected to routine hygiene and disinfection protocols performed by denture users, in addition to thermal and Ph variations that may affect the effect and durability of this experimental product. Thus, further *in vivo* investigations on the anti-adherent potential against biofilm-associated with the denture base are needed to prove its effectiveness against DS, determine its longevity, and whether it causes any damage to the structure of dentures.

5. Conclusions

A biological material such as a fibrin biopolymer facilitates the growth of *C. albicans* maybe due to the robust fibrin network formed. On the other hand, when this biological medicine is associated with molecules that are candidates for antifungal, such as chlorhexidine or *P. granatum*, there is an important inhibition of biofilm production. Future clinical trials will be need to reproduce these findings.

6. Patents

Not applicable.

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3. DISCUSSION

PMMA-based materials remain the most common products used on removable denture bases (JOHN; GANGADHAR; SHAH, 2001; UZUN; HERSEK; TINCER, 1999). Because of its high water absorption, rough and microporous surface, and hydrophobic nature, PMMA denture bases favor the accumulation of microbial biofilm (CAZZANIGA et al., 2015; NASSAR et al., 2000; IKEYA et al., 2016). Considering these characteristics of the removable denture, the modification of the surface properties of PMMA-based dentures with biocompatible coating adhesives can help increase the resistance to surface wear and/or chemical reactivity of the surface. This can affect biofilm development, making it a viable tool for the prevention of DS.

Cyanoacrylate adhesives exhibit interesting properties such as quick polymerization, strong adherence to the surfaces, appropriate polymerization in a moist environment, biocompatibility (ALKAN et al., 2007; GONZALES et al., 2000; DADASX et al., 2007), and bacteriostatic (HOWELL et al., 1995; KIM, 1997), hemostatic (LUMSDEN; HEYMAN, 2006), and embolic effects (TOKUDA et al., 2009). In the last few years, cyanoacrylate adhesives were studied to be a good alternative to conventional sutures, bone graft fixation, and denture coating (ESTEVES et al., 2014; TAVORA et al., 2019; SAQUIB et al., 2018).

This study showed that conventional ethyl cyanoacrylate and ethyl cyanoacrylate formulation gels obtained promising results in inhibiting *C. albicans* biofilms for up to 30 days. The effect of ethyl cyanoacrylate against *C. albicans* was also reported by Távora *et al.*, after 24 h of incubation. Similarly, Ali et al. (2013) and Dogan et al. (2019) evaluated the effect of cyanoacrylate adhesives on several *Candida spp*. This could be associated with possible changes in the surface energy and roughness of acrylic resins. This was explained by the strong electronegative charge of the polymer and the low viscosity of ethyl cyanoacrylate adhesives that provide a smoother and regular surface, thus, forming a mechanical barrier that prevents the accumulation and development of biofilm (MALHOTRA et al., 2016). In contrast, other authors suggest that this reduction in the amount of biofilm may be related to the release of by-products, such as formaldehyde and cyanoacetate compounds. This occurs during the process of degradation of cyanoacrylate adhesives, which could trigger an antifungal activity owing to their cytopathic effect (SOON; GIL; JONES, 2015) that tends to decrease in 14 days and remains with low reactivity for 28 days (THAKEB et al., 1995).

This degradation rate can be decreased according to the length of the alkyl chain (MULLER et al., 1990). Therefore, there is a slower degradation process that releases fewer toxic by-products per unit of time with the longer alkyl chain molecules compared with the faster rate of the shorter alkyl chain molecules (THUMWANIT; KEDJARUNE, 1999). In this study, the inhibitory effect of two short-chain adhesives was evaluated. Hence, suggesting that with the decrease in the cytopathic reaction due to accelerated degradation, *C. albicans* could adapt or colonize the surface as the coating completely or partially degrades. However, this was not observed in the present study, as the development of the *C. albicans* biofilm remained reduced for up to 30 days after cyanoacrylate application.

Another biocompatible adhesive called fibrin biopolymer derived from snake venom was studied recently. This fibrin sealant is a biological material composed of fibrinogen-rich cryoprecipitate extracted from *Bubalus bubalis* buffalo blood and a trombone-like enzyme extracted from *Crotalus durissus terrificus*. When in contact with calcium and factor XIII, thrombin converts fibrinogen to soluble fibrin. Thereby, providing a stable clot and simulating the final step of the coagulation cascade. This product has hemostatic, adhesive, and sealant properties. Thus, recent clinical and experimental studies have reported its use in drug administration and tissue management (BUCHAIM et al., 2019).

In this study, it was reported that the incorporation of *P. Granatum* into a fibrin biopolymer reduced biofilm formation on different PMMA surfaces. Almeida et al. (2018) and Da Silva et al. (2018) also reported reduced *C. albicans* biofilm development in specimens treated with *P. granatum* extracts. This plant has been widely used in folk medicine and is considered a reservoir of bioactive compounds (ISMAIL et al., 2012). The antifungal effect of the extract of *P. granatum* peel is related to the punicalagin components and ellagitannin derivatives, which are abundant in the crude hydroalcoholic extract of *P. granatum* peel (MENEZES; CORDEIRO; VIANA, 2006; ENDO et al., 2010; ANIBAL et al., 2013; GARCÍA-VILLALBA et al., 2015). According to Bassiri-Jahromi et al. (2015), the action of tannins against *Candida* could be due to their molecular structure and toxicity, astringent properties, or other mechanisms. In addition, Da Silva et al. (2018) reported that the action mechanism of *P. granatum* on *C. albicans* can also involve oxidative stress, energetic collapse, damage to the cell wall, and rupture of yeast cells.

This study shows that the incorporation of digluconate chlorhexidine into fibrin biopolymer inhibited biofilm development up to 72 h on both PMMA materials. These

findings are supported by Redding et al. (2009), who evaluated chlorhexidine-incorporated thin-film polymers on acrylic resin specimens after 24 h of incubation amd Garaicoa et al. (2018), that investigated the antifungal capacity of chlorhexidine incorporated into denture adhesives. Currently, chlorhexidine is a chemical agent widely studied and used in dentistry. It has important properties such as a wide spectrum of antimicrobial action (ZEHNDER, 2006; RUFF et al., 2006; SENA et al., 2006), substantivity (ROSENTHAL et al., 2004; DAMETTO et al., 2005), and low toxicity (EL KARIM et al., 2007). This substance can be used as a disinfectant or topical medication for the treatment and control of DS. Chlorhexidine is a cationic agent with antimicrobial activity. This cationic nature of chlorhexidine promotes connection with the ammonium compound on the microbial surface (the acidic phosphate groups in gram-positive and lipopolysaccharide in gram-negative bacteria), which can alter bacterial integrity. Modification of the permeability of the cytoplasmic membrane promotes precipitation of cytoplasmic proteins, alters the cell osmotic balance, affects metabolism, growth, and cell division, inhibits membrane ATPase, and inhibits anaerobic processes (ESTRELA et al., 2003).

Here, we evaluated the effect of fibrin biopolymer coating on *C. albicans* on different PMMA surfaces, although this adhesive favored biofilm formation. It was not possible to show similar data since this study is the first to use fibrin biopolymer as a denture coating and to evaluate its effect on *C. albicans.* Biofilm overgrowth could be related to the presence of proteins and peptides, as it is abundant in snake venom and constitutes 90-95% of its dry weight. These proteins and peptides have either enzymatic activity and/or have toxicological properties (WAHEED; MOIN; CHOUDHRY, 2017). Thus, the proteins could act as nutrient

reservoirs for *C. albicans* development on the resin surface. However, it is noteworthy that our data certified fibrin biopolymer as an efficient drug delivery system for antimicrobials or antifungals, tolerating a wet environment, and promoting local prevention and management of DS.

Two different PMMA materials (heat-polymerized and pre-polymerized resin CAD/CAM) were evaluated in this study with no statistical difference. However, the pre-polymerized resin presented less biofilm formation. Other studies have demonstrated that the surface of the heat-treated specimens exhibited significantly greater adherence of *C. albicans* than the CAD/CAM specimen surface, as well as greater roughness (MURAT et al., 2019; AL-FOUZAN; AL-MEJRAD; ALBARRAG, 2017). CAD/CAM-fabricated complete dentures have several advantages, including a decrease in porosity as the denture base is formed from a prepolymerized block of acrylic resin that is industrially polymerized under protocol conditions at high heat and pressure (BIDRA et al., 2013). This may affect the surface characteristics and microbial adhesion (STEINMASSL et al., 2017).

The present study suggests that cyanoacrylate adhesives have a durable inhibitory effect against *C. albicans* biofilms and that the incorporation of *P. granatum* and digluconate chlorhexidine on fibrin biopolymer adhesive has the potential to reduce *C. albicans* biofilm formation. Both experimental coatings seem promising for use in the prevention, control, and treatment of DS. However, for evaluating the pharmacological potential of these synthetic and biological adhesives and their possibility of clinical use, further investigations must be performed. In this pre-clinical trial, we highlighted the determination death curve, probable mechanism of action, and studies to evaluate the effects of these on the PMMA structure.

4. **Conclusion**

- Ethyl cyanoacrylate adhesive (liquid and gel) coating promoted the long-term (up to 30 days) effect on *C. albicans* biofilms.

- Coating with fibrin biopolymer alone induced *C. albicans* biofilm formation.

- Coatings with fibrin biopolymers incorporated into digluconate chlorhexidine or *P. granatum* reduced biofilm formation on heat and pre-polymerized surfaces.

- No statistical difference was observed in *C. albicans* adherence on heat and pre-polymerized surfaces, although the pre-polymerized group presented less number of biofilms.

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6. ANNEXES

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