Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba
Área de Prótese Dental

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Avaliação *In vitro* da adesão de *Candida spp* sobre a superfície de resinas acrílicas para base e reembasamento de próteses removíveis

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

Piracicaba
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É muito simples: só se vê bem com o coração.

O essencial é invisível aos olhos.

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Foi o tempo que perdeste com tua rosa que fez tua rosa tão importante."

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RESUMO

A candidose é a infecção oral fúngica mais comum diagnosticada em humanos, com prevalência de até 67% em usuários de prótese. Embora tenha sido inicialmente associada apenas a *Candida albicans*, outras espécies podem ser responsáveis por mais de 50% dos casos de infecção. Ainda, fatores como presença de saliva e bactérias parecem desempenhar importante papel na colonização por *Candida*. Assim, este estudo objetivou verificar a influência destes fatores na adesão de duas espécies de *Candida* (*Candida albicans e Candida glabrata*) sobre a superfície de resinas acrílicas e reembasadores. Corpos de prova (2,5x1,2x0,2 cm) confeccionados com duas resinas acrílicas (convencional e de microondas) e dois reembasadores (temporário e permanente) tiveram sua rugosidade (Ra) e energia livre de superfície (ELS) mensuradas, sendo aleatoriamente divididos de acordo com a exposição aos fatores: presença ou ausência de saliva, presença ou ausência de bactérias e espécie de *Candida*. Os espécimes foram levados a uma câmara de fluxo utilizando-se uma bomba peristáltica para perfusão de cultura de bactérias seguida por uma das espécies de *Candida*, ou apenas a cultura de uma das espécies de *Candida*. A contagem das células de *Candida* aderidas foi realizada em microscópio óptico (400x). Os dados foram submetidos à análise de variância para Ra e adesão, e ao teste de Kruskal-Wallis para ELS (a=0,05). O reembasador temporário apresentou a maior Ra, seguido do permanente, enquanto as resinas acrílicas exibiram as menores rugosidades ($p<0,0001$). Os valores de ELS foram similares para os materiais, mas diferentes do reembasador temporário ($p<0,0001$). A adesão de *C. albicans* e *C. glabrata* variou de 3,2 a 564,4 e 3,2 a 1400,4 cel/mm^2 respectivamente, com diferenças estatísticas ($p<0,05$) em alguns grupos. O reembasador temporário mostrou maiores níveis de adesão. A colonização foi diminuída pela saliva, enquanto na presença de bactérias e saliva houve aumento da adesão ($p<0,05$). Estes resultados sugerem que a adesão inicial das duas espécies de *Candida* foi fortemente afetada pela rugosidade, presença de saliva e bactérias, mas não pela energia livre de superfície.

ABSTRACT

Candida-associated stomatitis is reported in up to 67% of a population wearing dentures. Recently, disease-associated *Candida* species have shifted from *C. albicans* to non-*albicans* species. Since factors such as presence of saliva and oral bacteria appear to play a major role in the initial phases of yeasts adhesion, this study aimed to determine whether these factors produced differences in acrylic resins and denture liners *C. albicans* and *C. glabrata* adherence. Samples (2.5x1.2x0.2 cm) of two acrylic resins (heat and microwave-cured) and two denture liners (soft and hard) were prepared and had their surface free energy (SFE) and surface roughness (Ra) measured and were randomly divided according to their exposure to the following factors: saliva coating or uncoating, presence or absence of bacteria and *Candida* species. Specimens were assayed in a flow chamber connected to a peristaltic pump for perfusion of bacteria culture plus one of the *Candida* species culture or only the *Candida* culture (control). Adhesion was determined by count on a light microscope (400 x). Statistical analyses was performed by ANOVA (Ra and *Candida* species adhesion) and Kruskal-Wallis (SFE) (a=.05). Soft liner presented the roughest surface, followed by the hard liner, whereas acrylic resins exhibited the smoothest surfaces ($p<.0001$). The SFE values of all materials were similar but different from the soft liner ($p<.0001$). *C. albicans* and *C. glabrata* adhesion on the materials ranged from 3.2 to 564.4, and 3.2 to 1400.4 cells mm^{-2} respectively, with statistically significant differences ($p<.05$) in some cases. The soft liner exhibited the highest levels of adhesion. The overall colonization was significantly decreased by saliva ($p<.05$), while bacteria increased the adhesion in the presence of saliva. These results taken together suggest that initial adhesion of *Candida* species was strongly affected by the surface roughness, presence of saliva and bacteria, but not by surface free energy.

1 INTRODUÇÃO GERAL

A candidose é a infecção oral fúngica mais comum diagnosticada em humanos (Muzyka, 2005), apresentando-se como uma inflamação dos tecidos bucais, cuja prevalência é de até 67% nos usuários de próteses removíveis (Arendorf & Walker, 1987; Spiechowicz *et al.*, 1991; Radford *et al.*, 1999). Esta inflamação também é denominada estomatite induzida por prótese ou estomatite por dentaduras, sendo a *Candida albicans* fortemente associada como o principal agente etiológico desta patologia. Entretanto, hoje é sabido que espécies de *Candida* não-*albicans* (*C. tropicalis, C. parapsilosis*, *C. glabrata*, *C. krusei,* e *C. dubliniensis*) podem ser isoladas e responsáveis por mais de 50% dos casos de infecção (Samaranayake & Samaranayake, 1994; Coleman *et al.*, 1997; Viscoli *et al.*, 1999; Samaranayake & Samaranayake, 2001). Os motivos destes achados ainda não estão completamente esclarecidos, sendo em muitas circunstâncias relacionados a repetidas terapias antifúngicas, o que causaria mudanças nos hospedeiros (Procop & Roberts, 2004; Nucci & Marr, 2005).

Os fungos normalmente vivem como comensais inócuos e colonizam grande variedade de *habitats* humanos, como pele e mucosa (Samaranayake, 1992; McMullan-Vogel *et al.*, 1999). O crescimento sobre superfícies é natural no ciclo de vida das espécies de *Candida* (Kumamoto & Vinces, 2005), o que pode explicar a ocorrência comum da colonização fúngica nos usuários de próteses. As lesões da mucosa oral relacionadas às próteses removíveis são reações agudas ou crônicas decorrentes da presença de biofilme dental, leveduras, constituintes do material utilizado para a confecção das próteses, pouca retenção ou injúrias mecânicas (Budtz-Jörgensen, 1971; Budtz-Jörgensen, 1978; Dorey *et al.*, 1985). Entretanto, dentre esses fatores, os causados pela candidose podem interferir com o tratamento reabilitador e principalmente ser uma barreira para a saúde do paciente (Perezous, 2005), uma vez que as próteses podem servir como fonte de reinfecção (Muzyka, 2005).

Devido à alta prevalência e virulência desses microrganismos nos processos inflamatórios, Baysan *et al.* (1998), Radford *et al.* (1999), Egusa *et al.* (2000) e Nikawa *et al.* (2000b) dedicaram-se a estudar os fatores que interferem

na colonização e adesão de *Candida spp* sobre a superfície de próteses removíveis. Dentre estes fatores, incluem-se as propriedades físicas de rugosidade e energia de superfície das resinas acrílicas para bases de próteses. Alguns autores têm sugerido que a energia livre parece desempenhar um importante papel nas fases iniciais de adesão, (Minagi *et al*, 1985; van Dijk *et al*., 1987), embora estudos recentes apontem para uma ausência de correlação entre energia livre de superfície e adesão microbiana (Waters *et al*., 1997; Serrano-Granger *et al*., 2005). Por outro lado, a maior rugosidade de uma superfície favoreceria a adesão de microrganismos, uma vez que estes estão mais protegidos contra forças que tendem a deslocá-los nas fases iniciais da colonização (Quirynen & Bollen, 1995; Radford *et al*., 1999).

Entretanto, poucos estudos levam em consideração as diferenças entre os materiais em relação ao tipo de polimerização ou materiais rembasadores (Samaranayake *et al*., 1980; Minagi *et al*., 1985; Vasilas *et al*., 1992; Waters *et al*., 1997; Radford *et al*., 1998; Millsap *et al*., 1999). Vários problemas são relatados com os materiais reembasadores, sendo a colonização por *Candida* o mais freqüente. Entretanto, os resultados reportados na literatura são inconsistentes e controversos, já que Razek & Mohamed (1980) e Wright (1980) relataram haver redução ou ausência de colonização devido ao efeito fungicida decorrente da adição de componentes antifúngicos ou antibacterianos aos reembasadores, enquanto Wright *et al.* (1985), Graham *et al.* (1991) e Kulak & Kazazoglu (1998) identificaram expressiva presença de leveduras em próteses reembasadas com estes materiais.

Na cavidade bucal, durante o processo de colonização, o microrganismo, para alcançar e interagir com o substrato necessita interagir com a película adquirida, formada pela adsorção seletiva de glicoproteínas salivares, que se forma imediatamente após o contato da superfície da prótese com a saliva (de Jong *et al*., 1984; Quirynen & Bollen, 1995). A formação desta película está diretamente associada à sua capacidade de molhamento que é regulada pela energia livre de superfície (Sipahi *et al*., 2001), influenciando a adesão de *Candida* sobre o material (Quirynen & Bollen, 1995; Siphai *et al*., 2001).

O efeito de limpeza da saliva e componentes salivares, como lisozima, histatina, lactoferrina, calprotectina e IgA secretora dificulta a aderência dos microrganismos às superfícies bucais (Ueta *et al.*, 2000; Cannon *et al.*, 2001; Tanida *et al.*, 2001; Dodds *et al.*, 2004; Elguelzabal *et al.*, 2004), enquanto componentes como mucina (Nikawa & Hamada, 1990; Edgerton *et al.*, 1993; Nikawa *et al.*, 1993; Dodds *et al.*, 2004) e estaterina (Johansson *et al.*, 2000) facilitam a adsorção de microrganismos em resina acrílica e materiais reembasadores embebidos em saliva (Nikawa *et al.*, 2000a; Nikawa *et al.*, 2000b).

Contudo, estudos sobre a influência da saliva na aderência de *Candida* são contraditórios: enquanto é relatada maior prevalência de algumas espécies de *Candida* em pacientes com diminuição do fluxo salivar *in vivo* (Koseki *et al.*, 2004) ou reduções nos níveis de adesão na presença de saliva (Saramanayake *et al.*, 1980; Waters *et al.*, 1997; Radford *et al.*, 1999; Bosch *et al.*, 2003; Elguelzabal *et al.*, 2004), outros autores verificaram aumento da adesão inicial sucedido por efeito inibitório da saliva ao longo do tempo (San Millán *et al.*, 2000; Ramage *et al.*, 2004). Ainda, ausência de interferência da saliva nos valores de adesão tem sido reportada (Jin *et al.*, 2004).

Adicionalmente, as bactérias presentes no biofilme da prótese têm sido associadas à aderência de *Candida* (Budtz-Jörgensen, 1983; Gusberti *et al.*, 1985; Catalan *et al.*, 1987; Koopmans *et al.*, 1988; Vasilas *et al.*, 1992; Millsap *et al.*, 1999; Nikawa *et al.*, 2000b), sendo os estreptococos e actonomices as principais delas. Neste estudo as bactérias *S. mutans* e *A. naeslundii* foram as selecionadas porque elas aderem a superfícies duras na cavidade bucal, como dentes ou próteses, e estão envolvidas na formação e acúmulo de biofilme sobre as superfícies mencionadas (Carlsson *et al.*, 1969). Em acréscimo, deve ser destacado que essas bactérias correspondem a um terço de todas as espécies encontradas no biofilme de próteses (Nyvad & Kilian, 1987).

Diante do exposto é possível verificar que ainda não há um consenso sobre o papel da saliva e da presença de bactérias como *Streptococcus* e *Actinomyces* na adesão de *Candida* a superfícies de resina acrílica e reembasadores (Millsap *et al.*, 1999). Embora características superficiais sejam importantes para determinar

a energia livre de superfície e a composição da película adquirida e, portanto influenciar na aderência de *Candida*, poucos estudos compararam os tipos de resina e reembasadores considerando todos os fatores citados e, particularmente a adesão de outras espécies de *Candida*, além da *Candida albicans* (Minagi *et al.*, 1985; Luo & Samaranayake, 2002).

A alta prevalência de estomatite por dentadura associada ao aumento na população de indivíduos idosos, imunocomprometidos ou usuários de polifarmacia e portadores de próteses removíveis, justificam o esclarecimento das interações adesivas entre a *Candida* e algumas bactérias orais presentes em biofilmes formados sobre a superfície de resinas acrílicas e reembasadores. Assim, a proposta deste estudo foi determinar a energia livre e rugosidade de superfície de duas resinas acrílicas e dois reembasadores de prótese e relacionar essas propriedades com a adesão de *Candida albicans* e *Candida glabrata* quando na presença de saliva e de *Streptococcus mutans e Actinomyces naeslundii*, considerados importantes na adesão.

2 CAPÍTULO

Capítulo 1: *In vitro Candida* colonization on acrylic resins and denture liners: influence of surface free energy, roughness, saliva and adhering bacteria

In vitro Candida colonization on acrylic resins and denture liners: influence of surface free energy, roughness, saliva and adhering bacteria

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ABSTRACT

Purpose: Since factors such as surface roughness (Ra), surface free energy (SFE), saliva and oral bacteria appear to play a major role in yeasts adhesion in the development of denture stomatitis, this study aimed to determine whether these factors produced differences in the adherence of *Candida albicans* and *Candida glabrata* to acrylic resins and denture liners. ***Materials and methods:*** Samples (2.5x1.2x0.2 cm) of two acrylic resins and two denture liners were prepared and had their Ra and SFE measured. Specimens were randomly divided according to their exposure to the following factors: saliva coating or uncoating, presence or absence of bacteria (*S. mutans* and *A. naeslundii*) and *Candida* species. Specimens were assayed in a flow chamber connected to a peristaltic pump for bacteria perfusion culture plus one of the *Candida* species culture or only the *Candida* culture (control). Adhesion was determined by count under a light microscope (400x). Data were analyzed by ANOVA (Ra and *Candida* adherence) and Kruskal-Wallis (SFE) (a=.05). ***Results:*** The soft liner exhibited the roughest surface, followed by the hard liner, whereas the acrylic resins exhibited the smoothest surfaces ($p<.0001$). SFE values of all materials were similar except for the soft liner ($p<.0001$). *C. albicans* and *C. glabrata* adhesion to the materials ranged from 3.2 to 564.4, and 3.2 to 1400.4 cells mm^{-2} respectively, with significant differences ($p<.05$) in some cases. The soft liner exhibited the highest adhesion levels. The overall colonization was significantly decreased by saliva ($p<.05$), while bacteria increased the adhesion in the presence of saliva. ***Conclusions:*** These results suggest that initial *Candida* species adhesion was strongly affected by surface roughness but not by surface free energy. Additionally, the presence of saliva and pre-colonization with bacteria seems to influence yeasts adhesion.

Key words: *Candida* adherence; acrylic resins; denture liners, bacterial adhesion, saliva

Introduction

Candida-associated denture stomatitis is reported as the most common infection in the oral environment[1] existing in up to 67% of the population wearing dentures[2,3]. Multiple systemic factors may cause a predisposition to oral candidal infections such as immunosuppression, dietary factors, malignancies, broad-spectrum antibiotics, smoking, age, diabetes mellitus, iron and vitamin deficiencies, and salivary gland hypofunction[4,5]. It is also well known that dentures, exhibiting poorly fitting and poor hygiene are the most frequent cause of this opportunistic infection[1,6].

Although *C. albicans* is by far the predominant isolate in this condition[7], other non-*albicans* species such as *Candida glabrata*, the second most predominant and most virulent species, are frequently isolated both from acrylic denture surfaces and the palatal mucosa[8].

Moreover, while *Candida* species are pointed out to be the major cause of the disease[1,9], denture plaque is also unequivocally involved[10]. Plaque plays a causative role, together with *Candida* species, in the development of denture stomatitis[11,12]. Denture plaque is composed mainly of bacteria[11,13], *Streptococcus mutans* being one of prominent members on dentures[14] and *Actinomyces naeslundii* corresponding to one third of all bacteria found in dental plaque[15], while yeast apparently constitutes a minor part of the total microbial flora both in healthy patients and in patients with denture stomatitis[11]. The adhesion of *Candida* may also be dictated by non specific forces (i.e., van der Waals forces, London forces and hydrophobic interactions)[16,17] as well as specific factors such as receptor-ligand binding[17,18].

The use of denture liners for denture prostheses is advantageous in many clinical situations in which patients who have the greatest need for resilient liners usually have thin, sharp, or badly resorbed residual alveolar ridges[19] or chronic tissue irritation from dentures[20]. Even though these materials exhibited excellent tissue tolerance, one of the problems associated with them is the colonization of *Candida* spp on and within the material. Furthermore, these fungal growths destroy

the surface quality of the liner and may cause irritation of the oral tissues because of a combination of surface roughness and the concentration of exotoxins and metabolic products of the fungal colonies[19]. Nevertheless, conflicting results are seen in the literature on these materials. Some *in vitro* studies reported inhibitory effect on *C. albicans*[21], while others showed they have no fungal inhibitory effect[22,23].

Moreover, the role played by saliva in with the presence of bacteria during the initial colonization and subsequent biofilm formation is poorly understood. In fact, multiple studies have demonstrated that pre-treatment of acrylic resin samples with whole saliva decreases the initial adherence of *C. albicans*[24-26], while others showed an increased adherence[5,27,28], or no effect was observed[29]. Nonetheless, a reduction in the levels of *Candida albicans* adherence to acrylic resins or resilient materials in the presence of saliva was found by many authors[3,24,30,31].

Additionally, it has been reported that the physico-chemical properties of the solid surfaces may alter the composition of the denture pellicle and initial adherence of yeasts to these surfaces. However, limited attention has been paid to the interactions between yeasts, denture material surfaces, presence of saliva and bacteria other than the research that has been done on how average surface roughness and free energy affect retention[32-34].

As the growth and colonization of prosthesis by *Candida* are of such clinical importance, the purpose of this study was to determine the influence of surface free energy and surface roughness on the adhesion of two species of *Candida* to acrylic resins and denture liners. Also, saliva and bacteria pre-colonization on the materials were investigated. The null hypotheses tested was that there is no correlation between surface roughness or surface free energy and *Candida* adhesion, and also that *Candida* adhesion is not affected by either the presence of saliva or by bacteria pre-colonization.

Materials and Methods

Experimental Design

This *in vitro* study involved a complete randomized and blinded (regarding yeast counts) design, considering four materials (Polymethylmethacrylate - PMMA heat-cured acrylic resin, PMMA microwave-cured acrylic resin, soft denture liner or hard denture liner), saliva (coated or uncoated), bacteria (presence or absence) and *Candida* species (*C. albicans* or *C. glabrata*) as factors in the study. Yeast adhesion, surface roughness and surface free energy were the dependent variables in the study. This study was approved by the Research and Ethics Committee of FOP/UNICAMP (Protocol 111/2004).

Samples were prepared from two acrylic resin denture base materials and two denture liners. All samples had their surface roughness and surface free energy evaluated previously to the adherence assay. Specimens were tested in a flow chamber, using uncoated specimens and lack of exposure to bacteria as controls. First, bacteria were allowed to adhere to the sample surfaces from a flowing suspension and subsequently a yeast flowing suspension allowed yeast to adhere. Count of *Candida* adhesion was carried out under a light microscope. All procedures were performed under a laminar airflow hood to minimize the possibility of contamination.

Preparation of acrylic resins and denture liners specimens

The PMMA samples were prepared from two acrylic resin denture base materials, using heat-cured and microwave-cured acrylic resins (Denture acrylic and Ondacryl, Clássico Artigos Odontológicos Ltd, São Paulo, Brazil) by the compression-mould technique. Initially, rectangular patterns (2.5 x 1.2 x 0.2 cm) were cut of wax sheets and were invested in a flask and subsequently boiled out. Powder and liquid acrylic denture base materials were mixed and processed according to manufacturer's directions. Similarly, the denture liners (Coe Soft and Kooliner, GC America, Alsip, IL, USA) were prepared to the same uniform size by placing glass slides over them and firmly fixing both ends, then separating the glass plates after curing, as previously described[9]. One side of the samples were

finished by an automatic grinder by using 320, 400 and 600 grid-sized sandpaper under cooling water and polished with a brush disc with pumice slurry and a felt cone with chalk powder[35], except for the soft denture liner, where surface roughness was determined by the glass slides. Acrylic resin samples were immersed in distilled water at 37°C for 12 h for residual monomer release[36].

Surface roughness

Surface roughness (R_a) of the samples was measured using a profilometer (Surfcorder SE 1700 Kozaka Industry, Kozaka, Japan) with a 0.01 mm resolution, calibrated at sample length of 0.8 mm, 2.4 mm percussion of measure, and 0.5 mm/s. Three readings were taken for each sample and a mean value was calculated[37].

Surface free energy

To characterize the wetting properties of the acrylic resin surfaces, contact angles were measured for each sample. Water was chosen as the test liquid[32,38]. The experimental setup consisted of an adjustable stage on which the samples were placed, and a droplet (15 µL) of deionized distilled water was dispensed on 0-degree tilt specimen surface by a micropipette. Photographs (Sony Cybershot F-717, SONY, Tokyo, Japan) of the droplets were taken immediately under standard conditions and contact angles were measured (AutoCAD 2005, Autodesk Inc., USA) from the left boundaries of the magnified photographs to the point of air-water-sample intersection. The mean value of three measurements for each surface was used. Surface free energy was calculated (Maple 9.5, Waterloo Maple Inc., Canada) using the cos T [39] of contact angles using the following equation:

$$\cos(\Theta) = -1 + \frac{2\sqrt{\gamma_1/\gamma}\,\exp^{-\beta(\gamma_1-\gamma_2)^2}}{\gamma}$$

After surface roughness and surface free energy measurements were completed, the specimens were randomly assigned to one of the experimental

conditions (n=10). The contaminants presented in the samples were removed by sonication in sterilized deionized distilled water for 20 min[40].

Microorganisms and growth condition

A loopful of stock yeast culture of *C. albicans* (ATCC 90028) and *C. glabrata* (ATCC 2001) were streaked onto Sabouraud's glucose agar (Difco, Sparks, MD, USA) and incubated for 24h at 37?C. Cells were harvested, washed with saline solution and standardized to 1 to 5 x 10^6 cells mL^{-1}, ascertained spectrophotometrically (Bausch & Lomb Spectronic 20, San Pablo, CA, USA) at 530 nm, corresponding to the pattern 0.5 of the Mc Farland scale[5,10,27].

Streptococcus mutans UA 159 and *Actinomyces naeslundii* ATCC 12104 were recovered and a loopful of each stock culture was streaked onto Blood agar plates (Difco) incubated in a 10% CO_2 atmosphere. Before each experimental set bacteria were subcultured overnight in 1% glucose-enriched TSB broth (Difco). Growth curves were previously established to ascertain a microbial concentration of 10^8 bacteria mL^{-1} [5,27].

Human saliva collection and preparation for the adherence assay

Human whole saliva was collected from a healthy donor[41,42] who had not used antibiotics, mouth rinses or any other medication known to affect microorganisms or salivary composition and flow, in the past 3 months. Saliva was collected during masticatory stimulation with Parafilm M (American Co., Greenwich, Ct, USA) in a sterilized ice-chilled Erlenmeyer tube and clarified by centrifugation at 10 000 *g* for 10 min at 4?C[5,27]. The supernatant was collected and put into sterile Petri plates, where half of the samples rested for 30 min to form an acquired pellicle, while the other half were uncoated (control). After this period, the acrylic samples were removed from the Petri dishes and used in the adherence assay.

Adherence assay

Both *Candida* species were assayed in either the presence or absence (control) of bacteria strains and in the presence or absence of saliva. The specimens were inserted into a flow chamber connected to a flow system by a peristaltic pump operating at 0.5 mL min^{-1} (Econopump, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The bacterial suspension was perfused and bacteria were allowed to attach for 30 minutes. Flow was switched to PBS buffer in order to remove non-adhering bacteria from the chamber and system tubing. Subsequently, a *Candida albicans* or *Candida glabrata* suspension was perfused through the chamber for 2h and again switched to buffer to remove non-adhering yeasts and the chamber was then drained[5].

Specimens were removed, gently washed with PBS, then washed with ethanol at 80 %, stained for 1 min with crystal violet and once again washed with PBS[3,30,32].

Yeast counts

Adherent yeast cells in 15 fields of view in each sample (0.25 mm^2 per field) were enumerated and the results were expressed as cells mm^{-2}, using a light microscope (Leitz Ortholux, Leitz Wetzlar, Germany) at 400x magnification. Thus, the following parameters were used to standardize the counts: 1) budding yeast was considered as a unit cell if the daughter was smaller than the mother cell and 2) a hyphum was counted as a single cell[24].

Statistical analysis

Surface roughness data were analyzed by one-way ANOVA and Tukey test. Surface free energy data were assessed by Kruskal-Wallis one-way ANOVA on ranks test. Data from yeast counts underwent statistical analysis and the assumptions of homogeneity of variances and normal distribution of errors were tested for the response variables tested. Thus, data of yeast counts were transformed by log. Analysis of variance (ANOVA) followed by Tukey test considering four factors – material, exposure or not to saliva, exposure or not to bacterial suspension, and *Candida* species, and their interactions was performed.

All analyses were carried out at a 5% significance level (The SAS system for Windows 9.0, SAS Institute Inc., Cary, NC, USA).

Results

The surface roughness statistically differed amongst material, except for the acrylic resins, which presented the smoothest surfaces ($p<.0001$). The soft denture liner presented the roughest surface ($p<.0001$) (Table 1). Surface free energy values for the acrylic resins and the hard denture liner were very similar but were different from the values of the soft liner, which showed the lowest values ($p<.0001$) (Table 1). *Candida albicans* and *Candida glabrata* adhesion on the materials ranged from 3.2 to 564.4, and 3.2 to 1400.4 cells mm^{-2} respectively, with statistically significant differences ($p<.05$) in some cases depending on the factors involved (Tables 2 and 3). The soft liner exhibited the highest levels of adhesion. The overall colonization on all materials was significantly decreased by the presence of saliva ($p<.05$), while bacteria presence increased the adhesion of *Candida* in the presence of saliva. However, this depended on the interactions among factors in study (Tables 2 and 3).

Discussion

A flow cell system was used to assess the factors influencing adhesion of *Candida* to materials tested. All materials were exposed to identical laboratory environments so that responses could be compared. Moreover, the experimental model simulated oral environmental conditions by having saliva and bacteria present, both of which could affect *Candida* adherence. Other important factors, such as *Candida* growth conditions, can also influence adhesion. Hence all strains were identically prepared in order to eliminate this possibility.

In the present study, surface free energy seemed not to have a direct correlation on the adhesion of the *Candida* species corroborating other studies where no relationship between surface free energy and number of retained yeast

cells has been described[30,34]. However, other authors reported a positive relationship between surface free energy and adhesion[16,18,32]. Although previous reports have correlated surface free energy and microorganisms' adhesion[43], other important factors may influence adhesion and should be carefully considered, as cell surface factors, diet, salivary composition and secretion rates, and antibody titers which may be conditioning factors in rates of plaque formation[11].

Our results showed that surface roughness influenced both species adhesion, as the roughest surface material exhibited higher yeast counts. Furthermore, Quirynen *et al.*[44] described the existence of a threshold roughness (0.2 ?m) below which no further impact on the adhesion should be expected. In fact, to reach more consistent results, another experimental design should be carried out, considering different surface roughness and surface free energy in the same material. In our study, these variables assessed were carried out to characterize the material's surface and to assure standardization of the finishing and polishing procedures.

A wide variation in adhesion was found between and amongst groups, as described by other authors[3,34,37]. Clusters of yeasts were observed predominantly in the denture liners, which led to a difficult count. None of the materials appear to have an inhibitory influence on the adherence of either *Candida albicans* or *Candida glabrata*. Indeed, on the soft denture liner, depressions in the surface entrapped many cells, which necessitated counting cells at different focal planes. The porous surface texture of the materials, especially the soft denture liner, which exhibited the highest counts, may have harbored yeast cells, allowing increased colonization even though an antifungal effect would be expected since these materials have antifungal components in their composition (dibutultin dilaurate, vinyl silane, or zinc dimethyldithiocarbamate), according to the manufacturers information.

The soft denture liner showed a higher number of yeast counts in all conditions, corroborating other study where denture liners had no inhibitory effect on *Candida* adhesion[45]. Indeed, these findings suggest that denture liners seem to have a supporting effect on fungal growth, indicating the ability of the yeast to

penetrate the deepest confines of the materials, as shown in other studies[17,23]. However, previous studies have found some inhibitory effect on *Candida albicans* or have found a minimal inhibitory effect that decreased over time[21,45]. Likewise, the nutrient-rich environment of the oral cavity might overpower any inhibitory effect present in the denture liners[22]. Moreover, the constant "bath" of saliva in the mouth might possibly leach out the active antifungal ingredients. Probably the culture flow achieved by the peristaltic pump might be responsible for this non-inhibitory effect of the tested materials.

Candida glabrata showed higher counts than *C. albicans* in most of experimental conditions and materials, concurring with previous studies where the same trend has occurred[7,16,40]. The degree of adhesion of a certain species to biological surfaces may indicate their pathogenic potential[46]. These different adherence results may be explained by the complexity and phenotypic heterogeneity of the *Candida* species population within the oral cavity, expressed in different hidrophobicity, secretion of extracelular proteinases, hyphal formation and thigmotropism[27,40], that directly influence *Candida* adherence rates.

It is evident that the relationship between saliva pellicle on denture surfaces and candidal colonization is a complex subject. The role of saliva during the adherence and colonization process of *Candida* species is still not clear. It is well known that innate defenses, such as the flushing effect of saliva, and anti-*candida* salivary components, such as lysozyme, histatins, lactoferrin, calprotectin, and sIgA interact with *Candida* species and decrease its adherence to oral surfaces[4], while components in whole saliva including mucins, statherin and proline-rich-proteins have been reported to adsorb to *Candida albicans*, increasing its adherence to saliva-coated resins and resilient materials[28,42,47]. In the present study, there was a decrease in adherence of both *Candida* species to specimens pre-coated with saliva, as previously described[24,25]. One could suppose that saliva diminished the effect of surface roughness of the materials probably because saliva alters the surface free energy of the materials. Additionally, antimicrobial properties of saliva may have contributed to the lower adhesion found in this study in saliva presence.

Co-aggregation between a number of oral bacteria led to increased investigations in the two general dominants in early plaque development, *Streptococcus* and *Actinomyces*[48]. In general, the presence of both bacteria strains increased the yeast adhesion in the presence of saliva, as related by other studies[5,49], but decreased adhesion in the absence of saliva[24]. The overall results of our study corroborate these data, as our results support the antimicrobial properties of saliva. Other studies might not concur with ours probably due to varying protocols, making comparisons difficult.

Altogether, as fungal adhesion was greater, especially in the material presenting with higher surface roughness, it is reasonable to speculate the importance of the rehabilitation material surface properties in clinical situations where the oral cavity is re-colonized after antimycotic treatment withdrawal in patients with oral candidiasis, as the yeasts may be harbored in more remote sites of the material. Indeed, the presence of a rehabilitation material which could somewhat be more favorable than other to avoid the oral cavity re-colonization is mandatory. Hence, studies that could explore the factors related to initial re-colonization by *Candida* in different materials is nevertheless important. Therefore, the length and *in vitro* nature of this investigation may not account for changes inherent in the materials after long periods of use and other changes that might occur only in oral fluids conditions.

Finally, the null hypothesis tested was rejected since all factors under study (material, presence of saliva, bacteria pre-colonization and *Candida* species) influenced yeast adhesion. Additionally, surface roughness affected both *Candida* species adhesion. Surface free energy seemed of less importance than the factors mentioned above.

Conclusions

Within the limitations of this *in vitro* study, it is possible to conclude that initial adhesion of *Candida* species was strongly affected by the presence of saliva and bacteria, and influenced by surface roughness. In clinical terms, the selection of appropriate materials for a given function may affect *Candida* adhesion, and should be carefully considered in treatment protocols.

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Table 1. Surface roughness – Ra (μm) and surface free energy – SFE (erg cm^{-2}) for tested materials (Mean ± SD).

Material	Ra	SFE
PMMA (heat-cured)	0.15 ± 0.06 a	42.77 ± 3.75 a
PMMA (microwave-cured)	0.15 ± 0.06 a	42.42 ± 3.45 a
Kooliner (cold-cured)	0.56 ± 0.17 b	42.37 ± 3.58 a
CoeSoft (cold-cured)	1.49 ± 0.43 c	39.82 ± 4.57 b

For comparisons among materials, different letters mean significant differences ($p<.05$).

Table 2. *Candida albicans* adhesion, according to the combination of material, saliva and bacteria (Mean yeast per mm^2 ± SD).

	Uncoated		Coated with saliva	
Material	No bacteria	Bacteria	No bacteria	Bacteria
PMMA (heat-cured)	86.96 ± 113.52 abA	84.60 ± 66.49 aA	10.84 ± 7.39 aB	25.37 ± 13.25 aB
PMMA (microwave-cured)	91.04 ± 70.59 aA	56.60 ±35.02 aB	26.52 ± 27.23 aD	39.88 ± 43.49 aC
Kooliner	91.64 ± 72.69 aA	48.16 ± 35.57 aA	62.80 ± 41.65 abB	26.96 ± 21.64 aB
CoeSoft	195.92 ± 174.87 bA	185.20 ± 144.91 bA	63.44 ± 24.47 bC	84.60 ± 86.67 bB

Lower case letters indicate statistically significant differences ($p<.05$) among materials. Upper case letters indicate statistically significant differences ($p<.05$) between presence/absence of bacteria and coating with saliva.

Table 3. *Candida glabrata* adhesion, according to the combination of material, saliva and bacteria (Mean yeast per mm^2 ± SD).

	Uncoated		Coated with saliva	
Material	No bacteria	Bacteria	No bacteria	Bacteria
PMMA (heat-cured)	255.24 ± 230.49 aA	149.52 ± 86.75 aC	50.04 ± 47.42 aD	195.60 ± 432.82 abB
PMMA (microwave-cured)	266.16 ± 228.47 aA	192.68 ± 208.54 aB	60.36 ± 44.08 aD	123.20 ± 246.77 aC
Kooliner	416.24 ± 322.91 abA	355.48 ± 458.71aB	49.00 ± 46.14 aD	60.48 ± 77.99 aC
CoeSoft	483.36 ± 141.71 bA	131.57 ± 137.57 aD	236.60 ± 234.78 bC	270.92 ± 372.96 bB

Lower case letters indicate statistically significant differences ($p<.05$) among materials. Upper case letters indicate statistically significant differences ($p<.05$) between presence/absence of bacteria and coating with saliva.

3 CONCLUSÃO GERAL

Os resultados do presente estudo sugerem haver influência positiva da saliva e da pré-colonização por bactérias na adesão de *Candida*. Somado a isso, os resultados indicam que a rugosidade de superfície exerce papel no número de células de *Candida* aderidas aos materiais utilizados para base de prótese ou reembasamento. Estes fatores apontam para a necessidade da realização de novos estudos para melhor entendimento da candidose.

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* De acordo com a norma utilizada na FOP/Unicamp, baseada no modelo Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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ANEXO 1 – Certificado de aprovação do Comitê de Ética em Pesquisa

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

UNICAMP

CERTIFICADO

Certificamos que o Projeto de pesquisa "Avaliação *in vitro* da adesão de *Candida* sp sobre a superfície de resinas acrílicas e condicionadores teciduais", protocolo CEP nº ***111/2004***, dos Pesquisadores: ***Tatiana Pereira*** e ***Altair A. Del Bel Cury***, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde - MS e foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia - UNICAMP.

We certify that the research project "*In vitro* evaluation of *Candida* sp adhesion on acrylic resin and tissue conditioners surface", Register Number ***111/2004*** of ***Tatiana Pereira*** and ***Altair A. Del Bel Cury***, is in agreement with the recommendations of 196/96 Resolution of the National Health Committee - Brazilian Health Department and was approved by the Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas - UNICAMP.

Piracicaba - SP, Brazil, May 31 2004

Profa. Dra. Cinthia Pereira Machado Tabchoury
Secretaria
CEP/FOP/UNICAMP

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

ijp

Progress report 716

Title: In vitro Candida colonization on acrylic resins and denture liners: influence of surface free energy, roughness, saliva and adhering bacteria

Type: Short Communications

Author: Altair Antoninha Del Bel Cury

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APÊNDICE

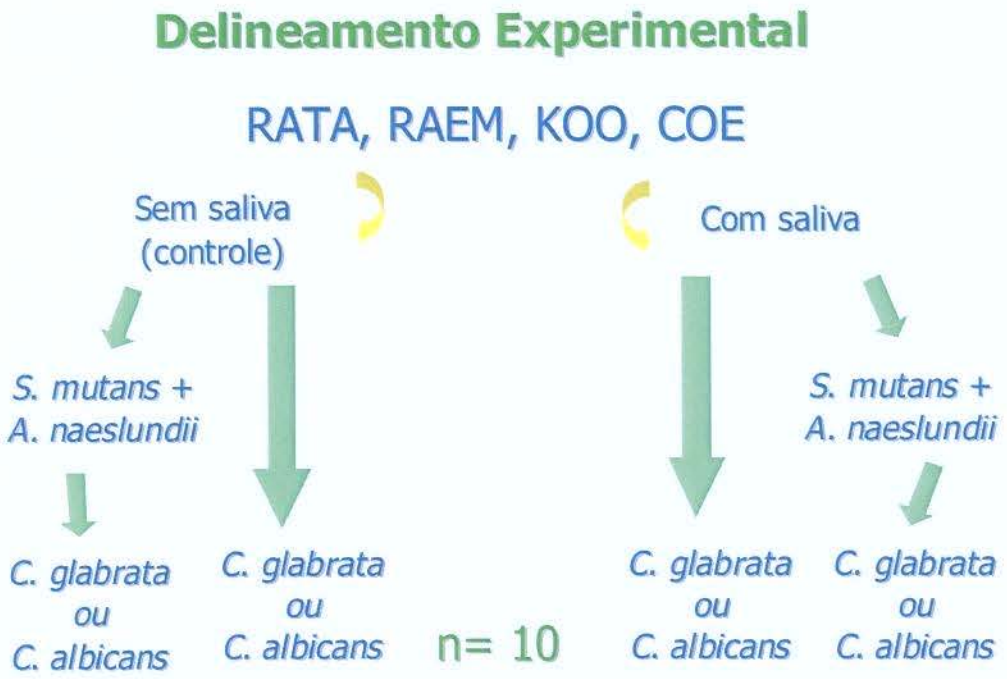

Figura 1. Divisão dos grupos de acordo com as condições experimentais em estudo.

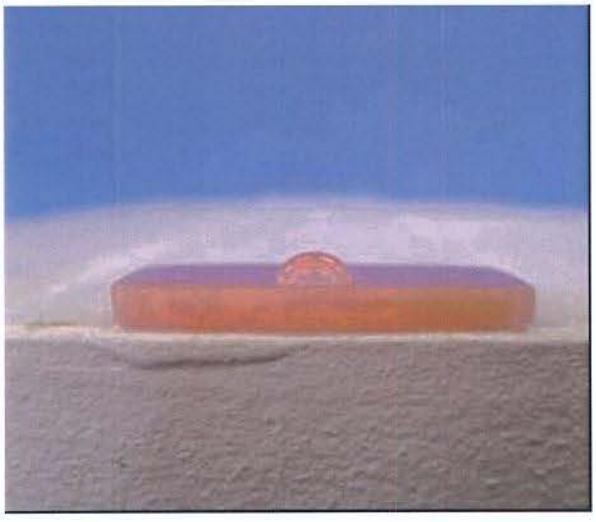

Figura 2. Espécime de resina acrílica colocado sobre o dispositivo para captura de imagem após deposição da gota.

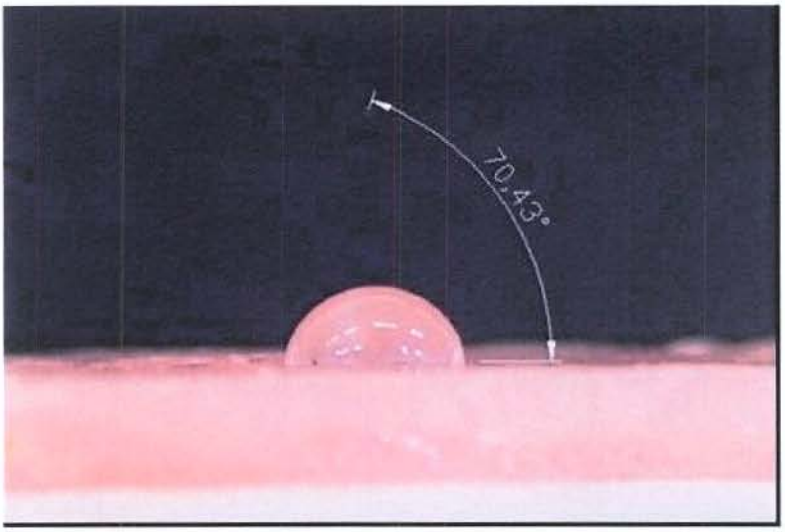

Figura 3. Mensuração do ângulo de contato em espécime de CoeSoft, para posterior obtenção da energia livre de superfície.

Figura 4. Mensuração da rugosidade de superfície.

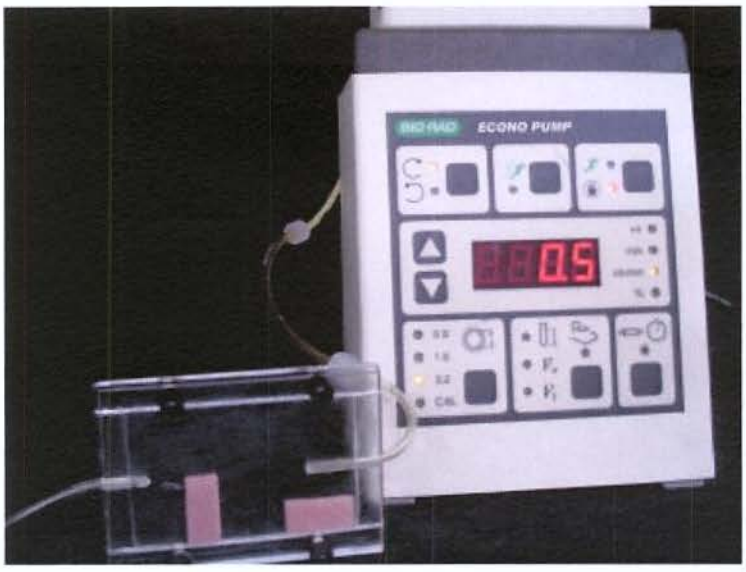

Figura 5. Câmara de fluxo ligada à bomba peristáltica para o ensaio de adesão.

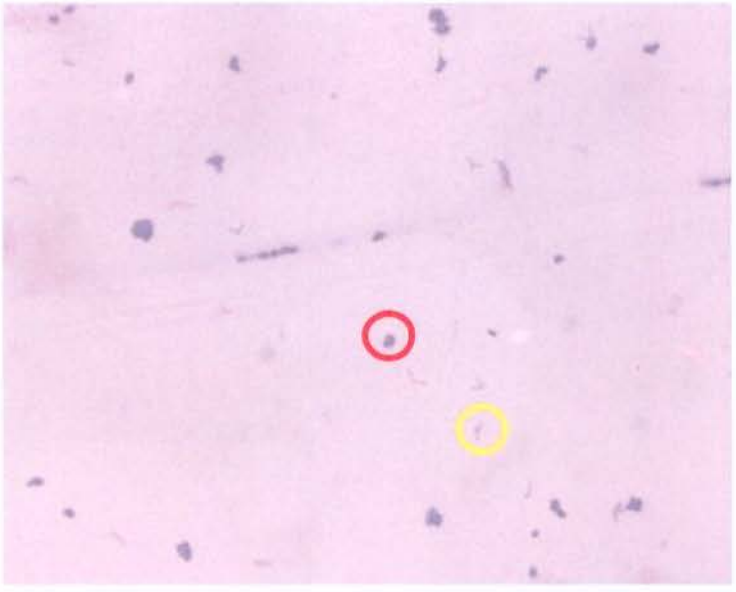

Figura 6. Adesão de *Candida glabrata* em resina acrílica polimerizada por energia de microondas com pré-colonização de bactérias e sem saliva (400x). No destaque em amarelo, bactérias; em vermelho, *Candida glabrata*.