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Efeitos de Diferentes Tratamentos a Plasma e de Variações na Coleta, Preparo e Pré-condicionamento com Saliva na Adesão de *Candida* a uma Resina para Base de Prótese

Tese apresentada ao Programa de Pós-graduação em Reabilitação Oral, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista "Júlio de Mesquita Filho", para a obtenção do título de Doutor em Reabilitação Oral.

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Dedicatória

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"Confia no SENHOR e faze o bem; habita na terra e alimenta-te da verdade. agrada-te do SENHOR, e Ele satisfará os desejos do teu coração. Entrega o teu caminho ao SENHOR, confia Nele, e o mais Ele fará"
(Salmos 37:3-5)

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"Com efeito, grandes cousas fez o SENHOR por nós, por isso estamos alegres"
(Salmos 126:3)

"Ainda que eu tenha o dom de profetizar e conheça todos os mistérios e toda a ciência; ainda que eu tenha tamanha fé, a ponto de transportar montes, se não tiver amor nada serei"
(I Coríntios 13:2)

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(...) Hoje me sinto mais forte,
Mais feliz, quem sabe
Eu só levo a certeza
De que muito pouco sei,
Ou nada sei

(...) É preciso amor
Pra poder pulsar
É preciso paz pra poder sorrir
É preciso a chuva para florir

(...) Cada um de nós compõe a sua história
Cada ser em si
Carrega o dom de ser capaz
De ser feliz...

(*Tocando em Frente*
Almir Sater e Renato Teixeira)

Sumário

Zamperini CA. Efeitos de diferentes tratamentos a plasma e variações na coleta, preparo e pré-condicionamento com saliva na adesão de *Candida* a uma resina para base de prótese [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2011.

Resumo

A adesão de *Candida* às superfícies protéticas é o passo inicial para ocorrência da estomatite protética. Entre os diversos fatores envolvidos na adesão de *Candida* spp. às superfícies poliméricas estão as interações hidrofóbicas e eletrostáticas, a rugosidade superficial e a película salivar. Assim, os objetivos deste estudo foram: investigar o potencial de diferentes tratamentos a plasma (Ar/50W; ArO_2/70W; AAt/130W; $ArSF_6$/70W) de modificar uma resina acrílica para base de prótese (VIPIWAVE) para reduzir a aderência de *Candida albicans* (ATCC 90028), avaliada pelo ensaio de XTT e cristal violeta. O efeito da rugosidade de superfície e do pré-condicionamento com saliva também foram avaliados; investigar se modificações de superfícies por meio de dois tratamentos a plasma (Ar/50W; AAt/130W) reduziriam a aderência de *Candida glabrata* (ATCC 2001), avaliada pela coloração cristal violeta, sobre superfícies lisas de resina acrílica. Além disso, o efeito do pré-condicionamento com saliva também foi avaliado; e ainda, avaliar se variações nos períodos de pré-condicionamento com saliva (0 min; 30 min; 60 min; 180 min; 720 min), nos parâmetros de centrifugação (velocidade e tempo) e número de doadores de saliva influenciariam os resultados de adesão de *Candida albicans* a uma resina acrílica para base de prótese, avaliada por meio do ensaio de XTT e coloração cristal violeta. Além disso, a correlação entre os dois métodos utilizados para avaliação da adesão de *Candida albicans* também foi avaliada. Os resultados obtidos demonstraram que os tratamentos a plasma são efetivos para modificação da hidrofobicidade de superfície ou incorporação de átomos de flúor na superfície da resina acrílica. Entretanto, após os tratamentos a plasma e imersão das amostras em água, houve

alterações significantes nos valores médios de ângulo de contato obtidos. Os grupos ArO_2/70W e $ArSF_6$/70 W apresentaram menores valores de absorbância para a adesão de *Candida albicans* comparados aos outros grupos. Nenhuma diferença significante foi observada entre os grupos tratados a plasma e o grupo controle, quando a adesão de *Candida albicans* foi avaliada por meio da coloração cristal violeta, independente da rugosidade superficial e presença ou ausência de saliva. O número de *Candida glabrata* aderida, avaliado pela coloração cristal violeta, foi significantemente menor no grupo tratado com Ar/50W comparado ao grupo controle, na ausência de saliva. Entretanto, na presença de pré-condicionamento com saliva, nenhuma diferença significante foi observada entre os grupos experimentais e controle para adesão de *Candida glabrata*. Os diferentes períodos de pré-condicionamento com saliva não influenciaram significantemente a adesão de *Candida albicans*, entretanto, os parâmetros de centrifugação (velocidade e tempo) e o número de doadores de saliva influenciaram significantemente os resultados de adesão de *Candida albicans* à resina acrílica avaliada. Nenhuma correlação significante foi encontrada entre os métodos utilizados para avaliação da adesão de *Candida albicans*, coloração cristal violeta e ensaio de XTT. Portanto, os tratamentos a plasma com ArO_2/70W e $ArSF_6$/70W demonstraram-se promissores para redução da adesão de *Candida albicans*, enquanto o tratamento a plasma com Ar/50W apresentou resultado promissor para redução da adesão de *Candida glabrata* à resina acrílica avaliada. Além disso, a película de saliva, dependendo das condições experimentais, pode aumentar a adesão de *Candida albicans*, mas não altera significantemente a adesão de *Candida glabrata*. As variações metodológicas relacionadas ao pré-condicionamento com saliva influenciaram os resultados de adesão de *Candida albicans*.

Palavras-chave: Aderência celular; biofilmes; *Candida albicans*; *Candida glabrata*; resinas acrílicas; saliva.

Zamperini CA. Effects of different plasma treatments and variations in the collection, preparation and preconditioning with saliva on *Candida* adhesion to a denture base acrylic resin [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2011.

Abstract

The adhesion of *Candida* to denture surfaces is the initial step for occurrence of denture stomatitis. Among the various factors involved on *Candida* adhesion to polymeric surfaces are the hydrophobic and eletrostatic interactions, surface roughness and pellicle salivary. Hence, the aims of this study were: to investigate the potential of different plasma treatments (Ar/50W; ArO_2/70W; AAt/130W; $ArSF_6$/70W) to modify a denture base acrylic resin (VIPIWAVE) to reduce the *Candida albicans* adhesion (ATCC 90028), evaluated by XTT reduction assay and crystal violet staining. The effect of surface roughness and saliva coating was also evaluated; to investigate the potential of two plasma treatments (Ar/50W; AAt/130W) to modify a denture base acrylic resin to reduce the *Candida glabrata* adhesion (ATCC 2001), evaluated by crystal violet staining. Moreover, the effect of saliva coating was also evaluated; and to assess the effect of different periods of preconditioning with saliva (0 min; 30 min; 60 min; 180 min; 720 min), variations in the centrifugation parameters (speed and time) and number of donors of saliva on *Candida albicans* adhesion to a denture base resin using crystal violet staining and XTT reduction assay. Additionally, the correlation between the two methods used for assessing *Candida albicans* adhesion was also evaluated. The results obtained demonstrated that the plasma treatments were effective in modifying hydrophobicity or incorporation of fluorine into acrylic resin. However, there were significant alterations in the contact angle measured after immersion in water. Groups ArO_2/70W and $ArSF_6$/70W showed significantly lower absorbance readings to *Candida albicans* adhesion than the other groups. No statistically significant difference in the

adherence of *Candida albicans*, evaluated by crystal violet staining, was observed between the plasma treated and control groups, irrespective of the presence or absence of saliva, and surface roughness. The number of adhered *Candida glabrata*, evaluated by counting after crystal violet staining, was significantly lower in Ar/50W group than the control group, in the absence of saliva. However, after preconditioning with saliva, *Candida glabrata* adherence in experimental and control groups did not differ significantly. The different periods of preconditioning with saliva had no significant influence in the *Candida albicans* adhesion, but the centrifugation parameters (speed and time) and number of donors of saliva influenced the results of *Candida albicans* adhesion to the denture base acrylic resin. No significant correlation was found between the two methods used for assessing *Candida albicans* adhesion, crystal violet staining and XTT reduction method. Thus, the results demonstrated that ArO_2/70W and $ArSF_6$/70W plasma treatments showed promising potential for reducing *Candida albicans* adhesion, while the Ar/50W plasma treatment showed promising potential for reducing *Candida glabrata* adhesion to denture base resins. Moreover, the saliva pellicle, depending of experimental conditions, may increase the *Candida albicans* adhesion, but it not significantly influences the *Candida glabrata* adhesion. The diverse methodological procedures regarding to preconditioning with saliva alter the results of *Candida albicans* adhesion.

Keywords: Cell adherence; biofilms; *Candida albicans*; *Candida glabrata*; acrylic resins; saliva.


Introdução

1 Introdução

A estomatite protética é um tipo de candidíase bucal que comumente afeta os usuários de prótese (Dagistan et al.[11], 2009). Essa condição patológica caracteriza-se pela presença de inflamação na mucosa, particularmente naquela que mantém contato com a superfície interna das próteses removíveis, totais ou parciais (Wilson[80], 1998; Barbeau et al.[2], 2003; Ramage et al.[60], 2004). Apesar da etiologia multifatorial (Wilson[80], 1998; Dagistan et al.[11], 2009), tem sido observado que *Candida albicans* é o microrganismo mais freqüentemente associado à estomatite protética (Dagistan et al.[11], 2009; Abaci et al.[1], 2010). Entretanto, recentemente, espécies não-*albicans* têm sido isoladas das superfícies protéticas e da mucosa oral (Dagistan et al.[11], 2009; Abaci et al.[1], 2010). Entre essas espécies, *Candida glabrata* foi a espécie mais comumente isolada em pacientes com estomatite protética, seguida pela *Candida pseudotropicalis*, *Candida Krusei*, *Candida tropicalis*, *Candida parapsilosis*, e outras (Dagistan et al.[11], 2009). Segundo Coco et al.[10] (2008), biofilmes mistos de *Candida albicans* e *Candida glabrata* foram associados com a ocorrência da estomatite protética, indicando que a *Candida glabrata* pode desempenhar um papel importante nessa patogênese. Além disso, nos últimos anos, a prevalência de infecções com *Candida glabrata* tem aumentado, principalmente em pacientes imunocomprometidos, o que merece atenção desde que essas infecções são, frequentemente, mais difíceis de tratar e apresentam maior taxa de mortalidade comparada às infecções com outras espécies não-*albicans* (Li et al.[29], 2007).

Os tratamentos mais comumente recomendados para a estomatite protética têm sido a utilização de medicamentos antifúngicos tópicos ou sistêmicos e a associação da escovação da prótese com a imersão em soluções desinfetantes (Budtz-Jorgensen[5], 1990; Chau et al.[9], 1995; Pavarina et al.[48], 2003). Outro método proposto para a desinfecção das próteses é a irradiação com energia de micro-ondas (Ribeiro et al.[62], 2009). Embora esses tratamentos sejam eficientes na redução dos sinais e sintomas da doença, eles apresentam alguns inconvenientes,

como: não eliminação do microrganismo (Lombardi et al.[31], 1993; Lamfon et al.[27], 2005); a indução de efeitos hepatotóxicos e nefrotóxicos (Lombardi et al.[31], 1993); a resistência dos microrganismos a esses medicamentos (Lamfon et al.[27], 2005); possíveis efeitos citotóxicos (Sagripanti et al.[64], 2000); e alterações nas propriedades físicas e mecânicas das resinas acrílicas utilizadas na confecção das próteses (Polyzois et al.[54], 1995; Ma et al.[33], 1997). Além disso, todos esses métodos visam à inativação dos microrganismos após sua adesão sobre a superfície das próteses. Essas limitações e desvantagens das terapias atuais enfatizam a importância de métodos de tratamento direcionados para a redução da adesão inicial dos microrganismos, desde que o pré-requisito para colonização e, consequentemente, ocorrência da estomatite protética é a adesão de *Candida* spp. às superfícies orais, incluindo mucosa e superfícies protéticas (Nikawa et al.[44], 1997; Verran, Maryan[77], 1997; Yildirim et al.[81], 2005).

Embora os mecanismos exatos por meio dos quais a adesão de *Candida* às superfícies acrílicas ocorre sejam desconhecidos, muitos fatores que podem afetar a adesão têm sido descritos, entre eles, a rugosidade superficial, a película de saliva e as interações hidrofóbicas e eletrostáticas.

Idealmente, um material deveria possuir uma superfície lisa e polida, a fim de que o acúmulo de biofilme fosse evitado ou minimizado (Zissis et al.[84], 2000). Entretanto, Zissis et al.[84] (2000), ao estudar diversas resinas para base de prótese e resinas reembasadoras, encontraram que a rugosidade de superfície dos materiais protéticos estudados variaram de 0,7 a 7,6 micrômetros. Em função dos valores de rugosidade obtidos e da grande variação entre os materiais, os autores concluíram que há possibilidade de acúmulo de biofilme em todos os materiais avaliados. Particularmente em relação à estomatite protética, a rugosidade está diretamente associada à retenção e aderência de *Candida* e desenvolvimento do biofilme dessas espécies (Pereira-Cenci et al.[51], 2008). Nesse contexto, a rugosidade superficial pode favorecer a fixação dos microrganismos, devido à maior área de superfície disponível para adesão, e ainda, por protegê-los contra as forças de remoção (Radford et al.[59], 1998; Taylor et al.[74], 1998; Radford et al.[58], 1999; Lamfon et al.[28], 2003).

Quando a prótese é inserida na cavidade oral, sua superfície é rapidamente recoberta por um fino filme de saliva denominado película salivar (Yildirim et al.[82], 2006). Tendo em vista que os microrganismos usualmente não se fixam diretamente nas superfícies das próteses, a presença e importância da saliva no processo de adesão e colonização fúngica são indiscutíveis, mas, o papel que ela desempenha ainda não é claro (Radford et al.[58], 1999; Nikawa et al.[40], 2001). A saliva é uma secreção exócrina produzida por diferentes glândulas salivares, consistindo de água, eletrólitos e proteínas (de Almeida et al.[12], 2008; Bräuer et al.[4], 2009). Várias funções têm sido atribuídas à saliva, entre elas as propriedades antimicrobianas, devido à presença de proteínas imunológicas e não imunológicas (de Almeida et al.[12], 2008). Entretanto, a saliva também possui proteínas que poderiam atuar como receptores para promover a adesão microbiana inicial (Edgerton et al.[14], 1993; Holmes et al.[23], 2006; Bürgers et al.[6], 2010), e/ou atuarem como fonte de água e nutrientes para o crescimento e reprodução dos microrganismos (De Jong, Van Der Hoeven[13], 1987). Assim, a influência da película salivar pode ser regulada por interações específicas entre a célula de *Candida* spp. e receptores presentes na saliva. Além disso, a película de saliva também pode influenciar a adesão por meio de alterações das características de superfície dos substratos envolvidas no processo de adesão, tais como a rugosidade superficial e a hidrofobicidade do material (Sipahi et al.[71], 2001; Yildirim et al.[81], 2005; Burgers et al.[7], 2009). Embora muitos estudos têm avaliado o papel da película salivar na adesão de *Candida* spp, os resultados obtidos até o presente momento são controversos. Tem sido sugerido que essa divergência entre os estudos pode estar relacionada às variações metodológicas (Pereira-Cenci et al.[51], 2008), tais como variações no número de doadores, tipo de saliva utilizada (estimulada ou não estimulada), parâmetros de centrifugação (tempo e velocidade), tempo de condicionamento com saliva, entre outros.

A correlação entre aderência fúngica e hidrofobicidade de superfície dos materiais também tem sido avaliada (Klotz et al.[26], 1985; Minagi et al.[36], 1985). Klotz et al.[26] (1985) encontraram uma relação linear entre o número de células aderidas por unidade de área e o ângulo de contato do substrato, ou seja, quanto

mais hidrofóbica a superfície, maior a aderência celular por unidade de área. Por outro lado, Minagi et al.[36] (1985) observaram que o aumento do ângulo de contato dos materiais estudados resultou em um aumento no número de células aderidas para *Candida tropicalis*, mas uma diminuição foi observada para *Candida albicans*. Apesar da contradição com relação à exata interação entre forças hidrofóbicas e a aderência de *Candida albicans*, esses autores concordaram com relação à importância da interação hidrofóbica na adesão inicial dos fungos aos substratos inertes, especialmente, às superfícies protéticas. Ainda nesse contexto, é importante considerar a hidrofobicidade de superfície da célula fúngica. Diversos autores afirmam que a maior hidrofobicidade de superfície celular fúngica associa-se à maior capacidade de aderência às superfícies acrílicas ou às células do hospedeiro (Hazen et al.[20], 1991; Samaranayake et al.[66], 1994; Samaranayake et al.[67], 1995; Panagoda et al.[46], 2001; Luo, Samaranayake[32], 2002; Blanco et al.[3], 2006) e que *Candida albicans*, comparada às outras espécies, apresenta uma das menores hidrofobicidades de superfície celular, ou seja, menores medidas de ângulos de contato (Minagi et al.[35], 1986; Samaranayake et al.[67], 1995; Luo, Samaranayake[32], 2002). Os resultados encontrados por Luo, Samaranayake[32] (2002) demonstraram que, tanto *Candida glabrata* como *Candida albicans* apresentaram boa aderência às superfícies acrílicas; entretanto, *Candida glabrata* apresentou maior aderência a essas superfícies quando comparada a *Candida albicans*, resultado que foi correlacionado à maior hidrofobicidade relativa de superfície celular dos isolados de *Candida glabrata*. Minagi et al.[35] (1986) estudaram a hidrofobicidade de superfície celular de seis espécies de *Candida*. Esses autores encontraram a seguinte seqüência, do maior para o menor ângulo de contato da célula fúngica: *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis*, *Candida albicans* e *Candida stellatoidea*. Assim, todos esses resultados sugerem que superfícies hidrofílicas poderiam inibir a adesão de *Candida* às superfícies acrílicas, particularmente de células relativamente hidrofóbicas (Yoshijima et al.[83], 2010).

Interações eletrostáticas também têm sido mencionadas como um fator que pode influenciar a aderência de *Candida* às superfícies poliméricas (Park et al.[47],

2003; Puri et al.[55], 2008). A interação entre polímeros e fungos sugere a presença de forças eletrostáticas, desde que as superfícies plásticas possuem um grau variado de carga de superfície negativa e, similarmente, todas as células vivas, incluindo os fungos, possuem carga de superfície negativa (Klotz et al.[26], 1985). Klotz et al.[26] (1985) avaliaram a influência das interações eletrostáticas negativas ao carregarem os fungos positivamente. Essa carga positiva nos fungos ocasionou alteração do comportamento de aderência, tornando-os, consideravelmente, mais aderentes. Diante disso, esses autores concluíram que as interações eletrostáticas repulsivas realmente existem, porque na ausência delas, a aderência é aumentada. Eles ainda puderam supor que essas interações eletrostáticas, embora presentes e capazes de influenciar a cinética de aderência, são menores quando comparadas às forças hidrofóbicas, considerando que mesmo na presença delas (forças repulsivas) a adesão ocorre (Klotz et al.[26], 1985) . Isso indica que tratamentos que resultem em superfícies negativamente carregadas poderiam reduzir a adesão de *Candida* spp.

Desde que as características dos substratos são importantes para a adesão de *Candida*, a modificação de superfícies visando inibir ou diminuir a adesão de microrganismos seria uma alternativa para prevenção da estomatite protética. Nesse contexto, o tratamento a plasma tem sido considerado um método de modificação de superfícies de materiais com aplicação em várias áreas (Yildirim et al.[81], 2005). Nessa técnica, um gás parcialmente ionizado é criado por uma descarga elétrica, e assim, um ambiente altamente reativo é gerado com presença de elétrons, íons e radicais livres (Hauser et al. [19], 2009). Além de ser um processo eficiente, outra vantagem dessa técnica é que ela permite a alteração de superfície sem indução de modificações profundas (Rangel et al.[61], 2004; Hodak et al.[22], 2008), preservando as propriedades físicas e mecânicas do material. Alguns autores têm demonstrado que o tratamento a plasma é um método efetivo para melhorar a hidrofilicidade (Rangel et al.[61], 2004; Yildirim et al.[81], 2005), modificar a composição química das superfícies (Hodak et al.[22], 2008; Suanpoot et al.[72], 2008) e diminuir a adesão bacteriana (Rad et al.[57], 1998). O tratamento a plasma também permite a incorporação de flúor no material (Guruvenket et al.[17],

2008), resultando em uma superfície carregada negativamente (Robinson et al.[63], 1997). Esses resultados sugerem que a superfície dos materiais utilizados na confecção de próteses removíveis totais ou parciais poderia ser modificada por meio do tratamento a plasma, prevenindo que tais superfícies atuem como um reservatório de infecção.


Proposição

2 Proposição

Os objetivos deste estudo in vitro foram:

1. Investigar o potencial de diferentes tratamentos a plasma de modificar a superfície de uma resina acrílica para base de prótese para reduzir a adesão de *Candida albicans* avaliada por meio do ensaio de XTT. Os efeitos da rugosidade superficial do substrato e pré-condicionamento com saliva também foram avaliados.
2. Investigar o potencial de diferentes tratamentos a plasma de modificar a superfície de uma resina acrílica para base de prótese para reduzir a adesão de *Candida albicans* avaliada por meio da contagem celular após coloração com cristal violeta. Os efeitos da rugosidade superficial do substrato e pré-condicionamento com saliva também foram avaliados.
3. Investigar o potencial de diferentes tratamentos a plasma de modificar a superfície de uma resina acrílica para base de prótese para reduzir a adesão de *Candida glabrata* avaliada por meio da contagem celular após coloração com cristal violeta. O efeito do pré-condicionamento com saliva também foi avaliado.
4. Avaliar o efeito de diferentes períodos de pré-condicionamento com saliva na adesão de *Candida albicans* a uma resina acrílica para base de prótese. Adicionalmente, a correlação entre os dois métodos utilizados para avaliação da adesão de *Candida albicans*, ensaio de XTT e contagem celular após coloração cristal violeta, foi investigada.
5. Avaliar o efeito de variações nos parâmetros de centrifugação e número de doadores de saliva na adesão de *Candida albicans* a uma resina acrílica para base de prótese, por meio do ensaio de XTT e contagem celular após coloração cristal violeta.


Capítulos

3 Capítulos

3.1 Capítulo 1

Adherence in vitro of *Candida albicans* to plasma treated acrylic resin. Effect of plasma parameters, surface roughness and salivary pellicle

Adherence of *Candida* to modified acrylic

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Abstract

The adhesion of *Candida albicans* to surfaces is the prerequisite for occurrence of denture stomatitis. Objective: Hence, this study investigated if surface modifications with plasma treatments could reduce the adherence of *Candida albicans* to a denture base resin. Methods: Specimens (n=180) with roughened and smooth surfaces were made and divided into five groups: control – specimens were left untreated; experimental groups – specimens were submitted to plasma treatments to obtain surfaces with different hydrophobicity (Ar/50 W; ArO_2/70 W; AAt/130 W) or incorporation of fluorine ($Ar/SF_6$70 W). Contact angle measurements were performed immediately after the treatments and after immersion in water for 48 hours. For each group, half of the specimens were incubated with saliva prior to the adhesion assay. The number of adherent yeasts was evaluated by XTT reduction method. Results: For the experimental groups, there was significant change in the mean contact angle after 48 hours of immersion in water. Groups ArO_2/70 W and $ArSF_6$/70 W showed significantly lower absorbance readings than the other groups, regardless the presence or absence of saliva and surface roughness. Conclusions: Results demonstrated that ArO_2/70 W and $ArSF_6$/70 W plasma treatments showed promising potential for reducing the adherence of *Candida albicans* to denture base resins.

Keywords: *Candida albicans*; denture acrylic; saliva; roughness; fungal adherence.

Introduction

The inability of current antifungal therapy to cure denture stomatitis emphasizes the importance of treatment methods directed towards reducing initial fungal attachment, since the prerequisite for colonization and, consequently, occurrence of denture stomatitis is the adhesion of *Candida albicans* to oral surfaces, including mucosa and denture surfaces [1-3]. Although the exact mechanisms by which the adhesion of *Candida* to acrylic surfaces occurs are unknown, many factors that affect *Candida* adherence have been described, among them surface roughness, salivary pellicle, and hydrophobic and electrostatic interactions. Surface roughness seems to favor microbial attachment and difficult detachment, probably because it provides a larger surface area and/or protection against shear forces [4]. The influence of salivary pellicle may be regulated by specific interactions between the *C. albicans* cellular adhesins and receptors in the pellicle [5]. Saliva may also alter the surface characteristics of the substrates involved in the adhesion process, such as roughness and hydrophobicity [3,5,6]. With regard to hydrophobic interactions, a nearly linear relationship between the number of *Candida albicans* adhering per unit area and the hydrophobicity of polymers (determined by the contact angle) has been observed [7]. In addition, it has been reported that the closer the surface free energy of the substrate surface and the yeast, the higher was the probability of adherence [8]. Electrostatic interaction has also been mentioned as a factor that can influence the adherence of *Candida* to polymers [9,10]. Yeasts whose surfaces had been electrically altered

(positive charge) were more adherent due to repulsive forces between negatively charged yeast cell and polymer surfaces [7].

Since surface characteristics of substratum are important to *Candida* adherence [3,6], chemical modification of the surface charge of denture base acrylic resins by copolymerization of methacrylic acid to methyl methacrylate [9;11] or incorporation of phosphate groups in the monomer [10,12] have been proposed to prevent denture stomatitis. Another approach is the application of coatings with or without incorporation of antifungal medications to change the hydrophobicity or discourage microbial attachment [11,13,14]. Although these methods have been effective in reducing the adhesion of *Candida albicans* to the acrylic surfaces, there are concerns regarding the biocompatibility and the physical properties of these modified polymers [9,10-12] as well as long-term durability [11,13,14]. Glow discharge plasma-based treatments have also been considered a potential method for surface modification of polymeric materials in many fields [3]. In this technique, a partially ionized gas is generated by an electrical discharge, and thus, a highly reactive environment is created with species like electrons, ions and free radicals. Besides time efficient process, another advantage of this technique is that it allows surface alteration without inducing bulk modifications [15,16], preserving the mechanical and physical-chemistry properties of the original materials. Some authors have demonstrated that the plasma treatment is an effective method to improve the hydrophilicity [3,15,17], modify the chemical composition of the surfaces [16,18], and decrease bacterial attachment [19]. Plasma treatment also allows the incorporation of fluorine-containing species to the material [20,21], resulting in

negatively charged surfaces [22]. These results suggest that the surface of materials used in removable complete and partial denture could be modified by plasma treatment, preventing such surfaces to act as infection reservoirs. However, information on the adhesion of *Candida albicans* to glow-discharge modified acrylic denture base polymers are scarce and only oxygen plasma treatment was evaluated [3]. Moreover, surface modification of denture base resins by fluorine plasma treatment still remains to be investigated.

The main purpose of the present in vitro study was to investigate the potential of different plasma treatments to modify a denture base acrylic resin to reduce the *Candida albicans* adhesion. The effect of substrate surface roughness and saliva coating was also evaluated.

Materials and Methods

Preparation of Acrylic Resin Specimens

The specimens (n=180) were fabricated from an acrylic resin denture base material (Vipi Wave - VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda Pirassununga, SP, Brazil) using a conventional flasking and pressure-pack technique. Initially, a metal mold was used to make disk-shaped silicone patterns Zetaplus/Indurent - Zhermack, Badia Polesine, Rovigo, Italy) measuring 13.8 X 2 mm. Half of the silicone patterns were invested in the flaks directly in dental stone, while the other half of the patterns were sandwiched between two glass slides before investing. These two types of investing techniques were used to obtain rough and smooth specimens, thus mimicking the tissue-fitting surface and the outer surface of dentures,

respectively. The flasks were separated, the silicone patterns were removed, and the stone surfaces were painted with a separating medium (Vipi Film - VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda Pirassununga, SP, Brazil). For each specimen, 1 g of powder and 0.47 mL of monomer liquid were mixed and processed according to the manufacturer's instructions. The mixture was packed into the molds, a trial pack was completed, and excess material was removed. A final pack was performed and held for 15 minutes. The denture base acrylic resin was processed in a 500 W domestic microwave oven (Brastemp – Brastemp da Amazonia SA, Manaus, AM, Brazil) for 20 minutes at 20% power, followed by 5 minutes at 90% power. The flasks were allowed to bench cool at room temperature, the specimens were deflasked, and excess flash was aseptically removed with a sterile bur (Maxi-Cut; Lesfils de August Malleifer SA, Ballaigues, Switzerland).

Surface Roughness Measurements

The surface roughness of all specimens was measured with a profilometer (Mitutoyo SJ 400 – Mitutoyo Corporation - Japan). Three measurements were made for each specimen and the average reading was designated as the Ra (μm) value of that specimen. Resolution was 0.01 μm, interval (cutoff length) was 0.8 mm, transverse length was 2.4 mm, the stylus speed was 0.5 mm/s, and the diamond stylus tip radius was 5 μm. All measurements were recorded by one operator.

Plasma Treatments

After roughness measurements, the specimens were cleaned in an ultrasonic cleaner using water and detergent bath for 15 minutes, then sonicated in distilled water for 15 minutes and dried in air. The specimens were then divided into five groups, each one including 18 specimens processed against stone and 18 polymerized in contact with glass. In the control group, the specimens were left untreated. For the four experimental groups, both specimen surfaces were exposed to plasmas generated under the following conditions: argon atmosphere at 50 W (group Ar/50 W); argon/oxygen atmosphere at 70 W (group ArO_2/70 W); atmospheric air at 130 W (AAt/130 W); argon atmosphere, followed by plasma treatment in a sulfur hexafluoride atmosphere, both performed at 70 W (group Ar/$SF_6$70 W). The plasma exposure time (5 minutes) and the position of the specimens within the plasma chamber were kept unchanged. To determine the plasma parameters used in the experimental groups, pilot experiments were performed in which various conditions of exposure time, atmosphere composition and pressure, and radiofrequency power were tested. For groups Ar/50 W, Ar/$O_2$70 W and AAt/130 W, the plasma parameters were chosen based on the degree of surface hydrophobicity. Parameters that produced surfaces with low hydrophobicity (contact angle close to zero) were used for group AAt/130 W. For groups Ar/50 W and Ar/$O_2$70 W, parameters that provided hydrophobicity values between those of the untreated specimens (higher hydrophobic) and those of the group AAt/130 W specimens were chosen. In the case of group Ar/$SF_6$70 W, the pilot experiments established the appropriate conditions for the incorporation of

fluorine into the surfaces. Fluorine incorporation was confirmed by photoelectron spectroscopy analysis (XPS), carried out in a UNI-SPECS UHV spectrometer using Mg K_α line (E = 1253.6 eV) and with the analyzer pass energy set to 10 eV. The inelastic background of the C 1s, F 1s, O 1s, and N 1s electron core-level spectra was subtracted using Shirley's method. The binding energies of the spectra were corrected using the hydrocarbon component of the polymer fixed at 285.0 eV. The composition of the surface layer was determined from the ratio of the relative peak areas corrected by sensitivity factors of the corresponding elements. The spectra were fitted without placing constraints using multiple Voigt profiles. The width at half maximum (FWHM) varied between 1.6 and 2.0 eV and the accuracy of the peak positions was ±0.1 eV. One specimen of untreated denture base acrylic resin and one of $ArSF_6$-treated specimen were analyzed.

Plasma treatments were performed by the application of radiofrequency power (13.56 MHz) to two parallel plate electrodes fitted inside a homemade stainless steel vacuum chamber. In this technique, gas temperature remains at room temperature, preserving the integrity of the material [23,24]. In addition, during plasma treatment, specific active agents such as, ultraviolet photons and radicals are generated, resulting in sterilization of the samples [25].

Contact Angle Measurements

The water contact angle has been measured to characterize the surface wettability [3,15]. This angle is defined as the angle at the intercept of a plane tangent to the drop and the plane containing the substrate-liquid interface. The

measurements were performed in an automated goniometer (Ramé-Hart, 100-00) using deionized water as test liquid. The goniometer comprises a CCD camera to record the image of a droplet placed onto the surface using a microsyringe and a dedicated image processing software to determine the contact angle. Measurements in two different positions were made for each specimen and the average was calculated. Specimens were then stored at room temperature in sterile distilled water for 48 h to release any residual monomer [26]. Afterwards, the contact angles of each specimen were again measured.

Saliva Collection

Unstimulated whole human saliva was collected from fifteen healthy adult volunteers. The saliva was expectorated into sterile 50 mL Falcon tubes on ice, pooled and clarified by centrifugation at 10000 *g* for 5 min at 4 ºC [26]. The saliva was prepared at 50% (vol/vol) in sterile PBS [27]. The resulting saliva was immediately stored at -70 ºC until use. The study was approved by the Ethics Committee of Araraquara Dental School (027/2007), and all subjects volunteered to participate and signed an informed consent form.

Adherence Assay

Candida albicans strain ATCC 90028 was used. Stock cultures were maintained at -70 ºC. After recovery this was maintained on YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) stored at 4 – 6 ºC during the experimental period. To prepare the yeast inoculum, a loopful of the stock culture was streaked onto YEPD medium and incubated at 37 ºC for 48 h. Two loopfuls

of this young culture were transferred to 20 mL of yeast nitrogen base (YNB) medium with 50 mM glucose and incubated at 37 ºC for 24 h. Cells of the resultant culture were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2) at 5000 *g* for 5 min and resuspended in YNB with 100mM glucose. *Candida* suspensions were spectrophotometrically standardized to a concentration of 1 x 10^7 cells/mL. Three mL of the standardized *C. albicans* cell suspension was added to each well containing the specimen. The cells were left to adhere for 90 min at 37 ºC [28]. The non-adherent cells were removed from the specimen by gently washing twice with 3 ml PBS. For all experimental conditions, the negative controls were acrylic specimens to which no cells were added. All experiments were performed in triplicate on three independent occasions.

Preconditioning with Saliva

To investigate the effect of the saliva on candidal adhesion to the denture acrylic resin, half of the specimens from each group (9 rough and 9 smooth) were incubated into the 12-well microtiter plates and coated with 3 mL of prepared saliva for 30 min at room temperature prior to the adhesion assay.

Measurement of Adherent *C. albicans*

To estimate the number of adherent yeasts, 9 specimens from each experimental condition were evaluated by XTT reduction assay, which evaluates cell viability of the adherent cells. XTT (Sigma, MO, USA) was prepared in ultrapure water at a final concentration of 1mg/mL. The solution was filter sterilized and stored at -70 ºC until use. Menadione (Sigma, MO, USA) solution

was prepared in acetone at 0.4 mM immediately before each assay. After washing, the specimens were transferred to new wells with 158 µl PBS with 200mM glucose, 40 µl XTT and 2 µl menadione were inoculated to each well. The plates were incubated for 3 h in the dark at 37 ºC [29]. The whole content of each well was transferred to a tube, and centrifuged at 5000 *g* for 2 minutes. The colorimetric change of the supernatant was measured using a microtiter plate reader (Thermo Plate – TP Reader) at 492 nm.

Differences in the metabolic activity (XTT assay) among the experimental conditions (smooth and rough surface, presence and absence of saliva), within each group, was evaluated by Kruskal-Wallis test. Because no significant differences were found, data from each group were then pooled together, and a Kruskal-Wallis non-parametric analysis was performed to detect differences among the groups. For each group, two-way repeated measure analysis of variance, followed by Tukey's test, was used to evaluate the effect of investing technique and time of measurement on the contact angle. Data from roughness measurements were analyzed by Kruskall-Wallis non-parametric test. A significance level of 0.05 was used for all statistical tests.

Results

Candida albicans adherence as determined by XTT assay is shown in Figure 1. Groups ArO_2/70 W and $ArSF_6$/70 W were not different from each other and both showed significantly lower absorbance readings than the other groups ($p<.05$), regardless the presence or absence of saliva and surface roughness

(smooth or roughened). All negative controls exhibited no metabolic activity (data not shown).

Table 1 shows the means and standard deviations of contact angle and the results of Tukey post hoc tests. It can be seen that there was significant change in the mean contact angle after 48 hours of immersion in water for all groups evaluated, with the exception of the control group, in which no significant difference was found. In control group, there was significant difference between the mean contact angle of smooth and roughened surfaces, regardless the time of measurement. Similar result was observed in group $ArSF_6$/70 W only in the 48 hours period.

Roughness values of all groups evaluated are presented in Table 2. There were no significant differences among the groups in each investing technique. For all groups, the mean roughness values of the specimens processed against stone were higher than those of the specimens processed against glass.

XPS analysis demonstrated the incorporation of fluorine into the surface of the specimens of $ArSF_6$/70 group. Figure 2 shows the signals assigned to CF_2 and CF_3 moieties and the envelope curve, which represents the total F(1s) area.

Discussion

The initial attachment of *Candida albicans* on the mucosal surface of the denture is essential in the colonization and development of denture stomatitis. Since many factors may influence the initial adherence of yeasts to acrylic surfaces, such as attractive hydrophobic interactions and repulsive electrostatic

forces, the development of the methods that reduce the adherence of *Candida* to these surfaces, could be a significant step toward treatment and prevention of denture stomatitis. Glow-discharge plasma, a type of cold plasma, has been often used as a method of surface modification; however, in dentistry it has received little attention. In this technique, gas temperature can remain as low as room temperature [24], preserving the integrity of polymer-based materials. This is of particular importance for denture base acrylic resins, in which the heating may cause dimensional changes and affect the fit of the denture bases to the supporting tissues [23].

In this study, the aim was to investigate whether surface modifications with different plasma treatments could decrease the adherence of *Candida albicans* to a denture base resin. One of these modifications was intended to decrease the surface hydrophobicity. The results revealed that plasma treatments with Ar/50 W, ArO_2/70 W, AAt/130 W decreased the hydrophobicity of all surfaces (rough and smooth) immediately after the plasma treatment, when compared to the untreated specimens. This occurred most likely because the plasma treatments generated free radicals in the material by inelastic collisions, mainly involving energetic electrons in the discharge and species on the polymer surfaces [15]. Chemical reactions that occur between these free radicals and species, such as atomic hydrogen or oxygen from the polymer or atmospheric contaminants, incorporate hydrophilic groups to the polymer surfaces [15], and the contact angle is reduced.

Another surface modification used in the present study involved the incorporation of fluorine in the resin surface. To the author's knowledge, to date this is the first study that has addressed this issue. The results have shown that the contact angle, immediately after plasma treatment with $ArSF_6$/70 W, increased considerably compared to control group, which is in agreement with the studies of Guruvenket et al. [21] and Rangel et al. [20]. This was probably due to the replacement of hydrophilic species by fluorine atoms [16]. As a result, the hydrogen bonds between water molecules and surface groups decrease, reducing the hydrophilicity [16]. X-ray photoelectron spectroscopy (XPS) was used to confirm the chemical changes on surfaces treated with $ArSF_6$/70 W. The incorporation of fluorine occurred, as demonstrated by the presence of F(1s) peak in XPS spectra. There was a decrease in the atomic concentrations of carbon from 75.3 at.% to 55.4 at.%, oxygen from 23.0 at.% to 14.1 at.%, and fluorine incorporation of 29.6 at.%.

In this study, the adhesion of *Candida albicans* was quantified using the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. This colorimetric method is based on metabolic activity [30] and has been widely used for the quantification of yeasts [27,30,31]. The results revealed that ArO_2/70W plasma treatment significantly reduced the yeast adhesion. These results do not agree with the data reported by Yildirim et al. [3], who have found higher counts of *Candida albicans* in plasma treated surfaces than in the unmodified control group. One possible reason for this disagreement could be that, in the study of Yildirim et al. [3], different plasma parameters were used (oxygen atmosphere at 50 or 100 W, during 15 minutes). The decrease of

adherence observed in the present study could not be related to hydrophobic interactions. Although ArO_2/70W plasma treatment resulted in a more hydrophilic surface (Table 1), after immersion of the specimens in water for 48 hours, the contact angles were similar to those of the control specimens. A possible explanation for this recovery could be that the decrease in the water contact angle obtained with ArO_2/70 W enhanced the surface energy. Under such a situation, it has been observed the polymer surfaces submitted to plasmas tended to return to their original hydrophobicity [15]. This was attributed to movement of polar groups from the surface to the polymer bulk.

The results also demonstrated that the adherence of *C. albicans* to $ArSF_6$/70W plasma treated specimens was significantly reduced compared to control. To the author's knowledge to date, the potential of fluorine plasma treatment to reduce adhesion of *Candida albican*s to denture base material has yet not been addressed. Similarly to the ArO_2/70W treatment, it was not possible to correlate the reduction in *C. albicans* adhesion promoted by $ArSF_6$/70W treatment with surface hydrophobicity. After the $ArSF_6$/70W plasma treatment, the sample surfaces became hydrophobic exhibiting the highest contact angle values. However, after water immersion for 48 hours, a decrease in the contact angle values was observed and the values were close to those obtained in the other groups, including control. Despite these changes, the fluorine was still present in the surface, as demonstrated by XPS analysis. Hence, the reduction of the adherence of *C. albicans* with $ArSF_6$/70W plasma treatment could be attributed to repulsive electrostatic forces between the fungal cells and specimens in which

fluorine was incorporated. Robinson et al. [22] found that increasing the degree of fluorination the surfaces became more negative due to the presence of the electronegative fluorine atoms. It has been reported that surface-charged resins may alter the ionic interaction between the denture base and *Candida* spp [9-11]. Negatively charged resin surfaces showed significantly lower levels of *Candida* than the untreated ones [9,11].

Roughness has been considered an important factor that affects the adhesion and some studies have found that an increase in surface roughness facilitated the yeast retention [1,32-34]. Thus, in this study, in all groups half of the specimens were processed against stone and half were polymerized in contact with glass for obtaining rough and smooth surfaces, respectively. However, no significant differences were observed in the *Candida albicans* adhesion (Figure 1). These results are in accordance with those reported in recent studies where no significant influence of roughness on adherence of *Candida albicans* was verified. [5,26,35-37]. Nevertheless, other studies should be conducted using specimens with prepared surfaces that cover a wide range of roughness values.

Since all intra-oral surfaces are coated by saliva, it is important to consider its effects on adhesion. Hence, in the present investigation, half of the specimens of each group were preconditioned with saliva prior to inoculation. Adhesion of *Candida albicans* to untreated and treated specimens was not influenced by saliva. A comparison among *in vitro* studies reveals contradictory results. While some authors observed that salivary pellicle promoted fungal colonization on the materials [2,28,38-41], others have found that the pretreatment with saliva had no

effect [27,30,42-44] or decreased the *Candida* adherence [26,44,45-48]. These divergent results could be attributed to different methodologies used, including the number of donors and possible individual variations, the use of stimulated or unstimulated saliva, filtered or whole saliva, undiluted or diluted saliva, different speed and time of saliva centrifugation and incubation periods and temperatures. These factors could result in different compositions and viscosities, affecting the role of saliva in adherence. The different results could also be related to the materials evaluated in each study, such as resilient denture lining materials [2,39,40,42,47,48], maxillofacial polymeric materials [41], acrylic surfaces [26,28,38,41,43-48], and polystyrene [27,30]. It has been observed that surfaces with small differences in their chemical composition differ in respect to adsorption of salivary proteins [6]. These variations in methodologies make comparison among studies difficult and point to the need of standardization. Nevertheless, the results of this study are similar to those reported by Ramage et al. [27], Jin et al. [30] and Thein et al. [43] who observed that the presence of saliva did not interfere with *Candida albicans* adherence.

This study has limitations since only one strain of *Candida albicans* and one heat-polymerized denture base were used. In addition, other plasma parameters and/or atmospheres should be evaluated. Despite these limitations, the results demonstrated that $ArSF_6$/70W e ArO_2/70 plasma treatments showed promise, justifying further investigation.

Within the limitations of this in vitro study, the following conclusions can be drawn:

1) The adherence of *Candida albicans* was significantly reduced by ArO_2/70 W and $ArSF_6$/70 W plasma treatments when compared to the control group, regardless the presence or absence of saliva and surface roughness (smooth or roughened).

2) The hydrophobicity (high water contact angle) of the acrylic resin evaluated was altered by the plasma treatments. However, after 48 hours of immersion in water, the mean contact angles of the treated specimens were similar to those of control specimens.

3) No significant effect of surface roughness and saliva on the adherence of *Candida albicans* was detected for all groups evaluated.

Acknowledgements

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Tables

Table 1 – Means and standard deviations (SD) of contact angles (º) obtained immediately after plasma treatments and after 48 hours of immersion in water.

Groups	Surfaces	Time of measurement	
		Immediately after plasma treatment	After 48 h in water
Control	Smooth	57.06 (3.05) [a]	55.67 (2.62) [a]
	Roughened	60.34 (4.04) [b]	59.86 (4.38) [b]
AAt/130 W	Smooth	1.88 (2.58) [a]	56.92 (6.38) [b]
	Roughened	0.45 (0.92) [a]	56.20 (5.52) [b]
Ar/50 W	Smooth	43.82 (5.17) [a]	45.82 (7.26) [b]
	Roughened	40.03 (6.24) [a]	46.85 (6.81) [b]
ArO_2/70 W	Smooth	23.00 (4.18) [a]	58.60 (6.11) [b]
	Roughened	25.01 (6.29) [a]	56.68 (7.57) [b]
$ArSF_6$/70 W	Smooth	95.83 (7.95) [a]	67.38 (6.48) [b]
	Roughened	98.91 (8.74) [a]	56.27 (6.94) [c]

Means with equal letters within the same group are not different at a level of $p<0.05$.
No comparisons were made among groups.

Table 2 – Means and standard deviations (SD) of roughness (Ra-µm) for groups and surfaces (n=18).

Groups	Surfaces	
	Smooth	Roughened
Control	0.27 (0.08) [a]	1.76 (0.83) [b]
AAt/130 W	0.33 (0.11) [a]	2.08 (0.40) [b]
Ar/50 W	0.33 (0.09) [a]	1.86 (0.63) [b]
ArO_2/70 W	0.29 (0.09) [a]	1.75 (0.51) [b]
$ArSF_6$/70 W	0.29 (0.08) [a]	1.82 (0.52) [b]
Kruskall – Wallis	p= 0.287	p= 0.239

Means followed by the same superscript lower case letters within each column are not significantly different at p = 0.05.

Figures

Figure 1

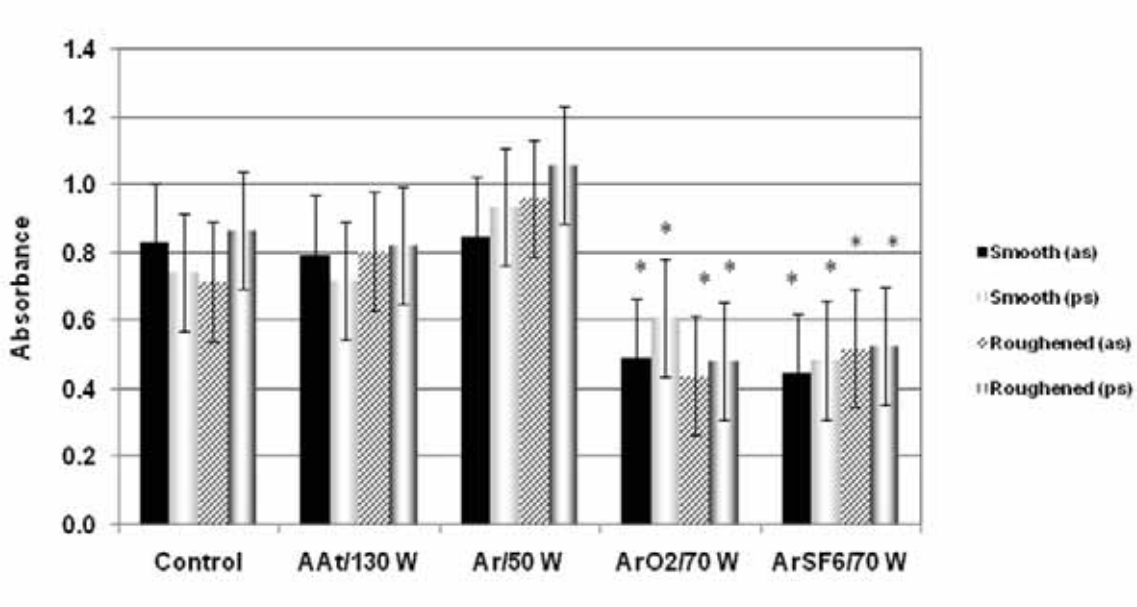

Figure 2

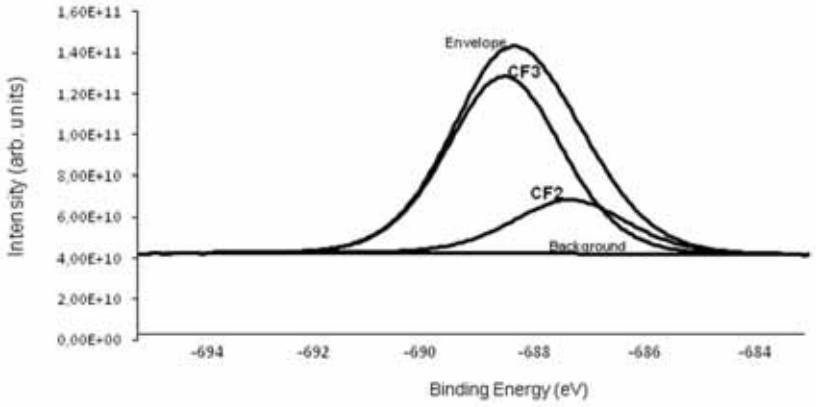
1.60E+11
1.40E+11
1.20E+11
1.00E+11
8.00E+10
6.00E+10
4.00E+10
2.00E+10
0.00E+00
Intensity (arb. units)
Envelope
CF3
CF2
Background
-694
-692
-690
-688
-686
-684
Binding Energy (eV)

Figure captions

Figure 1 – Mean absorbance (OD at 492 nm) and 95% confidence intervals for all groups.

as = absence of saliva; ps = presence of saliva.

* = statistically different means compared to control, AAt/130 and Ar/50 groups.

Figure 2 - XPS analysis of the specimens of $ArSF_6$/70 group. Envelope represents total F(1s) area.

3.2 Capítulo 2

Evaluation of fungal adherence to plasma-modified polymethylmethacrylate

Fungal adherence to polymethylmethacrylate

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Summary

There is a propensity for fungal adherence to the polymethylmethacrylate used for making denture bases. Therefore, this study investigated whether surface modifications with plasma treatments would reduce the adherence of *C. albicans* to a denture base resin. Samples (n=180) with smooth and rough surfaces were

made and divided into five groups: control – non treated; experimental groups – submitted to plasma treatments to obtain surfaces with different hydrophobicities (Ar/50 W; ArO_2/70 W; AAt/130 W) or with incorporated fluoride ($Ar/SF_6$70 W). Contact angles were measured immediately after treatments and after samples were immersed in water for 48 hours. For each group, half the samples were incubated with saliva before the adherence test. The number of adhered *C. albicans* was evaluated by counting after violet crystal staining. The plasma treatments were effective in modifying the polymethylmethacrylate surface. However, there was a significant alteration in the contact angle measured after immersion in water. No statistically significant difference in the adherence of *C. albicans* was observed between the experimental and control groups, irrespective of the presence or absence of saliva, and surface roughness.

Keywords: *Candida* spp; *Candida albicans*; fungal adherence; denture stomatitis; cell-surface-hydrophobicity.

Introduction

Polymers are widely used materials in different areas [1, 2]. In dentistry, polymethylmethacrylate is the polymer of choice for making removable denture bases for the purpose of rehabilitating partially or completely edentulous patients. Nevertheless, in spite of the good mechanical and esthetic properties of this material, microorganisms, particularly *Candida albicans*, have a propensity for

adhering to denture surfaces [1]. This adhesion capacity of *Candida albicans* to denture surfaces is the first step, considered essential, for the development of denture stomatitis [3-5], a common type of oral candidiasis among denture wearers [6-8]. Therefore, the development of methods that could modify these surfaces in order to prevent the adhesion of *Candida albicans*, would be a significant advancement in the treatment of this pathology.

Although the exact mechanisms involved in the adherence of microorganisms to dentures are not completely known, various factors may influence this process, among them, surface roughness and the presence of saliva. The increase in surface roughness has been correlated with the greater ease of fungal retention [3, 9]. On the other hand, the effect of saliva on this process is not clear and the results are controversial [10-12]. Some authors [13-15] also point out the influence of hydrophobic interactions. Minagi et al. [14] observed that the closer the surface energy of the fungal cell and the substrate are, the greater the probability of adherence occurring. Klotz et al. [13] observed a linear relationship between the number of *Candida albicans* adhered per unit of area and the hydrophobicity of polymers. Electrostatic interactions have also been mentioned as another factor that could influence the adherence of microorganisms to polymers [11, 13, 16]. Fungal cells, whose surfaces were electrically altered with a positive charge, were shown to be more adherent, suggesting the action of repulsive forces present between the fungi and polymers [13].

Thus, considering that the characteristics of the substrate are important for *Candida* adherence [4, 17], the surface modification of the

polymethylmethacrylate used for making denture bases could reduce the adhesion of *Candida albicans*. In this context, the modification of biomaterial surfaces by means of plasma treatment has been proposed [1, 4, 15]. Among the various results achieved by this technique, is the increase in the hydrophilicity of biomaterials [2, 4, 18], modification of the chemical composition of surfaces [19, 20] and the reduction in bacterial adhesion [21]. Plasma treatment also allows fluoride to be incorporated into the materials [22, 23], which could result in a negatively charged surface [24] and reduce the adhesion of *Candida* [11, 13].

In view of this, the object of the present study was to verify whether surface modifications with different plasma treatments would diminish the adherence of *Candida albicans* to a polymethylmethacrylate used for denture bases.

Materials and Methods

Preparation of Acrylic Resin Specimens

The specimens (n=180) were fabricated from an acrylic resin denture base material (Vipi Wave - VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda Pirassununga, SP, Brazil) using a conventional flasking and pressure-pack technique. Initially, a metal mold was used to make disk-shaped silicone patterns Zetaplus/Indurent - Zhermack, Badia Polesine, Rovigo, Italy) measuring 13.8 X 2 mm. Half of the silicone patterns were invested in the flasks directly in dental stone, while the other half of the patterns were sandwiched between two glass slides before investing. These two types of investing techniques were used to obtain rough and smooth specimens, thus

mimicking the tissue-fitting surface and the outer surface of dentures, respectively. The flasks were separated, silicone patterns removed, and the stone surfaces were painted with a separating medium (Vipi Film - VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda Pirassununga, SP, Brazil). For each specimen, 1 g of powder and 0.47 ml of monomer liquid were mixed and processed according to the manufacturer's instructions. The mixture was packed into the molds, a trial pack was completed, and excess material was removed. A final pack was performed and held for 15 minutes. The denture base acrylic resin was processed in a 500 W domestic microwave oven (Brastemp – Brastemp da Amazônia SA, Manaus, AM, Brazil) for 20 minutes at 20% power, followed by 5 minutes at 90% power. The flasks were allowed to bench cool at room temperature, the specimens were deflasked, and excess flash was aseptically removed with a sterile bur (Maxi-Cut; Lesfils de August Malleifer SA, Ballaigues, Switzerland).

Surface Roughness Measurements

The surface roughness of all specimens was measured with a profilometer (Mitutoyo SJ 400 – Mitutoyo Corporation - Japan). Three measurements were made for each specimen and the average reading was designated as the Ra (μm) value of that specimen. The resolution was 0.01 μm, the interval (cutoff length) of 0.8 mm, the transverse length of 2.4 mm; the stylus speed 0.5 mm/s, and the diamond stylus tip radius was 5 μm. All measurements were recorded by one operator.

Plasma Treatments

After roughness measurements, the specimens were cleaned in an ultrasonic cleaner using water and detergent bath for 15 minutes, then sonicated in distilled water for 15 minutes and air dried. The specimens were then divided into five groups, each including 18 specimens processed against stone and 18 polymerized in contact with glass. In the control group, the specimens were left untreated. For the four experimental groups, both specimen surfaces were exposed to plasmas generated under the following conditions: argon atmosphere at 50 W (group Ar/50 W); argon/oxygen atmosphere at 70 W (group ArO_2/70 W); atmospheric air at 130 W (AAt/130 W); argon atmosphere, followed by plasma treatment in a sulfur hexafluoride atmosphere, both performed at 70 W (group Ar/$SF_6$70 W). The plasma exposure time (5 minutes) and the position of the specimens within the plasma chamber were kept unchanged. To determine the plasma parameters used in the experimental groups, pilot experiments were performed, in which various conditions of exposure time, atmosphere composition and pressure, and radiofrequency power were tested. For groups Ar/50 W, Ar/$O_2$70 W and AAt/130 W, the plasma parameters were chosen based on the degree of surface hydrophobicity. Parameters that produced surfaces with low hydrophobicity (contact angle close to zero) were used for group AAt/130 W. For groups Ar/50 W and Ar/$O_2$70 W, parameters that provided hydrophobicity values between those of the untreated specimens (higher hydrophobic) and those of the group AAt/130 W specimens were chosen. In the case of group Ar/$SF_6$70 W, the pilot experiments established the appropriate conditions for the incorporation of fluorine into the surfaces. Fluorine incorporation was confirmed by photoelectron

spectroscopy analysis (XPS), carried out in a UNI-SPECS UHV spectrometer using Mg K_α line (E = 1253.6 eV) and with the analyzer pass energy set to 10 eV. The inelastic background of the C 1s, F 1s, O 1s, and N 1s electron core-level spectra was subtracted using Shirley's method. The binding energies of the spectra were corrected using the hydrocarbon component of the polymer fixed at 285.0 eV. The composition of the surface layer was determined from the ratio of the relative peak areas corrected by sensitivity factors of the corresponding elements. The spectra were fitted without placing constraints using multiple Voigt profiles. The width at half maximum (FWHM) varied between 1.6 and 2.0 eV and the accuracy of the peak positions was ±0.1 eV. One specimen of untreated denture base acrylic resin and one of $ArSF_6$-treated specimen were analyzed.

Plasma treatments were performed by the application of radiofrequency power (13.56 MHz) to two parallel plate electrodes fitted inside a homemade stainless steel vacuum chamber.

Contact Angle Measurements

The water contact angle has been measured to characterize the surface wettability [4, 18]. This angle is defined as the angle at the intercept of a plane tangent to the drop and the plane containing the substrate-liquid interface. The measurements were performed in an automated goniometer (Ramé-Hart, 100-00) using deionized water as test liquid. The goniometer comprises a CCD camera to record the image of a droplet placed onto the surface using a microsyringe and a dedicated image processing software to determine the contact angle. Measurements in two different positions were made for each specimen and the

average was calculated. Specimens were then stored at room temperature in sterile distilled water for 48 h to release any residual monomer [10]. Afterwards, the contact angles of each specimen were again measured.

Saliva Collection

Unstimulated whole human saliva was collected from fifteen healthy adult volunteers. The saliva was expectorated into sterile 50 ml Falcon tubes on ice, pooled and clarified by centrifugation at 10,000 *g* for 5 min at 4 ºC [10]. The saliva was prepared at 50% (vol/vol) in sterile PBS [25]. The resulting saliva was immediately stored at -70 ºC until use. The study was approved by the Ethics Committee of Araraquara Dental School (027/2007), and all subjects volunteered to participate and signed an informed consent form.

Adherence Assay

Candida albicans strain ATCC 90028 was used. Stock cultures were maintained at -70 ºC. After recovery this was maintained on YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) stored at 4 – 6 ºC during the experimental period. To prepare the yeast inoculum, a loopful of the stock culture was streaked onto YEPD medium and incubated at 37 ºC for 48 h. Two loopfuls of this young culture were transferred to 20 ml of yeast nitrogen base (YNB) medium with 50 mM glucose and incubated at 37 ºC for 24 h. Cells of the resultant culture were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2) at 5000 *g* for 5 min and resuspended in YNB with 100 mM glucose. *Candida* suspensions were spectrophotometrically standardized to a concentration of 1 x 10^7 cells/ml. Three ml of the standardized *C. albicans* cell

suspension was added to each well containing the specimen. The cells were left to adhere for 90 min at 37 ºC [26]. The non-adherent cells were removed from the specimen by gently washing twice with 3 ml PBS. The negative controls were acrylic specimens to which no cells were added. All experimental conditions were performed in triplicate on three independent occasions.

Preconditioning with Saliva

To investigate the effect of the saliva on candidal adhesion to the denture acrylic resin, half of the specimens from each group (9 rough and 9 smooth) were incubated in 12-well microtiter plates and coated with 3 ml of prepared saliva for 30 min at room temperature prior to the adhesion assay.

Measurement of Adherent *C. albicans*

Nine specimens from each experimental condition (rough and smooth surfaces, plasma treatment and control, presence and absence of saliva) were evaluated by crystal violet staining assay. After the non-adherent cells were removed by washing, the specimens were fixed in 80% ethanol, stained with crystal violet for 1 minute and washed with PBS [27]. Adherent yeast cells were counted in 10 different fields for each specimen, using a light microscope (Olympus BX51, Japan) at 400 x magnification and the mean values were calculated. The results were expressed as cells/mm^2.

Data from roughness measurements were analyzed by Kruskall-Wallis non-parametric test. For each group, two-way repeated measure analysis of variance, followed by Tukey's test were used to evaluate the effect of investing technique and time of measurement on the contact angle. Differences in the

adherent yeast cells (crystal violet staining assay) among the experimental conditions were evaluated by two-way measure analysis of variance. Data of yeast counts (cells per mm^2) were transformed by log. A significance level of 0.01 was used for all statistical tests.

Results

Roughness values of all groups evaluated are presented in Table 1. For all groups, the mean roughness values of the specimens processed against glass were lower than those of the specimens processed against stone. There were no significant differences among the groups in each investing technique.

Table 2 shows the means and standard deviations of contact angle and the results of Tukey post hoc tests. It can be observed that there was significant change in the mean contact angle after 48 hours of immersion in water for all groups, with the exception of the control group, in which no significant difference was found. In ArO_2/70 W and $ArSF_6$/70 W groups, there was significant difference between the mean contact angle of smooth and roughened surfaces, in the 48-hour period. XPS analysis demonstrated the incorporation of fluorine into the surface of the specimens of the $ArSF_6$/70 group (Fig. 1).

Candida albicans adherence, as determined by crystal violet staining assay is shown in Fig. 2. Experimental and control groups did not differ from each other ($p<.01$), irrespective of the presence or absence of saliva and surface roughness (smooth or roughened).

Discussion

Initial adherence of *Candida albicans* on the surface of the polymethylmethacrylate used for making denture bases, is essential in the pathogenesis of denture stomatitis. In view of this, the development of methods that reduce this adherence would be an important step in the treatment and prevention of denture stomatitis. Plasma treatment has been widely used as a method for modifying surfaces. In this technique, a partially ionized gas is created by an electrical discharge, and consequently, a highly reactive environment is formed. Thus, the purpose of this study was to investigate whether surface modifications by means of different plasma treatments would diminish the adherence of *Candida albicans* on a denture base acrylic resin.

Bearing in mind the possible influence of surface roughness on the initial adherence of microorganisms [3, 28, 29], two test specimen fabrication methods were used in this study to obtain rough and smooth surfaces, simulating, respectively, the internal and external surfaces of dental prostheses. According to Quirynen et al. [28], roughness could increase the area available for adhesion and provide niches for microorganisms, protecting them from the action of the forces of removal. In this study, the mean Ra values of rough surfaces were higher than those of the smooth surfaces for all the groups. Nevertheless, no statistically significant effect of roughness on the adherence of *Candida albicans* was detected. It has been demonstrated that the roughness of denture base materials can vary considerably, and values between 3.4 and 7.6 μm have been observed [30], which are higher than those obtained in the rough samples used in this study. Moreover, Taylor et al. [31] observed that although a small increase in roughness

had resulted in greater bacterial adhesion, higher increases in roughness diminished adherence. Results such as these indicate the need for further studies that evaluate the effect of surface roughness on the adhesion of *Candida albicans*, using resin samples with different pre-established roughness values that cover a wide range of variation. In any event, the results observed in the present study are in agreement with those of recent researches [10, 27, 32-34] in which no significant influence of roughness on the adherence of *Candida albicans* was observed.

The surface modifications made in the samples of the present study include the reduction of surface hydrophobicity, obtained by means of the plasma treatments with AAt/130 W, ArO_2/70 W, Ar/50 W, as well as the incorporation of fluoride into the resin surface, achieved in the group treated with $ArSF_6$/70 W. The groups AAt/130 W, ArO_2/70 W and Ar/50 W presented a decrease in the angles of contact values, indicating that immediately after the treatments, the surfaces became more hydrophilic. These results could be attributed to the fact that during plasma treatments, inelastic collisions involving energy electrons from the discharge and species from the polymer surface create free radicals, which in turn react chemically with species from the polymer or atmospheric air, incorporating hydrophilic groups into the polymeric surfaces [18]. This incorporation of hydrophilic groups increases the surface energy, consequently diminishing the angles of contact values [18].

Similar to the observations made by other authors [22, 23], plasma treatment with $ArSF_6$/70 W increased the angle of contact of the samples. This

increase probably occurred by means of substitutions of hydrophilic species by fluoride atoms [19]. As a result, the hydrogen bonds between the water molecules and the superficial groups of the polymer diminished, reducing the hydrophilicity of the samples [19]. X-ray photoelectron spectroscopy (XPS) was performed to confirm the chemical changes in specimens treated with $ArSF_6$/70 W. The incorporation of fluorine occurred, as demonstrated by the presence of F(1s) peak in XPS spectra. There was a decrease in the atomic concentrations of carbon from 75.3 at.% to 55.4 at.%, oxygen from 23.0 at.% to 14.1 at.%, and fluorine incorporation of 29.6 at.%.

When the adherence of *Candida albicans* was considered, no significant difference was found between the control and experimental groups. This result differs from those reported by Yildirim et al. [4], who found greater adhesion of *Candida albicans* on surfaces submitted to plasma treatments, when compared with the control group. A possible explanation for this divergence could be attributed to the different plasma treatment parameters used in the two studies. In the research by Yildirim et al. [4], the samples were submitted to treatments in oxygen atmospheres at 50 or 100 W, for 15 minutes. The absence of difference between the groups observed in the present study could be explained by the fact that after the samples were immersed in water for 48 hours, there was an alteration in the angles of contact values for the experimental groups; that is, the hydrophobicity of the samples submitted to plasma treatments came close to those presented by the samples in the control group.

In the groups AAt/130 W, ArO_2/70 W and Ar/50 W, this alteration in hydrophobicity occurred due to the increase in surface energy. Under this condition, it has been observed that polymeric surfaces submitted to plasmas tend to return to their original hydrophobicity [18]. This could be attributed to the movement of polar groups from the surface to the internal part of the polymer. Although the plasma treatment with $ArSF_6$/70W created hydrophobic surfaces, or surfaces with low surface energy, after the samples had been immersed in water the angles of contact values of this group also came close to the values of the others, including those of the control group. In spite of this alteration in hydrophobicity, the fluoride remained present on the surface, as demonstrated by the XPS analysis. Robinson et al. [24] observed that the increase in the degree of fluorination of the surfaces became more negative due to the presence of electronegative atoms of fluoride. Moreover, it has been suggested that resins with charged surfaces can alter the ionic interaction between the denture base and the microorganisms [11, 16, 35]. However, no reduction in the adherence of *Candida albicans* by means of plasma treatment with $ArSF_6$/70W was detected in this study.

When the denture is inserted into the oral cavity, its surface is rapidly covered by a fine film of saliva known as the acquired pellicle [36]. This film is capable of altering the properties of the surfaces exposed to it [4], in the same way as the chemical characteristic of the surface of biomaterials is also capable of influencing the formation and composition of the acquired film [4, 10, 36]. Thus, surfaces with small differences in their chemical compositions differ with regard

to the adsorption of salivary proteins [4, 17]. In spite of the importance of saliva in the process of adhesion and colonization of microorganisms, the role it plays is still not clear [37]. In this study, half the samples of each group were pre-conditioned in saliva before the fungal adherence test. The results demonstrated that the adhesion of *Candida albicans* to the surfaces was not influenced by the saliva, under all the experimental conditions evaluated. A comparison between in vitro studies revealed contradictory results [4]. While some authors observed that the film of saliva promoted fungal colonization on the materials [5, 7, 26, 37-39], others found that pre-treatment with saliva did not significantly affect [25, 40-43] or diminish the adherence of *Candida* [8-10, 43-45). These divergent results could be attributed to the different methodologies used in each study, including different numbers of donors, the use of stimulated or unstimulated saliva, filtered or total saliva, diluted or undiluted saliva, different centrifugation times and speeds, as well as different incubation periods and temperatures. In the present investigation, the saliva used was diluted with PBS, according to the methodology of Ramage et al. [25], which could have contributed to the absence of the effect of pre-conditioning on fungal adherence observed in the two studies.

In addition to the factors related to the saliva itself, the different results found in the literature could also be related to the different materials evaluated in the studies of *Candida albicans* adherence, such as resilient denture relining materials [5, 9, 38, 39, 41, 45], maxillofacial polymeric materials [37], acrylic surfaces [7-10,26, 37, 42-45], hydroxyapatite [7] and polystyrene [25, 40]. These methodological variations make comparison between the studies difficult and

point out the need for standardization. Nevertheless, the results of the present study are similar to those related by Ramage et al. [25], Jin et al. [40] and Thein et al. [42], in which the presence of saliva did not interfere significantly in the adherence of *Candida albicans*.

This study has limitations, considering that only one strain of *Candida albicans* and one heat polymerizable denture base material were used. In spite of the adherence of *Candida albicans* not having been altered by the plasma treatments, other parameters and treatment atmospheres may provide different results. Moreover, an increase in the incidence of other species, among them *Candida glabrata*, which present greater hydrophobicity than *Candida albicans*, has been observed particularly in immunosuppressed patients. These aspects must be considered in future studies.

Within the limitations of this in vitro study, the following conclusions were drawn:

1) The hydrophobicity (contact angle) of the acrylic resin evaluated was altered by the plasma treatments used. However, the mean contact angles of the treated specimens were similar to those of control specimens, after 48 hours of immersion in water.

2) The adherence of *Candida albicans* was not significantly reduced by plasma treatments when compared with the control, irrespective of the presence or absence of saliva and surface roughness (smooth or roughened).

3) For all groups evaluated, no significant effect was detected with regard to the influence of surface roughness and saliva on the adherence of *Candida albicans*.

Acknowledgements

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Tables

Table 1 – Means and standard deviations (SD) of roughness (Ra - μm) of groups and surfaces (n=18).

Groups	Surfaces	
	Smooth	Roughened
Control	0.30 (0.07) [a]	1.68 (0.56) [b]
AAt/130	0.28 (0.09) [a]	1.95 (0.56) [b]
Ar/50	0.30 (0.09) [a]	1.86 (0.52) [b]
ArO_2/70	0.28 (0.08) [a]	1.61 (0.45) [b]
$ArSF_6$/70	0.28 (0.08) [a]	1.79 (0.54) [b]
Kruskall – Wallis	p= 0.734	p= 0.724

Means followed by the same superscript lower case letters within each column are not significantly different at p = 0.01.

Table 2 – Means and standard deviations (SD) of contact angles (°) obtained immediately after plasma treatments and after 48 hours of immersion in water

		Time of measurement	
Groups	Surfaces	Immediately after plasma treatment	After 48 h in water
Control	Smooth	58.15 (2.97)[a]	56.15 (2.62)[a]
	Roughened	59.75 (4.95)[a]	58.31 (4.83)[a]
AAt/130 W	Smooth	2.77 (3.08)[a]	58.97 (6.38)[b]
	Roughened	1.15 (1.65)[a]	57.38 (5.52)[b]
Ar/50 W	Smooth	41.77 (5.34)[a]	45.72 (7.26)[b]
	Roughened	38.46 (4.33)[a]	47.04 (6.81)[b]
ArO_2/70 W	Smooth	24.21 (3.84)[a]	63.51 (6.11)[b]
	Roughened	26.27 (5.51)[a]	49.61 (7.57)[c]
$ArSF_6$/70 W	Smooth	95.84 (5.88)[a]	63.52 (6.48)[b]
	Roughened	100.72 (7.44)[a]	55.44 (6.94)[c]

Means followed by the same superscript lower case letters within the same group are not different at a level of $p<0.01$.

No comparisons were made among groups.

Figures

Figure 1

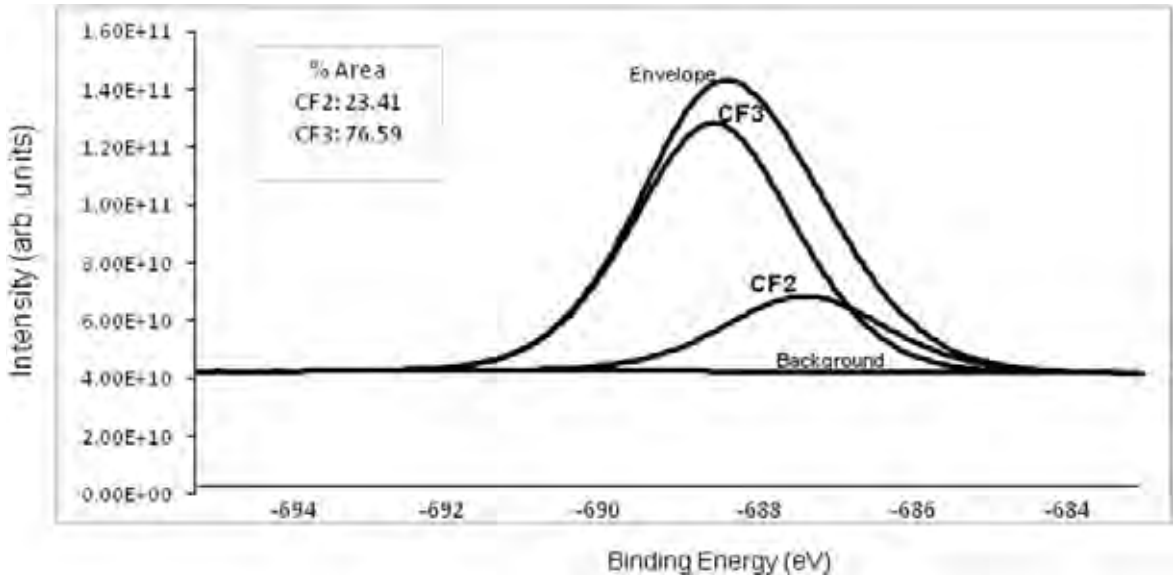

Figure 2

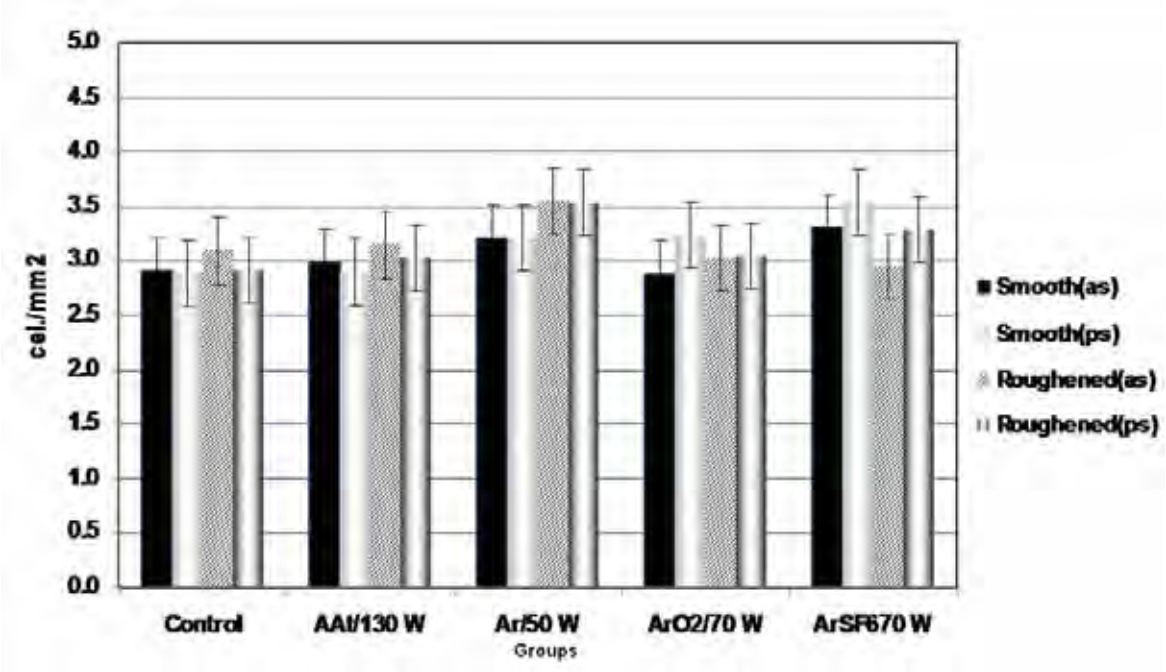
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
cel./mm2
Control
AAl/130 W
Ar/50 W
ArO2/70 W
ArSF670 W
Groups
Smooth(as)
Smooth(ps)
Roughened(as)
Roughened(ps)

Figure Legends

Figure 1 – XPS analysis of the specimens of $ArSF_6$/70 group. Envelope represents total F(1s) area.

Figure 2 – Mean log numbers (cells/mm^2) and 95% confidence intervals for all groups.

as = absence of saliva; ps = presence of saliva.

3.3 Capítulo 3

In vitro adhesion of *Candida glabrata* to denture base acrylic resin modified by glow-discharge plasma treatment

Running title: *C. glabrata* adhesion to a modified resin

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Abstract

Candida adhesion to polymeric surfaces has been related to hydrophobic interactions. Objective: Therefore, this study evaluated if surface modifications with plasma treatments would reduce *Candida glabrata* adhesion to a denture base resin. Methods: Specimens (n=54) with smooth surfaces were made and divided into three groups (n=18): control – non-treated; experimental groups – specimens submitted to glow-discharge plasma treatment to obtain hydrophilic surfaces (Ar/50 W; AAt/130 W). Contact angles measurements were performed immediately after the treatments and after immersion in water for 48 h. For each group, half (n=9) of the specimens were precondicionated with saliva before the adhesion assay. The number of adhered *C. glabrata* was evaluated by counting after crystal violet staining. Results: Ar/50W group showed significantly lower *C. glabrata* adherence than the control group, in the absence of saliva. After preconditioning with saliva, *C. glabrata* adherence in experimental and control groups did not differ significantly. The plasma treatments were effective in modifying the acrylic surface. However, there were significant changes in the contact angles after 48 h of immersion in water. Conclusions: The results demonstrated that Ar/50 W plasma treatment showed promising potential for reducing *C. glabrata* adhesion to denture base resins.

Keywords: *Candida*; *Candida glabrata*; saliva; acrylic resins.

Introduction

The ability of *Candida* to grow attached to oral surfaces in communities known as biofilms is an important factor in the development of denture stomatitis. Although *Candida albicans* still is the microorganism most often associated with this infection, non-*albicans* species have been isolated from denture surfaces and oral mucosa [1]. *Candida glabrata* was the second most commonly isolated pathogen in patients with denture-induced stomatitis, followed by *C. pseudotropicalis*, *C. Krusei*, *C. tropicalis*, *C. parapsilosis* and others [1]. In addition, mixed *Candida albicans* and *Candida glabrata* biofilms have been associated with denture stomatitis [2], indicating that *Candida glabrata* may also play an integral role in this pathogenesis [2]. Moreover, in recent years, the prevalence of *C. glabrata* infections has increased, mainly in compromised patients [3]. This fact must receive attention because the mortality rate of *C. glabrata* infections is higher compared with infection with other non-*albicans Candida* and are more often difficult to treat [3].

Since the adhesion of *Candida* spp. to surfaces is a prerequisite for the formation of biofilm and development of denture stomatitis, the inhibition of this process could be effective to treat or prevent this pathology [4]. Many factors that affect *Candida* adherence have been described, among them the hydrophobic interactions. It has been demonstrated that these interactions are involved in the adherence of *Candida* to acrylic [4-8]. The closer the surface free energy of the substrate and the yeast, the higher was the probability of adherence [6]. A significant positive correlation between cell surface hydrophobicity and adhesion

to acrylic surfaces of *Candida glabrata*, *Candida krusei* and *Candida albicans* has also been observed [7,8]. Moreover, higher cell surface hydrophobicity and greater avidity to acrylic of *Candida glabrata* as compared with *Candida albicans* has been observed [8]. Thus, these results suggest that hydrophilic surfaces could inhibit the adherence of *Candida* to acrylic surfaces, particularly the adherence of relatively hydrophobic fungal cells [4], such as *Candida glabrata*.

Efforts have been made to modify acrylic resins in order to decrease the adherence of *Candida* spp [4,9-14]. However, few studies evaluate the effectiveness of these approaches against the adhesion of *Candida glabrata* [4]. Among the methods for surface modification, there is the glow-discharge plasma treatment, a type of cold plasma. This treatment is a gaseous mixture comprising high energy electrons, ions, ultraviolet photons and reactive neutral species with energy to break covalent bonds on the material surface and, thus, to change its characteristics [15]. As gas temperature can remain as low as room temperature [16], an advantage of this technique is that it allows the treatment of materials that cannot be subjected to high temperatures [15], such as denture base acrylic resins. Despite these advantages, in dentistry, the glow-discharge plasma treatment has received little attention [17,18]. Moreover, there is no information about the adhesion of *Candida glabrata* to plasma treated denture base acrylic resin.

Some studies also have demonstrated that the salivary pellicle is involved in adherence of *Candida* to acrylic. However, the role of saliva during initial adhesion and biofilm formation of *Candida* is poorly understood. While some studies have demonstrated that the saliva pellicle increased the colonization of

Candida [19-23], others have showed that preconditioning the materials with saliva either did not affect [24-27] or reduced *Candida* adhesion [28-32]. Furthermore, few studies evaluated the effect of saliva on *Candida glabrata* adhesion [29,31,32].

Therefore, the main purpose of this in vitro study was to investigate the potential of two plasma treatments to modify a denture base acrylic resin to reduce the *Candida glabrata* adhesion. Moreover, the effect of saliva coating was also evaluated.

Materials and Methods

Preparation of acrylic resin specimens

The specimens (n=54) were fabricated from a microwave denture base acrylic resin (Vipi Wave - VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda Pirassununga, SP, Brazil) using a conventional flasking and pressure-pack technique. Initially, with the use of a metal mold, disk-shaped silicone patterns (Zetaplus/Indurent - Zhermack, Badia Polesine, Rovigo, Italy) were made with dimensions 13.8 × 2 mm. For surface standardization, the silicone patterns were invested between two glass slides in dental stone in microwave flaks. After the stone had set, the flasks were separated and the silicone patterns were removed. For each specimen, 1 g of powder and 0.47 ml of monomer liquid were mixed and processed according to the manufacturer's instructions. The mixture was packed into the molds, a trial pack was completed, and excess material was removed. A final pack was performed and held for 15

minutes. The denture base acrylic resin was processed in a 500 W domestic microwave oven (Brastemp – Brastemp Amazonia SA, Manaus, AM, Brazil) for 20 minutes at 20% power, followed by 5 minutes at 90% power. The flasks were allowed to bench cool at room temperature, the specimens were deflasked, and excess flash was aseptically removed with a sterile bur (Maxi-Cut; Lesfils de August Malleifer SA, Ballaigues, Switzerland).

Surface roughness measurements

The surface roughness of all specimens was measured with a profilometer (Mitutoyo SJ 400 – Mitutoyo Corporation – Tokyo, Japan). Four measurements were made for each specimen and the average reading was designated as the Ra (μm) value of that specimen. Resolution was 0.01 μm, interval (cutoff length) was 0.8 mm, transverse length was 2.4 mm, the stylus speed was 0.5 mm/s, and the diamond stylus tip radius was 5 μm. All measurements were recorded by one operator.

Plasma treatments

After roughness measurements, the specimens were cleaned in ultrasonic water and detergent bath for 15 minutes, then sonicated in distilled water for 15 minutes and dried in air. The specimens were then divided into three groups, each one including 18 specimens. In the control group, the specimens were left untreated. For the two experimental groups, both specimen surfaces were exposed to generated plasmas using the following conditions: argon atmosphere at 50 W (group Ar/50 W); atmospheric air at 130 W (AAt/130 W). The plasma exposure

time (5 minutes) and the position of the specimens within the plasma chamber were kept unchanged. For experimental groups, the plasma parameters were chosen based on the degree of surface hydrophobicity. Parameters that produced surfaces with low hydrophobicity (contact angle close to zero) were used for group AAt/130 W. For the group Ar/50 W, parameters that provided hydrophobicity values between those of the untreated specimens (higher hydrophobic) and those of the group AAt/130 W specimens were chosen.

Plasma treatments were performed through the application of radiofrequency power (13.56 MHz) to two parallel plate electrodes fitted inside a homemade stainless steel vacuum chamber. In this technique, gas temperature remains at room temperature, preserving the integrity of the material [16,33]. In addition, during plasma treatment, specific active agents such as, ultraviolet photons and radicals are generated, resulting in sterilization of the samples [34].

Contact angle measurements

The contact angle is defined as the angle at the intercept of a plane tangent to the drop and the plane containing the substrate-liquid interface. Water contact angle has been measured to characterize the surface wettability [17,35]. The measurements were performed in an automated goniometer (Ramé-Hart, 100-00) using deionized water as test liquid. The goniometer comprises a CCD camera to record the image of a droplet placed onto the surface using a microsyringe and an dedicated image processing software to determine the contact angle. Measurements in two different positions were made for each specimen and the

average was calculated. Specimens were then stored at room temperature in sterile distilled water 48 h to release any residual monomer [31]. Afterwards, contact angles of each specimen were again measured.

Saliva Collection

Whole human unstimulated saliva was collected from 15 healthy adult volunteers. The saliva was expectorated into sterile 50 ml Falcon tubes on ice, pooled and clarified by centrifugation at 10000 g for 5 min at 4 ºC [31] and then filtered through membrane filter of 0.22 μm pore size [23,26,36]. The resulting saliva was immediately stored at -70 ºC until use. The study was approved by the Ethics Committee of Araraquara Dental School (21/2008), and all subjects volunteered to participate and signed an informed consent form.

Adherence assay

Candida glabrata strain ATCC 2001 was used. Stock cultures were maintained at -70 ºC. After recovery this was maintained on YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) stored at 4 – 6 ºC during the experimental period. For preparation of the yeast inoculum, two loopfuls of the stock culture were streaked onto YEPD medium and incubated at 37 ºC for 48 h. Two loopfuls of this young culture were transferred to 20 ml of yeast nitrogen base (YNB) medium with 50 mM glucose and incubated at 37 ºC for 24 h. Cells of the resultant culture were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2) at 5000 *g* for 5 min and resuspended in YNB with 100 mM glucose. *Candida* suspensions were standardized to a concentration of 1×10^7

cells/ml, spectrophotometrically. Three ml of the standardized *C. glabrata* cell suspension was added to each well containing the specimen. The cells were left to adhere for 90 min at 37 ºC [23]. The non-adherent cells were removed from the specimen by gently washing with 3 ml PBS twice. For all experimental conditions, the negative controls were acrylic specimens to which no cells were added. All experiments were performed in triplicate on three independent occasions.

Preconditioning with saliva

To investigate the effect of the saliva on *Candida glabrata* adhesion to the denture acrylic resin, half of specimens from each group (n=9) were incubated into the 12-well microtiter plates and coated with 3 ml of prepared saliva for 30 min at room temperature prior to the adhesion assay.

Measurement of adherent *C. glabrata*

Crystal violet staining

After the non-adherent cells were removed by washing, all specimens were fixed in 80% ethanol, stained with crystal violet for 1 minute and washed with PBS [37]. Adherent yeast cells were counted in 10 different fields for each specimen, using a light microscope (Olympus BX51, Tokyo, Japan) at 400 x magnification and the mean values were calculated. Adherent yeast cells were counted in a “blind” manner to avoid subjective bias. The results were expressed as cells/mm^2.

Statistical Analysis

Differences in the adherent yeast cells (crystal violet staining assay) among the experimental conditions were evaluated by two-way analysis of variance followed by Tukey's test. Data of yeast counts (cells mm^{-2}) were transformed by log. For each group, statistical analysis was performed using the paired Student's t test to evaluate the effect of the time of measurement on the contact angle. One-way analysis of variance, followed by Tukey's test, was used to determine differences in the contact angle among groups after 48 hours of immersion in water. Comparison of the roughness values among the groups was performed by the non-parametric Kruskal-Wallis test. A significance level of 0.05 was used for all statistical tests.

Results

Candida glabrata adherence as determined by crystal violet staining assay is shown in Figure 1. In the absence of saliva, group Ar/50W showed significantly lower *Candida glabrata* adherence than the control group ($P<.05$). Experimental and control groups did not differ ($P>.05$), in the presence of saliva. Figure 1 also shows that, within each group, there was no significant difference between absence and presence of saliva. All negative controls exhibited no metabolic activity (data not shown). Table 1 shows the means and standard deviations of contact angle. There was significant change in the mean contact angle after 48 h of immersion in water, for all groups evaluated ($P<.05$). It can be observed that, after 48 h of immersion in water, control group demonstrated higher contact angle value than the experimental groups ($P<.05$), which did not differ from each other

(P>.05). Roughness maximum, median and minimum values are presented in Table 2. There were no significant differences in the median roughness values among all groups evaluated.

Discussion

Among the virulence attributes of *Candida*, the ability of adherence to acrylic is a prerequisite for colonization and development of biofilms on denture surfaces. Although this attribute has been extensively studied in *C. albicans*, few studies evaluated *C. glabrata* adhesion, mainly to modified surfaces [4]. This is particularly important because, in the last years, the prevalence of *C. glabrata* has increased in human infections as a consequence of the emergence of the acquired immunodeficiency syndrome and the wide use of immunosuppressive medications [3]. Moreover, a high mortality rate has been observed when *C. glabrata* is associated with systemic infections [3]. Therefore, in the present study we evaluate the potential of two plasma treatments to modify a denture base acrylic resin in order to reduce the *C. glabrata* adhesion. To the author's knowledge, this has yet not been investigated.

Various advantages have been attributed to plasma treatment. This technique is dry, cold, fast and allows altering the surface properties of a wide variety of materials [15]. Another positive aspect of this method is that the bulk properties and function of the material are usually kept, as the depth of plasma treatment is limited to a few nanometers of the surface [15,17,35]. In this study, the plasma treatments intended to decrease the surface hydrophobicity to reduce

the *C. glabrata* adhesion. The measurements made immediately after the treatments indicated that, since the decrease in contact angle values is related with the plasma atmosphere and the power applied, it was possible to regulate surface hydrophilicity as planned [14,18].

In the absence of saliva, the results demonstrated that the plasma treatment with Ar/50 W significantly reduced *C. glabrata* adhesion. One possible explanation for this reduction could be the fact that Ar/50 W group specimens presented the lowest contact angle, after immersion in water. Since hydrophobic interactions are involved in the adhesion process [4-8], the hydrophilic surface observed in Ar/50W group could have inhibited the adherence of *C. glabrata*. Although the characteristic of cell surface hydrophobicity (CSH) is not species specific [4,38], *C. glabrata* has been considered a relatively hydrophobic *Candida* [8,39]. When compared to *C. albicans*, *C. glabrata* presented higher CSH and higher tendency to adhere to acrylic surface [8]. Additionally, the closer the surface free energy of the surface and the microorganism, is higher the probability of *Candida* adherence [6]. Thus, the results obtained suggest that hydrophilic acrylic surfaces could inhibit the adherence of *Candida glabrata*.

The decrease in the hydrophobicity of the surfaces, observed immediately after the plasma treatments, can be attributed to energetic electrons generated during the procedure that collide on the acrylic surface. These collisions can result in chemical bonds breakage creating free radicals in the surface [15,35]. The reactions between the free radicals and species from material or atmosphere can increase the surface free energy that is reflected in a decrease of the contact angle

values [15,35]. Similar results were also reported in other investigations in which plasma treatments were made to modify polymeric surfaces [14,17,18,35,40].

The results also showed that, after immersion in water for 48 hours, an alteration of the contact angles of all groups evaluated was detected. This demonstrates that the hydrophobicity of the specimens submitted to plasma treatments came close to that observed for the control group specimens. This alteration occurs because the modification caused by plasma treatment increases the surface energy [15,35]. Under this condition, it has been observed that the polymers have a tendency to recover their hydrophobicity, which has been attributed to the rotation of polar groups around the polymeric backbone into the material bulk [35]. Despite the increase in the contact angle values observed in experimental groups, they remained significantly more hydrophilic compared to control after 48 hours of immersion in water.

In this study, when the acrylic surfaces were preconditioned with saliva, there were no significant differences in *Candida glabrata* adhesion among all groups evaluated. It has been demonstrated that the salivary coating is an important factor in determining the wettability properties of denture materials and, after conditioning with saliva, wettability characteristics of biomaterials can be altered [18,41]. The surface free energy of various materials, including a microwave-cured acrylic resin, was decreased in approximately 10% when the specimens were coated with saliva [41]. This may help explain the absence of any plasma effect in *Candida glabrata* adhesion after preconditioning with saliva.

One other important observation is that, within each group, there was no significant difference between absence and presence of saliva. This result is in accordance with those obtained in previous investigations [29,31] which evaluated the effect of saliva on *C. glabrata* adhesion. These findings suggest that the salivary pellicle did not increase the *Candida glabrata* adhesion to polymer surfaces, as has been found with *Candida albicans* [19,23]. Moreover, there are only a few studies dealing with interactions between salivary pellicle and *Candida glabrata* [29,31,32,42]. Hence, further studies are needed on this subject.

The surface roughness is an important factor that can affect the adhesion of *Candida* to the material surfaces [32,43,44]. Thus, in this study, the specimens were made between glass slides in order to obtain smooth and standardized surfaces. As can be observed in Table 2, the results demonstrated that there were no significant differences in the median roughness values among all groups evaluated.

In conclusion, this in vitro study demonstrates that hydrophilic surfaces, as those obtained with Ar/50W plasma treatment, have potential for reducing the adhesion of *Candida glabrata*, which may be involved in the pathogenesis of denture stomatitis and related infections. Additionally, the saliva pellicle did not significantly increase *Candida glabrata* adhesion. However, as this study is limited only to one strain and one heat-polymerized denture base, more comprehensive investigations using other strains as well as different denture base materials are required.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1 - Means and standard deviations (SD) of contact angles (º) obtained immediately after plasma treatments and after 48 hours of immersion in water.

Groups	Time of measurement	
	Immediately after plasma treatment	After 48 h in water
Control	65.79 (8.66) ^{A}	58.84 (4.50) ^{Ba}
Ar/50W	47.04 (5.80) ^{A}	51.40 (4.82) ^{Bb}
AAt/130W	2.61 (2.69) ^{A}	54.14 (5.21) ^{Bb}

Horizontally, means with different capital superscript letters are significantly different (P<.05). Vertically, means with different small superscript letters are significantly different (P<.05). Immediately after plasma treatment, no comparison was made among groups.

Table 2 - Median (maximum to minimum) roughness values (Ra-µm) for all groups.

Groups	Roughness	
Control	Median	0.23
	Maximum	0.39
	Minimum	0.10
Ar/50 W	Median	0.22
	Maximum	0.35
	Minimum	0.10
AAt/130 W	Median	0.26
	Maximum	0.39
	Minimum	0.09

No significant differences were found among all groups evaluated ($P>.05$).

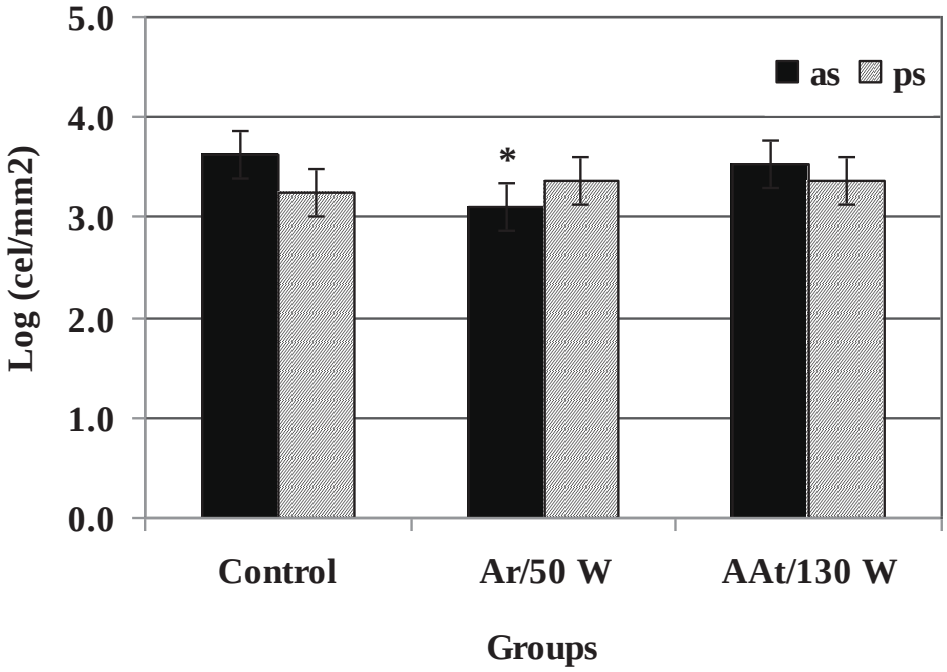
5.0
4.0
3.0
2.0
1.0
0.0
Log (cel/mm2)
as
ps
*
Control
Ar/50 W
AAt/130 W
Groups

Figure 1

Figure Caption

Figure 1. Mean log numbers (cells/mm^2) and 95% confidence intervals for all groups. as: absence of saliva; ps: presence of saliva. (*) Statistically different mean compared to control.

3.4 Capítulo 4

Effect of different periods of preconditioning with saliva on adhesion of *C. albicans* to a denture base resin by crystal violet staining and XTT assay

Short title: Effect of saliva on adhesion of *Candida*

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Abstract

The role of saliva on *Candida* adhesion to biomaterials is not clearly defined. Aim: this study investigated whether different periods of preconditioning with saliva would influence the adhesion of *Candida albicans* to a denture base resin. Methods: samples (n=90) with smooth surfaces were made and then divided into five groups: 1 control - without saliva; 4 experimental groups – conditioned in saliva for periods of: 30, 60, 180 or 720 minutes. *Candida* adhesion was evaluated by crystal violet staining and XTT assay. Results: the one-way analyses of variance revealed that there were no significant differences among the mean numbers of adherent cells or among the mean absorbances for all groups. No significant correlation was found between the two methods used for assessing *C. albicans* adhesion. Conclusions: the different periods of preconditioning with saliva had no significant influence on the adhesion of *Candida albicans* to the denture base acrylic resin.

Key words: Acrylic Resins; Biofilms; Adhesion; *Candida albicans*; Saliva.

Introduction

The presence of *Candida albicans* biofilms on removable denture surfaces plays an important role in the etiology of denture stomatitis. The capacity of this fungus to adhere to surfaces is the first stage in the biofilm formation process, which is followed by colony formation and cell organization, secretion of extracellular matrix, maturation and dissemination of the biofilm [1].

It is known that the adhesion of *Candida* cells to denture surfaces is mediated by a salivary conditioning film or pellicle [2,3,4] that provides receptors for microbial adhesion [5]. However, the role of saliva on *Candida albicans* adhesion to biomaterials is, as yet, not fully established, as conflicting results have been reported. Some authors have observed that the saliva pellicle promoted *Candida albicans* colonization on the materials [6,7,8,9,10]. Conversely, others have found that preconditioning the materials with saliva either did not affect [11,12,13,14] or reduced *Candida albicans* adhesion [15,16,17,18,19]. The different periods of preconditioning with saliva could interfere in the adhesive capacity of *Candida* [20] and contribute to the inconsistent findings from these studies. In a number of researches, the materials were incubated with saliva for short periods such as 30 minutes [16,17,18,19], 1 hour [7,8,9], 2 to 3 hours [4,10] and 4 hours [11,13]. Longer incubation periods, including overnight [21], 18 hours [15] and 24 hours [22] have also been used to create a salivary conditioning film. However, to date, no information is available concerning how different periods of preconditioning with saliva affect the adhesion of *Candida* cells to denture materials.

One method used for evaluating adhesion of *Candida* is the cell staining assay [15,18,19,23,24]. In this method, the adhered cells on the material surface are fixed, stained and counted in a microscope. The dye commonly used in this technique is the crystal violet, which is basic and binds to negatively charged extracellular molecules, including cell surface molecules and polysaccharides in the extracellular matrices [25]. Another method that has been widely used is the XTT assay [1,10,11,16], which evaluates the metabolic activity of viable cells [26]. This assay is based on the reduction of a water-soluble tetrazolium salt [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium-hydroxide] to a formazan product by the mitochondrial dehydrogenases of the live cells.

Therefore, the aim of this study was to assess the effect of different periods of preconditioning with saliva on the adhesion of *C. albicans* to a denture base resin using crystal violet staining and XTT assay. Additionally, the correlation between the two methods used for assessing *C. albicans* adhesion was evaluated.

Materials and Methods

Preparation of acrylic resin specimens

The specimens (n=90) were fabricated from a microwave denture-base acrylic resin (Vipi Wave - VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda Pirassununga, SP, Brazil) using a conventional flasking and pressure-pack technique. Initially, with the use of a metal mold, disk-shaped silicone patterns Zetaplus/Indurent - Zhermack, Badia Polesine, Rovigo,

Italy) measuring 13.8 X 2 mm were made. To obtain smooth and standardized surfaces, the silicone patterns were invested in dental stone in microwave flasks and positioned between two glass slides. After the stone had set, the flasks were separated and the silicone patterns were removed. For each specimen, 1 g of powder and 0.47 ml of monomer liquid were mixed, packed into the molds, and processed in a 500 W domestic microwave oven (Brastemp – Brastemp da Amazonia SA, Manaus, AM, Brazil) according to the manufacturer's instructions (20 minutes at 20% power, followed by 5 minutes at 90% power). The flasks were allowed to bench cool at room temperature, the specimens were deflasked, and excess flash was aseptically removed with a sterile bur (Maxi-Cut; Lesfils de August Malleifer SA, Ballaigues, Switzerland).

Surface roughness measurements

The surface roughness of all specimens was measured with a profilometer (Mitutoyo SJ 400 – Mitutoyo Corporation - Japan). Four measurements were made for each specimen and the mean reading was designated as the Ra (μm) value of that specimen. The measurements were recorded by one operator. Resolution was 0.01 μm, interval (cutoff length) was 0.8 mm, transverse length was 2.4 mm, the stylus speed was 0.5 mm/s, and the diamond stylus tip radius was 5 μm.

Saliva Collection

Whole human unstimulated saliva was collected from 15 healthy adult volunteers. The saliva was expectorated into sterile 50 ml Falcon tubes on ice,

pooled and clarified by centrifugation at 10000 g for 5 min at 4 ºC [18] and then sterilized using membrane filter with a 0.22 μm pore size [10,13,27,28]. The resulting saliva was immediately stored at -70 ºC until use. The study was approved by the Ethics Committee of Araraquara Dental School, and all subjects volunteered to participate and signed an informed consent form.

Test specimen sterilization

Before the microbiological tests, the test specimens were kept in distilled water at ambient temperature for 48 hours, in order to eliminate residual monomers [18]. After this, they received an ultrasound bath for 20 minutes and were exposed to ultraviolet light in the laminar flow chamber for 20 minutes on each side [29].

Preconditioning with saliva

The 90 test specimens were divided into five groups (n=18), as follows: 1 control (without preconditioning in saliva) and 4 experimental groups that were conditioned with saliva for periods of: 30 minutes (30 min); 1 hour (60 min); 3 hours (180 min); or 12 hours (720 min). The specimens of the four experimental groups were incubated in 12-well microtiter plates with 3 ml of the saliva preparation at room temperature [17] prior to the adherence assay.

Adherence assay

The stock culture of *Candida albicans* strain ATCC 90028 was maintained in YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) stored at

4 – 6 ºC during the experimental period. For preparation of the yeast inoculum, two loopfuls of the stock culture were streaked onto YEPD medium and incubated at 37 ºC for 48 h. Two loopfuls of this young culture were transferred to 20 ml of yeast nitrogen base (YNB) medium with 50 mM glucose and incubated at 37 ºC for 21 h. Cells of the resultant culture were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2) at 5000 g for 5 min and resuspended in YNB with 100 mM glucose. *Candida* suspensions were standardized to a concentration of 1 x 10^7 cells/ml, spectrophotometrically. Three ml of the standardized *C. albicans* cell suspension was added to each well containing the specimen. The cells were left to adhere for 90 min at 37 ºC [10]. The non-adherent cells were removed from the specimen by gently washing with 3 ml PBS twice. The negative controls were acrylic specimens to which no cells were added. All experiments were performed in triplicate on three independent occasions.

Crystal violet staining

Nine specimens from each group were evaluated by crystal violet staining assay. After the non-adherent cells were removed by washing, the specimens were fixed in 80% ethanol, stained with crystal violet for 1 minute and washed with PBS [24]. Adherent yeast cells were counted in 10 different fields for each specimen, using a light microscope (Olympus BX51, Japan) at 400 x magnification and the mean values were calculated. Adherent yeast cells were counted in a "blind" manner to avoid subjective bias. The results were expressed as cells/mm^2.

XTT assay

Nine specimens from each group were evaluated by XTT reduction assay, as described elsewhere [1]. Briefly, XTT (Sigma, MO, USA) was prepared in ultrapure water (1mg/ml), filter sterilized and stored at -70 ºC until used. Menadione (Sigma, MO, USA) solution was prepared in acetone at 0.4 mM immediately before each assay. After washing, the specimens were transferred to new wells with 158 µl PBS with 200mM glucose, 40 µl XTT and 2 µl menadione were inoculated to each well. The plates were incubated for 3 h in the dark at 37 ºC. The whole content of each well was centrifuged at 5000 g for 2 minutes and the colorimetric change of the supernatant was measured using a microtiter plate reader (Thermo Plate – TP Reader) at 492 nm.

Statistical Analysis

Comparison of the roughness values among the groups was performed by the non-parametric Kruskal-Wallis test. For each adhesion assay used, one-way analysis of variance with Welch's correction was performed to evaluate the effect of time of preconditioning with saliva on *Candida albicans* adhesion. For the crystal violet technique, data of yeast counts (cells per mm^2) were transformed by log. A significance level of 0.05 was used for all statistical tests. To evaluate the correlation between the two methods used for assessing *C. albicans* adherence, Pearson's coefficient of correlation was used.

Results and Discussion

Candida adhesion to dentures, an essential step in biofilm formation and development of denture stomatitis, may be influenced by saliva. This influence may be regulated by specific interactions between the cellular adhesins and receptors in the salivary pellicle [2,4,5], as well as, by the action of salivary proteins as a source of nutrients for microbiological growth [30]. On the other hand, these proteins may also act by blocking the locations of adhesion originally present on substrates [4,11]. It has also been observed that saliva may alter the surface characteristics of the substrates involved in the adhesion process, such as roughness and hydrophobicity [4,28,31,32]. Conversely, some authors have reported that the original substratum surface properties may be transferred even through the protein layer on the substratum surface and still influence microbial adhesion [4,28]. In spite of several studies that have investigated the influence of the salivary pellicle in the adhesion process [6,7,8,9,10,11,12,13,14,15,16,17,18,19,21], the role it plays is not yet clear. The divergences found in the literature could be related to the different methodologies, including the different periods of preconditioning with saliva [20]. Thus, the aim of this study was to evaluate whether different periods of preconditioning with saliva would have an influence on the adhesion of *Candida albicans* to a denture base acrylic resin using crystal violet staining and XTT assay. To the author's knowledge, to date this is the first study that has addressed this issue.

Considering that surface roughness can influence the adhesion of *Candida albicans* to the substrate [19,33,34,35], the samples were made between glass slides in order to obtain smooth, standardized surfaces. The results demonstrated that there

were no significant differences in the mean roughness values among all groups evaluated, for both crystal violet staining and XTT assay (Table 1).

The results from crystal violet staining and XTT assay revealed that the differences among all experimental groups were not significant, indicating that the periods of preconditioning with saliva evaluated in the present investigation did not influence the adhesion of *Candida albicans* (Table 2). These findings suggest that in studies of *Candida albicans* adhesion to acrylic resins, shorter periods of preconditioning with saliva, such as 30 or 60 minutes could be used, making the adhesion assay procedures easier and quicker to perform. Nevertheless, it is important to point out that the saliva used in this study was filter sterilized [27] to obtain more reproducible results [17]. Given that salivary pellicle formation on biomaterials is a selective process [31], the filtration process may have depleted some of the protein complexes, such as certain mucin complexes involved in *Candida* adhesion, that are present in human saliva [17]. Therefore, further studies should be conducted to evaluate the influence of different periods of preconditioning the substrates on *Candida* adhesion, when using whole saliva.

The coefficient of correlation between crystal violet staining and XTT assay was low ($r=0.223$, $P = 0.141$), showing no significant correlation between the two methods used for assessing *C. albicans* adherence. The results obtained by staining demonstrated that there were no significant differences between the experimental groups and the control, indicating that preconditioning with saliva did not influence the fungal adhesion (Table 2). For XTT assay, although the statistical analysis failed to find significant differences among the groups, the

absorbance values obtained after preconditioning with saliva were higher than those of the control group. The two methods used in the present study to determine the adhesion of *C. albicans* to the denture base resin are based on different principles. In the crystal violet staining method, the stained cells are quantified by counting in selected fields of the substrate [15,18,19,23,24], whereas in the XTT assay, the metabolic activity of all viable cells is measured [1,11,21,26]. It is worth noting that most of the studies that have found an increase in *Candida albicans* colonization on denture materials after pre-incubation with saliva have used tests of monitoring the pH of the growth medium, adenosine triphosphate (ATP) analysis and XTT assay [6,7,8,9,10]. Conversely, when the adherent cells were quantified by microscopy or by colony forming unit (UFC) counts, the majority of the studies on *Candida albicans* colonization on denture materials observed an absence of significant effect of saliva [12,13,14] or reduced adhesion [14,15,16,17,18,19]. Hence, it is suggested that the controversial results regarding the role of saliva on the adhesion of *Candida* to denture materials may at least partially be accounted for by different adhesion assays used. This highlight that the effect of saliva on the adhesion and biofilm development of *Candida* should not be studied by a single quantification method.

The divergent results among studies may also be attributable to the various materials that have been used as substrates, among them acrylic surfaces [9,10,13,14,15,16,17,18,19,22], denture reline materials [6,7,8,12,17,19], maxillofacial polymeric materials [5,9] and polystyrene [11,21]. It has been reported that the exposed chemical groups of the solid surface probably play the most important role in determining

the selectivity of the adsorption process [4,31]. Yildirim et al. (2006) [36] observed that acrylic resin surfaces modified by plasma treatments adsorbed different amounts of high molecular weight mucin. Therefore, the composition of the salivary pellicle may vary among the materials used. As there are only few data in the literature about the resulting protein concentrations formed on different prosthetic materials [37], further studies are necessary to evaluate these differences.

In conclusion, this study demonstrated that different periods of preconditioning with saliva did not influence the adhesion of *Candida albicans* to the denture-base acrylic resin as evaluated by crystal violet staining and XTT assay. Further, the coefficient of correlation between the results obtained by crystal violet staining and XTT assay was not significant. Finally, as the current study was confined only to one denture-base acrylic resin and one strain of *Candida albicans* future studies with other *Candida* species and denture base materials are warranted. Besides, in addition to the period of preconditioning with saliva, other experimental conditions, such as number of donors, speed and time of centrifugation have varied considerably among studies and the influence of these differences on *Candida* adhesion to denture materials needs further investigations.

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Tables

Table 1

Table 1 – Median (maximum – minimum) roughness values (Ra-µm) for all groups in the two methods used for assessing *C. albicans* adherence.

Methods	Groups				
	Control	30 min	60 min	180 min	720 min
Crystal violet staining					
Median	0.21	0.20	0.26	0.16	0.16
Maximum	0.47	0.38	0.30	0.33	0.34
Minimum	0.12	0.14	0.15	0.09	0.11
XTT assay					
Median	0.14	0.25	0.23	0.18	0.23
Maximum	0.38	0.39	0.33	0.40	0.36
Minimum	0.10	0.08	0.09	0.12	0.11

For both, Crystal violet staining and XTT assay, no significant differences were found among all groups evaluated ($P > 0.05$).

Groups: control - without saliva; experimental groups – conditioned in saliva for periods of: 30, 60, 180 or 720 minutes.

Table 2

Table 2 - Mean log numbers of cells/mm^2 (crystal violet staining) and mean absorbance values at 492 nm (XTT assay) for all groups evaluated. Standard deviations are in parentheses.

Methods	Groups				
	Control	30 min	60 min	180 min	720 min
Crystal violet staining	2.59 (0.13)	2.57 (0.38)	2.66 (0.33)	2.65 (0.35)	2.68 (0.39)
XTT assay	0.20 (0.07)	0.31 (0.15)	0.35 (0.21)	0.32 (0.19)	0.33 (0.23)

For both, Crystal violet staining and XTT assay, no significant differences were found among all groups evaluated ($P > 0.05$).

Groups: control - without saliva; experimental groups – conditioned in saliva for periods of: 30, 60, 180 or 720 minutes.

3.5 Capítulo 5

The effect of human whole saliva on the in vitro adhesion of *Candida albicans* to a denture base acrylic resin: a focus on collection and preparation of saliva samples

Short title: Effect of human saliva on adhesion of *Candida*

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Summary

The effect of saliva on *Candida albicans* adhesion still remains controversial. The diverse protocols used for collection and preparation of saliva samples may contribute to the conflicting results. Thus, this study investigated whether variations in the centrifugation parameters and number of donors of saliva would influence the adhesion of *C. albicans* (ATCC 90028) to a denture base resin. Samples (n=72) with smooth surfaces were made and then divided into four groups: 1 control (C) - without saliva; 3 experimental groups – G1: saliva from 15 volunteers centrifuged at 10000 rpm for 5 min; G2: saliva from 15 volunteers centrifuged at 12000 rpm for 30 min; G3: saliva from 1 volunteer centrifuged at 10000 rpm for 5 min. *Candida* adhesion was evaluated by XTT reduction assay and crystal violet staining. Data were analyzed by one-way analyses of variance (P=0.05). For XTT assay, groups G_2, G_3 and control were not significantly different, while group G_1 showed significantly higher absorbance value than control. For crystal violet staining, there were no significant differences among the mean log numbers of adherent cells for all groups. The results indicated that variations in the centrifugation parameters and number of donors may influence the effect of saliva on *Candida albicans* adhesion to denture base resins.

Key words: Acrylic Resins; Biofilms; *Candida*; *Candida albicans*; Saliva.

Introduction

An important step in the pathogenesis of denture stomatitis is the attachment of *Candida albicans* to denture surfaces, followed by biofilm formation [1-2]. The physicochemical characteristics of the denture materials, such as roughness [3-5], electrostatic charge [1, 6] and surface free energy [1, 7-8], may considerably influence *C. albicans* adhesion. In the oral environment, however, the denture surfaces are coated by a thin film of saliva known as salivary pellicle. Saliva is an exocrine secretion produced by different salivary glands [9], consisting of water, electrolytes, and proteins [9-10]. Various functions have been attributed to saliva, among them antimicrobial properties due to the presence of immunologic and non-immunologic proteins [10]. However, saliva also possesses proteins that could act as receptors, to promote the initial microbial adhesion [11-13], and as a source of water and nutrients for growth and reproduction of microorganisms [14]. Thus, besides non-specific surface properties, yeast adhesion can also be influenced by specific receptors in the acquired salivary pellicle [11-13].

In this context, to develop new strategies for preventing denture stomatitis, it is essential to evaluate the influence of salivary pellicle on *Candida* adhesion to denture surfaces. However, the interactions between biomaterials, salivary pellicle and *C. albicans* are complex [15], and the effect of saliva on *C. albicans* adhesion still remains controversial. While some researchers have found that the salivary pellicle increases *C. albicans* colonization on the materials [11-13, 16-21], others

have observed that the preconditioning with saliva either does not significantly affect [22-26] or decreases *C. albicans* colonization [4, 27-31].

In these studies, diverse protocols have been used for collection and preparation of saliva samples, with differences in the nature (e.g. the quality of saliva from different individuals) and in the centrifugation parameters. It is therefore conceivable that these factors may have affected the outcomes and that this variability may have accounted partly for the conflicting data reported by different authors [32]. All these show the importance of quality control of saliva in studies of this nature and point out the need for standardization [22]. However, to date, no information is available concerning how different methods of collection and preparation of saliva samples affect the adhesion of *Candida* spp. cells to denture materials.

Different methods have been used to investigate the adhesion of *Candida* to biomaterials, among them XTT and cell staining assays. For XTT assay, the water-soluble tetrazolium salt [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium-hydroxide] is taken up by living cells and is reduced by mitochondrial dehydrogenase to colored tetrazolium formazan products that are determined spectrophotometrically [20, 22, 26, 33-35]. In the staining assay, the fungal cells that adhered to the material surface are fixed, stained with a basic dye, crystal violet, and then counted under a microscope [1, 26-27, 30-31, 36-37].

Therefore, the aim of the present study was to evaluate the effect of variations in the centrifugation parameters and number of donors of saliva on *C.*

albicans adhesion to a denture base resin using crystal violet staining and XTT reduction assay.

Materials and Methods

Preparation of acrylic resin specimens

The specimens (n=72) were fabricated from a microwave denture base acrylic resin (Vipi Wave - VIPI Indústria e Comércio Exportação e Importação de Produtos Odontológicos Ltda Pirassununga, SP, Brazil) using a conventional flasking and pressure-pack technique. Initially, with the use of a metal mold, disk-shaped silicone patterns (Zetaplus/Indurent - Zhermack, Badia Polesine, Rovigo, Italy) were made with dimensions 13.8 X 2 mm. For surface standardization, the silicone patterns were invested between two glass slides in dental stone in microwave flaks [15, 38]. After the stone had set, the flasks were separated and the silicone patterns were removed. For each specimen, 1 g of powder and 0.47 ml of monomer liquid were mixed and processed according to the manufacturer's instructions. The mixture was packed into the molds, a trial pack was completed, and excess material was removed. A final pack was performed and held for 15 minutes. The denture base acrylic resin was processed in a 500 W domestic microwave oven (Brastemp – Brastemp Amazonia SA, Manaus, AM, Brazil) for 20 minutes at 20% power, followed by 5 minutes at 90% power. The flasks were allowed to bench cool at room temperature, the specimens were deflasked, and excess flash was aseptically removed with a sterile bur (Maxi-Cut; Lesfils de August Malleifer SA, Ballaigues, Switzerland).

Surface roughness measurements

The surface roughness of all specimens was measured with a profilometer (Mitutoyo SJ 400 – Mitutoyo Corporation – Tokyo, Japan). Four measurements were made for each specimen and the average reading was designated as the Ra (μm) value of that specimen. Resolution was 0.01 μm, interval (cutoff length) was 0.8 mm, transverse length was 2.4 mm, the stylus speed was 0.5 mm/s, and the diamond stylus tip radius was 5 μm. All measurements were recorded by one operator. Only samples with an average surface roughness ≤ 0.2 μm were selected for this study.

Specimen sterilization and group assignment

Before the microbiological tests, the specimens were kept in distilled water at ambient temperature for 48 hours, in order to eliminate residual monomers [30]. After this, they received an ultrasound bath for 20 minutes and were exposed to ultraviolet light in the laminar flow chamber for 20 minutes on each side [39].

Hence, the 72 specimens were divided into four groups (n=18), as follows: 1 control (C), without preconditioning in saliva, and 3 experimental groups (G_1, G_2 and G_3) that were conditioned with saliva prepared as described below.

Saliva collection and preparation

For the experimental groups G_1 and G_2, whole human unstimulated saliva from 15 healthy adult volunteers was expectorated into sterile 50 ml Falcon tubes on ice, pooled and clarified by centrifugation. Thereafter, for group G_1, the saliva was centrifuged at 10000 rpm for 5 min at 4 ºC [30], while for group G_2, saliva was centrifuged at 12000 rpm for 30 min at 4 ºC [18-19, 22, 25, 27-28].

For group G_3, whole human unstimulated saliva was collected from one healthy adult volunteer. The saliva was expectorated into sterile 50 ml Falcon tubes on ice, pooled and clarified by centrifugation at 10000 rpm for 5 min at 4 ºC [16-17, 25, 30-31].

For all experimental groups, the supernatant of saliva was sterilized using membrane filter with a 0.22 μm pore size [20, 24]. The resulting saliva was immediately stored at -70 ºC until use. The study was approved by the Ethics Committee of Araraquara Dental School, and all subjects volunteered to participate and signed an informed consent form.

In vitro salivary pellicle formation

The specimens of the three experimental groups were incubated in 12-well microtiter plates with 3 ml of the saliva preparation at room temperature for 30 minutes prior to the adherence assay [29].

Preparation of *Candida* suspension and adherence assay

The stock culture of *Candida albicans* strain ATCC 90028 was maintained in YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) stored at 4 – 6 ºC during the experimental period. For preparation of the yeast inoculum, two loopfuls of the stock culture were streaked onto YEPD medium and incubated at 37 ºC for 48 h. Two loopfuls of this young culture were transferred to 20 ml of yeast nitrogen base (YNB) medium with 50 mM glucose and incubated at 37 ºC for 21 h. Cells of the resultant culture were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2), centrifuged at 4000 g for 5 min and resuspended in YNB with 100 mM glucose. *Candida* suspensions were

standardized to a concentration of 1 x 10^7 cells/ml, spectrophotometrically. Three ml of the standardized *C. albicans* cell suspension was added to each well containing the specimen. The cells were left to adhere for 90 min at 37 ºC in a shaker at 75 rpm [20]. The non-adherent cells were removed from the specimen by gently washing with 3 ml PBS twice. The negative controls were acrylic specimens to which no cells were added. All experiments were performed in triplicate on three independent occasions.

XTT reduction assay

Nine specimens from each group were evaluated by XTT reduction assay, as described elsewhere [22]. Briefly, XTT (Sigma, MO, USA) was prepared in ultrapure water (1mg/ml), filter sterilized and stored at -70 ºC until used. Menadione (Sigma, MO, USA) solution was prepared in acetone at 0.4 mM immediately before each assay. After washing, the specimens were transferred to new wells with XTT solution in the following proportion: 158 µl PBS with 200 mM glucose, 40 µl XTT and 2 µl menadione. The plates were incubated for 3 h in the dark at 37 ºC. The whole content of each well was centrifuged at 5000 g for 2 minutes and the colorimetric change of the supernatant was measured using a microtiter plate reader (Thermo Plate – TP Reader) at 492 nm.

Crystal violet staining

Nine specimens from each group were evaluated by cell counting after crystal violet staining. After the non-adherent cells were removed by washing, the specimens were fixed in 80% ethanol, stained with crystal violet for 1 minute and washed with PBS [36]. Adherent yeast cells were counted in 10 different fields for

each specimen, using a light microscope (Olympus BX51, Japan) at 400 x magnification and the mean values were calculated. Adherent yeast cells were counted in a "blind" manner to avoid subjective bias. The results were expressed as cells/mm^2.

Statistical Analysis

For each adhesion assay used, one-way analysis of variance, followed by Tukey's test, was performed to evaluate the effect of variations in the centrifugation parameters and number of donors of saliva on *Candida albicans* adhesion. For the crystal violet technique, data of yeast counts (cells/mm^2) were transformed by log. A significance level of 0.05 was used for all statistical tests.

Results

Candida albicans adhesion determined by XTT reduction assay is shown in Fig. 1. One-way analysis of variance demonstrated that groups G_2, G_3 and control were not significantly different, while group G_1 showed significantly higher absorbance readings than the control group ($p<.034$).

Candida albicans adhesion, as determined by crystal violet staining assay, is shown in Fig. 2. One-way analysis of variance revealed that there were no significant differences among the mean log numbers of adherent cells for all groups evaluated.

All negative controls exhibited no metabolic activity (data not shown).

Discussion

Although several studies have evaluated the influence of the salivary pellicle in the *C. albicans* adhesion process to biomaterials [4, 11-13, 16-31], the

results are conflicting and the role it plays is not yet clear. Among other factors, these divergences could be related to the diverse methodological procedures used in these studies. Recently, the effect of different periods of preconditioning with saliva on *Candida albicans* adhesion to one denture base acrylic resin was investigated, and no significant effect was found [26]. However, to the best of our knowledge to date there are no studies that have addressed the effect of variations in the methods used for collection and preparation of saliva samples. Thus, the aim of this study was evaluate whether variations in the centrifugation speed and time and number of donors of saliva would influence on the *Candida albicans* adhesion to a denture base acrylic resin using crystal violet staining and XTT assay.

Since the attachment of microorganisms on surfaces is related to surface roughness [3-5, 31], the specimens of this study were made between glass slides and their roughness were measured to ensure that smooth and standardized surfaces were obtained [15, 38].

XTT reduction assay and crystal violet staining assays were selected for this study because they are simple and versatile methods that have been frequently used by investigators to investigate the adhesion of *C. albicans* to biomaterials [1, 20, 22, 26-27, 30-31, 33-37].

The results obtained by XTT reduction assay demonstrated that the preconditioning with saliva collected from various donors and centrifuged at 10000 rpm for 5 minutes increased significantly the metabolic activity of *Candida albicans* in comparison to control (Fig. 1). However, in the groups in which saliva

was collected from one donor or centrifuged longer and at a higher speed, no significant differences were detected when they were compared to control. The yeast-surface recognition systems involve different ligand-receptor mechanisms based on protein or carbohydrate moieties existing in this interaction [11]. Moreover, it was suggested salivary mucins also bind to *C. albicans*, and they promote yeast adhesion to polymethylmethacrylate [11]. Higher centrifugal forces might separate significant amounts of the high molecular weight mucins [4, 32]. Thus, the centrifugation must be made without causing considerable effects on the biochemical and biophysical properties of saliva. It has been observed that centrifugation at 10000 g for 5 minutes at 4 °C had minimal impact normal saliva protein profiling [40]. These may help explain, at least in part, the significantly higher absorbance value of group G1 when compared to control. Another aspect to be considered is that saliva composition varies greatly inter-individually [32]. Hence, the use of saliva collected from several donors and pooled may have minimized this variation and, consequently, its influence on the adhesion results.

Differently from the results obtained by the XTT assay, the crystal violet staining revealed that the adhered cell number was higher in group G_1 compared to control and the other experimental groups, but the differences did not reach statistical significance. It is important to emphasize that the two methods used in this investigation for assessing *C. albicans* adherence to the denture base resin are based on different principles. The XTT reduction assay is a method in which the metabolic activity of viable cells is measured [22, 26, 33-35]. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of XTT, yielding

colored formazan crystals, which may be measured spectrophotometrically [41]. It has been reported that the XTT method correlates well with other quantitative technique, such as adenosine tri-phosphate (ATP) and colony forming units (CFU) methods [22, 42]. For the other assay, the adhered cells are fixed, stained with the basic dye crystal violet, and quantified by counting in selected fields of the substrate surface [1, 26-27, 30-31, 36-37]. Therefore, because cells (both living and dead) are stained by crystal violet, this assay does not allow differentiating between living and dead cells [43]. These differences between XTT and crystal violet assays may help explain the results obtained in this study and may have accounted, at least in part, for the controversial results reported by others. Therefore, when possible, it is recommended to use more than one method to evaluate the effect of saliva on *Candida* adhesion to surfaces, particularly if they are based on different principles.

It has been reported that exposed chemical groups of the material surface have important role in determining the selectivity of the adsorption process [44]. Hence, considering that solid surfaces differ in respect to adsorption of salivary proteins, the pellicle composition may vary among diverse materials [44]. An analysis of the literature reveals that a variety of materials has been used as substrate in vitro studies that evaluate the effect of saliva on *Candida* adhesion [4, 12-13, 16-20, 23-31]. This variety of materials makes the comparison of results difficult and can partially contribute to the conflicting results regarding the effect of preconditioning with saliva on *Candida* adhesion.

In conclusion, this study focused mainly on the effect of variations in the collection and preparation of saliva on the *Candida albicans* adhesion. The results demonstrated that variations in the centrifugation parameters and number of donors can influence the effect of saliva on *Candida albicans* adhesion, thus emphasizing that the protocols must be standardized. As the present study evaluated only one denture base acrylic resin, further investigations with other materials are necessary. Moreover, other *Candida* species must also be evaluated in future studies. Besides, in addition to the speed and time of centrifugation and number of donors, the use of filtered or unfiltered saliva has varied among studies and the influence of this difference on *Candida* adhesion to denture materials needs further investigations.

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Figures

Figure 1

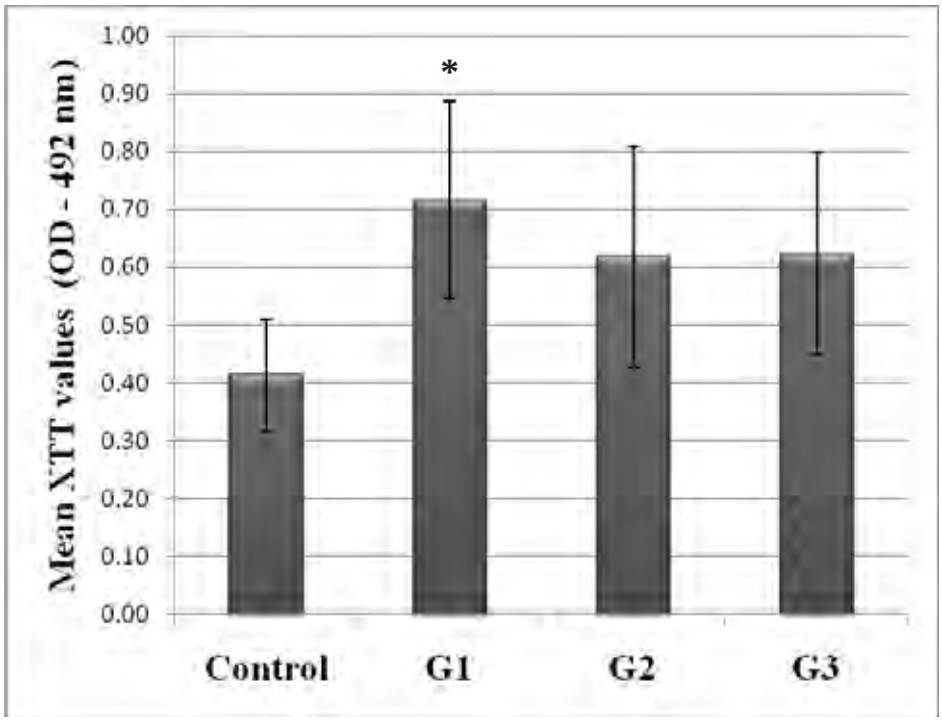

Figure 2

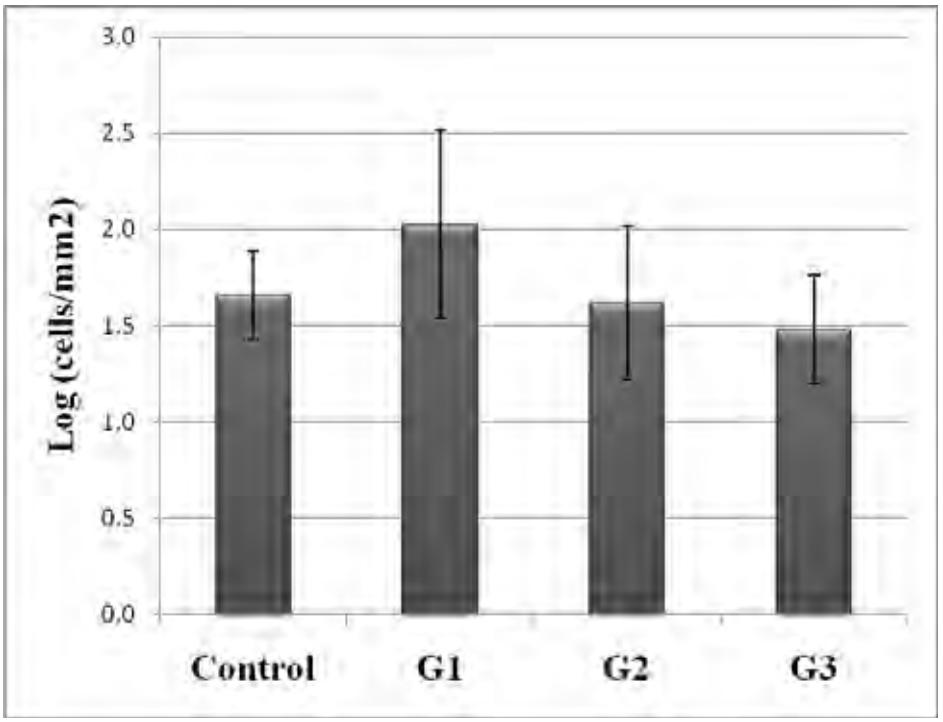

Figure Legends

Figure 1. Mean absorbance values at 492 nm and 95% confidence intervals for all groups. Groups: C - control - without saliva; experimental groups – conditioned in saliva prepared as follows: G_1 – 10000 g/5 min – 15 donors; G_2 – 12000 g/30 min – 15 donors; G_3 – 10000 g/5 min – 1 donor. Statistically different mean compared to control group.

Figure 2. Mean log numbers (cells mm^{-2}) and 95% confidence intervals for all groups. For abbreviations, see legend of Figure 1.


Discussão

4 Discussão

Para discussão dos resultados obtidos no presente estudo, os fatores avaliados foram divididos em tópicos e serão abordados a seguir.

Adesão de *Candida* spp.

A aderência inicial de *Candida* nas superfícies das próteses removíveis é essencial na colonização dessas superfícies e, consequentemente, no desenvolvimento da estomatite protética. Desde que muitos fatores podem influenciar o fenômeno de adesão inicial dos fungos às superfícies acrílicas, tais como interações hidrofóbicas atrativas e forças eletrostáticas repulsivas, o desenvolvimento de métodos que alterem as características superficiais e reduzam a aderência de *Candida* a essas superfícies, seria um passo importante no tratamento e prevenção da estomatite protética.

O tratamento a plasma tem sido utilizado como um método de modificação de superfícies[22,81]. Esse tipo de tratamento caracteriza-se por ser seco, frio e rápido, o que permite a alteração das propriedades de superfície de uma ampla variedade de materiais[19]. Outro aspecto positivo desse método é que as propriedades e a função dos materiais são preservadas, considerando-se que a profundidade do tratamento a plasma é limitada a poucos nanômetros da superfície[19,61,81]. Além disso, nessa técnica, a temperatura permanece tão baixa quanto a temperatura ambiente[30]. Esse aspecto é particularmente importante para as resinas acrílicas para base de prótese, nas quais o aquecimento pode causar alterações dimensionais e afetar a adaptação das bases aos tecidos de suporte[53]. Nos estudos apresentados, os tratamentos a plasma objetivaram diminuir a hidrofobicidade, bem como, incorporar flúor na resina acrílica visando à diminuição da adesão de *Candida albicans* e *Candida glabrata*.

Os resultados obtidos com relação à adesão de *Candida albicans* demonstraram que os grupos $ArSF_6$/70W e ArO_2/70W não apresentaram diferenças entre si e os níveis de atividade metabólica neles obtidos foram

significantemente menores quando comparados aos outros grupos tratados e ao controle, conforme indicado pelo ensaio de XTT. Essa observação difere do resultado obtido por Yildirim et al.[81] (2005) que observaram aumento da aderência de *Candida albicans* a uma resina para base de prótese após tratamentos a plasma. Uma possível explicação para essa diferença seriam os parâmetros determinados no estudo de Yildirim et al.[81] (2005), no qual foi utilizado o gás oxigênio a 50 e 100 W durante 15 minutos. A modificação de superfície observada no grupo $ArSF_6$/70W envolveu a incorporação de flúor na superfície da resina acrílica. Os resultados demonstraram que os valores de ângulos de contato, imediatamente após o tratamento a plasma com $ArSF_6$/70W, aumentaram consideravelmente comparados aos valores do grupo controle, estando de acordo com as observações de Guruvenket et al.[17] (2008). Esse fato provavelmente ocorreu devido à substituição de espécies hidrofílicas por átomos de flúor[22]. Consequentemente, as ligações de hidrogênio entre as moléculas de água e os grupos presentes na superfície diminuíram, reduzindo a hidrofilicidade das amostras tratadas com $ArSF_6$/70W[22]. Assim, a redução da adesão de *Candida albicans* no grupo tratado com $ArSF_6$/70W pode ser atribuída à presença de flúor na resina acrílica como demonstrado pela análise de XPS. Essa redução da adesão de *Candida albicans* poderia ser atribuída ao aumento das forças eletrostáticas repulsivas entre as células fúngicas e as amostras contendo átomos de flúor. Robinson et al.[63] (1997) relataram que a incorporação de flúor torna as superfícies mais negativas, devido à presença de átomos eletronegativos de flúor. Tem sido relatado que superfícies de resina acrílica carregadas negativamente apresentaram níveis significantemente menores de *Candida* aderidas comparadas às superfícies não tratadas, ou seja, superfícies carregadas negativamente podem alterar a interação iônica entre a base de prótese e a célula de *Candida* spp.[47,55].

Os resultados obtidos no estudo apresentado no capítulo 3 demonstraram também que o tratamento a plasma com Ar/50W reduziu significantemente a adesão de *Candida glabrata,* na ausência de saliva. Uma possível explicação para essa redução poderia ser o fato de as amostras do grupo Ar/50W apresentarem o menor valor de ângulo de contato, após 48 horas de imersão em água.

Considerando-se que as interações hidrofóbicas estão envolvidas no processo de adesão[26,32,36,66,83], as superfícies hidrofílicas observadas no grupo Ar/50W podem ter inibido a adesão de *Candida glabrata*. Embora a característica de hidrofobicidade de superfície celular (HSC) não seja específica para cada espécie[46,83], *Candida glabrata* tem sido considerada uma espécie relativamente hidrofóbica[32,35]. Quando comparada com *Candida albicans*, *Candida glabrata* apresentou maior HSC e maior tendência de se aderir às superfícies acrílicas[32]. Adicionalmente, quanto mais próxima a energia livre de superfície do substrato e do microrganismo, maior é a probabilidade de adesão de *Candida*[36]. Assim, os resultados obtidos sugerem que superfícies acrílicas hidrofílicas poderiam inibir a adesão de *Candida glabrata*. Entretanto, na presença de saliva, não foi possível observar o mesmo efeito após o tratamento a plasma com Ar/50W. Tem sido observado que a cobertura com saliva é um importante fator na determinação das propriedades de molhabilidade dos materiais protéticos, ou seja, após o condicionamento com saliva, as características de molhabilidade dos biomateriais podem ser alteradas[71,83]. A energia livre de superfície de vários materiais, incluindo uma resina acrílica polimerizada por meio de micro-ondas, diminuiu em aproximadamente 10% quando as amostras foram recobertas com saliva[71]. Isso poderia ajudar a explicar a ausência de efeito do tratamento a plasma na adesão de *Candida glabrata* depois do pré-condicionamento das amostras de resina acrílica com saliva.

As mensurações dos ângulos de contato dos grupos imediatamente após os tratamentos a plasma demonstraram que os diferentes graus de hidrofilicidade propostos no presente estudo foram obtidos. A diminuição na hidrofobicidade das superfícies, observadas imediatamente após os tratamentos a plasma Ar/50W, ArO_2/70W, AAt/130W, pode ser atribuída aos elétrons energéticos criados durante o tratamento que colidem na superfície acrílica. Essas colisões podem resultar em quebra de ligações químicas criando radicais livres na superfície[19,61]. As reações entre os radicais livres e espécies do material ou da atmosfera, tais como hidrogênio ou oxigênio, podem incorporar grupos hidrofílicos na superfície do polímero, aumentando sua energia livre de superfície e, consequentemente,

diminuindo os valores de ângulo de contato[19,61]. Resultados similares também foram relatados em outras investigações em que tratamentos a plasma foram utilizados para modificar superfícies poliméricas[45,61,81-82].

Entretanto, foi observado que, quando as amostras tratadas a plasma foram imersas em água por 48 horas, houve uma tendência de recuperação, ou seja, os valores de ângulos de contato aproximaram-se dos valores obtidos nas amostras do grupo controle. Esse aspecto, provavelmente, pode ter contribuído para a igualdade entre as médias de adesão de *Candida albicans* e *Candida glabrata* observada entre o grupo controle e os demais grupos tratados a plasma. Para *Candida albicans*, foi observada igualdade entre todos os tratamentos e o controle, quando a adesão foi avaliada pela coloração cristal violeta, e entre os grupos AAt/130W, Ar/50W e o controle, quando avaliados pelo ensaio de XTT. Para adesão de *Candida glabrata*, os resultados não demonstraram diferença entre as amostras tratadas com plasma AAt/130W e aquelas não tratadas, nas duas condições avaliadas (com e sem pré-condicionamento com saliva). Devido à alteração nos ângulos de contato das amostras tratadas, após sua imersão em água, também não foi possível relacionar a redução da adesão de *Candida albicans*, observada pelo ensaio de XTT, para os grupos $ArSF_6$/70W e ArO_2/70W, com a hidrofobicidade das superfícies. A estabilidade das superfícies hidrofílicas obtidas por meio de tratamentos a plasma foi estudada por Rangel et al.[61], em 2004. Esses autores observaram que embora superfícies de silicone submetidas a diferentes tratamentos a plasma apresentaram uma diminuição dos valores de ângulos de contato, elas retornaram à hidrofobicidade original, após a exposição ao ar atmosférico. Uma possível explicação para essa alteração é que a diminuição do ângulo de contato obtida por meio do tratamento a plasma aumenta a energia de superfície da resina acrílica[19,61]. Nessa condição, tem sido observado que as superfícies poliméricas tendem a retornar à hidrofobicidade original, devido à rotação dos grupos polares ao redor da cadeia polimérica, da superfície em direção ao interior do material[61].

Nos estudos apresentados nos capítulos 1, 2 e 3, os valores de ângulo de contato, após 48 horas, demonstraram que os resultados obtidos com os

tratamentos a plasma sobre superfícies de resina acrílica também não foram estáveis e alteraram-se após imersão em água, embora o tratamento a plasma com Ar/50W tenha apresentado valores menores de ângulos de contato comparados aos demais grupos.

Rugosidade Superficial

Zissis et al.[84] (2000), ao estudar diversas resinas para base de prótese e resinas utilizadas para reembasamento, observaram que a rugosidade de superfície dos materiais protéticos pode variar consideravelmente, tendo sido obtidos valores de 0,7 a 7,6 micrômetros. Assim, tem sido sugerido que a rugosidade superficial pode favorecer a fixação dos microrganismos, devido à maior área de superfície disponível para adesão, e ainda, por proteger os microrganismos contra as forças de remoção[28,58-59,74,77]. Assim, visando avaliar o efeito da rugosidade superficial sobre a adesão de *Candida albicans*, dois métodos de confecção das amostras foram utilizados nos estudos apresentados nos capítulos 1 e 2, os quais permitiram a obtenção de superfícies com diferentes valores de rugosidades superficiais. Dessa forma, nesses estudos, todos os grupos eram compostos de metade das amostras processada contra o gesso e a outra metade polimerizada em contato com o vidro, a fim de se obterem, respectivamente, superfícies rugosas e lisas. Para todos os grupos avaliados, os valores médios de rugosidade obtidos nas amostras processadas contra o gesso foram sempre superiores aqueles das amostras processadas contra o vidro. Entretanto, a avaliação do efeito da rugosidade superficial sobre a aderência de *Candida albicans* não revelou diferenças estatisticamente significantes. Esses resultados discordam dos obtidos em diversos estudos[28,59,74,77], em que números significantemente maiores de *Candida albicans* foram encontrados em superfícies rugosas comparadas às superfícies lisas. Por outro lado, recentemente, outros autores[7,15,37,39,43] também não observaram influência significante da rugosidade sobre a aderência de *Candida albicans*, concordando com os resultados observados no presente estudo. Essa divergência aponta para necessidade de mais estudos que avaliem o efeito de diferentes valores de rugosidade na adesão de *Candida* spp.

Nos estudos apresentados nos capítulos 3, 4 e 5, todas as amostras foram confeccionadas entre duas lâminas de vidro para obtenção de superfícies lisas e padronizadas[38,78]. Os resultados obtidos demonstraram que não houve diferenças significativas nos valores de rugosidade entre todos os grupos avaliados.

Película Salivar

Desde que todas as superfícies orais são recobertas pela película salivar, foi considerado importante avaliar o efeito da saliva no processo de adesão fúngica. Estudos in vitro que avaliam esse efeito têm apresentado resultados contraditórios. Alguns autores[6,8,14,21,23,40-42,44,56] observaram aumento da colonização fúngica ao estudarem o efeito da película de saliva na adesão de *Candida albicans.* Outros estudos[25,34,37,50,65,79], por outro lado, encontraram que a saliva resultou em diminuição nos valores de adesão fúngica sobre as superfícies acrílicas, silicone e materiais reembasadores. Ao considerar o efeito da saliva, Ramage et al.[60] (2004) observaram que esse foi dependente do período de avaliação, ou seja, eles observaram um aumento da adesão de *Candida albicans* na fase de aderência inicial, mas um efeito mínimo ou de diminuição após 24 horas. Segundo Thein et al.[75] (2007), a saliva humana pode modular o processo de adesão e colonização dependendo da natureza e número de espécies envolvidas. Os resultados obtidos nos estudos apresentados nos capítulos 1 e 2 indicaram que a presença da película salivar não alterou significantemente o processo de adesão de *Candida albicans* à resina acrílica avaliada, o que concorda com os estudos realizados por Tari et al.[73] (2007) e Jin et al.[24] (2004). Entretanto, é importante ressaltar que a saliva utilizada nesses dois estudos foi diluída em PBS[60], o que pode ter influenciado os resultados obtidos.

Outra observação importante é que a saliva não aumentou a adesão de *Candida glabrata* como demonstrado no estudo apresentado no capítulo 3. Foi possível observar que não houve nenhuma diferença significantemente entre presença e ausência da película salivar na adesão de *Candida glabrata*. Esses resultados estão de acordo com aqueles obtidos em investigações anteriores[34,37,50,52] que avaliaram o efeito da saliva na adesão de *Candida glabrata*.

Dessa forma, os resultados sugerem que a película salivar não aumenta a adesão de *Candida glabrata* às superfícies poliméricas, ao contrário do que tem sido observado por alguns autores para *Candida* albicans[8,40-42,44]. Além disso, considerando que poucos estudos avaliaram a interação entre a película salivar e *Candida glabrata*[34,37,50,52], mais investigações ainda são necessárias para avaliar essa questão.

As divergências encontradas na literatura com relação ao desempenho da película salivar no processo de adesão de *Candida albicans* têm sido, em parte, atribuídas às variações metodológicas entre os estudos, incluindo os diferentes períodos de pré-condicionamento com saliva e as variações na coleta e preparo das amostras de saliva[51]. Assim, os objetivos dos estudos apresentados nos capítulos 4 e 5 foram avaliar se diferentes períodos de pré-condicionamento com saliva e variações na coleta e preparo das amostras de saliva influenciariam os resultados de adesão de *Candida albicans* a uma resina para base de prótese utilizando-se dois métodos de análise, o ensaio de XTT e a coloração cristal violeta.

A influência da película salivar na adesão de *Candida* e no desenvolvimento do biofilme protético pode ocorrer por meio de interações específicas entre as adesinas celulares e receptores específicos presentes na saliva[7,14,23], e ainda, pela atuação das proteínas salivares como fontes de nutrientes para o crescimento microbiológico[13]. Por outro lado, essas proteínas também podem atuar bloqueando os locais de adesão originalmente presentes nos substratos[7,24]. Tem sido observado ainda que a saliva pode alterar as características superficiais dos substratos envolvidas no processo de adesão, como rugosidade e hidrofobicidade superficiais[7,18,71,81], embora existam também autores que relatem que as propriedades superficiais dos materiais são transferidas através da película proteica, mantendo sua influência sobre a adesão microbiana[18,23].

Os resultados obtidos após os diferentes períodos de pré-condicionamento em saliva revelaram que as diferenças entre todos os grupos não foram significativas, ou seja, os diferentes períodos de pré-condicionamento em saliva não influenciaram a adesão de *Candida albicans,* tanto pelo ensaio de XTT como

pela coloração cristal violeta. Esses resultados sugerem que, em estudos de adesão de *Candida albicans* em resinas acrílicas, períodos mais curtos de pré-condicionamento em saliva, como 30 ou 60 minutos, poderiam ser utilizados, tendo em vista a maior facilidade de execução quando comparados a períodos mais longos.

Ainda com relação a esses resultados, o coeficiente de correlação entre os dois métodos de análise utilizados, coloração cristal violeta e ensaio de XTT, foi baixo. Os resultados obtidos por meio da coloração demonstraram que não houve diferenças significativas entre os grupos experimentais e o controle, indicando que o pré-condicionamento em saliva não alterou a adesão fúngica. Por outro lado, no ensaio de XTT, embora a análise estatística não tenha encontrado diferenças significantes entre os grupos, os valores de absorbância obtidos após o condicionamento em saliva foram numericamente maiores comparados aos valores do grupo controle. Um aspecto importante a ser considerado é que os dois métodos de análise baseiam-se em princípios diferentes. Na coloração cristal violeta, as células aderidas às superfícies da resina acrílica são fixadas, coradas com o corante básico cristal violeta e quantificadas por meio da contagem celular em campos selecionados na superfície do substrato[15,26,37,50,65]. Desde que células (viáveis e não viáveis) são coradas pelo corante cristal violeta, esse método não permite diferenciação entre células vivas e mortas[49]. Por outro lado, o ensaio de XTT é um método em que a atividade metabólica das células viáveis é medida[24,60,70]. Tem sido relatado que esse método correlaciona bem com outras técnicas quantitativas, tais como, adenosina trifosfato (ATP) e contagem das unidades formadoras de colônias viáveis (UFC/mL)[24,76]. Uma análise da literatura revela que a maioria dos estudos que encontraram aumento da colonização de *Candida albicans* sobre materiais protéticos após pré-condicionamento com saliva utilizou testes que avaliam o metabolismo fúngico[8,40-42,44]. Por outro lado, quando métodos de quantificação celular por meio de microscopias ou contagem de unidades formadoras de colônias (UFC/mL) foram utilizados, a maioria dos estudos sobre colonização de *Candida albicans* sobre materiais protéticos observou ausência de efeito significativo da saliva[25,73,75] ou diminuição da

adesão[25,34,37,50,79]. Diante disso, é possível que as divergências encontradas nos estudos que avaliam o efeito da saliva na adesão de *Candida albicans* possam também estar relacionadas, pelo menos parcialmente, com os diferentes métodos de avaliação de adesão utilizados. Os resultados obtidos no presente estudo apontam que os efeitos da saliva na adesão e no desenvolvimento de biofilmes de *Candida* deveriam ser avaliados utilizando-se mais de um método de quantificação, particularmente se eles são baseados em princípios diferentes.

Com relação à coleta e preparo das amostras de saliva, os resultados obtidos pelo ensaio de XTT demonstraram que o pré-condicionamento com saliva coletada de vários doadores e centrifugada a 10.000 g por 5 minutos aumentou significantemente a atividade metabólica de *Candida albicans* quando comparada ao grupo controle (sem saliva). Entretanto, nos grupos em que a saliva foi coletada de um único doador, ou centrifugada por tempo e velocidade maiores, nenhuma diferença significante foi detectada quando comparados ao grupo controle. Os sistemas de reconhecimento fungo-superfície envolvem diferentes mecanismos baseados em carboidratos e proteínas existentes nesta interação[14]. Tem sido sugerido também que mucinas salivares aderem-se às células de *Candida albicans*, promovendo a adesão fúngica ao polimetilmetacrilato[14]. Considerando-se que maiores forças de centrifugação podem separar quantidades significantes de mucinas de alto peso molecular[58,69], a centrifugação deveria ser feita sem causar efeitos consideráveis nas propriedades bioquímicas e biofísicas da saliva. Tem sido observado que a centrifugação a 10.000 g por 5 minutos a 4 °C teve impacto mínimo no perfil protéico da saliva[68]. Esses achados ajudam a explicar, pelo menos parcialmente, o valor de absorbância significantemente maior do grupo G1 (saliva de vários doadores centrifugada a 10.000 g por 5 minutos a 4 °C) quando comparado ao grupo controle. Outro aspecto a ser considerado é que a composição da saliva varia consideravelmente entre os indivíduos[69]. Assim, o uso de amostras de saliva coletada de vários doadores pode minimizar essa variação e, consequentemente, sua influência nos resultados relacionados à adesão de *Candida albicans*.

Diferentemente dos resultados obtidos por meio do ensaio de XTT, a coloração cristal violeta revelou que o número de células aderidas foi maior para o grupo G1 comparado ao grupo controle e aos demais grupos experimentais, mas essas diferenças não alcançaram significância estatística. É importante enfatizar novamente que os dois métodos utilizados para avaliar a adesão de *Candida albicans* à resina acrílica são baseados em princípios diferentes. As diferenças apontadas entre o ensaio de XTT e a coloração cristal violeta podem ajudar a explicar os resultados obtidos neste estudo e podem explicar, pelo menos parcialmente, os resultados controversos encontrados na literatura. Assim, quando possível, é recomendado utilizar mais de um método para avaliar o efeito da saliva na adesão de *Candida* às superfícies.

Finalmente, as divergências de resultados entre os estudos podem também ser atribuídas aos diferentes materiais que têm sido utilizados como substratos, entre eles, superfícies acrílicas[8,21,25,34,37,40,50,65,75,79], materiais protéticos reembasadores[41-42,44,50,73,79], materiais poliméricos maxilofaciais[23,40] e poliestireno[24,60]. Tem sido relatado que os grupos químicos expostos nas superfícies sólidas desempenham papel importante na seletividade do processo de adsorção[6,71]. Yildirim et al.[82] (2006) observaram que superfícies de resina acrílica modificadas por meio de tratamentos a plasma adsorveram quantidades diferentes de mucina de alto peso molecular. Portanto, é possível afirmar que a composição da película salivar pode variar entre os diversos materiais avaliados. Tendo em vista que as concentrações proteicas resultantes nos diferentes materiais protéticos foram pouco avaliadas[16], estudos adicionais são necessários para analisar essas diferenças.

Os estudos apresentados apresentam limitações desde que uma resina acrílica para base de prótese e uma cepa de *Candida albicans* e *Candida glabrata* foram utilizadas. Além disso, tratamentos a plasma utilizando outros parâmetros também devem ser avaliados. Apesar dessas limitações, os resultados obtidos sugerem que os tratamentos utilizados nos grupos $ArSF_6$/70W e ArO_2/70W tem potencial para redução da aderência de *Candida albicans* sobre as superfícies das bases de próteses e devem ser melhor analisados em estudos futuros. Além disso,

superfícies hidrofílicas são efetivas para redução da aderência de *Candida glabrata* sobre superfícies acrílicas. Os diferentes períodos de pré-condicionamento em saliva não influenciaram os resultados de adesão de *Candida albicans* a uma resina acrílica para base de próteses. Entretanto, variações na coleta e preparo das amostras de saliva são fatores que podem influenciar nos resultados de adesão de *Candida albicans*.


Conclusão

5 Conclusão

Dentro das limitações deste estudo, as seguintes conclusões podem ser feitas:

- Os tratamentos a plasma utilizados neste estudo foram eficientes para alterar o grau de hidrofobicidade ou incorporar flúor na superfície da resina acrílica;
- A diminuição da atividade metabólica de *Candida albicans* sobre a resina acrílica estudada foi observada nos tratamentos $ArSF_6$/70W e ArO_2/70W, quando analisada pelo método XTT;
- A aderência de *Candida glabrata* foi diminuída sobre superfícies hidrofílicas obtidas por meio do tratamento a plasma com Ar/50W, quando avaliada pela coloração cristal violeta;
- A saliva não alterou significantemente a aderência de *Candida glabrata*.
- Os diferentes períodos de pré-condicionamento em saliva não influenciaram significativamente a aderência de *Candida albicans* sobre a resina acrílica avaliada;
- O coeficiente de correlação entre os resultados obtidos com a coloração cristal violeta e o ensaio de XTT foi baixo;
- As variações na coleta e preparo das amostras de saliva utilizadas são fatores que podem influenciar os resultados de aderência de *Candida albicans*.
- A rugosidade superficial não alterou significantemente a aderência de *Candida albicans*.


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Anexos

7 Anexos

Anexo 1

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Adherence in vitro of *Candida albicans* to plasma treated acrylic resin. Effect of plasma parameters, surface roughness and salivary pellicle

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ABSTRACT

The adhesion of Candida albicans to surfaces is the prerequisite for occurrence of denture stomatitis.

Objective: Hence, this study investigated if surface modifications with plasma treatments could reduce the adherence of *C. albicans* to a denture base resin.

Methods: Specimens (n = 180) with roughened and smooth surfaces were made and divided into five groups: control—specimens were left untreated; experimental groups—specimens were submitted to plasma treatments to obtain surfaces with different hydrophobicity (Ar/50 W; ArO_2/70 W; AAt/130 W) or incorporation of fluorine (Ar/SF_6/70 W). Contact angle measurements were performed immediately after the treatments and after immersion in water for 48 h. For each group, half of the specimens were incubated with saliva prior to the adhesion assay. The number of adherent yeasts was evaluated by XTT reduction method.

Results: For the experimental groups, there was significant change in the mean contact angle after 48 h of immersion in water. Groups ArO_2/70 W and $ArSF_6$/70 W showed significantly lower absorbance readings than the other groups, regardless the presence or absence of saliva and surface roughness.

Conclusions: Results demonstrated that ArO_2/70 W and $ArSF_6$/70 W plasma treatments showed promising potential for reducing the adherence of *C. albicans* to denture base resins.

1. Introduction

The inability of current antifungal therapy to cure denture stomatitis emphasizes the importance of treatment methods directed towards reducing initial fungal attachment, since the prerequisite for colonization and, consequently, occurrence of denture stomatitis is the adhesion of *Candida albicans* to oral surfaces, including mucosa and denture surfaces.[1–3] Although the exact mechanisms by which the adhesion of Candida to acrylic surfaces occurs are unknown, many factors that affect *Candida* adherence have been described, among them surface roughness, salivary pellicle, and hydrophobic and electrostatic interactions. Surface roughness seems to favour microbial attachment and difficult detachment, probably because it provides a larger surface area and/or protection against shear forces.[4] The

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Anexo 2

mycoses

Original article

Evaluation of fungal adherence to plasma-modified polymethylmethacrylate

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Summary

There is a propensity for fungal adherence to the polymethylmethacrylate used for making denture bases. Therefore, this study investigated whether surface modifications with plasma treatments would reduce the adherence of *Candida albicans* to a denture base resin. Samples ($n = 180$) with smooth and rough surfaces were made and divided into five groups: control – non-treated; experimental groups – submitted to plasma treatments to obtain surfaces with different hydrophobicities (Ar/50 W; ArO_2/70 W; AAr/130 W) or with incorporated fluoride ($Ar/SF_6$70 W). Contact angles were measured immediately after treatments and after samples were immersed in water for 48 h. For each group, half the samples were incubated with saliva before the adherence test. The number of adhered *C. albicans* was evaluated by counting after crystal violet staining. The plasma treatments were effective in modifying the polymethylmethacrylate surface. However, there was a significant alteration in the contact angle measured after immersion in water. No statistically significant difference in the adherence of *C. albicans* was observed between the experimental and control groups, irrespective of the presence or absence of saliva, and surface roughness.

Key words: *Candida* spp, *Candida albicans*, fungal adherence, denture stomatitis, cell-surface hydrophobicity.

Introduction

Polymers are widely used materials in different areas.[1–3] In dentistry, polymethylmethacrylate is the polymer of choice for making removable denture bases for the purpose of rehabilitating partially or completely edentulous patients. Nevertheless, in spite of the good mechanical and aesthetic properties of this material, microorganisms, particularly *Candida albicans*, have a propensity for adhering to denture surfaces.[3] This adhesion capacity of *C. albicans* to denture surfaces is the first step, considered essential, for the development of denture stomatitis,[3–5] a common type of oral candidiasis among denture wearers.[6–8] Therefore, the development of methods that could modify these surfaces to prevent the adhesion of *C. albicans*, would be a significant advancement in the treatment of this pathology.

Although the exact mechanisms involved in the adherence of microorganisms to dentures are not completely known, various factors may influence this process, among them, surface roughness and the presence of saliva. The increase in surface roughness has been correlated with the greater ease of fungal retention.[4,9] On the other hand, the effect of saliva on this process is not clear and the results are controversial.[10–12] Some authors[13–15] also point out the influence of hydrophobic interactions. Minagi *et al.* [14] observed that the closer the surface energy of the fungal cell and the substrate are, the greater the probability of adherence occurring. Klotz *et al.* [13] observed a linear relationship between the number of *C. albicans* adhered per unit of area and the hydrophobicity of polymers. Electrostatic interactions have also been mentioned as another factor that could influence the adherence of microorganisms to

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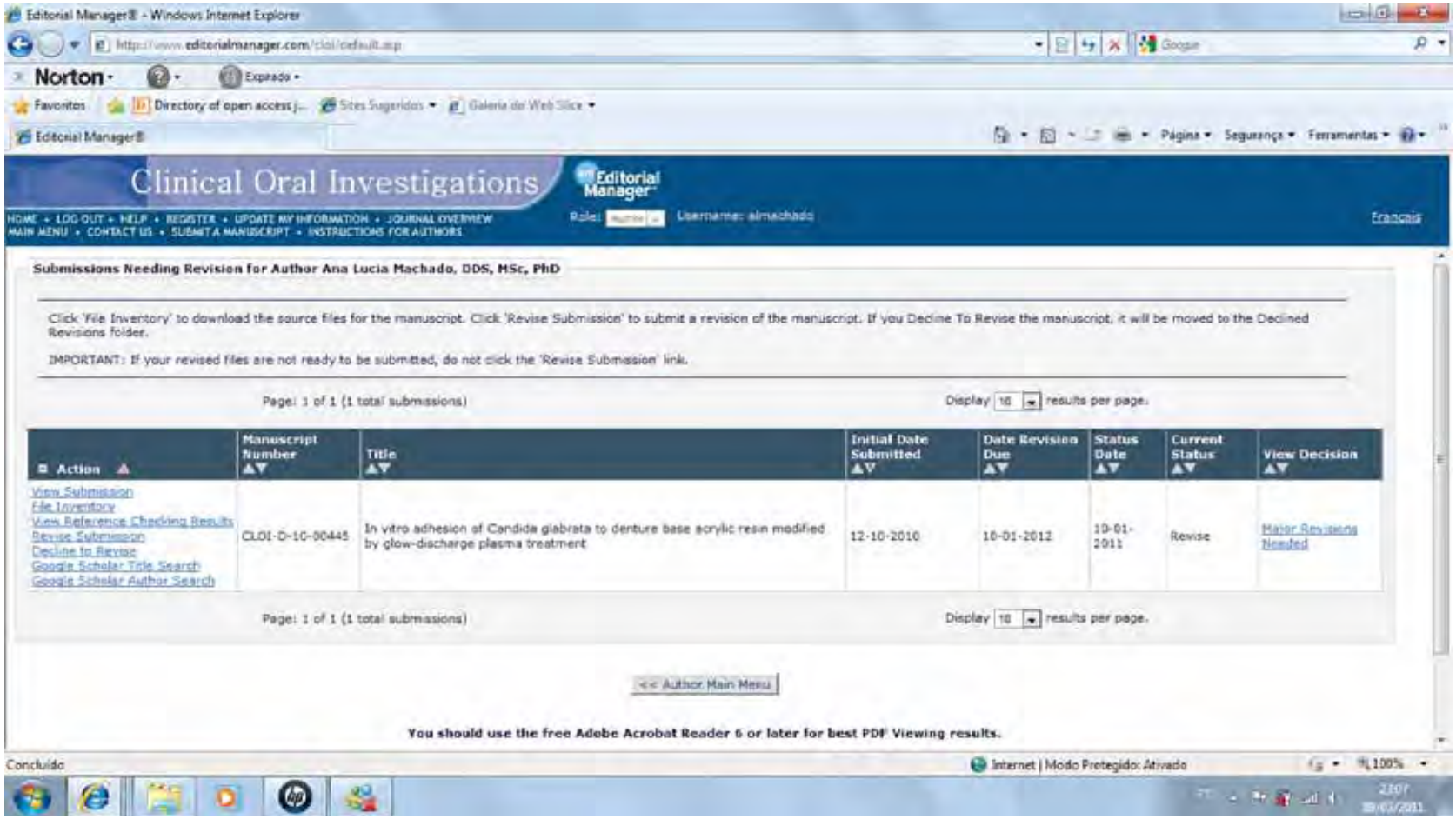
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Anexo 3

Anexo 4

Journal of Investigative and Clinical Dentistry (2010), **1**, 114–119

ORIGINAL ARTICLE

Oral Microbiology

Effect of different periods of preconditioning with saliva on *Candida albicans* adhesion to a denture base resin by crystal violet staining and XTT assay

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Keywords
acrylic resin, adhesion, biofilm, *Candida albicans*, saliva.

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Abstract

Aim: The role of saliva on *Candida* adhesion to biomaterials has not been clearly defined. The present study investigates whether different periods of preconditioning with saliva would influence the adhesion of *Candida albicans* to a denture base resin.

Methods: Ninety samples of acrylic resin with smooth surfaces were made and then divided into five groups: one control without saliva, and four experimental groups conditioned in saliva for periods of 30 min, 1, 3, or 12 h. *Candida* adhesion was evaluated by crystal violet staining and 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-5-([phenylamino] carbonyl)-2H-tetrazolium-hydroxide assay.

Results: The one-way analysis of variance revealed that there were no significant differences among the mean number of adherent cells or among the mean absorbance for all groups. No significant correlation was found between the two methods used for assessing *Candida albicans* adhesion.

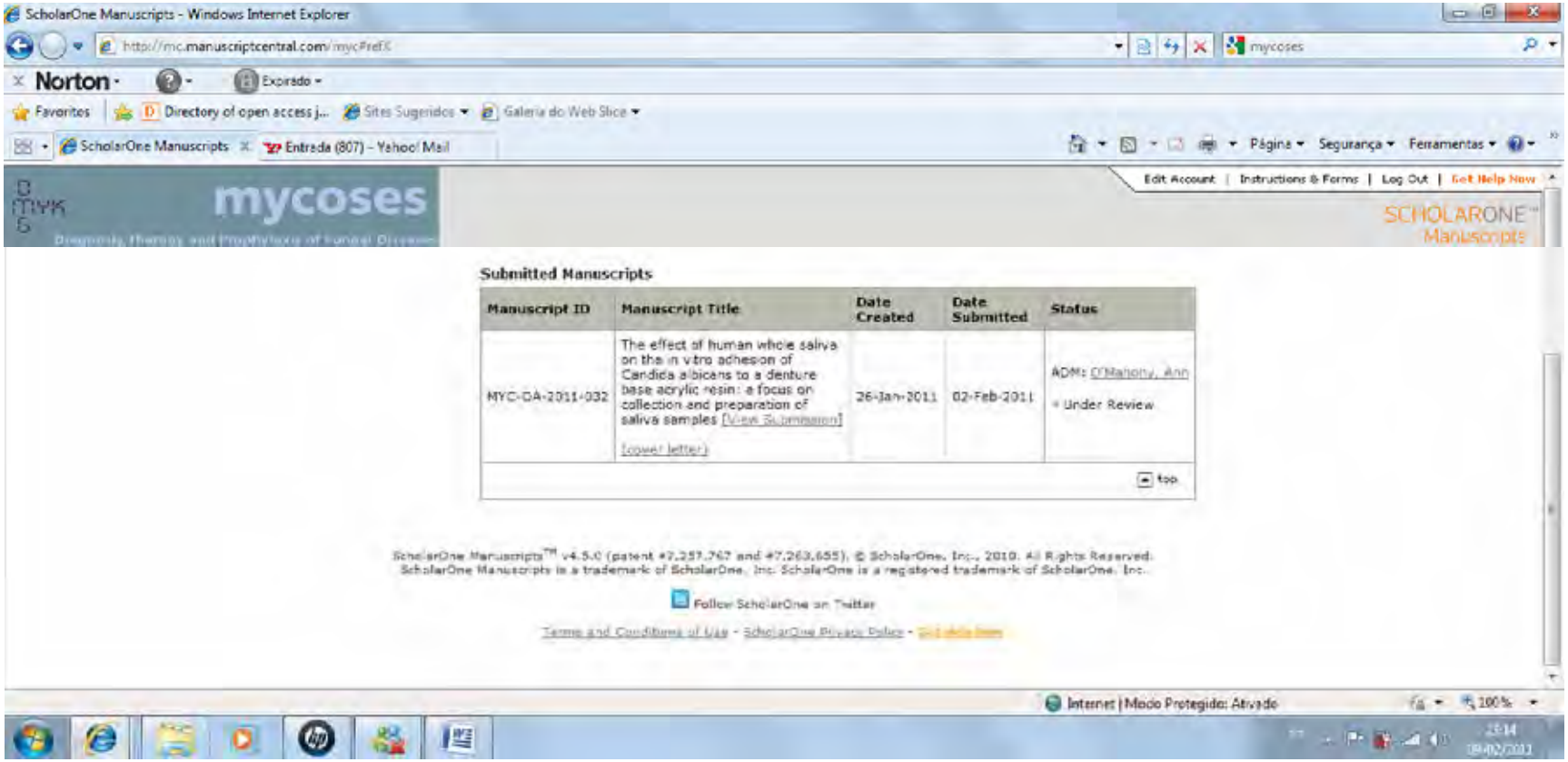
Conclusion: The different periods of preconditioning with saliva had no significant influence on the adhesion of *Candida albicans* to the denture base acrylic resin.

Introduction

The presence of *Candida albicans* biofilms on removable denture surfaces plays an important role in the etiology of denture stomatitis. The capacity of this fungus to adhere to surfaces is the first stage in the biofilm formation process, which is followed by colony formation and cell organization, the secretion of the extracellular matrix, maturation, and dissemination of the biofilm.[1]

It is known that the adhesion of *Candida* cells to denture surfaces is mediated by a salivary conditioning film or pellicle[2–4] that provides receptors for microbial adhesion.[5] However, the role of saliva on *Candida albicans* adhesion to biomaterials has not yet been fully established, as conflicting results have been reported. Some authors have observed that the saliva pellicle promotes *Candida albicans* colonization on the materials.[6–10] Conversely, others have found that preconditioning the materials with saliva either does not affect[11–14] or reduces *Candida albicans* adhesion.[15–19] The different periods of preconditioning with saliva could interfere in the adhesive capacity of *Candida*[20] and contribute to the inconsistent findings from these studies. In a number of studies, the materials were incubated with saliva for short periods, such as 30 min,[16–19] 1 h,[7–9] 2–3 h,[4,10] and 4 h.[11,13] Longer incubation periods, including overnight,[21] 18 h,[15] and 24 h,[22] have also been used to create a salivary conditioning film. However, to date, no information is available concerning how different periods of preconditioning with saliva affect the adhesion of *Candida* cells to denture materials.

One method used for evaluating the adhesion of *Candida* is the cell-staining assay.[15,18,19,23,24] In this method, the adhered cells on the material surface are fixed, stained,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Coordenadora

Anexo 6

UNIVERSIDADE ESTADUAL PAULISTA " JÚLIO DE MESQUITA FILHO"

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Certificado

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Prof. Dr. Mauricio Meirelles Nagle

Coordenador

Anexo 7

UNIVERSIDADE ESTADUAL PAULISTA " JÚLIO DE MESQUITA FILHO"

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Comitê de Ética em Pesquisa

Certificado

Certificamos que o projeto de pesquisa intitulado *"INFLUÊNCIA DO TEMPO DE PRÉ-EXPOSIÇÃO À SALIVA NA ADESÃO DE CANDIDA ALBICANS EM UMA RESINA PARA BASE DE PRÓTESE"*, sob o protocolo nº **25/08** e o relatório final de responsabilidade do Pesquisador (a) *ANA LÚCIA MACHADO*, estão de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa–FOAr.

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Anexo 8


Apêndices

8 Apêndice

Apêndice 1

Os resultados obtidos durante as leituras de rugosidade superficial das amostras processadas entre vidros, ângulo de contato (imediatamente após os tratamentos e 48 horas após a imersão em água destilada estéril), bem como, os valores de absorbância relativos à adesão de *Candida albicans* nos diferentes grupos estão apresentados nas Tabelas 1A a 5A.

Tabela 1A - Grupo controle (não submetido ao tratamento a plasma)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
Controle	Ausente	0,28	53,80	51,91	0,63
Controle	Ausente	0,32	60,31	59,87	1,25
Controle	Ausente	0,29	55,30	56,90	0,83
Controle	Ausente	0,31	55,30	53,22	1,00
Controle	Ausente	0,35	53,80	51,91	0,67
Controle	Ausente	0,20	57,68	56,18	0,79
Controle	Ausente	0,35	56,62	59,83	0,97
Controle	Ausente	0,34	57,78	55,20	0,37
Controle	Ausente	0,31	55,35	52,87	1,00
Controle	Presente	0,18	57,68	56,18	0,78
Controle	Presente	0,23	56,62	59,83	1,01
Controle	Presente	0,16	57,78	55,20	0,96
Controle	Presente	0,15	55,35	52,87	0,54
Controle	Presente	0,39	51,80	53,68	0,57
Controle	Presente	0,25	65,20	56,85	0,37
Controle	Presente	0,36	61,17	58,23	0,74
Controle	Presente	0,20	57,78	55,20	0,96
Controle	Presente	0,19	57,68	56,18	0,78

Tabela 2A - Grupo submetido ao tratamento com ar atmosférico (AAt/130W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
AAt	Ausente	0,49	7,59	56,33	0,78
AAt	Ausente	0,39	0,00	56,65	0,76
AAt	Ausente	0,41	3,00	47,95	1,22
AAt	Ausente	0,23	7,00	55,36	0,89
AAt	Ausente	0,15	3,00	59,09	0,54
AAt	Ausente	0,47	0,00	58,08	0,75
AAt	Ausente	0,26	0,00	60,85	0,79
AAt	Ausente	0,32	0,00	55,47	0,73
AAt	Ausente	0,25	1,00	63,77	0,69
AAt	Presente	0,47	2,80	54,59	0,86
AAt	Presente	0,41	6,00	62,25	1,03
AAt	Presente	0,47	0,00	47,95	0,94
AAt	Presente	0,31	0,00	58,51	0,33
AAt	Presente	0,22	2,42	53,46	0,52
AAt	Presente	0,31	0,00	45,66	0,62
AAt	Presente	0,23	0,00	52,17	0,76
AAt	Presente	0,35	1,00	66,24	0,75
AAt	Pres	0,22	0,00	70,11	0,70

Tabela 3A - Grupo submetido ao tratamento com uma mistura de gás argônio e oxigênio (ArO_2/70W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
ArO_2	Ausente	0,38	23,02	56,34	0,84
ArO_2	Ausente	0,34	21,28	59,21	0,33
ArO_2	Ausente	0,20	27,46	56,49	0,35
ArO_2	Ausente	0,25	19,46	68,80	0,34
ArO_2	Ausente	0,21	22,31	52,87	0,31
ArO_2	Ausente	0,33	29,54	58,43	0,32
ArO_2	Ausente	0,30	22,02	59,38	0,76
ArO_2	Ausente	0,18	26,99	57,32	0,61
ArO_2	Ausente	0,17	25,31	43,32	0,57
ArO_2	Presente	0,22	16,82	57,79	0,86
ArO_2	Presente	0,28	20,28	59,46	0,10
ArO_2	Presente	0,47	17,85	60,20	0,35
ArO_2	Presente	0,45	32,22	50,86	0,53
ArO_2	Presente	0,37	22,69	56,52	0,31
ArO_2	Presente	0,31	20,24	68,59	0,31
ArO_2	Presente	0,27	26,61	63,01	1,12
ArO_2	Presente	0,29	19,87	59,66	0,94
ArO_2	Presente	0,24	20,01	66,51	0,96

Tabela 4A - Grupo submetido ao tratamento com gás argônio (Ar/50W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
Ar	Ausente	0,25	39,08	35,26	0,62
Ar	Ausente	0,22	41,85	52,10	0,87
Ar	Ausente	0,33	39,69	58,62	1,19
Ar	Ausente	0,49	49,84	37,26	1,08
Ar	Ausente	0,31	41,63	46,71	1,14
Ar	Ausente	0,32	44,05	44,27	0,93
Ar	Ausente	0,23	32,69	56,08	1,06
Ar	Ausente	0,15	48,13	48,51	0,38
Ar	Ausente	0,39	48,02	34,62	0,37
Ar	Presente	0,40	49,19	36,49	0,91
Ar	Presente	0,31	38,52	41,07	0,91
Ar	Presente	0,42	44,79	43,29	0,71
Ar	Presente	0,27	53,15	49,92	0,97
Ar	Presente	0,45	43,47	41,73	1,15
Ar	Presente	0,38	48,17	50,54	0,99
Ar	Presente	0,27	44,05	51,76	1,20
Ar	Presente	0,45	37,25	53,19	1,04
Ar	Presente	0,25	45,21	43,32	0,55

Tabela 5A - Grupo submetido ao tratamento com gás argônio seguido pelo tratamento com o gás hexafluoreto de enxofre ($ArSF_6$/70W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
$ARSF_6$	Ausente	0,28	98,13	70,09	0,24
$ARSF_6$	Ausente	0,21	96,86	67,58	0,37
$ARSF_6$	Ausente	0,24	98,71	74,55	0,18
$ARSF_6$	Ausente	0,30	86,79	60,18	0,34
$ARSF_6$	Ausente	0,33	97,15	69,92	0,33
$ARSF_6$	Ausente	0,22	105,67	53,03	0,33
$ARSF_6$	Ausente	0,26	103,27	69,39	0,70
$ARSF_6$	Ausente	0,43	94,53	61,31	0,92
$ARSF_6$	Ausente	0,30	98,98	64,41	0,60
$ARSF_6$	Presente	0,27	105,05	66,79	0,43
$ARSF_6$	Presente	0,36	91,82	71,90	0,32
$ARSF_6$	Presente	0,21	84,97	73,48	0,10
$ARSF_6$	Presente	0,13	93,26	57,44	0,98
$ARSF_6$	Presente	0,26	103,55	67,43	0,32
$ARSF_6$	Presente	0,30	108,52	71,21	0,33
$ARSF_6$	Presente	0,36	79,21	62,54	0,74
$ARSF_6$	Presente	0,40	90,85	77,64	0,47
$ARSF_6$	Presente	0,41	87,68	73,86	0,69

Os resultados obtidos durante as leituras de rugosidade superficial das amostras processadas entre gesso, ângulo de contato (imediatamente após os tratamentos e 48 horas após a imersão em água destilada estéril), bem como, os valores de absorbância relativos à adesão de *Candida albicans* nos diferentes grupos estão apresentados nas Tabelas 6A a 10A.

Tabela 6A - Grupo controle (não submetido ao tratamento a plasma)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
Controle	Ausente	2,50	57,34	60,41	0,59
Controle	Ausente	2,13	61,88	54,28	0,76
Controle	Ausente	1,15	69,10	67,03	0,72
Controle	Ausente	1,41	58,31	64,85	0,42
Controle	Ausente	1,06	63,29	59,59	0,67
Controle	Ausente	1,10	58,85	56,71	0,69
Controle	Ausente	3,16	58,31	64,85	0,81
Controle	Ausente	1,19	53,15	59,92	0,96
Controle	Ausente	1,14	57,34	60,41	0,83
Controle	Presente	2,36	58,31	64,85	0,66
Controle	Presente	3,11	63,29	59,59	1,15
Controle	Presente	1,12	58,85	52,71	0,88
Controle	Presente	2,61	59,08	63,49	0,53
Controle	Presente	3,11	57,34	60,41	0,62
Controle	Presente	1,01	61,88	54,28	1,31
Controle	Presente	1,08	69,10	67,03	1,11
Controle	Presente	1,27	61,88	54,28	0,64
Controle	Presente	1,12	58,85	52,71	0,88

Tabela 7A - Grupo submetido ao tratamento com ar atmosférico (AAt/130W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
AAt	Ausente	2,24	0,00	49,98	1,00
AAt	Ausente	2,33	0,00	60,49	1,19
AAt	Ausente	1,71	0,00	57,02	1,21
AAt	Ausente	2,19	3,06	49,84	0,51
AAt	Ausente	1,42	1,53	48,46	0,57
AAt	Ausente	1,18	0,00	60,18	0,34
AAt	Ausente	2,30	1,29	62,59	0,83
AAt	Ausente	1,93	0,00	57,07	0,74
AAt	Ausente	2,24	0,00	61,27	0,87
AAt	Presente	2,47	0,00	60,29	0,84
AAt	Presente	2,66	0,00	45,17	1,13
AAt	Presente	2,39	0,00	62,52	0,93
AAt	Presente	2,11	0,00	52,98	0,48
AAt	Presente	2,13	0,00	59,33	0,94
AAt	Presente	2,53	0,00	54,11	0,89
AAt	Presente	1,52	0,00	63,55	0,73
AAt	Presente	2,26	0,00	52,09	0,82
AAt	Presente	1,90	2,16	54,65	0,64

Tabela 8A - Grupo submetido ao tratamento com uma mistura de gás argônio e oxigênio (ArO_2/70W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
ArO_2	Ausente	1,28	26,40	70,50	0,14
ArO_2	Ausente	1,32	16,31	52,54	0,14
ArO_2	Ausente	1,01	19,01	59,30	0,12
ArO_2	Ausente	2,32	28,40	69,15	0,52
ArO_2	Ausente	2,06	26,25	62,54	0,39
ArO_2	Ausente	2,46	32,36	51,02	0,31
ArO_2	Ausente	2,66	34,59	50,76	1,18
ArO_2	Ausente	2,52	20,58	47,08	0,74
ArO_2	Ausente	1,78	28,65	52,40	0,42
ArO_2	Presente	1,70	17,79	59,49	0,16
ArO_2	Presente	2,18	17,95	70,18	0,21
ArO_2	Presente	1,43	21,23	58,25	0,14
ArO_2	Presente	1,02	17,85	60,20	0,79
ArO_2	Presente	1,30	32,22	50,86	0,68
ArO_2	Presente	1,49	22,69	56,52	0,31
ArO_2	Presente	1,65	34,81	52,56	0,68
ArO_2	Presente	1,75	31,15	47,83	0,57
ArO_2	Presente	1,57	21,91	49,03	0,80

Tabela 9A - Grupo submetido ao tratamento com gás argônio (Ar/50W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
Ar	Ausente	1,42	29,60	57,05	0,87
Ar	Ausente	1,32	41,82	59,60	0,70
Ar	Ausente	2,52	39,81	49,52	0,92
Ar	Ausente	1,37	35,81	39,26	1,32
Ar	Ausente	2,55	47,41	39,84	0,94
Ar	Ausente	1,44	32,01	48,43	1,17
Ar	Ausente	2,51	42,45	40,33	1,10
Ar	Ausente	2,64	36,75	50,19	0,88
Ar	Ausente	1,47	48,05	46,10	0,75
Ar	Presente	1,02	46,64	51,04	0,97
Ar	Presente	1,14	37,97	48,66	1,20
Ar	Presente	2,59	44,88	52,28	0,73
Ar	Presente	1,80	39,51	46,77	1,09
Ar	Presente	1,44	51,62	47,56	1,24
Ar	Presente	2,36	30,65	37,98	1,33
Ar	Presente	1,96	37,22	44,48	0,87
Ar	Presente	1,08	35,35	32,89	0,93
Ar	Presente	2,85	43,00	51,38	1,16

Tabela 10A - Grupo submetido ao tratamento com gás argônio seguido pelo tratamento com gás hexafluoreto de enxofre ($ArSF_6$/70W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	XTT (Abs)
$ARSF_6$	Ausente	1,67	92,47	49,62	0,17
$ARSF_6$	Ausente	1,71	92,91	54,96	0,36
$ARSF_6$	Ausente	1,91	84,56	50,61	0,16
$ARSF_6$	Ausente	1,83	95,32	71,40	0,56
$ARSF_6$	Ausente	2,33	90,56	58,44	0,36
$ARSF_6$	Ausente	3,13	108,35	61,52	0,53
$ARSF_6$	Ausente	1,71	94,92	56,74	0,57
$ARSF_6$	Ausente	1,07	110,34	68,60	0,90
$ARSF_6$	Ausente	1,76	106,36	57,43	1,08
$ARSF_6$	Presente	1,02	93,73	49,23	0,21
$ARSF_6$	Presente	1,43	90,22	52,17	0,15
$ARSF_6$	Presente	2,04	87,25	54,24	0,25
$ARSF_6$	Presente	2,06	112,59	56,44	0,37
$ARSF_6$	Presente	1,40	109,19	53,17	0,31
$ARSF_6$	Presente	1,82	103,90	60,43	1,04
$ARSF_6$	Presente	1,34	104,84	55,63	1,19
$ARSF_6$	Presente	1,82	105,76	41,73	0,74
$ARSF_6$	Presente	2,62	97,13	60,49	0,49

Apêndice 2

Os resultados obtidos durante as leituras de rugosidade superficial das amostras processadas entre vidros, ângulo de contato (imediatamente após os tratamentos e 48 horas após a imersão em água destilada estéril), bem como, os valores de células/mm^2 relativos à adesão de *Candida albicans* nos diferentes grupos estão apresentados nas Tabelas 11A a 15A.

Tabela 11A - Grupo controle (não submetido ao tratamento a plasma)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
Controle	Ausente	0,24	55,30	53,22	278,21	2,45
Controle	Ausente	0,24	51,80	53,68	2289,74	3,36
Controle	Ausente	0,31	60,31	51,87	2603,85	3,42
Controle	Ausente	0,30	58,10	55,90	306,41	2,49
Controle	Ausente	0,38	54,73	60,23	520,51	2,72
Controle	Ausente	0,32	57,80	55,44	1514,10	3,18
Controle	Ausente	0,46	58,60	57,11	3205,13	3,51
Controle	Ausente	0,41	55,57	51,84	398,72	2,60
Controle	Ausente	0,33	58,10	55,90	315,38	2,50
Controle	Presente	0,36	58,31	64,85	703,85	2,85
Controle	Presente	0,24	63,29	59,59	319,23	2,51
Controle	Presente	0,23	58,85	52,71	1097,44	3,04
Controle	Presente	0,25	64,25	60,90	665,38	2,82
Controle	Presente	0,23	59,11	57,47	3130,77	3,50
Controle	Presente	0,23	60,31	51,87	2730,77	3,44
Controle	Presente	0,26	55,30	56,90	333,33	2,52
Controle	Presente	0,36	58,83	55,23	703,85	2,85
Controle	Presente	0,21	58,10	55,90	283,33	2,45

Tabela 12A - Grupo submetido ao tratamento com ar atmosférico (AAt/130W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
AAt	Ausente	0,23	3,20	62,92	1230,77	3,09
AAt	Ausente	0,42	3,40	55,76	456,41	2,66
AAt	Ausente	0,42	2,00	58,14	371,79	2,57
AAt	Ausente	0,29	0,00	62,35	207,69	2,32
AAt	Ausente	0,35	0,00	65,52	1454,65	3,16
AAt	Ausente	0,27	0,00	62,86	550,00	2,74
AAt	Ausente	0,18	6,00	64,61	3342,31	3,52
AAt	Ausente	0,16	9,00	58,21	3116,67	3,49
AAt	Ausente	0,24	0,00	68,97	2361,54	3,37
AAt	Presente	0,23	3,55	54,81	419,23	2,62
AAt	Presente	0,27	4,76	55,76	624,36	2,80
AAt	Presente	0,40	5,00	59,73	78,21	1,90
AAt	Presente	0,45	0,00	51,47	219,23	2,34
AAt	Presente	0,19	0,00	55,73	1219,23	3,09
AAt	Presente	0,20	0,00	38,99	534,62	2,73
AAt	Presente	0,28	4,00	68,62	3052,56	3,48
AAt	Presente	0,23	9,00	51,15	3471,79	3,54
AAt	Presente	0,17	0,00	65,82	4143,59	3,62

Tabela 13A - Grupo submetido ao tratamento com uma mistura de gás argônio e oxigênio (ArO_2/70W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
ArO_2	Ausente	0,27	22,41	62,28	1391,03	3,14
ArO_2	Ausente	0,39	19,32	56,48	915,38	2,96
ArO_2	Ausente	0,42	16,59	75,16	2444,87	3,39
ArO_2	Ausente	0,24	28,96	59,63	294,87	2,47
ArO_2	Ausente	0,23	22,65	57,34	179,49	2,26
ArO_2	Ausente	0,25	28,27	63,78	625,64	2,80
ArO_2	Ausente	0,28	24,87	71,40	583,33	2,77
ArO_2	Ausente	0,20	25,56	50,53	933,33	2,97
ArO_2	Ausente	0,27	28,45	54,18	1619,23	3,21
ArO_2	Presente	0,20	24,87	72,79	1335,90	3,13
ArO_2	Presente	0,45	22,44	72,33	6423,08	3,81
ArO_2	Presente	0,21	22,90	58,38	4506,41	3,65
ArO_2	Presente	0,22	22,06	66,89	732,05	2,87
ArO_2	Presente	0,31	26,78	72,29	564,10	2,75
ArO_2	Presente	0,39	25,78	50,22	515,38	2,71
ArO_2	Presente	0,25	29,75	62,04	2467,95	3,39
ArO_2	Presente	0,23	26,80	73,38	1812,82	3,26
ArO_2	Presente	0,30	17,33	64,15	3147,44	3,50

Tabela 14A - Grupo submetido ao tratamento com gás argônio (Ar/50W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
Ar	Ausente	0,29	50,80	54,16	229,49	2,36
Ar	Ausente	0,46	39,41	37,64	4541,03	3,66
Ar	Ausente	0,41	30,19	33,80	1883,33	3,28
Ar	Ausente	0,31	44,03	46,75	4288,46	3,63
Ar	Ausente	0,14	41,22	52,02	6888,46	3,84
Ar	Ausente	0,42	48,58	51,57	14302,56	4,16
Ar	Ausente	0,29	39,16	39,82	464,10	2,67
Ar	Ausente	0,24	32,54	55,08	506,41	2,71
Ar	Ausente	0,21	43,67	36,36	389,74	2,59
Ar	Presente	0,34	46,10	43,16	221,79	2,35
Ar	Presente	0,28	48,83	39,59	5087,18	3,71
Ar	Presente	0,42	39,85	41,47	387,18	2,59
Ar	Presente	0,16	41,57	49,75	10615,38	4,03
Ar	Presente	0,20	40,39	51,04	9042,31	3,96
Ar	Presente	0,35	36,60	46,74	5243,59	3,72
Ar	Presente	0,28	42,58	43,86	988,46	3,00
Ar	Presente	0,21	45,50	54,70	355,13	2,55
Ar	Presente	0,38	40,81	45,48	971,79	2,99

Tabela 15A - Grupo submetido ao tratamento com gás argônio seguido pelo tratamento com gás hexafluoreto de enxofre ($ArSF_6$/70W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
$ARSF_6$	Ausente	0,31	94,30	60,80	1861,54	3,27
$ARSF_6$	Ausente	0,21	96,05	61,06	1966,67	3,29
$ARSF_6$	Ausente	0,23	90,08	67,60	2235,90	3,35
$ARSF_6$	Ausente	0,23	101,28	66,38	805,13	2,91
$ARSF_6$	Ausente	0,22	110,79	70,10	1346,15	3,13
$ARSF_6$	Ausente	0,22	92,47	71,41	2921,79	3,47
$ARSF_6$	Ausente	0,33	92,29	65,12	1448,72	3,16
$ARSF_6$	Ausente	0,49	97,19	70,37	2955,13	3,47
$ARSF_6$	Ausente	0,35	98,14	64,68	5019,23	3,70
$ARSF_6$	Presente	0,25	97,12	64,87	7378,21	3,87
$ARSF_6$	Presente	0,23	92,96	59,27	5830,77	3,77
$ARSF_6$	Presente	0,29	91,69	58,27	5484,62	3,74
$ARSF_6$	Presente	0,24	91,02	55,63	2694,87	3,43
$ARSF_6$	Presente	0,22	97,55	72,03	7082,05	3,85
$ARSF_6$	Presente	0,24	102,36	64,85	3565,38	3,55
$ARSF_6$	Presente	0,32	100,18	51,43	1548,72	3,19
$ARSF_6$	Presente	0,41	83,49	61,67	3424,36	3,53
$ARSF_6$	Presente	0,20	96,09	57,74	779,49	2,89

Os resultados obtidos durante as leituras de rugosidade superficial das amostras processadas entre gesso, ângulo de contato (imediatamente após os tratamentos e 48 horas após a imersão em água destilada estéril), bem como, os valores de células/mm^2 relativos à adesão de *Candida albicans* nos diferentes grupos estão apresentados nas Tabelas 16A a 20A.

Tabela 16A - Grupo controle (não submetido ao tratamento a plasma)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
Controle	Ausente	1,90	53,15	49,92	948,72	2,98
Controle	Ausente	1,51	49,19	56,49	764,10	2,88
Controle	Ausente	2,84	57,34	60,41	573,08	2,76
Controle	Ausente	1,28	66,70	50,45	1985,90	3,30
Controle	Ausente	1,30	62,01	65,27	5202,56	3,72
Controle	Ausente	1,07	49,19	56,49	719,23	2,86
Controle	Ausente	1,33	62,01	65,27	438,46	2,64
Controle	Ausente	1,54	65,04	63,31	1296,15	3,11
Controle	Ausente	1,86	59,10	64,82	3930,77	3,59
Controle	Presente	1,05	61,88	54,28	156,41	2,20
Controle	Presente	1,86	58,85	52,71	889,74	2,95
Controle	Presente	1,46	64,25	60,90	5752,56	3,76
Controle	Presente	1,53	61,88	54,28	1244,87	3,10
Controle	Presente	3,10	58,85	52,71	1184,62	3,07
Controle	Presente	1,86	59,08	63,49	106,41	2,03
Controle	Presente	2,07	64,25	60,90	156,41	2,20
Controle	Presente	1,39	62,07	62,59	3365,38	3,53
Controle	Presente	1,20	60,68	55,24	2430,77	3,39

Tabela 17A - Grupo submetido ao tratamento com ar atmosférico (AAt/130W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
AAt	Ausente	2,47	2,73	57,26	316,67	2,50
AAt	Ausente	1,87	0,97	49,67	4965,38	3,70
AAt	Ausente	2,07	0,00	59,32	2325,64	3,37
AAt	Ausente	1,53	0,00	72,71	517,95	2,72
AAt	Ausente	1,69	0,00	61,52	617,95	2,79
AAt	Ausente	1,98	0,00	63,53	505,13	2,70
AAt	Ausente	2,64	5,10	61,88	3375,64	3,53
AAt	Ausente	1,11	3,92	66,35	3367,95	3,53
AAt	Ausente	2,48	0,00	56,79	3360,26	3,53
AAt	Presente	1,81	3,36	56,93	839,74	2,92
AAt	Presente	2,32	0,00	45,11	388,46	2,59
AAt	Presente	1,04	0,00	57,47	1024,359	3,01
AAt	Presente	1,05	0,00	53,85	397,4359	2,60
AAt	Presente	1,71	2,35	60,53	415,38	2,62
AAt	Presente	2,22	0,44	55,78	526,92	2,72
AAt	Presente	2,02	0,00	51,50	9457,69	3,98
AAt	Presente	3,09	0,00	49,70	2876,92	3,46
AAt	Presente	1,96	1,87	52,92	2037,18	3,31

Tabela 18A - Grupo submetido ao tratamento com uma mistura de gás argônio e oxigênio (ArO_2/70W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
ArO_2	Ausente	1,41	26,26	58,81	1520,51	3,18
ArO_2	Ausente	1,29	31,48	36,75	1841,03	3,27
ArO_2	Ausente	1,76	18,30	52,47	2292,31	3,36
ArO_2	Ausente	2,32	27,86	52,69	735,90	2,87
ArO_2	Ausente	1,51	22,77	47,99	2485,90	3,40
ArO_2	Ausente	1,42	22,61	42,99	433,33	2,64
ArO_2	Ausente	1,04	21,47	36,59	775,64	2,89
ArO_2	Ausente	1,56	29,02	51,15	810,26	2,91
ArO_2	Ausente	2,05	31,34	49,69	580,77	2,76
ArO_2	Presente	2,47	20,29	43,63	1905,13	3,28
ArO_2	Presente	1,01	20,31	49,57	3485,90	3,54
ArO_2	Presente	2,10	21,65	55,63	1947,44	3,29
ArO_2	Presente	1,97	23,82	43,36	278,21	2,45
ArO_2	Presente	1,79	32,10	51,12	1461,54	3,17
ArO_2	Presente	1,05	28,43	55,99	657,69	2,82
ArO_2	Presente	1,67	23,55	61,59	280,77	2,45
ArO_2	Presente	1,47	38,49	47,93	903,85	2,96
ArO_2	Presente	1,02	33,11	55,09	2832,05	3,45

Tabela 19A - Grupo submetido ao tratamento com gás argônio (Ar/50W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
Ar	Ausente	1,74	29,61	53,29	1980,77	3,30
Ar	Ausente	1,55	47,90	40,09	7442,31	3,87
Ar	Ausente	1,85	36,83	48,70	6889,74	3,84
Ar	Ausente	1,45	39,89	47,96	8961,54	3,95
Ar	Ausente	2,73	41,82	47,46	16185,90	4,21
Ar	Ausente	1,46	40,00	31,07	11607,69	4,06
Ar	Ausente	1,49	37,64	51,71	485,90	2,69
Ar	Ausente	1,81	39,61	55,28	3017,95	3,48
Ar	Ausente	2,16	37,21	34,08	333,33	2,52
Ar	Presente	1,18	31,55	63,31	1334,62	3,13
Ar	Presente	1,49	36,92	45,89	8550,00	3,93
Ar	Presente	1,51	40,60	48,18	21423,08	4,33
Ar	Presente	2,42	35,34	51,94	4655,13	3,67
Ar	Presente	3,08	37,12	53,34	4411,54	3,64
Ar	Presente	1,96	36,90	43,35	7676,92	3,89
Ar	Presente	1,18	36,89	42,04	1529,49	3,18
Ar	Presente	2,10	45,71	38,11	2538,46	3,40
Ar	Presente	2,30	40,70	50,92	484,62	2,69

Tabela 20A - Grupo submetido ao tratamento com gás argônio seguido pelo tratamento com hexafluoreto de enxofre ($ArSF_6$/70W)

Grupo	Saliva	Rugosidade (Ra-µm)	Ângulo de contato após tratamento (º)	Ângulo de contato 48 horas (º)	cel/mm^2	log (cel)
$ARSF_6$	Ausente	2,03	92,91	51,80	957,69	2,98
$ARSF_6$	Ausente	1,11	110,21	44,44	987,18	2,99
$ARSF_6$	Ausente	2,99	90,22	52,18	3705,13	3,57
$ARSF_6$	Ausente	1,72	103,60	54,25	578,21	2,76
$ARSF_6$	Ausente	2,19	102,12	48,19	648,72	2,81
$ARSF_6$	Ausente	1,20	104,94	58,02	346,15	2,54
$ARSF_6$	Ausente	1,05	93,44	49,30	1467,95	3,17
$ARSF_6$	Ausente	2,00	110,32	46,26	679,49	2,83
$ARSF_6$	Ausente	2,03	112,53	69,54	875,64	2,94
$ARSF_6$	Presente	1,01	97,56	48,80	3307,69	3,52
$ARSF_6$	Presente	1,97	97,15	57,80	2164,10	3,34
$ARSF_6$	Presente	1,70	99,40	52,21	2033,33	3,31
$ARSF_6$	Presente	2,34	85,52	60,34	2371,79	3,38
$ARSF_6$	Presente	1,82	98,57	55,78	4098,72	3,61
$ARSF_6$	Presente	2,04	109,96	70,51	1282,05	3,11
$ARSF_6$	Presente	1,71	101,38	49,29	6216,67	3,79
$ARSF_6$	Presente	1,10	98,52	57,02	206,41	2,32
$ARSF_6$	Presente	2,28	104,66	72,14	1671,79	3,22

Apêndice 3

Os resultados obtidos durante as leituras de rugosidade superficial das amostras processadas entre vidro, ângulo de contato (imediatamente após os tratamentos e 48 horas após a imersão em água destilada estéril), bem como, os valores de células/mm^2 relativos à adesão da *Candida glabrata* nos diferentes grupos estão apresentados nas Tabelas 21A a 26A.

Tabela 21A – Grupo controle (não submetido ao tratamento a plasma), na ausência de saliva

Grupo	Rugosidade média (Ra-µm)	Ângulo de contato após tratamento (°)	Ângulo de contato após 48 horas (°)	Células/mm^2
	0,13	58,44	51,27	4039,74
	0,31	56,24	55,48	5376,92
	0,11	53,97	59,57	5180,77
Controle	0,11	65,57	64,86	958,97
	0,26	66,24	57,85	4969,23
	0,37	70,20	59,44	3393,59
	0,16	69,49	56,92	6524,41
	0,23	74,18	64,98	5771,79
	0,31	78,81	61,46	4915,38

Tabela 22A – Grupo controle (não submetido ao tratamento a plasma), na presença de saliva

Grupo	Rugosidade média (Ra-µm)	Ângulo de contato após tratamento (°)	Ângulo de contato após 48 horas (°)	Células/mm^2
	0,12	56,40	56,75	3506,41
	0,37	51,40	58,48	3443,59
	0,10	61,50	64,42	1151,28
Controle	0,10	67,72	53,44	2782,05
	0,25	66,70	52,78	307,69
	0,36	80,63	59,17	1987,18
	0,17	78,79	65,34	1317,95
	0,23	63,14	63,20	2211,54
	0,39	64,80	53,77	2373,08

Tabela 23A – Grupo submetido ao tratamento com gás argônio (Ar/50 W), na ausência de saliva

Grupo	Rugosidade média (Ra-µm)	Ângulo de contato após tratamento (°)	Ângulo de contato após 48 horas (°)	Células/mm^2
	0,22	42,60	46,38	5967,95
	0,19	44,63	57,94	3452,56
	0,25	44,82	45,66	5617,95
Ar/50 W	0,16	39,18	53,83	1055,13
	0,23	53,60	47,33	1061,54
	0,34	43,12	51,71	437,18
	0,17	48,40	46,88	1015,38
	0,20	35,96	43,96	270,51
	0,35	54,65	53,76	570,51

Tabela 24A – Grupo submetido ao tratamento com gás argônio (Ar/50 W), na presença de saliva

Grupo	Rugosidade média (Ra-µm)	Ângulo de contato após tratamento (°)	Ângulo de contato após 48 horas (°)	Células/mm^2
	0,10	49,68	48,94	7246,15
	0,25	47,46	54,52	3869,23
	0,11	42,16	44,70	3412,82
Ar/50 W	0,11	40,71	57,88	2724,36
	0,22	50,26	52,24	642,31
	0,30	47,33	49,78	1176,92
	0,16	56,61	58,22	1719,23
	0,28	53,26	55,57	2671,79
	0,34	52,25	55,98	2335,90

Tabela 25A – Grupo submetido ao tratamento com ar atmosférico (AAt/130W), na ausência de saliva

Grupo	Rugosidade média (Ra-µm)	Ângulo de contato após tratamento (°)	Ângulo de contato após 48 horas (°)	Células/mm^2
	0,33	3,41	52,99	5557,69
	0,28	0,00	50,93	6569,23
	0,10	6,06	52,21	6143,59
AAt/130 W	0,10	0,00	63,35	2717,95
	0,28	7,30	61,88	883,33
	0,30	1,94	48,81	1503,85
	0,14	0,00	54,20	2414,10
	0,24	2,12	57,90	5932,05
	0,35	7,50	57,91	4315,38

Tabela 26A – Grupo submetido ao tratamento com ar atmosférico (AAt/130W), na presença de saliva.

Grupo	Rugosidade média (Ra-µm)	Ângulo de contato após tratamento (°)	Ângulo de contato após 48 horas (°)	Células/mm^2
	0,10	0,00	53,48	7587,18
	0,31	3,19	47,20	1725,64
	0,17	2,35	51,60	5069,23
AAt/130 W	0,09	0,00	46,56	730,77
	0,28	3,96	58,50	1510,26
	0,32	0,00	45,62	1928,21
	0,12	0,00	54,10	7184,62
	0,24	6,37	58,58	656,41
	0,39	2,77	58,67	2716,67

Apêndice 4

Os resultados obtidos durante as leituras de rugosidade superficial das amostras e os valores de células/mm^2 relativos à adesão da *Candida albicans* nos diferentes grupos estão apresentados nas Tabelas 27A a 31A.

Tabela 27A – Grupo controle (sem pré-condicionamento com saliva)

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
Grupo controle	A1	0,29	580,77
	A2	0,47	400,00
	A3	0,12	328,21
	A4	0,37	505,13
	A5	0,14	202,56
	A6	0,21	370,51
	A7	0,12	396,15
	A8	0,23	385,90
	A9	0,20	467,95

Tabela 28A – Grupo submetido ao pré-condicionamento em saliva por 30 minutos

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
Grupo 30 minutos	A1	0,38	210,26
	A2	0,36	578,21
	A3	0,16	1221,79
	A4	0,20	434,62
	A5	0,37	217,95
	A6	0,14	174,36
	A7	0,15	100,00
	A8	0,34	388,46
	A9	0,17	1289,74

Tabela 29A – Grupo submetido ao pré-condicionamento em saliva por 1 hora

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
Grupo 1 hora	A1	0,15	197,44
	A2	0,15	548,72
	A3	0,26	279,49
	A4	0,27	207,69
	A5	0,27	374,36
	A6	0,17	250,00
	A7	0,27	1132,05
	A8	0,30	942,31
	A9	0,16	1373,08

Tabela 30A – Grupo submetido ao pré-condicionamento em saliva por 3 horas

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
	A1	0,29	301,28
	A2	0,09	708,97
	A3	0,28	335,90
Grupo 3 horas	A4	0,14	394,87
	A5	0,13	1273,08
	A6	0,20	1548,72
	A7	0,15	426,92
	A8	0,33	219,23
	A9	0,16	134,62

Tabela 31A – Grupo submetido ao pré-condicionamento em saliva por 12 horas

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
	A1	0,15	289,74
	A2	0,12	185,90
	A3	0,33	255,13
Grupo 12 horas	A4	0,14	1675,64
	A5	0,25	621,79
	A6	0,34	2000,00
	A7	0,31	248,72
	A8	0,11	769,23
	A9	0,16	228,21

Os resultados obtidos durante as leituras de rugosidade superficial das amostras e os valores de absorbância relativos à adesão da *Candida albicans* nos diferentes grupos estão apresentados nas Tabelas 32A a 36A.

Tabela 32A – Grupo controle (sem pré-condicionamento com saliva)

Grupo	Amostras	Rugosidade média (Ra-µm)	Absorbância
Grupo controle	A1	0,11	0,239
	A2	0,12	0,202
	A3	0,14	0,213
	A4	0,28	0,269
	A5	0,12	0,223
	A6	0,37	0,063
	A7	0,10	0,115
	A8	0,38	0,243
	A9	0,20	0,244

Tabela 33A– Grupo submetido ao pré-condicionamento em saliva por 30 minutos

Grupo	Amostras	Rugosidade média (Ra-µm)	Absorbância
Grupo 30 minutos	A1	0,08	0,349
	A2	0,22	0,588
	A3	0,25	0,276
	A4	0,21	0,408
	A5	0,39	0,236
	A6	0,11	0,163
	A7	0,26	0,141
	A8	0,37	0,454
	A9	0,27	0,181

Tabela 34A – Grupo submetido ao pré-condicionamento em saliva por 1 hora

Grupo	Amostras	Rugosidade média (Ra-µm)	Absorbância
Grupo 1 hora	A1	0,09	0,757
	A2	0,23	0,410
	A3	0,18	0,407
	A4	0,29	0,219
	A5	0,33	0,137
	A6	0,15	0,211
	A7	0,26	0,258
	A8	0,27	0,167
	A9	0,13	0,603

Tabela 35A – Grupo submetido ao pré-condicionamento em saliva por 3 horas

Grupo	Amostras	Rugosidade média (Ra-µm)	Absorbância
Grupo 3 horas	A1	0,14	0,276
	A2	0,18	0,281
	A3	0,14	0,189
	A4	0,27	0,794
	A5	0,26	0,339
	A6	0,33	0,377
	A7	0,40	0,196
	A8	0,14	0,205
	A9	0,12	0,180

Tabela 36A – Grupo submetido ao pré-condicionamento em saliva por 12 horas

Grupo	Amostras	Rugosidade média (Ra-µm)	Absorbância
Grupo 12 horas	A1	0,11	0,107
	A2	0,23	0,394
	A3	0,11	0,654
	A4	0,24	0,388
	A5	0,34	0,543
	A6	0,36	0,576
	A7	0,32	0,086
	A8	0,11	0,139
	A9	0,20	0,110

Apêndice 5

Os resultados obtidos durante as leituras de rugosidade superficial das amostras e os valores de células/mm^2 relativos à adesão da *Candida albicans* nos diferentes grupos estão apresentados nas Tabelas 37A a 40A.

Tabela 37A – Grupo controle (sem pré-condicionamento com saliva)

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
Grupo 1	A1	0,25	142,50
	A2	0,35	74,20
	A3	0,17	84,50
	A4	0,27	31,50
	A5	0,31	31,70
	A6	0,08	22,50
	A7	0,26	49,50
	A8	0,16	45,60
	A9	0,25	14,70

Tabela 38A – Grupo 2 - Vários doadores (10.000/5min)

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
Grupo 2	A1	0,18	261,20
	A2	0,38	963,20
	A3	0,11	17,60
	A4	0,16	12,70
	A5	0,33	126,30
	A6	0,23	39,60
	A7	0,05	54,40
	A8	0,37	170,20
	A9	0,26	520,60

Tabela 39A – Grupo 3 - Vários doadores (12.000/30min)

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
	A1	0,24	9,40
	A2	0,24	21,00
	A3	0,29	403,00
Grupo 3	A4	0,35	14,80
	A5	0,33	34,00
	A6	0,08	15,00
	A7	0,20	70,10
	A8	0,21	155,00
	A9	0,20	37,60

Tabela 40A – Grupo 4 – Um doador (10.000/5min)

Grupo	Amostras	Rugosidade média (Ra-µm)	Células/mm^2
	A1	0,38	34,60
	A2	0,08	9,10
	A3	0,28	79,60
Grupo 4	A4	0,19	42,30
	A5	0,34	30,80
	A6	0,15	9,40
	A7	0,21	114,50
	A8	0,07	24,60
	A9	0,35	15,70

Os resultados obtidos durante as leituras de rugosidade superficial das amostras e os valores de absorbância relativos à adesão da *Candida albicans* nos diferentes grupos estão apresentados nas Tabelas 41A a 44A.

Tabela 41A – Grupo controle (sem pré-condicionamento com saliva)

Grupo	Amostras	Rugosidade média (Ra-µm)	Absorbância
Grupo 1	A1	0,28	0,360
	A2	0,35	0,281
	A3	0,24	0,474
	A4	0,15	0,266
	A5	0,38	0,422
	A6	0,27	0,413
	A7	0,34	0,371
	A8	0,22	0,442
	A9	0,08	0,690

Tabela 42A – Grupo 2 - Vários doadores (10.000/5min)

Grupo	Amostras	Rugosidade média (Ra-µm)	Absorbância
Grupo 2	A1	0,26	0,633
	A2	0,20	0,577
	A3	0,19	0,673
	A4	0,14	0,435
	A5	0,33	0,615
	A6	0,19	0,514
	A7	0,16	0,998
	A8	0,40	0,980
	A9	0,17	1,012

Tabela 43A – Grupo 3 - Vários doadores (12.000/30min)

Grupo	Amostras	Rugosidade média (µm)	Absorbância
	A1	0,18	0,497
	A2	0,07	0,382
	A3	0,37	0,376
Grupo 3	A4	0,28	0,715
	A5	0,23	0,520
	A6	0,29	0,360
	A7	0,27	0,758
	A8	0,07	1,040
	A9	0,36	0,910

Tabela 44A – Grupo 4 - Um doador (10.000/5min)

Grupo	Amostras	Rugosidade média (Ra-µm)	Absorbância
	A1	0,32	0,404
	A2	0,09	0,380
	A3	0,40	0,477
Grupo 4	A4	0,30	0,496
	A5	0,06	0,549
	A6	0,37	0,648
	A7	0,17	0,965
	A8	0,17	1,001
	A9	0,36	0,693

Autorizo a reprodução deste trabalho.

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