TATIANA PEREIRA CENCI

AVALIAÇÃO DA FORMAÇÃO DE BIOFILME DE ESPÉCIES DE *CANDIDA* SOBRE A SUPERFÍCIE DE RESINAS ACRÍLICAS PARA BASE E REEMBASAMENTO DE PRÓTESES REMOVÍVEIS

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

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Piracicaba

2008

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecária: Marilene Girello – CRB-8ª. / 6159

C332a

Cenci, Tatiana Pereira.
Avaliação da formação de biofilme de espécies de *candida* sobre a superfície de resinas acrílicas para base e reembasamento de próteses removíveis. / Tatiana Pereira Cenci. -- Piracicaba, SP : [s.n.], 2008.

Orientador: Altair Antoninha Del Bel Cury.
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Saliva. 2. Bactérias. 3. Microscopia confocal. I. Del Bel Cury, Altair Antoninha. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

(mg/fop)

Título em Inglês: Evaluation of Candida species biofilm formation on acrylic resin and denture liners used in prosthodontics

Palavras-chave em Inglês (Keywords): 1. Saliva. 2. Bacteria. 3. Microscopy, confocal
Área de Concentração: Prótese Dental
Titulação: Doutor em Clínica Odontológica
Banca Examinadora: Altair Antoninha Del Bel Cury, Dalva Cruz Laganá, Fernanda Faot, Lourenço Correr Sobrinho, Livia Maria Andaló Tenuta

Data da Defesa: 09-05-2008
Programa de Pós-Graduação em Clínica Odontológica

UNICAMP

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 09 de Maio de 2008, considerou a candidata TATIANA PEREIRA CENCI aprovada.

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PROFa. DRa. LÍVIA MARIA ANDALÓ TENUTA

A **Deus**, por sempre iluminar meus caminhos.

À minha mãe, **Sandra**, minhas irmãs **Talita** e **Taciana**, ao **Tio Artur**, e meu avô **Carlos Bresser da Silveira**, os responsáveis pelo meu caráter e que, com suas palavras de conforto sempre me estimularam a continuar. Transmitiram ensinamentos, trocaram experiências. Estiveram comigo nos momentos felizes e tristes, nos momentos de força e fraqueza. Obrigada seria pouco pelo muito que tenho, e pelo muito que passamos.

Ao meu marido **Max**, meu grande companheiro. Seria absolutamente impossível chegar até aqui sem você. Levaria toda a minha vida para agradecer pelos conselhos e ajuda durante o desenvolvimento deste trabalho, pelo companheirismo, pela união em todos os momentos em que caminhamos juntos. Amor é a palavra mais simples que poderia dizer à você.

AGRADECIMENTO ESPECIAL

À minha orientadora, **Profa. Dra. Altair Antoninha Del Bel Cury**, pela amizade e pelo carinho, pelo exemplo de trabalho, de rigidez e pulso firme para formar e desenvolver minha auto-crítica científica durante estes anos de convivência. Obrigada por ter me desafiado a pensar. Através de seu incentivo, vislumbrei um ideal nesta profissão e mais do que nunca, acredito que a ousadia e o erro são os caminhos para as grandes realizações. É impossível, apenas em palavras, agradecer por todas as oportunidades. A senhora é meu espelho como pesquisadora e educadora.

AGRADECIMENTOS

À **Universidade Estadual de Campinas** por meio do seu Magnífico Reitor, Prof. Dr. José Tadeu Jorge.

À **Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas**, por meio de seu Diretor, Prof. Dr. Francisco Haiter Neto.

À **Fundação de Amparo a Pesquisa do Estado de São Paulo**, **FAPESP**, e à **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES**, pelas bolsas concedidas (Bolsa de Doutorado 06/00396-8, Auxílio à Pesquisa 06/03043-9 e Programa de Doutorado com Estágio no Exterior BEX 1482/06-8).

Ao Coordenador dos Cursos de Pós-Graduação da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, **Prof. Dr. Mario Alexandre Coelho Sinhoreti**.

À Coordenadora do Programa de Pós-Graduação em Clínica Odontológica da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, **Profa. Dra. Renata Cunha Matheus Rodrigues Garcia**, a quem agradeço também, por toda ajuda, apoio e pelo exemplo a ser seguido.

Ao **Prof. Dr. Jaime Aparecido Cury**, responsável pelo laboratório de Bioquímica Oral da Faculdade de Odontologia de Piracicaba, UNICAMP, pelos ensinamentos e exemplo de profissionalismo. Agradeço imensamente por toda ajuda e uso das instalações.

À **Profa. Dra. Cínthia Pereira Machado Tabchoury**, por toda a ajuda e suporte durante o desenvolvimento deste trabalho.

Ao **Prof. Dr. Jacob Martien "Bob" ten Cate**, pela orientação durante estágio realizado no Academic Centre for Dentistry Amsterdam, ACTA. Agradeço pelos preciosos ensinamentos durante a confecção de parte desta tese e pela confiança depositada em mim. Jamais me esquecerei do seu mais sábio conselho: *Geen Shakespeare, alstublieft*.

Aos técnicos do Departamento de Cariologia, Endodontia e Odontopediatria da ACTA, **Rob Exterkate, Mark Buijs** e **Michel Hoogenkamp** pelos muitos ensinamentos, amizade, ajuda com as atividades de laboratório, e produtivas discussões científicas e não científicas durante o período de estágio realizado junto àquele departamento. Obrigada por fazerem do estágio no exterior uma das melhores experiências científicas que tive.

Aos queridos **Wim Crielaard, Egija Zaura**, **Monique van der Veen, Suzanne Lupens, Cor van Loveren, Guus van Strijp, Efije Kraneveld, Linda Kruiten, Anja Prosperi, Zewdu Terefwork, Chi Pham, Hok Lim, Duygu Kara** e **Rifat Özok** por compartilharem sabedoria e pelo agradável convívio em Amsterdam.

Às minhas amigas-irmãs **Milena Newhook**, **Hiromi Teruya e Beatriz Mizerkowski**, pela amizade, carinho e apoio constantes.

À amiga **Fabiana Gouveia Straioto**, pelas conversas sobre o "nosso futuro" e pela ajuda enquanto estive fora durante o PDEE. Obrigada seria pouco para lhe agradecer.

Aos meus grandes amigos **Leonardo Henrique Vadenal Panza**, e **Wander José da Silva** pelas inúmeras conversas – científicas ou não – durante esta jornada.

Aos queridos **Valdete Bissani Cenci** e **Sérgio Luis Cenci**, por todo apoio e carinho durante este trajeto.

Aos meus grandes e eternos amigos do Centrinho **Ana Lúcia Almeida**, **Fernanda Ferrari**, **Aline Siqueira**, **Celene de Oliveira**, **Juliana Nicolielo**, **Kazuza Ferreira**, **Lígia Ustulin** e **Marcelo Hamata**, grandes companheiros, pessoas muitos especiais que sempre me incentivaram e ajudaram nessa jornada, meu muito obrigada por todo apoio.

À amiga **Lívia Maria Andaló Tenuta**, pelo companheirismo e exemplo de pesquisadora.

À Sra. **Joselena Casati**, responsável técnica pelo Laboratório de Prótese Removível, pela imensa ajuda durante todo o Doutorado e pela agradável convivência.

Aos amigos **Erick Souza, Vanessa Camila da Silva, Euler Rocha** e **Fernanda Brighenti,** pela preciosa amizade que perdura mesmo após nossa volta ao Brasil. Obrigada pelo carinho.

Aos amigos e colegas da Pós-Graduação, **Priscila Serrano, Carolina Aires**, **Rafael Moraes, Luciano Gonçalves, Maria Áurea Ferreira, Lucíola Vasconcelos, Antônio Pedro Ricomini, Frederico Fernandes, William Custódio, Simone Gomes, Juliana Moura, Fernanda Faot, Gláuber Vale, Renzo Vasquez, Karla Oliveira, Rodrigo Arthur, Thais Negrini, Annicele Andrade, Gustavo Gameiro**, **Stela Pereira**, **Gisele Moi, Cláudia Zamataro, Anna Papa** e **Carolina Nóbrega**, pela convivência e por me proporcionarem vários momentos felizes. Muito obrigada a cada um de vocês.

À técnica do Laboratório de Farmacologia, **Eliane Mello Franco** por todo carinho e ajuda. Obrigada!

Aos técnicos do Laboratório de Bioquímica **Waldomiro Vieira Filho** e J**osé Alfredo da Silva** agradeço por toda a ajuda para confecção deste estudo.

À **Érica Alessandra Pinho Sinhoreti** e **Raquel Q. Marcondes Cesar Sacchi** secretárias da Coordenadoria Geral dos Programas de Pós-graduação; **Emílio Carlos Salles**, secretário do Programa de Pós-Graduação em Clínica Odontológica; meu sincero agradecimento pela atenção e gentileza dispensada durante esses anos de convívio como aluna de pós-graduação.

Aos voluntários que aceitaram participar deste estudo, minha eterna gratidão.

A todos que indiretamente contribuíram para a realização deste trabalho.

“When apparently we have reached the limits of possibility, new avenues of progress and advancement are opened to our view and advances which shall make our knowledge of today seem in the light of the future to be but the densest ignorance"

William Jarvie, 1905. *In*: Journal of the William Jarvie Society, 2005

RESUMO

A candidose é a infecção oral fúngica mais comum diagnosticada em humanos, com prevalência de até 77,5% em usuários de próteses removíveis. Embora tenha sido inicialmente associada apenas à *Candida albicans*, outras espécies de *Candida* podem ser responsáveis por mais de 50% dos casos de infecção. Ainda, fatores como presença de saliva, bactérias e características de materiais utilizados para confecção de próteses removíveis parecem desempenhar importante papel na adesão, colonização e formação de biofilme por *Candida*. Assim, este trabalho objetivou (i) discutir os fatores que controlam a adesão inicial, colonização e formação de biofilme de *Candida* em um artigo de revisão, no intuito de apontar diretrizes para estudos futuros e ainda, mostrar de que forma estes fatores podem ser controlados, ajudando na prevenção da doença; (ii) verificar a influência *in vitro* de alguns dos fatores supracitados na formação de biofilme de *C. albicans* sobre a superfície de hidroxiapatita, resina acrílica e reembasador temporário e; (iii) avaliar *in situ* a formação de biofilme sobre espécimes de resina acrílica e reembasadores de próteses inseridos nas próteses totais de 21 voluntários. Para avaliação da formação de biofilme de *C. albicans*, espécimes de diversos materiais foram confeccionados e alocados aleatoriamente em grupos de acordo com a exposição à presença ou ausência de saliva, presença ou ausência de *Streptococcus mutans* e *Candida glabrata*. O biofilme foi formado sobre os espécimes por 24 h. Após este período, as células viáveis de *C. albicans* e *C. glabrata* foram quantificadas (UFC/cm^2), sendo o biofilme e a formação de hifas de *C. albicans* analisados estruturalmente através de microscopia confocal. Os dados obtidos foram submetidos à análise de variância (α=0,05) para biofilme (*C. albicans* e *C. glabrata*) e número de hifas. Para o terceiro objetivo, espécimes (4 x 4 x 2mm) de resina acrílica (n=252) e reembasadores (temporário; n=126 e permanente; n=126) foram fabricados e tiveram sua rugosidade e energia livre de superfície mensurados através de um rugosímetro e da mensuração da imagem da gota séssil formada sobre o espécime, respectivamente. A seguir, estes foram inseridos em recessos realizados na superfície vestibular das próteses inferiores dos voluntários, para formação de biofilme em um estudo do tipo cruzado. Após 2,

7 e 14 dias, o biofilme formado sobre os espécimes foi analisado em relação à contagem de microrganismos totais, estreptococos totais, estreptococos do grupo mutans, *Actinomyces* e espécies de *Candida*. A seguir, os espécimes foram reavaliados quanto à rugosidade e energia livre de superfície. No estudo *in vitro*, o reembasador temporário apresentou menor número de células viáveis, seguido da resina acrílica e hidroxiapatita (ANOVA; $p<0,05$). Houve menor recuperação de *C. glabrata* em biofilmes formados sobre espécimes com saliva (ANOVA; $p<0,05$). A presença de *S. mutans* inibiu o crescimento de hifas de *C. albicans*., enquanto que biofilmes com as duas espécies de *Candida* não mostraram interações competitivas. O estudo *in situ* mostrou que, de maneira geral, as propriedades dos materiais testados se modificaram durante o experimento, o mesmo ocorrendo em relação às contagens de microrganismos. O percentual de espécies de *Candida* e *C. glabrata* recuperados do biofilme aumentaram após 14 dias (ANOVA; $p<0,05$). Houve diferenças na contagem de estreptococos totais, *Actinomyces*, microrganismos totais e percentuais de *Actinomyces* em relação aos microrganismos totais, onde pode ser observado aumento de contagem após 7 e 14 dias (ANOVA; $p<0,05$). Diferentes espécies de *Candida* foram observadas no biofilme simultaneamente, enquanto a *C. glabrata* foi a única espécie avaliada a mostrar aumento de contagem do segundo ao décimo quarto dia, mostrando progressiva colonização. Neste estudo *in vitro*, os biofilmes de *Candida* foram afetados pelos fatores avaliados, saliva, tipo de substrato e presença de outros microrganismos. Os resultados também indicam o efeito facilitador do substrato no desenvolvimento do biofilme.

Palavras chave: *Candida albicans, Candida glabrata*, biofilme, resina acrílica, reembasadores

ABSTRACT

Candida-associated stomatitis is the most common fungal oral infection in humans, with a prevalence reported in up to 77.5% of a population wearing dentures. Disease-associated *Candida* species have shifted from *C. albicans* to non-*albicans* species, these latter being responsible for more than 50% of the infections. Additionally, several factors as the presence of saliva, bacteria and dental prostheses materials' characteristics seem to be related to the adhesion, colonization and biofilm formation of *Candida*. This study aimed (i) to discuss the factors that govern initial adherence, colonization and biofilm formation of *Candida* by means of a review article, in order to suggest future research and show how these factors may be controlled, therefore helping to prevent the disease; (ii) to verify the influence of several of these factors in the biofilm formation of *C. albicans in vitro*, on hydroxyapatite, acrylic resin and soft denture liner; (iii) to evaluate *in situ* biofilm formed on acrylic resin and denture liner specimens inserted in the lower dentures of 21 volunteers. For *C. albicans* biofilm formation evaluation, specimens of several materials were manufactured and randomly assigned according to the following groups/factors: presence or absence of saliva and presence or absence of *S. mutans* and *C. glabrata*. Biofilm was formed for 24 h and viable cells of *C. albicans* and *C*. *glabrata* were quantified (CFU/cm^2). The biofilm structure and *C. albicans* hyphae formation were analyzed by confocal scanning laser microscopy. Data were analyzed by ANOVA for biofilm (*C. albicans* e *C. glabrata*) and hyphae (*C. albicans*) quantification (α=0.05). For the third aim, acrylic resin (n=252) and denture liner (hard; n=126 and soft; n=126) specimens (4 x 4 x 2mm) were prepared and had their surface roughness (Ra) and free energy (SFE) evaluated using a profilometer and the sessile drop technique, respectively. They were inserted in the buccal surface of the mandibular dentures of the volunteers for biofilm formation in a crossover study. After 2, 7 and 14 days, specimens and biofilm were collected. Specimens were re-evaluated for Ra and SFE and the biofilm quantified for total streptococci, mutans streptococci, *Actinomyces* and *Candida* species. The *in vitro* study showed that the soft liner had the lower number of viable cells, followed by acrylic resin and hydroxyapatite

(p<0.05). There was a lower *C. glabrata* recovery in biofilms formed on saliva coated specimens (p<0.05). The presence of *S. mutans* suppressed *C. albicans* hyphae formation, while dual *Candida* species biofilms did not show competitive interactions. Regarding the *in situ* study, substratum surfaces changed throughout the experiment, as happened with biofilm counts for several of the studied micro-organisms. Percentages of *Candida* species and *C. glabrata* recovered from the biofilm were higher after 14 days (ANOVA; p<0.05). There were differences in total streptococci, *Actinomyces*, total micro-organisms and percentages of *Actinomyces* in relation to total micro-organisms, where higher counts could be observed after 7 and 14 days (ANOVA; p<0.05). *Candida* species showed simultaneous colonisation, while *C. glabrata* was the only species evaluated to show rising counts from the 2nd to the 14th day, progressively colonising the biofilm. *Candida* biofilm formed *in vitro* was affected by all factors under study, i.e., saliva, substratum type and presence of other micro-organisms. Our results also indicate the supportive effect of substrata on biofilm development.

Key words: *Candida albicans, Candida glabrata*, biofilm, acrylic resin, denture liner

SUMÁRIO

INTRODUÇÃO GERAL

A epidemiologia das infecções causadas por fungos tem se modificado nos últimos 20 anos, tendo sido evidenciado que a incidência aumentou e a população de risco se expandiu, principalmente considerando-se o aumento do número de idosos na população (McMichael *et al*., 2004). Esta expansão da população de risco inclui ainda uma vasta lista de condições médicas, como transplantes, cânceres, terapia imunosupressiva, AIDS, parto prematuro, idade avançada e grandes cirurgias (Nucci e Marr, 2005; Cheng *et al*., 2005). Essa população de risco, frente à candidose e em condição de enfermidade e/ou imunosupressão está sujeita à alta mortalidade (30-40%), mas principalmente o agravamento da enfermidade pela candidose pode aumentar o tempo de permanência hospitalar e como conseqüência os custos (Wey *et al*., 1988; Leleu *et al*., 2002; Cheng *et al*., 2005).

A candidose é a infecção oral fúngica mais comum diagnosticada em humanos (Muzyka, 2005), apresentando-se como uma inflamação dos tecidos orais, cuja prevalência varia de 15 até 77,5% (Budtz-Jörgensen, 1981; Jeganathan e Lin, 1992; Espinoza *et al*., 2003; Emami *et al*., 2007) nos usuários de próteses removíveis. Esta inflamação também é denominada de estomatite induzida por prótese ou estomatite por dentaduras, sendo a *Candida albicans* fortemente associada como o principal agente etiológico desta patologia (Pires, 2002). Da mesma forma, usuários de próteses removíveis que não desenvolvem a doença possuem a *C. albicans* como espécie mais frequentemente isolada (Zaremba *et al*., 2006). Entretanto, hoje é sabido que espécies de *Candida* não-*albicans* podem ser responsáveis por mais de 50% dos casos de infecção. Espécies como a *C. glabrata, C. krusei* e *C. oralis* podem ser frequentemente isoladas em indivíduos com ou sem próteses removíveis (Zaremba *et al*., 2006). Os motivos desta mudança na prevalência de diferentes espécies ainda não estão completamente esclarecidos, sendo em muitas circunstâncias relacionados à repetidas profilaxias antifúngicas, o que causaria mudanças nos hospedeiros (Procop e Roberts, 2004; Nucci e Marr, 2005). Adicionalmente, é sabido que técnicas mais precisas de

identificação celular e molecular tornaram possível a identificação de outras espécies que outrora eram desconhecidas.

A predisposição para infecção por *Candida* pode ser o resultado de múltiplos fatores que podem ser divididos em orais e sistêmicos. Os fatores sistêmicos incluem imunosupressão (Tylenda *et al*., 1989; McCarthy, 1992; Flaitz e Hicks, 1999), dieta rica em carboidratos (Scully e Cawson, 1998), processos malignos (Bodey, 1984), antibióticos de amplo espectro (Seelig, 1966; Tylenda *et al*., 1989), xerostomia (McCarthy, 1992), idade (em especial os mais jovens e os mais velhos), diabetes mellitus, deficiências em ferro e vitaminas (Odds *et al*., 1978; Samaranayake, 1986; Soysa *et al*., 2006) e gravidez (Sarifakioglu *et al*., 2006). Os fatores locais incluem fumo (Soysa e Ellepola, 2005; Kreher *et al*., 1991), hipofunção de glândulas salivares (Samaranayake, 1990), uso de antibióticos tópicos, tratamento com esteróides, coexistência de doenças na mucosa oral (Budtz-Jörgensen, 1990) e especialmente a utilização de próteses removíveis (Budtz-Jörgensen, 1978; Moskona e Kaplan, 1992; Zegarelli, 1993).

O crescimento sobre a superfície de próteses é natural no ciclo de vida da *Candida* (Kumamoto e Vinces 2005), o que pode explicar a ocorrência comum da colonização fúngica nos usuários de próteses. As lesões da mucosa oral relacionadas às próteses removíveis são reações agudas ou crônicas decorrentes de biofilme dental, leveduras, constituintes do material utilizado para a confecção das próteses, pouca retenção ou injúrias mecânicas (Budtz-Jörgensen, 1978; Budtz-Jörgensen 1981; Dorey *et al*., 1985). Entretanto, de todas as lesões citadas, aquelas ocasionadas pela candidose podem interferir com o tratamento protético e principalmente ser uma barreira para a saúde do paciente (Perezous, 2005), uma vez que as próteses podem servir como fonte de microrganismos para a nova infecção (Muzyka, 2005). Devido à alta prevalência e virulência desses microrganismos nos processos inflamatórios, diversos autores (Baysan *et al*., 1998; Radford *et al*., 1999; Egusa *et al*., 2000; Nikawa *et al*., 2000) dedicaram-se a estudar os fatores que interferem na adesão, colonização e formação de biofilme de várias espécies de *Candida* (Verran e Motteram, 1987; Radford e Radford,

1993; Moura *et al*., 2006; Thein *et al*., 2006; Avon *et al*., 2007; Pereira-Cenci *et al*., 2007; Thein *et al*., 2007a; Thein *et al*., 2007b).

Dentre estes fatores, incluem-se as propriedades de rugosidade e energia livre de superfície das resinas acrílicas para base e reembasamento de próteses. Entretanto, poucos estudos levam em consideração as diferenças entre os vários materiais ou em relação à presença de agentes antifúngicos incorporados aos materiais rembasadores (temporários ou permanentes) (Samaranayake *et al*., 1980; Minagi *et al*., 1985; Vasilas *et al*., 1982; Waters *et al.*, 1985; Radford *et al*., 1998; Millsap *et al*., 1999).

A adesão inicial de microrganismos sobre a superfície da prótese ocorre por interações específicas como ligações covalentes, iônicas e pontes de hidrogênio. Posteriormente, no caso dos fungos, pode ocorrer o tigmotropismo das hifas, fixando-se sobre a resina e iniciando-se a fase de colonização da superfície, onde ocorre o desenvolvimento de micro-colônias e a formação de biofilme (Quirynen e Bollen, 1995; Nikawa *et al.*, 1997; Radford *et al*., 1999).

Durante o processo de colonização, o microrganismo, para alcançar e interagir com o substrato necessita remover a película adquirida, formada pela adsorção seletiva de glicoproteínas salivares, que se forma imediatamente após o contato da saliva com a superfície da prótese (de Jong *et al.*, 1984; Quirynen e Bollen, 1995). A formação desta película sobre a superfície da prótese está diretamente associada à sua capacidade de molhamento que é regulada pela energia livre de superfície (Sipahi *et al*., 2001). Assim, a presença da camada de compostos orgânicos interfere com a superfície de resina acrílica, influenciando a adesão de *Candida* sobre o material (Quirynen e Bollen, 1995; Sipahi *et al*., 2001). Estudos têm demonstrado que a energia livre de superfície parece ter um importante papel nas fases iniciais de adesão de *Candida*, especialmente para materiais contendo polimetilmetacrilato em sua composição, induzindo uma maior adesão de microrganismos quando esta energia está aumentada (Minagi *et al*, 1985; Van Dijk *et al*., 1987; Serrano-Granger *et al*., 2005). Da mesma forma, a maior rugosidade de uma superfície favorece a adesão de microrganismos, uma

vez que estes estão mais protegidos contra forças que tendem a deslocá-los nas fases iniciais da colonização (Quirynen e Bollen, 1995; Radford *et al*., 1999).

Adicionalmente, alguns autores relataram que materiais reembasadores resilientes são de fácil colonização por várias espécies de *Candida*. Entretanto, os resultados apresentados são inconsistentes e controversos, já que alguns autores relataram haver efeito fungicida (Razek e Mohamed, 1980), enquanto outros identificaram fungos em próteses reembasadas com estes materiais (Wright *et al.* 1985; Graham *et al.,* 1991; Kulak e Kazazoglu, 1998). Assim, parece haver uma importante diferença de colonização e manutenção de *Candida* em materiais utilizados para bases de prótese nos estudos *in vitro* e *in vivo*, já que estudos prévios sugerem que as bactérias presentes dentro de um biofilme oral estariam igualmente envolvidas no processo inflamatório causado por estomatite induzida por próteses (Budtz-Jörgensen, 1983; Gusberti *et al.*, 1985; Catalan *et al*., 1987; Koopmans *et al*., 1988).

A comunicação entre bactérias e fungos é crucial no processo de adesão e colonização. Os microrganismos presentes no ambiente oral interagem entre si de diversas maneiras, tais como a utilização de produtos metabólicos uns dos outros, através de comunicação via moléculas sinalizadoras, ajudando no processo de adesão e conseqüente colonização e formação de biofilme (Blankenship e Mitchell, 2006). Esta cooperação leva à adaptação frente a respostas de estresse e resultam em uma microflora balanceada (Palkova e Vachova, 2006; Mikelsaar e Mandar, 1993; McFarland 2000; Perdigon *et al*., 2001).

Dessa forma, considerando ser comum a presença de *Candida* em pacientes usuários de próteses removíveis, e tendo-se em vista os aspectos apresentados, torna-se importante analisar a adesão e a formação de biofilme de *Candida* e outros microrganismos em diferentes materiais utilizados para base e reembasamento de próteses removíveis. Considerando-se ainda que estudos recentes apontam para a importância de biofilmes multi-espécie no início e progressão da doença, é importante que se compreenda como estes biofilmes interagem com as superfícies e desta forma, entender seu crescimento e

possibilitar o estabelecimento de estratégias para prevenção e tratamento. A relação entre espécies de *Candida*, outros microrganismos e superfícies colonizáveis pode ser melhor compreendida pelo estudo da formação de biofilmes *in vitro* e *in situ*, o que possibilitaria também a avaliação do tempo necessário para a colonização inicial dessas superfícies e como isto contribuiria para a patogenicidade dos biofilmes formados sobre materiais protéticos.

Assim, este trabalho de tese objetivou:

(i) discutir os fatores que controlam a adesão inicial, colonização e formação de biofilme de *Candida* através de um artigo de revisão, no intuito de apontar diretrizes para futuros estudos e ainda, mostrar de que forma estes fatores podem ser controlados, ajudando na prevenção da doença;

(ii) verificar a influência *in vitro* de fatores tais como tipo de substrato, presença de saliva, e presença de outros microrganismos na formação de biofilme de *C. albicans* sobre a superfície de hidroxiapatita, resina acrílica e reembasador temporário;

(iii) avaliar *in situ* a formação de biofilme sobre materiais reembasadores de prótese e como esses materiais influenciariam a composição de biofilmes formados por até 14 dias, quando comparados à resina acrílica.

Development of *Candida*-associated denture stomatitis: new insights

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Abstract

Despite therapeutic progress, opportunistic oral fungal infectious diseases have increased in prevalence, especially in denture wearers. The combination of entrapment of yeast cells in irregularities in denture-base and denture-relining materials, poor oral hygiene and several systemic factors is the most probable cause for the onset of this infectious disease. Hence colonization and growth on prostheses by *Candida* species are of clinical importance. The purpose of this review is to critically discuss several key factors controlling the adhesion of *Candida* species which are relevant to denture-associated stomatitis. Although there is some consensus on the role of surface properties, studies on several other factors, as the use of denture liners, salivary properties and yeast-bacterial interactions, have shown contradictory findings. A comprehensive fundamental understanding is hampered by conflicting findings due to the large variations in experimental protocols, while other factors have never been thoroughly studied. Surface free energy and surface roughness control the initial adherence, but temporal changes have not been reported. Neither have *in vivo* studies shown if the substratum type is critical in dictating biofilm accumulation during longer periods in the oral environment. The contribution of saliva is unclear due to factors like variations in its collection and handling. Initial findings have disclosed that also bacteria are crucial for the successful establishment of *Candida* in biofilms, but the clinical significance of this observation is yet to be confirmed. In conclusion, there is a need to standardize experimental procedures, to bridge the gap between laboratory and *in vivo* methodologies and findings and – in general – to thoroughly investigate the factors that modulate the initial attachment and subsequent colonization of denture-base materials and the oral mucosa of patients subjected to *Candida* infections. Information on how these factors can be controlled is required and this may help to prevent the disease. The societal impact of such information is significant given the magnitude of the candidosis problem worldwide.

Uniterms

Candida albicans, Biofilm, Denture, Saliva, Bacteria

Introduction

Candida infections receive increasing attention, presumably due to the increased prevalence worldwide. Numerous studies have shown that several *Candida* species possess a multitude of virulence mechanisms leading to successful colonization and infection of the host when suitable conditions occur. The recognition that *Candida* is an important pathogen has led to many laboratory studies evaluating these virulence attributes in an attempt to clarify the pathogenesis of the disease. The progress made in understanding some of these features, such as the mechanisms that result in adherence to surfaces[1], cell surface hydrophobicity[2], and saliva[3] is very impressive though yet in many aspects inconclusive. Knowledge about how the adherence and biofilm formation process takes place and how to avoid or at least diminish *Candida* colonization are mandatory in clinical practice. This review aims to critically discuss several key factors controlling the adhesion of *Candida* species which are relevant to denture-associated stomatitis, to highlight areas of current controversy and to suggest future research.

Role of surface properties on *Candida* colonization

Fungi normally live as innocuous commensals and colonize various habitats in humans, notably skin and mucosa[4,5]. Commensal existence of oral *Candida* species varies from 20% to 50% in a healthy dentulous population[4,6]. As growth on surfaces is a natural part of the *Candida* lifestyle[7], one can expect that *Candida* colonizes denture.

There is a large body of evidence indicating that *Candida* is able to adhere to acrylic resin dentures. This is the first step that may lead to the development of the infectious process and that may ultimately result in varying degrees of denture stomatitis of the adjacent mucosa[3,8,9]. *Candida* adheres directly or via a layer of denture plaque to denture base (polymethylmethacrylate – PMMA)[10-12]. Without this adherence, micro-organisms would be removed from the oral cavity when saliva or food is being swallowed.

It is well-known that innumerable factors are involved in the adhesion of *Candida* to the acrylic resin base, though contradictory results have been reported

from *in vitro* studies[13-15]. Substrate surface properties, as surface charge, surface free energy, hydrophobicity, and roughness have all been reported to influence the initial adhesion of micro-organisms[16,17]. Microbial adhesion on biomaterial surfaces depends on the surface structure and composition of biomaterials, and on the physicochemical properties of the microbial cell surface, again its surface charge and hydrophobicity[18,19]. Components of the resilient denture liners and acrylic resin may reduce the adhesion and inhibit the growth of *Candida*[20-22].

(a) Surface free energy and surface roughness

Surface free energy is one of the main factors related to the development of denture related candidosis[23]. It is defined as the interaction between the forces of cohesion and adhesion and predicts whether or not wetting occurs[24]. A linear relationship between contact angle measurements on various types of substratum and *Candida albicans* adherence has been demonstrated, i.e. the higher the surface free energy, the higher will be the adhesion of micro-organisms and alternatively, the more hydrophobic the surface, the less cell adherence is expected[23,20,25].

Although the cited reports have found correlations between surface free energy and microbial' adhesion[26], other factors should also be considered, such as cell surface factors, diet, salivary composition and secretion rates, and antibody titers, which are all controlling factors in plaque formation[27] and could therefore influence yeast attachment. These many confounding factors might explain why recent studies have failed to show a direct correlation between surface free energy values and the adhesion of *Candida* species[13-15,28].

Higher adherence of particular *Candida* species, e.g. *C. tropicalis*, *C. glabrata* and *C.dubliniensis*, when compared with *C. albicans*, might be attributed to their relative surface free energy values, since hydrophobic micro-organisms seem to be more adherent to acrylic surfaces. While there are no studies regarding hydrophobicity of *C. tropicalis* and *C. dubliniensis*, Luo and Samaranayake[29] (2002) stated that *C. glabrata* is more hydrophobic than *C. albicans*.

Commonly used biomaterials exhibit significant differences in surface free energy. Heat-polymerized acrylic resin was reported to be more wettable than microwave-polymerized acrylic resin, due to acid-base interactions[14,30].

Surface roughness is calculated as the arithmetic average deviation of the surface valleys and peaks of a given surface[31]. It directly influences micro-organisms initial adherence to surfaces, biofilm development, and *Candida* species colonization. Materials with the roughest surface usually exhibit higher yeast counts[15,21,32,33]. This happens because surfaces may serve as a reservoir, with surface irregularities providing an increased chance of micro-organism retention and protection from shear forces, even during denture cleaning. In addition, these irregularities sometimes allow the entrapped microbial cells time to attach irreversibly to a surface[34].

Quirynen *et al*.[1] (1990) postulated a threshold roughness value (0.2 μm) below which no effect on the adhesion should be expected. Smooth and highly polished surfaces are of utmost importance not only for patient's comfort but also for denture/restoration longevity, good aesthetical results, oral hygiene and low plaque retention[35].

The presence of saliva is known to change this scenario. The nature of the substratum may influence the formation and the composition of the salivary pellicle, which layer may then become more relevant than the surface properties of the dental material itself[36]. It has been shown that saliva immersion decreases the surface roughness[32] and surface free energy[30] of acrylic resins. This might explain the general decrease of *Candida* species in those studies where specimens were coated with saliva. Saliva, its components and properties on *Candida* adherence and colonization is thoroughly discussed in the following paragraph *Role of the salivary properties on Candida colonization*.

The available studies on surface properties raise questions regarding the role of surface free energy and surface roughness. There is general agreement that the hydrophobicity of the cell surface and substratum is an important predictor in the adhesion process, i.e. surface free energy indicates the ease with which saliva spreads over a surface[23,30]. There is also consensus on the role of surface

roughness and the initial adherence process, i.e. surface roughness is positively correlated with the rate of bacterial/fungal colonization of biomaterials. If such rougher surfaces become exposed to the oral environment, they may be more susceptible to micro-organisms adhesion and biofilm formation and lead to infections. However, no studies on the application of certain treatments on different substratum types have been reported (i.e. application of different treatments diminishes the number of yeasts but may lead to detrimental changes of the substratum). *In vivo* studies may lead to different outcomes when compared with *in vitro* studies.

(b) Denture liners surface and characteristics

New materials have been developed in order to reduce and redistribute occlusal forces from dentures that might damage the underlying mucosal tissues[37,38]. In recent years, the use of denture liners, either hard or soft, has increased.

Liners are needed in many clinical situations in which patients have thin, sharp, or badly resorbed residual alveolar ridges or chronic tissue irritation from dentures[37,39]. Even though these materials exhibit excellent tissue tolerance, one of the problems is the colonization of *Candida* spp. on and within the material. Fungal growth is known to destroy the surface properties of the liner and this may lead to irritation of the oral tissues. This is due to a combination of increased surface roughness and high concentrations of exotoxins and metabolic products produced by the fungal colonies[39]. This observation is the rationale why attempts have been undertaken to incorporate antifungal agents or antiseptics in these materials.

Unfortunately, conflicting adherence/colonization results are reported on these lining materials. Some in vitro studies reported significant inhibitory effects on *C. albicans*[40,41]. More recent studies, however, showed only limited antifungal properties and no significant reduction on *Candida* adherence and colonization[15,40,42-49].

As can be seen in Figure 1 and as was also reported previously[21], denture liners, especially the soft ones, introduce a higher surface roughness. The porous surface texture of the material will entrap yeast cells (Figure 2), leading to an

increased (re)colonization in spite of the antifungals. Concomitantly, the nutrient-rich environment of the oral cavity might overrule any inhibitory effect induced by antifungals released from the denture liners[42].

Even though some *in vitro* studies have shown limited inhibitory effects, a reasonable explanation on why lining materials do not keep their antifungal characteristics could be the constant bathing in saliva in the mouth. Saliva extracts the antifungal ingredients, possibly even within a short time after the denture is placed in the oral environment, or dilutes the concentration near the denture surface to below fungicidal concentrations. Moreover, the antifungal included might not be effective against the particular *Candida* species (or mixture of micro-organisms, see below) that is causing the infection. Judging the literature the need emerges to systematically evaluate liners against various *Candida* species in relevant assays, e.g. involving various *Candida* and bacterial mixtures and saliva.

Role of salivary properties on *Candida* colonization

The role of human saliva in the *Candida* adhesion process is still controversial[14,50]. Saliva shows a physical cleaning effect and innate defence molecules, including lysozyme, histatin, lactoferrin, calprotectin and IgA[51,52], interact with *Candida* species, thereby decreasing adherence to and colonization of oral surfaces. Other components in whole saliva, including mucins[52,53], statherin[54] and proline-rich-proteins[3,51] have been reported to adsorb to *C. albicans*, thereby facilitating adherence to saliva-coated acrylic resins[55].

However, studies regarding the influence of **whole** saliva on *Candida* adherence are mutuality contradictory and no consensus can be found in the literature (Table 1). Several investigators reported that a saliva coating reduces the adherence of *C. albicans* in acrylic resin based materials[10,14,15,28,56-60]. Others showed increased adherence rates with saliva coating[12,57,61,62]. Three other research groups found no effect at all of a saliva coating[38,56,63]. A dynamic effect, depending on the morphological phase of *C. albicans* was also found[9,64], where initially adherence was increased, but subsequently decreased after 24 hours.

Several reasons might explain these divergent results. The most important are probably differences in the use of stimulated versus unstimulated saliva,

resulting in different protein composition and viscosity, hence protection[65]. Furthermore, different incubation periods, use of filtered or whole saliva, different saliva temperatures when performing the study, and the presence or absence of nutrients in the different studies may have interfered with cell viability and adherence capacity[10,32,52,63]. Obviously inter-individual variations in the composition of saliva affect the outcome of three component adherence system studies of substratum, saliva and yeast[14,15,50,53,66].

In the oral cavity a denture is coated with a salivary pellicle, which provides receptor sites for the adherence of micro-organism[67]. Again surface roughness and surface free energy are confounding factors in the coating. Although surface characteristics are important in determining the final composition of an acquired pellicle and hence can dictate colonization of *Candida* species, there are only few studies where the effects of different types of acrylic resins on this process are compared[23,32].

Studies dealing with the effect of saliva on adherence of *Candida* species, other than *C. albicans*, to acrylic resins *in vitro* and *in vivo*, indicate variable adherence levels[14,15,58]. *C. dubliniensis* counts have been shown to decrease[53], increase[68] or show no effect[14] in the presence of saliva, while *C. glabrata* counts were not influenced by saliva in one study[14] but decreased in another report[15].
Thus there is contradicting evidence with regard to the relationship *in vitro* between saliva and *Candida* adhesion. In general it may be concluded that low molecular weight proteins are related to the adherence levels of *Candida*[69]. This is in agreement with clinical studies[51,52,70,71], where patients with low or impaired salivary flow and/or composition presented higher *Candida* species counts when compared with saliva from patients with normal salivary flow. Collectively this confirms the regulating role of saliva in inhibiting *Candida* species adherence.

Candida species' shift

The *Candida* species most often reported to be associated with oral mucosal lesions is *Candida albicans*. But *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *C. dubliniensis* have also been isolated from diseased tissues[72-75]. Recently a shift in disease-associated *Candida* species from *Candida albicans*

towards these non-*albicans* species was observed[76-78]. While *C. albicans* is still by far the predominant isolate under inflammatory conditions[79], *C. glabrata* emerges as the second most prevalent species, frequently isolated from acrylic denture surfaces and the palatal mucosa[75]. *Candida glabrata* used to be considered a non-pathogenic *Candida* species, but the increased use of immunosuppressive drugs, as a cure of the immunosuppressive syndrome, have now led to increasing *C. glabrata* infections with high mortality rates[80]. The explanation for this trend towards morbidity due to ‘‘less pathogenic’’ yeasts remains to be established, but it has already been suggested that the increased worldwide use of antifungals has contributed to this phenomenon[81,82]. Besides the shift from *C. albicans* to *C. glabrata*, there is increasing evidence that more than one *Candida* species may simultaneously colonize mucosal habitats, as reported for the oral mucosa[83], tongue and palate[81], both in healthy and diseased subjects.

Bacteria and *Candida* interactions

Microbial cell to cell communication plays an important role in the colonization process. Micro-organisms present in the oral environment interact with each other in many ways, such as by using each other’s metabolic end-products, or by communicating more directly through signalling molecules[84]. Understanding the complex interactions between surfaces, saliva, eukaryotic and prokaryotic micro-organisms during infections is crucial in developing prevention and treatment strategies. In studies on *Candida* biofilm formation and *Candida* susceptibility, the characteristics of the oral environment in which the biofilms are naturally formed should be mimicked as closely as feasible[85].

The multicellular lifestyle of bacterial and yeast biofilms[86,87] is induced by environmental stress and/or restricted nutrient supplies[88]. These cooperation lead to adaptation to natural stress responses and result in a balanced microflora[88-91]. In addition to various forms of metabolic dependence micro-organisms may co-aggregate, with two or more genetically distinct strains interacting through specific cell to cell recognition[92]. Such co-aggregation has been observed between *C. albicans* and several other oral micro-organisms[93-95] and is an important factor in the microbial colonization and progression of infections in the oral cavity.

Bacteria and yeasts also interact via quorum sensing (QS). Quorum sensing is a polymicrobial coordination within a microbial community, based on excreted small molecules triggering a genetic response when present in sufficiently high concentrations. QS occurs both in single species bacterial communities and in complex mixed bacterial-yeast communities[96,97]. A recent study[98] showed that *Candida* hyphal formation can be modulated by Gram negative bacterial quorum sensing molecules. Particularly in the multispecies biofilm communities QS molecules may accumulate to high concentrations and hence are important in controlling physiology and homeostasis[99].

Although studies on biofilm development and species interactions have, so far, focused largely on bacterial species it has become clear that synergistic interactions among micro-organisms increase the efficiency of the impropagation[100,101]. Oral biofilm are not random mixtures of micro-organisms; but organized structures though varying in space and time while modulating adherence and metabolic properties[102]. Immediately after brushing or prophylaxis, the surface will be recoated with salivary pellicle and the first pioneer bacteria will colonize. These “early colonizers” are followed by the “late colonizers”, if the conditions of/in the biofilm become amenable for other species to survive[103].

Although there is variability in composition of an oral biofilm community depending on patient dependent characteristics, the mere presence of a specific micro-organism does not induce pathology. Typically this depends on a complex of micro-organisms-host interactions that modulate the host’s response leading to inflammation. Depending on the local conditions, bacteria may provide fungi with compounds that activate virulence determinants of fungi[104]. This is not only important for *Candida* infections but also why *Candida* may be responsible for non-*Candida* infections induced by the patient’s indigenous microflora[105].

Several researchers have studied interactions among *Candida* and bacteria in an attempt to determine how oral bacteria may modulate *Candida* adherence and colonization. The influence of *Streptococcus salivarius* has been reported to decrease *Candida* adherence[10], while cooperation between several *Streptococci* and *Candida albicans* has also been reported[11,106]. Other research groups

assessed *in vivo* biofilms, with various plaque collection methods generally destructive to the biofilm structure[9,107-110]. In contrast, the new confocal scanning laser microscopy using molecular biological staining techniques may elucidate unsolved issues or even identify artefacts arising from traditional methodologies. A recent study using acrylic resin samples of denture wearers *in vivo* has shown that different subjects present different biofilm formation rates, architecture and densities[111]. Unfortunately, the only substratum tested was acrylic resin and there was no attempt to characterize the surface properties, which might have resulted in a better understanding of the process. Clearly, understanding the biofilm behaviour of *Candida* species under various environmental conditions is the key to the development of effective preventive measures for *Candida* infections[112]. Further studies are needed to establish whether or not these interactions are strain-specific and on which other parameters they depend. As a result it may be possible to identify the stages when *C. albicans* and other emerging pathogenic species can be targeted in treatment and prevention.

Future research and final remarks

From the literature the picture emerges that many factors determine *Candida* harbouring biofilms. These factors include surface properties, micro-organisms interactions, biofilm architecture, and saliva. Obviously it is tempting to study the individual parameters in simple mechanistic studies. However, the level of contradictions in the pertaining literature should be interpreted by assuming multiple interactions between the various factors. A meaningful study of *Candida* biofilms thus only seems possible when the various factors are studied in a comprehensive experimental design.

As recent studies are pointing to the role of multi-species biofilms on the onset of the disease, studies that may explain how such biofilms interact with surfaces and how to prevent their growth are important. Fungal adhesion may be greater in materials presenting higher surface roughness. Consequently, the rehabilitation material chosen in clinical situations has to be carefully considered. When the oral cavity is re-colonized after antimycotic treatment withdrawal in

patients with oral candidiasis, the yeasts may be harboured in more remote sites of the material.

While the initial adhesion of *Candida* species is influenced by surface roughness, and may be influenced by the materials' surface free energy (question still under discussion), these characteristics should be evaluated in *in vivo*-like conditions. Indeed, the presence of a rehabilitation material that could favour health and avoid the oral cavity re-colonization is mandatory. Therefore, studies that could explore the factors related to initial re-colonization by *Candida* in different materials are of utmost importance. The relationship of denture base materials and their effect on fungal growth requires further investigation through epidemiologic, clinical, and basic research. These new studies may include surface characteristics, but other important matters discussed on this review are fundamental to facilitate treatment protocols. New research should be on multispecies biofilm, as close as possible to the *in vivo* situation. Furthermore, other emerging fungal pathogens, such as *Candida glabrata*, should be under investigation, as the results found for one *Candida* species (mainly *Candida albicans*) may not generally hold, again in experimental setups where other organisms and saliva are present.

References

1. Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Darius PL, van Steenberghe D. The influence of surface free energy and surface roughness on early plaque formation. An *in vivo* study in man. J Clin Periodontol. 1990;17:138-44.
2. Hazen KC, Brawner DL, Riesselman MH, Jutila MA, Cutler JE. Differential adherence of hydrophobic and hydrophilic Candida albicans yeast cells to mouse tissues. Infect Immun. 1991;59:907-12.
3. Cannon RD, Chaffin WL. Oral colonization by *Candida albicans*. Crit Rev Oral Biol Med. 1999;10:359-83.
4. Samaranayake LP. Oral mycoses in HIV infection. Oral Surg Oral Med Oral Pathol. 1992;73:171-80.

5. McMullan-Vogel CG, Jüde HD, Ollert MW, Vogel CW. Serotype distribution and secretory acid proteinase activity of *Candida albicans* isolated from the oral mucosa of patients with denture stomatitis. Oral Microbiol Immunol. 1999;14:183-89.
6. Radford DR, Challacombe SJ, Walter JD. Denture plaque and adherence of *Candida albicans* to denture-base materials *in vivo* and *in vitro*. Crit Rev Oral Biol Med. 1999;10:99-116.
7. Kumamoto CA, Vinces MD. Alternative *Candida albicans* lifestyles: growth on surfaces. Annu Rev Microbiol. 2005;59:113–33.
8. Chandra J, Mukherjee PK, Leidich SD, Faddoul FF, Hoyer LL, Douglas LJ, Ghannoum MA. Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. J Dent Res. 2001;80:903-8.
9. Ramage G, Tomsett K, Wickes BL, Lopez-Ribot JL, Redding SW. Denture stomatitis: a role for *Candida* biofilms. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;98:53-9.
10. Samaranayake LP, MacFarlane TW. An *in vitro* study of the adherence of *Candida albicans* to acrylic surfaces. Arch Oral Biol. 1980;25:603-9.
11. Branting C, Sund ML, Linder LE. The influence of *Streptococcus mutans* on adhesion of *Candida albicans* to acrylic surfaces *in vitro*. Arch Oral Biol. 1989;34:347-53.
12. Edgerton M, Scannapieco FA, Reddy MS, Levine MJ. Human submandibular-sublingual saliva promotes adhesion of *Candida albicans* to polymethylmethacrylate. Infect Immun. 1993;61:2644-52.
13. Serrano-Granger R, Campo-Trapero J, Del Río-Highsmith J. *In vitro* study of the adherence of *Candida albicans* to acrylic resins: relationship to surface energy. Int J Prosthodont. 2005;18:392-8.
14. Moura JS, da Silva WJ, Pereira T, Del Bel Cury AA, Rodrigues Garcia RC. Influence of acrylic resin polymerization methods and saliva on the adherence of four *Candida* species. J Prosthet Dent. 2006;96:205-11.
15. Pereira-Cenci T, Cury AA, Cenci MS, Rodrigues-Garcia RC. *In vitro Candida* colonization on acrylic resins and denture liners: influence of surface

free energy, roughness, saliva, and adhering bacteria. Int J Prosthodont. 2007;20:308-10.

16. Verheyen CC, Dhert WJ, de Blieck-Hogervorst JM, van der Reijden TJ, Petit PL, de Groot K. Adherence to a metal, polymer and composite by Staphylococcus aureus and Staphylococcus epidermidis. Biomaterials. 1993;14:383-91.

17. Bridgett MJ, Davies MC, Denyer SP, Eldridge PR. In vitro assessment of bacterial adhesion to Hydromer-coated cerebrospinal fluid shunts. Biomaterials. 1993;14:184-8.

18. Bellon-Fontaine MN, Mozes N, van der Mei HC, Sjollema J, Cerf O, Rouxhet PG, Busscher HJ. A comparison of thermodynamic approaches to predict the adhesion of dairy microorganisms to solid substrata. Cell Biophys. 1990;17:93-106.

19. Busscher HJ, Cowan MM, van der Mei HC. On the relative importance of specific and non-specific approaches to oral microbial adhesion. FEMS Microbiol Rev. 1992;8:199-209.

20. Klotz SA, Drutz DJ, Zajic JE. Factors governing adherence of *Candida* species to plastic surfaces. Infect Immun. 1985;50:97-101.

21. Verran J, Maryan CJ. Retention of *Candida albicans* on acrylic resin and silicone of different surface topography. J Prosthet Dent. 1997;77:535-9.

22. Waltimo T, Tanner J, Vallittu P, Haapasalo M. Adherence of *Candida albicans* to the surface of polymethylmethacrylate--E glass fiber composite used in dentures. Int J Prosthodont. 1999;12:83-6.

23. Minagi S, Miyake Y, Inagaki K, Tsuru H, Suginaka H. Hydrophobic interaction in *Candida albicans* and *Candida tropicalis* adherence to various denture base resin materials. Infect Immun. 1985;47:11-4.

24. Young T. *Philosophical Transactions of the Royal Society of London*, 9th ed. London 1805; 255.

25. Hazen KC. Participation of yeast cell surface hydrophobicity in adherence of *Candida albicans* to human epithelial cells. Infect Immun. 1989;57:1894-1900.

26. Busscher HJ, Weerkamp AH, van der Mei HC, van Pelt AW, de Jong HP, Arends J. Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. Appl Environ Microbiol. 1984;48:980-3.
27. Budtz-Jorgensen E, Theilade E, Theilade J. Quantitative relationship between yeast and bacteria in denture-induced stomatitis. Scand J Dent Res. 1983; 91:134-42.
28. Waters MG, Willians DW, Jagger RG, Lewis MA. Adherence of *Candida albicans* to experimental denture soft lining materials. J Prosthet Dent. 1997;3:306-12.
29. Luo G, Samaranayake LP. *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. APMIS. 2002;110:601-10.
30. Sipahi C, Anil N, Bayramli E. The effect of acquired salivary pellicle on the surface free energy and wettability of different denture base materials. J Dent. 2001;29:197-204.
31. Anusavice KJ. *Philips' science of dental materials*, 10th ed. Pennsylvania: W.B. Saunders, 1996:273–300.
32. Radford DR, Sweet SP, Challacombe SJ, Walter JD. Adherence of *Candida albicans* to denture-base materials with different surface finishes. J Dent. 1998;26:577-83.
33. Nevzatoğlu EU, Ozcan M, Kulak-Ozkan Y, Kadir T. Adherence of *Candida albicans* to denture base acrylics and silicone-based resilient liner materials with different surface finishes. Clin Oral Investig. 2007;11:231-6.
34. Taylor R, Maryan C, Verran J. Retention of oral microorganisms on cobalt–chromium alloy and dental acrylic resin with different surface finishes. J Prosthet Dent. 1998;80:592– 7.
35. Ulusoy M, Ulusoy N, Aydin AK. An evaluation of polishing techniques on surface roughness of acrylic resins. J Prosthet Dent. 1986;56:107-12.
36. Gocke R, Gerath F, von Schwanewede H. Quantitative determination of salivary components in the pellicle on PMMA denture base material. Clin Oral Investig. 2002;6:227-35.

37. McCabe JF. A polyvinylsiloxane denture soft lining material. *J Dent* 1998; 26: 521-526.
38. Tari BF, Nalbant D, Dogruman Al F, Kustimur S. Surface roughness and adherence of *Candida albicans* on soft lining materials as influenced by accelerated aging. J Contemp Dent Pract. 2007;8:18-25.
39. Masella RP, Dolan CT, Laney WR. The prevention of the growth of *Candida* on silastic 390 soft liner for dentures. J Prosthet Dent. 1975;33:250-7.
40. Douglas WH, Walker DM. Nystatin in denture liners- an alternative treatment of denture stomatitis. Br Dent J. 1973;135:55–9.
41. Wright PS. The effect of soft lining materials on the growth of *Candida albicans*. J Dent. 1980;8:144-51.
42. Graham BS, Jones DW, Burke J, Thompson JP. *In vivo* fungal presence and growth on two resilient denture liners. J Prosthet Dent. 1991;65:528-32.
43. el-Charkawi H, el-Said EA, Safouh HM, el-Raghi N. Effect of addition antimicrobial agents to denture reliners. Egypt Dent J. 1994;40:785-90.
44. Matsuura T, Abe Y, Sato Y, Okamoto K, Ueshige M, Akagawa Y. Prolonged antimicrobial effect of tissue conditioners containing silver– zeolite. J Dent. 1997;25:373–7.
45. Nikawa H, Yamamoto T, Hamada T, Rahardjo MB, Murata H, Nakanoda S. Antifungal effect of zeolite-incorporated tissue conditioner against *Candida albicans* growth and/or acid production. J Oral Rehabil. 1997;24:350–7.
46. Kulak Y, Kadir T. *In vitro* study of fungal presence and growth on three tissue conditioner materials. J Marmara Univ Dent Fac. 1997;2:682-4.
47. Kulak Y, Kazazoglu E. *In vivo* and *in vitro* study of fungal presence and growth on three tissue conditioning materials on implant supported complete denture wearers. J Oral Rehabil. 1998;25:135-8.
48. Chow CK, Matear DW, Lawrence HP. Efficacy of antifungal agents in tissue conditioners in treating candidiasis. Gerodontology. 1999;16:110–8.
49. Lefebvre CA, Wataha JC, Cibirka RM, Schuster GS, Parr GR. Effects of triclosan on the cytotoxicity and fungal growth on a soft denture liner. J Prosthet Dent. 2001;85:352–6.

50. Nikawa H, Jin C, Hamada T, Makihira S, Kumagai H, Murata H. Interactions between thermal cycled resilient denture lining materials, salivary and serum pellicles and *Candida albicans in vitro*. Part II. Effects on fungal colonization. J Oral Rehabil. 2000;27:124-30.
51. Tanida T, Ueta E, Tobiume A, Hamada T, Rao F, Osaki T. Influence of aging on candidal growth and adhesion regulatory agents in saliva. J Oral Pathol Med. 2001;30:328-35.
52. Dodds MW, Johnson DA, Yeh CK. Health benefits of saliva: a review. J Dent. 2005;33:223-33.
53. Elguezabal N, Maza JL, Ponton J. Inhibition of adherence of *Candida albicans* and *Candida dubliniensis* to a resin composite restorative dental material by salivary secretory IgA and monoclonal antibodies. Oral Dis. 2004;10:81-6.
54. Johansson I, Bratt P, Hay DI, Schluckebier S, Stromberg N. Adhesion of *Candida albicans*, but not *Candida krusei*, to salivary statherin and mimicking host molecules. Oral Microbiol Immunol. 2000;15:112-8.
55. Arendorf TM, Walker DM. Denture stomatitis: a review. J Oral Rehabil 1987; 14: 217-227.
56. Nikawa H, Iwanaga H, Kameda M, Hamada T. *In vitro* evaluation of *Candida albicans* adherence to soft denture-lining materials. J Prosthet Dent. 1992;68:804-8.
57. Millsap KW, Bos R, van der Mei HC, Busscher HJ. Adhesion and surface-aggregation of *Candida albicans* from saliva on acrylic surfaces with adhering bacteria as studied in a parallel plate flow chamber. Antonie Van Leeuwenhoek. 1999;75:351-9.
58. Millsap KW, Bos R, van der Mei HC, Busscher HJ. Adhesive interactions between voice prosthetic yeast and bacteria on silicone rubber in the absence and presence of saliva. Antonie Van Leeuwenhoek. 2001;79:337-43.
59. Maza JL, Elguezabal N, Prado C, Ellacuría J, Soler I, Pontón J. *Candida albicans* adherence to resin-composite restorative dental material: influence of

whole human saliva. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2002;94:589-92.

60. Bosch JA, Turkenburg M, Nazmi K, Veerman EC, de Geus EJ, Nieuw Amerongen AV. Stress as a determinant of saliva-mediated adherence and coadherence of oral and nonoral microorganisms. Psychosom Med. 2003;65:604-12.

61. Nikawa H, Hayashi S, Nikawa Y, Hamada T, Samaranayake LP. Interactions between denture lining material, protein pellicles and *Candida albicans*. Arch Oral Biol. 1993;38:631-4.

62. Vasilas A, Molina L, Hoffman M, Haidaris CG. The influence of morphological variation on *Candida albicans* adhesion to denture acrylic in vitro. Arch Oral Biol. 1992;37:613-22.

63. Jin Y, Samaranayake LP, Samaranayake Y, Yip HK. Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. Arch Oral Biol. 2004;49:789-98.

64. San Millán R, Elguezabal N, Regulez P, Moragues MD, Quindos G, Ponton J. Effect of salivary secretory IgA on the adhesion of *Candida albicans* to polystyrene. Microbiology. 2000;146:2105-12.

65. Veerman EC, van den Keybus PA, Vissink A, Nieuw Amerongen AV. Human glandular salivas: their separate collection and analysis. Eur J Oral Sci. 1996;104:346-52.

66. Dar-Odeh NS, Shehabi AA. Oral candidosis in patients with removable dentures. Mycoses. 2003;46:187-91.

67. Garcia RM, Léon BT, Oliveira VB, Del Bel Cury AA. Effect of a denture cleanser on weight, surface roughness, and tensile bond strength of two resilient denture liners. J Prosthet Dent. 2003;89:489-94.

68. Ramage G, Vandewalle K, Wickes BL, Lopez-Ribot JL. Characteristics of biofilm formation by *Candida albicans*. Rev Iberoam Micol. 2001;18:163-70.

69. Busscher HI, Geertsema-Doornbusch GI, van der Mei HC. Adhesion to silicone rubber of yeasts and bacteria isolated from voice prostheses: influence of salivary conditioning films. J Biomed Mater Res. 1997;34:201-10.

70. Radfar L, Kleiner DE, Fox PC, Pillemer SR. Prevalence and clinical significance of lymphocytic foci in minor salivary glands of healthy volunteers. Arthritis Rheum. 2002;47:520-4.
71. Nikawa H, Jin C, Makihira S, Hamada T, Samaranayake LP. Susceptibility of *Candida albicans* isolates from the oral cavities of HIV-positive patients to histatin-5. J Prosthet Dent. 2002;88:263-7.
72. MacPhail LM, Greenspan D, Dodd CL, Heinic GS, Beck C, Ekoku E. Association of fungal species with oral candidiasis in HIV infection. J Dent Res. 1993;72:353.
73. Samaranayake YH, Samarnayake LP. *Candida krusei*: biology, epidemiology, pathogenicity and clinical manifestations of an emerging pathogen. J Med Microbiol. 1994;41:295–310.
74. Coleman DC, Sullivan DJ, Bennett DE, Moran GP, Barry HJ, Shanley DB. Candidiasis: the emergence of a novel species, *Candida dubliniensis*. AIDS. 1997;11:557–67.
75. Samaranayake YH, Samaranayake LP. Experimental oral candidiasis in animal models. Clin Microbiol Rev. 2001;14:398-429.
76. Samaranayake LP. *Candida krusei* infections and fluconazole therapy. Hong Kong Med J. 1997;3:312–4.
77. Viscoli C, Girmenia C, Marinus A, Collette L, Martino P, Vandercam B, Doyen C, Lebeau B, Spence D, Krcmery V, De Pauw B, Meunier. Candidemia in cancer patients: a prospective, multicenter surveillance study by the Invasive Fungal Infection Group (IFIG) of the European Organization for Research and Treatment of Cancer (EORTC). Clin Infect Dis. 1999;28:1071-9.
78. Krcmery V, Barnes AJ. Non-*albicans Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. J Hosp Infect. 2002;50:243-60.
79. He XY, Meurman JH, Kari K, Rautemaa R, Samaranayake LP. *In vitro* adhesion of *Candida* species to denture base materials. Mycoses. 2006;49:80-4.
80. Krcmery K Jr. *Torupsis glabrata* – an emerging yeast pathogen in cancer patients. Int J Antimicrob Agents. 1999;11:1-6.

81. Schmidt-Westhausen AM, Bendick C, Reichart PA, Samaranayake LP. Oral candidosis and associated *Candida* species in HIV-infected Cambodians exposed to antimycotics. Mycoses. 2004;47:435–41.
82. Snydman DR. Shifting patterns in the epidemiology of nosocomial *Candida* infections. Chest. 2003;123:500S–3S.
83. Dronda F, Alonso-Sanz M, Laguna F, Chaves F, Martinez-Suarez JV, Rodriguez-Tudela JL, Gonzalez-Lopez A, Valencia E. Mixed oropharyngeal candidiasis due to *Candida albicans* and non-*albicans Candida* strains in HIV-infected patients. Eur J Clin Microbiol Infect Dis. 1996;15:446–52.
84. Blankenship JR, Mitchell AP. How to build a biofilm: a fungal perspective. Curr Opin Microbiol. 2006;9:588-94.
85. Lamfon H, Porter SR, McCullough M, Pratten J. Formation of *Candida albicans* biofilms on non-shedding oral surfaces. Eur J Oral Sci. 2003;111:465–71.
86. Kierek-Pearson K, Karatan E. Biofilm development in bacteria. Adv Appl Microbiol. 2005;57:79-111.
87. Mukherjee PK, Zhou G, Munyon R, Ghannoum MA. *Candida* biofilm: a well-designed protected environment. Med Mycol. 2005;43:191-208.
88. Palkova Z, Vachova L. Life within a community: benefit to yeast long-term survival. FEMS Microbiol Rev. 2006;30:806-24.
89. Mikelsaar M, Mandar R. Development of individual lactic acid microflora in the human microbial ecosystem. In: Salminen S, von Wright A, eds. *Lactic Acid Bacteria*, 1st Edn. New York: Marcel Dekker, 1993:256–60.
90. McFarland LV. Normal flora: diversity and functions. Microb Ecol Health Dis. 2000:12:193–207.
91. Perdigon G, Fuller R, Raya R. Lactic acid bacteria and their effect on the immune system. Curr Issues Intest Microbiol. 2001:2:27–42.
92. James A, Beaudette L, Costerton W. Interspecies bacterial interactions in biofilms. J Industrial Microbiol. 1995;15:257–62.

93. Hsu LY, Minah G, Peterson DE, Wingard JR, Merz WG, Altomonte V, Tylenda CA. Coaggregation of oral *Candida i*solates with bacteria from bone marrow transplant recipients. J Clin Microbiol. 1990;28:2621–6.
94. Holmes AR, Gopal PK, Jenkinson HF. Adherence of *Candida albicans* to a cell surface polysaccharide receptor on *Streptococcus gordonii*. Infect Immun. 1995;63:1827–34.
95. Jenkinson HF, Lala HC, Shepherd MG. Coaggregation of *Streptococcus sanguis* and other streptococci with *Candida albicans*. Infect Immun. 1990;58:1429–36.
96. Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM. Structural identification of a bacterial quorum-sensing signal containing boron. Nature. 2002;415:545-9.
97. Keller L, Surette MG: Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol. 2006;4:249-58.
98. Hogan DA, Vik A, Kolter R. A Pseudomonas aeruginosa quorum-sensing molecule influences *Candida albicans* morphology. Mol Microbiol. 2004;54:1212-23.
99. Koo H, Schobel BD, Scott-Anne K, Watson G, Bowen WH, Cury JA, Rosalen PL, Park YK. Apigenin and tt-farnesol with fluoride effects on *S. mutans* biofilms and dental caries. J Dent Res. 2005;84:1016-20.
100. Garcia de Viedma D, Lorenzo G, Cardona PJ, Rodriguez NA, Gordillo S, Serrano MJ, Bouza E. Association between the infectivity of *Mycobacterium tuberculosis* strains and their efficiency for extrarespiratory infection. J Infect Dis. 2005;192:2059-65.
101. Li L, Redding S, Dongari-Batgtzoglou. *Candida glabrata*, an emerging oral opportunistic pathogen. J Dent Res. 2007;86:204-15.
102. ten Cate JM. Biofilms, a new approach to the microbiology of dental plaque. Odontology. 2006;94:1-9.
103. Jenkinson HF, Lamont RJ. Oral microbial communities in sickness and in health. Trends Microbiol. 2005;13:589-95.

104. Wargo MJ, Hogan DA. Fungal--bacterial interactions: a mixed bag of mingling microbes. Curr Opin Microbiol. 2006;9:359-64.
105. Fridkin SK, Jarvis WR. Epidemiology of nosocomial fungal infections. Clin Microbiol Rev. 1996,9:499-511.
106. Verran J, Motteram KL. The effect of adherent oral streptococci on the subsequent adherence of *Candida albicans* to acrylic *in vitro*. J Dent. 1987;15:73-6.
107. Catalan A, Herrera R, Martinez A. Denture plaque and palatal mucosa in denture stomatitis: scanning electron microscopic and microbiologic study. J Prosthet Dent. 1987;57:581-6.
108. Frank RM, Steuer P. Transmission electron microscopy of plaque accumulations in denture stomatitis. J Prosthet Dent. 1985;53:115-24.
109. Radford DR, Radford JR. A SEM study of denture plaque and oral mucosa of denture-related stomatitis. J Dent. 1993;21:87-93.
110. Wood S R, Kirkham J, Marsh P D, Shore R C, Nattress B, Robinson C. Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. J Dent Res. 2000;79:21–7.
111. Avon SL, Goulet JP, Deslauriers N. Removable acrylic resin disk as a sampling system for the study of denture biofilms *in vivo*. J Prosthet Dent. 2007;97:32-8.
112. Thein ZM, Samaranayake YH, Samaranayake LP. *In vitro* biofilm formation of *Candida albicans* and non-*albicans Candida* species under dynamic and anaerobic conditions. Arch Oral Biol. 2007;52:761-7.
113. McCourtie J, MacFarlane TW, Samaranayake LP. Effect of saliva and serum on the adherence of *Candida* species to chlorhexidine-treated denture acrylic. J Med Microbiol. 1986;21:209-13.

Table 1. The effect of saliva on *Candida* species adherence/biofilm formation on acrylic surfaces, according to published data.

Authors	Saliva Collection	Saliva Type	*Candida* Species	Effect on *Candida* spp.
Samaranayake *et al*.[10], 1980	Unstimulated	Whole	*C. albicans*	Reduction
	Stimulated	Parotid	*C. albicans*	No effect
MacCourtie *et al*.[113], 1986	Unstimulated	Whole	*C. albicans*	Reduction
Nikawa *et al*.[56], 1992	Unstimulated	Whole	*C. albicans*	No effect
Vasilas *et al*.[62], 1992	Stimulated	Whole	*C. albicans*	Increase
		Parotid	*C. albicans*	Increase
		Submandibular-Sublingual	*C. albicans*	Increased/reduced[1]
Edgerton *et al*.[12], 1993	Stimulated	Submandibular-Sublingual	*C. albicans*	Increase
		Mucin-free	*C. albicans*	No effect
Nikawa *et al*.[61], 1993	Unstimulated	Whole	*C. albicans*	Increase
Waters *et al*.[28], 1997	Unstimulated	Whole	*C. albicans*	Reduction
Millsap *et al*.[57], 1999	Stimulated	Whole	*C. albicans*	Reduction/Increase[2]
San Millán *et al*.[64], 2000	Unstimulated	Whole	*C. albicans*	Increased/reduction[3]
Millsap *et al*.[58], 2001	Stimulated	Whole	*C. albicans*	Reduction
			C. krusei	Reduction
			C. tropicalis	Reduction
Ramage *et al*.[68], 2001	Stimulated	Whole	*C. dubliniensis*	Increase
Maza *et al*.[59], 2002	Unstimulated	Whole	*C. albicans*	Reduction
Bosch *et al*.[60], 2003	Unstimulated	Whole	*C. albicans*	Reduction
Jin *et al*.[63], 2004	Unstimulated	Whole	*C. albicans*	No effect
Ramage *et al*.[9], 2004	Stimulated	Whole	*C. albicans*	Increase[4]
Moura *et al*.[14], 2006	Stimulated	Whole	*C. albicans*	Reduction
			C. glabrata	No effect
			C. dubliniensis	Reduction/no effect[5]
			C. tropicalis	Reduction
Pereira-Cenci *et al*.[15], 2007	Stimulated	Whole	*C. albicans*	Reduction
			C. glabrata	Reduction
Tari *et al*.[38], 2007	Stimulated	Whole	*C. albicans*	No effect

[1]dependent upon the donor; [2]dependent upon the co-existence with other bacteria; [3]dependent on *Candida* morphological phase; [4]but decreased over time. [5]dependent upon the substratum

Legends to Figures

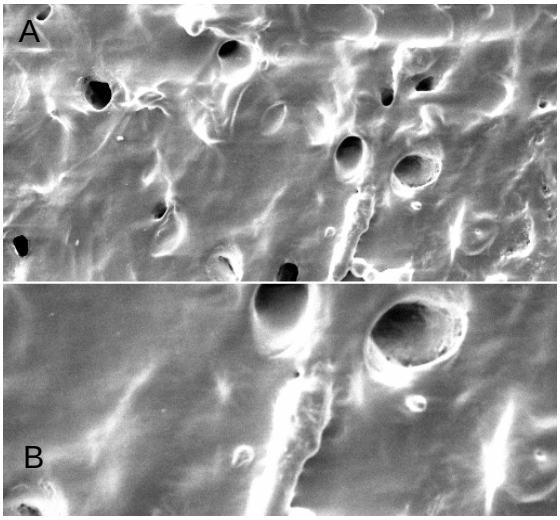

Figure 1. Scanning electron microscopy of a soft denture reliner showing the extents of defect; it is notable to observe that the material not only exhibits porosities, but also show surface irregularities, which may turn into adhesion sites (A: x 40; B: x 100). *Sample analyzed was prepared according to the manufacturer's directions (CoeSoft, GC America, Alsip IL, USA). It was subsequently mounted on a stub, air-dried, sputtercoated with gold (Balzers Union MED 010 evaporator), and examined with a Zeiss (Thornwood, NY) DSM940A scanning electron microscope at an accelerating voltage of 20.0 kV for surface characterization.*

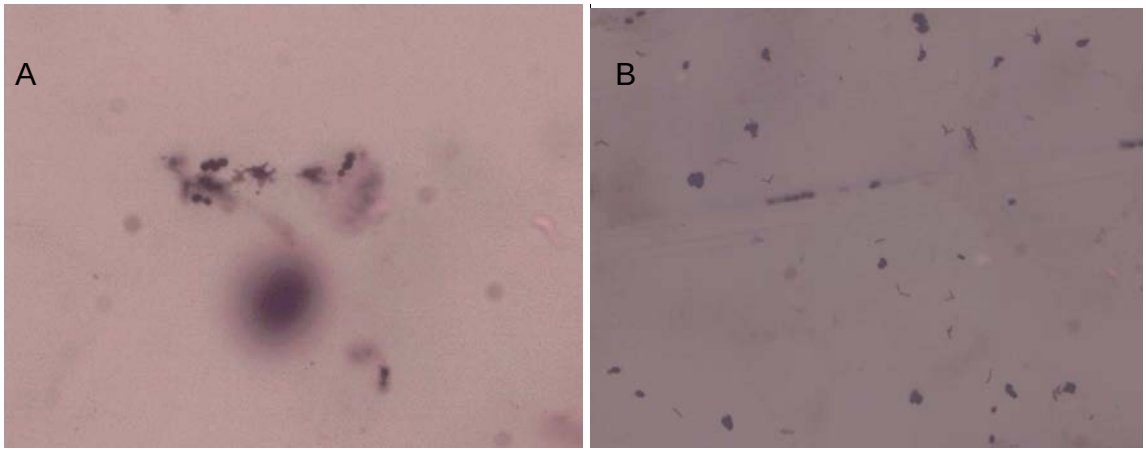

Figure 2. Adherence of *Candida albicans* and bacteria on a soft denture liner coated with saliva.
A – Note that bacteria and fungi are united. B – The sample was not coated with saliva; note that bacteria and fungi do not seem connected when compared to the coated sample.

The effect of *Streptococcus mutans* and *Candida glabrata* on *Candida albicans* biofilms formed on different surfaces

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Running title: *Candida* biofilms on different surfaces

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Abstract Although *Candida* containing biofilms contribute to the development of oral candidosis, the characteristics of multi-species *Candida* biofilms and how oral bacteria modulate these biofilms is poorly understood. The aim of this study was to investigate interactions between *Candida albicans* and either *Candida glabrata* or *Streptococcus mutans* in biofilms grown on various surfaces, with or without saliva. Hydroxyapatite (HA), polymethylmetacrylate (PMMA) and soft denture liner (SL) discs were used as substratum. Counts of viable micro-organisms in the accumulating biofilm layer were determined and converted to colony forming units per unit surface area. Confocal laser scanning microscopy was used to characterize biofilms and to quantitate the number of hyphae in each condition tested. Viable counts of *C. albicans* and *C. glabrata* per mm^2 decreased in the order HA > PMMA > SL ($p<0.05$). Biofilms grown on saliva-coated specimens harboured fewer *C. glabrata* than uncoated specimens ($p<0.05$). Glucose and the presence of *S. mutans* suppressed *C. albicans* hyphal formation. Dual *C.* species biofilms did not show competitive interaction between the two species. We conclude that *Candida* biofilms are significantly affected by saliva, substratum type and by the presence of other micro-organisms.

Key words: *Candida albicans*, *Candida glabrata*, dual-species biofilm, *Streptococcus mutans*, confocal scanning laser microscopy

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Introduction

Candida species are the main pathogens responsible for the development of denture stomatitis, which is the most common infection in denture wearers (1). Poorly fitting dentures and poor oral hygiene are the most frequent cause of this opportunistic infection (2). Especially in elderly patients, several predisposing factors may accumulate (i.e. dietary factors, malignancies, use of broad-spectrum antibiotics, smoking, age, diabetes mellitus, iron and vitamin deficiencies, and salivary gland dysfunction (2,3), which often leads to candidal infections.

Although *Candida albicans* is the predominant isolate in these infections (4), other non-*albicans* species, in particular *Candida glabrata*, are also frequently isolated from acrylic surfaces and the palatal mucosa (5). Moreover, while *Candida* species are identified as the major pathogens, bacteria from denture biofilms are generally also involved (6).

Denture biofilms are composed mainly of bacteria (7,8), with *Streptococcus mutans* showing a high prevalence, while yeast constitutes a minor part of the total microbial flora (9,10). On a given surface, the formation of multi-species biofilms increases the chance of survival for many micro-organisms in the oral environment. Although the oral cavity consists of many habitats, in terms of growth sites and growth conditions, each of which favours a specific group of bacteria, micro-organisms interact to ensure their individual survival (11,12).

C. albicans virulence is attributed to its ability to grow in the full range of vegetative morphologic forms: yeast, pseudohyphae and true hyphae. The observations that elongated hyphae evade or escape phagocytic cells and that yeast cells disseminate in the tissue and bloodstream suggest that morphology contributes as a major factor in the survival of *C. albicans* at various sites or conditions (13,14). Hyphae formation is required for robust biofilm formation, as well as cell–substrate and cell–cell interactions, and extracellular matrix production, which are key steps in biofilm development (15,16). Not only *C. albicans* properties but also interactions with co-habitating oral micro-organisms may determine *C. albicans* virulence characteristics and together this justifies studying multi-species biofilms on surfaces.

Very little is known on substratum effects on the interactions between *Candida* species and other oral micro-organisms, specifically denture liners substrata surfaces containing or releasing antifungals. Fungal growth is known to destroy the surface properties of denture liners and this may lead to irritation of the oral tissues. This observation is the rationale why attempts have been undertaken to incorporate antifungal agents or antiseptics in these materials. The use of denture liners for denture prostheses is needed in clinical situations in which patients have thin, sharp, or resorbed residual alveolar ridges, chronic tissue irritation from dentures or have received implant treatment (17). Even though these materials show excellent tissue tolerance, one of the problems is the colonization of *Candida* spp. on and in the material. Similarly, the role of saliva during the initial colonization and subsequent multi-species biofilm formation is poorly understood. Several studies have demonstrated that pre-treatment of samples with whole saliva decreased the initial adherence of *C. albicans* (18-22), while other studies showed either an increased adherence (23,24), or no effect (25).

While bacterial biofilms are currently being extensively studied, few studies have addressed fungal-bacterial biofilms. The complex interactions between yeasts, substratum surfaces, presence of saliva and oral bacteria have been studied superficially (10,22,26), but many questions have remained unanswered. Since colonization, growth and differentiation of *Candida* spp. in the oral cavity are of significant clinical importance, the purpose in our study was to analyse single and dual-species biofilm formation on various substratum types (one containing an antifungal agent), and to determine the effects of whole saliva and *S. mutans* on this process.

Material and methods

Experimental design

This *in vitro* study had a completely randomized and blinded design (regarding CFU counts), with substratum type (hydroxyapatite - HA, polymethylmetacrylate - PMMA or soft denture liner - SL), saliva (coated or uncoated), biofilm type (single species biofilms: *Candida albicans* and *Candida glabrata*; and dual species biofilms: *C. albicans* plus *Streptococcus mutans*, *C.*

glabrata plus *S. mutans* and *C. albicans* plus *C. glabrata*) and type of carbohydrate (glucose or sucrose) as factors. CFU counts of *C. albicans* and *C. glabrata* and number of hyphae (*C. albicans*) were the dependent variables. Scanning electron microscopy (SEM) was used to characterize substratum surfaces and confocal scanning laser microscopy (CLSM) was used to visualize the biofilm structure and to quantify hyphae formation.

HA, PMMA and SL discs were used as substrata, using 24-well polystyrene tissue culture plates. Discs without yeast or bacterial cells served as controls. Single and dual species biofilms were formed for 24 hours. After this period, discs with biofilms were removed from the wells and CFU counts of each micro-organism were calculated.

Preparation of PMMA and SL discs

Soft denture liner (Coe Soft, GC America, Alsip, IL, USA) and polymethylmetacrylate (Rebaron, GC Dental Products Corp., Aichi, Japan) discs were prepared according to the manufactures specifications at room temperature (20 ± 1.0 ºC and 50 ± 5% relative humidity), under aseptic conditions, using a Teflon mould (10.6 mm in diameter and 1.5-2.0 mm in thickness). A uniform surface was ensured by placing glass slides on both sides of the mould and firmly fixing both ends, and separating the glass slides after curing, after preparation (27). Discs were used immediately. The soft denture liner contained undecylenic acid (1-5%) as the antifungal ingredient.

Inoculum and media

The micro-organisms used in this study were *S. mutans* PDM15 (28): a mutant of *S. mutans* UA159 containing a green fluorescent protein (GFP) coding gene fragment, *C. albicans* ATCC 90028 and *C. glabrata* ATCC 90030. To prepare the inocula, *S. mutans* was first grown anaerobically on Todd-Hewitt yeast extract (THY; Difco, Sparks, MD, USA) agar plates, supplemented with 10 µg/ml erythromycin, for 2 days. *C. albicans* and *C. glabrata* were both grown aerobically on CHROMagar™ (CHROMagar™ *Candida*, Paris, France) plates for 24 hours. The modified semi-defined medium (pH 7.0) used in this study (29) contained 76 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH4)_2SO_4$, 35 mM NaCl, and 2 mM $MgSO_4$

$\cdot 7H_2O$ and was supplemented with filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 µM riboflavin, 0.3 µM thiamine HCl, and 0.05 µM D-biotin), amino acids (4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, and 0.1 mM L-tryptophan), 0.3% (w/v) yeast extract. This medium was selected for its constant pH (6.8), as pH is known to affect hyphal development. Also, the medium allows both species to grow together (data not shown) and diminishes background interference on CLSM. As it was one of our aims to check the role of the carbohydrates, we selected a medium with a single added source of carbon.

Subsequently, single colonies were inoculated into 10 ml of the semi-defined medium (18 mM glucose-enriched) individually for each micro-organism and incubated anaerobically for *S. mutans* and aerobically for *Candida* species at 37 ^{o}C overnight. Cells were harvested in the late exponential growth phase, washed with phosphate buffered saline (PBS; pH 7.2) and resuspended spectrophotometrically to a concentration of 10^8 cells/ml (0.35 at 600nm) for bacteria and 10^7 cells/ml for *Candida* species (0.38 at 520 nm). A standard curve of turbidity against colony forming unit (CFU) was used to obtain the number of cells (25).

Biofilm assays

Biofilm assays were performed with single-species biofilms of *C. albicans* or *C. glabrata*, and dual-species biofilms of *S. mutans* plus *C. albicans*, *S. mutans* plus *C. glabrata* and *C. albicans* plus *C. glabrata*. Discs of the three materials, prepared as previously described, were placed on the bottom of 24-well (15 mm diameter each well) polystyrene tissue culture plates (bio-one; Greiner, Frickenhausen, Germany). Subsequently, 2 ml of each cell suspension (10^8 CFUs *S. mutans* and/or 10^7 CFUs *C. albicans*/*C. glabrata* in the semi-defined medium (18 mM glucose or 24.35 mM sucrose), was added to each well.

Biofilms were formed on saliva-coated or non-coated hydroxyapatite discs (sHA or nHA), polymethylmetacrylate discs (sPMMA or nPMMA) and/or soft denture liner discs (sSL or nSL). Disc surface areas were 2.7 ± 0.2 cm^2. The sHA, sPMMA and sSL discs were prepared by incubation with clarified human whole

saliva for 1 hour at 37℃. Human whole saliva was collected from a single healthy volunteer during masticatory stimulation with Parafilm M (American Can Co., Greenwich. CT, USA) in an ice-chilled polypropylene tube and clarified by centrifugation at 10,000g for 10 minutes at 4^{o}C (30). For every experiment the saliva sample was collected at the same time of day and the volume limited to 50 ml per collection period, such as to account for the circadian rhythm in saliva composition (31). The supernatant was removed and immediately used.

All biofilm assays were performed in duplicate in at least four independent experiments on different days. The organisms were grown undisturbed (i.e. no dynamic growth condition such as the use of a rotary/orbital shaker or other source of shear forces that would disturb the biofilms was used) during 24 hours to allow biofilm formation. Additional biofilms were grown for biofilm analysis by means of CLSM.

Biofilm analyses

In all experiments, after the biofilm development phase (24 h), each disc was aseptically removed and washed twice with PBS in a standard fashion to remove loosely adherent material, by gentle insertion in a new well containing 2mL of sterilized PBS for 2 seconds. Discs were subsequently processed and vortexed for 1 minute in cysteine peptone water (CPW), to dissociate chains and aggregates of micro-organisms, as described elsewhere (4). The suspensions were subsequently serially diluted in PBS and 20 µl samples were plated in triplicate on Trypticase Yeast-Extract Cysteine Sucrose Bacitracin agar (TYCSB), CHROMagar™, and blood agar, (the latter to rule out possible contamination). The plates were incubated at 37 ℃, under anaerobic (blood agar and TYCSB agar), or aerobic (CHROMagar™) conditions for 24–72 h. Colony-forming units (CFU) were counted using a stereomicroscope, and the results were expressed in colony-forming units per area.

Scanning Electron microscopy and Confocal Scanning Laser Microscopy

For SEM, discs of all materials tested were mounted on a stub, air-dried, sputter-coated with gold (Balzers Union MED 010 evaporator) and examined with a

Zeiss (Thornwood, NY) DSM940A scanning electron microscope at an accelerating voltage of 20.0 kV for surface characterization prior to the biofilms assays.

For CLSM, the discs were carefully removed from the wells (after 24 h of biofilm formation), placed (face down) on a 35-mm-diameter glass-bottom Petri-dish (MatTek Corp., Ashland, MS, USA) containing 1 ml of PBS and 25 µg/ml of concanavalin A conjugate (ConA-rhodamine, Invitrogen, The Netherlands) and incubated for 15 min at 37^{o}C. ConA binds to glucose and mannose residues of the yeast cell wall polysaccharides as indicated by red fluorescence in CLSM. Biofilms were observed by CLSM (LSM510, Carl Zeiss, Jena, Germany) mounted on an inverted microscope (20x objective lens, Axiovert100 M, Zeiss). This microscope was equipped with an Ar-ion laser tuned at 488 nm and a 543 nm HeNe laser for simultaneous measurement of GFP (green; 505-530 nm bandpass) and conA (red; 560 nm longpass) in multitrack mode. To assess the structure of the biofilms, a series of optical sections was taken throughout the full depth of the biofilm. All images were captured by direct acquisition with Z-step ranging from 0.5 to 2 µm. Data were subsequently processed using ImageJ and ObjectJ (for display of 3D images; maximum pixel intensity for projection of Z series), and the number of hyphae was counted. Individual hyphal elements were enumerated within the 3D image sections by marking each element. This marking step allowed counting without repetition, as each counted element would appear with a mark generated by the computer program and therefore, avoided overlapping count. Using the Z-step, each hyphal element was verified in order to allow hyphal branching counts. As a parameter to standardize the counts, if in the 3D movement of the step, the image was characterized by branched hyphae without any separation, this was counted as a single element.

Statistical analysis

Statistical analyses were done using SAS software (SAS Institute Inc., version 9.0, Cary, N.C., USA) employing a significance level fixed at 5%. The null hypothesis assumed no differences among sugars, saliva, substrata or dual or single species biofilms. Data that violated the assumptions of equality of variances

and normal distribution of errors were transformed. Data of hyphae and CFU counts were analyzed by ANOVA, followed by Tukey test.

Results

Assessment of the various materials with SEM showed different degrees of surface irregularities. Remarkably, large amounts of porosities and irregularities were observed in the soft liner samples, while HA and PMMA surfaces were smoother (Figure 1).

All tested biofilms displayed significantly higher growth on HA ($p<0.002$), followed by PMMA and SL respectively, irrespective of the sugar type (glucose or sucrose) or the biofilm combination (single or dual-species; Tables 1 and 2; $p<0.0001$). *C. glabrata* showed higher CFU counts compared to *C. albicans* under all experimental conditions ($p<0.05$). Saliva coating resulted in lower CFU counts only for some of the conditions chosen for *C. glabrata* biofilm growth ($p<0.05$). *C. albicans* biofilms were not affected by saliva (Table 1; $p>0.05$).

When compared to the other types of biofilms, *C. albicans* co-cultured with *S. mutans* showed higher counts for all substrata tested ($p<0.001$). Dual *Candida* species, however, did not differ from single-species *C. albicans* biofilms with respect to *C. albicans* counts ($p>0.05$). Both *C. albicans* and *C. glabrata* biofilms grown with glucose showed higher CFU counts when compared with the sucrose-grown biofilms (Tables 1 and 2; $p<0.001$). Under all experimental conditions *C. glabrata* showed higher counts when grown together with *C. albicans*, when compared with the other biofilms under study ($p<0.05$).

Hyphae counts differed depending on sugar type and the presence of *S. mutans* for all experimental conditions (Table 3; $p<0.05$) and depended on saliva coating for PMMA discs ($p<0.001$). Regarding CLSM, it interestingly revealed that the holes in the SL material harboured many yeast and bacterial cells (Fig. 2A). The dual-species biofilm (*C. albicans* plus *S. mutans*) formed on the soft liner was composed of two layers: one near the material surface consisting almost completely of *S. mutans* cells (Fig 2B), and the second, the outer/top surface of the biofilm, containing only *Candida* cells (Fig. 2C; the layer of *S. mutans* cells is still visible under the yeast layer).

Effects of carbon source and saliva on biofilm structure were also observed (Fig. 3). Comparing panels A and B revealed that when biofilms were grown on glucose the formation of hyphae was suppressed in comparison with sucrose-grown biofilms (Table 3; $p<0.05$). Comparing Figure 3 panels B and D (yeast single and yeast-bacteria dual species), showed that hyphal inhibition by glucose was dependent also on the presence of *S. mutans*.

Saliva coated specimens showed a less dense biofilm structure, harbouring fewer micro-organisms, (Fig. 3C and Fig 4A). Dual *Candida* species biofilms showed a compact structure when grown on HA discs (Fig 4B). Grown on sucrose containing medium, hyphal elements were seen in the dual *Candida* species biofilm, as previously seen with *S. mutans* (Fig. 4A).

Discussion

Our study has shown that *C. albicans* biofilm formation is influenced by a multitude of interacting environmental conditions. The extent and morphology of biofilm formation were found to depend on the sugar used for growth, the substratum type, the presence of other micro-organisms and saliva. These findings emphasize the necessity to study these interactions in complex systems mimicking the oral cavity. We have shown that *S. mutans* increases *Candida* biofilm formation, and that *C. albicans* displays synergism with *C. glabrata*. Our study is the first to show formation of two layers (surface associated *S. mutans* cells separate from *C. albicans* cells) on a substratum containing an antifungal agent. The understanding of biofilm formation under different conditions, especially considering the presence of other micro-organisms may be a factor key in the development of therapies to prevent *Candida*-related diseases (9,10, 32).

Novel assays on quantification of *Candida* biofilms are based on assessing metabolic activity rather than viability (25,32,33). Of these, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium salt and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) used in reduction assays are colorimetric methods that quantify metabolic activity. We nevertheless still preferred CFU counts for several reasons. First, MTT or XTT can only be used for comparing conditions while using one yeast strain (33). Since

it was one of our aims to compare biofilm growth of *C. albicans* and *C. glabrata*, these assays were not applicable; moreover inclusion of the prokaryotic *S. mutans* would further complicate interpretations. Second, although quantification of CFU of resuspended biofilm cells is time-consuming and laborious, it directly enumerates the cell numbers, which unlike XTT/MTT assays, are not influenced by their metabolic status (25).

We used Confocal Laser Scanning Microscopy to gain understanding of (dual-species) biofilms formed on the different materials. We chose this technique instead of SEM because the fixation and dehydration required for SEM severely distorts biofilm architecture and shrinks any aqueous phase, whereas live-cell CLSM preserves the intact structure of biofilms (34). Although light penetration depth is restricted to about 100 µm in single photon CLSM, this was not a limitation for our thin biofilms. The CLSM observations revealed that other micro-organisms, not sensitive to undecylenic acid (or other antifungal), can first attach and grow on the SL after which *C. albicans* was able to colonize on this layer of *S. mutans*. We presume that other 'early colonizers' may also serve this purpose. Evaluation of (novel) denture liners should take this finding into account.

The soft denture liner with antifungal used in this study resulted in a lower amount of viable *C. albicans* cells in the surface biofilms under all conditions tested, corroborating studies where denture liners showed an inhibitory effect on C*andida* accumulation (27). However, conflicting results on single species *Candida* are found in the literature reporting both inhibitory and no antifungal effect (27,35,36,37,38). Colonization and growth of a certain species on biological surfaces is an indicator for this species' pathogenic potential. Even though previous studies showed a similar trend on the initial growth effects of antifungals on *C. albicans* and *C. glabrata* (21,22,39,40), the degree of growth found in our study, specifically in the case of the denture liner, indicates lack of activity of undecylenic acid against *C. glabrata*. Differences in growth inhibition may be explained by different models used to study fungal biofilms (single or multi-species), and by the complex phenotypic heterogeneity of a *Candida* population in the oral cavity. This heterogeneity is displayed by a variable surface

hydrophobicity, the absence or presence of secreted extracellular proteinases, hyphae formation and/or thigmotropism (5,39), all directly influencing *Candida* adherence. Although our results should be interpreted with care, since the nutrient-rich environment of the oral cavity does not (fully) match the *in vitro* nature of our study, they do point towards important clues on how *Candida* biofilms behave in the presence of an antifungal. Specific attention should be given to *C. glabrata*, which formed biofilms with higher cell counts than *C. albicans* under most of the experimental conditions and used materials.

Proportions of yeasts and hyphal cells have been shown to be dependent on the nutrient source in single species (*Candida* spp.) biofilms (6,25,32). Comprehensive studies on the effect of dietary sugars on modulation of oral *Candida* colonization and biofilm formation have already been reported on (25,41). The current multi-species study, showed that higher yeast counts were found in the presence of glucose when compared with sucrose.

From our data it is evident that the relationship between saliva pellicle on different substrata and *Candida* colonization is complex. Innate defence mechanisms, such as the flushing effect of saliva, and anti-*Candida* salivary components affect *Candida* physiology and decrease *Candida* adherence to oral surfaces (42). Other components in whole saliva have been reported to adsorb to *C. albicans* thereby increasing adherence to saliva-coated resins and resilient materials (24). Antimicrobial properties of saliva may contribute to the lower counts of micro-organisms. In addition, the nature of the substratum may influence the composition and the formation of the pellicle, which may be more important than the surface properties of the dental materials (43). Another observation is that the use of a saliva coating may in fact have little effect on biofilm formation (25), which agrees with our results, where saliva had no effect on *C. albicans* counts.

To study *Candida*-bacteria interactions we selected *S. mutans* because this bacterium is regularly found in denture plaque and is directly related to dental caries (44). We observed that *S. mutans* increased growth of both *Candida* species under all experimental conditions. These results not only suggest that there may be mutual growth stimulation of these micro-organisms, but also that they can co-

aggregate with each other, which may enhance the adhesion process (45). In contrast to the previously reported competition between *C. albicans* and *C. dubliniensis* (46) we observed a stimulatory effect when *C. glabrata* was co-cultured with *C. albicans*.

An important observation is the fact that *S. mutans* leads to suppression of hyphae formation of *C. albicans*. Most likely this is a result of the biofilm-growth benefits that *S. mutans* displays on *C. albicans*. This finding that *S. mutans* affects prominent virulence parameters of *C. albicans* should be considered in studies dealing with prevention of oral manifestations of *C. albicans*.

Our null hypothesis tested was rejected since the combined results show that all factors under study influenced yeast counts. It is justified to speculate on the importance of the rehabilitation material in clinical situations. Since yeasts may reside metabolically dormant in more remote sites of this material, the oral cavity will be quickly re-colonized after antimycotic treatment in patients with oral candidosis. In most societies the use of such rehabilitation materials cannot be avoided, so special care to avoid oral cavity re-colonization is mandatory. Further studies with a larger number of yeast strains and more oral bacterial species are needed to further increase our understanding of the oral ecosystem and the clinically important micro-organisms/materials interactions.

Acknowledgements

We thank Wander J. Silva and Emilena M. Lima for their valuable input in the SEM evaluations. The first author thanks CAPES for the scholarship during the "Sandwich" PhD Program at the Academic Centre for Dentistry Amsterdam (ACTA, The Netherlands).

References

1. Thein ZM, Samaranayake YH, Samaranayake LP. Characteristics of dual species *Candida* biofilms on denture acrylic surfaces. *Arch Oral Biol* 2007; **52**: 1200-1208.

2. Wilkieson C, Samaranayake LP, MacFarlane TW, Lamey PJ, MacKenzie D. Oral candidosis in the elderly in long term hospital care. *J Oral Pathol Med* 1991; **20**: 13-16.
3. Tylenda CA, Larsen J, Yeh CK, Lane HC, Fox PC. High levels of oral yeasts in early HIV-1 infection. *J Oral Pathol Med* 1989; **18**: 520-524.
4. Thein ZM, Samaranayake YH, Samaranayake LP. Effect of oral bacteria on growth and survival of *Candida albicans* biofilms. *Arch Oral Biol* 2006; **51**: 672-680.
5. Li L, Redding S, Dongari-Bagtzoglou A. *Candida glabrata*: an emerging oral opportunistic pathogen. *J Dent Res* 2007; **86**: 204-125.
6. Chandra J, Mukherjee PK, Leidich SD, Faddoul FF, Hoyer LL, Douglas LJ, Ghannoum MA. Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. *J Dent Res* 2001; **80**: 903-908.
7. Catalan A, Herrera R, Martinez A. Denture plaque and palatal mucosa in denture stomatitis: scanning electron microscopic and microbiologic study. *J Prosthet Dent* 1987; **57**: 581-586.
8. Koopmans AS, Kippuw N, de Graaff J. Bacterial involvement in denture-induced stomatitis. *J Dent Res* 1988; **67**: 1246-1250.
9. Kulak Y, Arikan A, Kazazoglu E. Existence of *Candida albicans* and microorganisms in denture stomatitis patients. *J Oral Rehabil* 1997; **24**: 788-790.
10. Baena-Monroy T, Moreno-Maldonado V, Franco-Martinez F, Aldape-Barrios B, Quindos G, Sanchez-Vargas LO. *Candida albicans*, *Staphylococcus aureus* and *Streptococcus mutans* colonization in patients wearing dental prosthesis. *Med Oral Patol Oral Cir Bucal* 2005; **1**: E27-39.
11. Chesson P. General theory of competitive coexistence in spatially varying environments. *Theor Popul Biol* 2000; **58**: 211–37.
12. ten Cate JM. Biofilms, a new approach to the microbiology of dental plaque. *Odontology* 2006; **94**: 1-9.

13. Brown AJ. Morphogenetic signaling pathways in *Candida albicans*. In: Calderone R ed. *Candida* and candidiasis. Washington, DC: ASM Press, 2002; 95-106.
14. Bergman J. Morphogenesis and cell cycle progression in *Candida albicans*. *Curr Opin Microbiol* 2006; **9**: 595-601.
15. Blankenship JR, Mitchell AP. How to build a biofilm: a fungal perspective. *Curr Opin Microbiol* 2006; **9**: 588-594.
16. d'Enfert C. Biofilms and their role in the resistance of pathogenic *Candida* to antifungal agents. *Curr Drug Targets* 2006; **7**: 465-470.
17. Malmstrom HS, Mehta N, Sanchez R, Moss ME. The effect of two different coatings on the surface integrity and softness of a tissue conditioner. *J Prosthet Dent* 2002; **87**: 153-157.
18. Samaranayake LP, McCourtie J, MacFarlane TW. Factors affecting the *in vitro* adherence of *Candida albicans* to acrylic surfaces. *Arch Oral Biol* 1980; **25**: 611-615.
19. Waters MG, Williams DW, Jagger RG, Lewis MAO. Adherence of *Candida albicans* to experimental denture soft lining materials. *J Prosthet Dent* 1997; **77**; 306-312.
20. Serrano-Granger C, Cerero-Lapiedra R, Campo-Trapero J, Del Rio-Highsmith J. *In vitro* study of the adherence of *Candida albicans* to acrylic resins: relationship to surface energy. *Int J Prosthodont* 2005; **18**: 392-398.
21. Moura JS, da Silva WJ, Pereira T, Del Bel Cury AA, Rodrigues Garcia RC. Influence of acrylic resin polymerization methods and saliva on the adherence of four *Candida* species. *J Prosthet Dent* 2006; **96**: 205-211.
22. Pereira-Cenci T, Del Bel Cury AA, Cenci MS, Rodrigues-Garcia RC. *In vitro Candida* colonization on acrylic resins and denture liners: influence of surface free energy, roughness, saliva and adhering bacteria. *Int J Prosthodont* 2007; **20**: 308-310.
23. Millsap KW, Bos R, van der Mei HC, Busscher HJ. Adhesion and surface–aggregation of *Candida albicans* from saliva on acrylic surfaces with

adhering bacteria as studied in a parallel plate flow chamber. *Antonie van Leeuwenhoek* 1999; **75**: 351-359.

24. Edgerton M, Scannapieco FA, Reddy MS, Levine MJ. Human submandibular-sublingual saliva promotes adherence of *Candida albicans* to polymethylmethacrylate. *Infect Immun* 1993; **61**: 2644-2652.
25. Jin Y, Samaranayake LP, Samaranayake Y, Yip HK. Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. *Arch Oral Biol* 2004; **49**: 789-798.
26. Yildirim MS, Hasanreisoglu U, Hasirci N, Sultan N. Adherence of *Candida albicans* to glow-discharge modified acrylic denture base polymers. *J Oral Rehabil* 2005; **32**: 518-525.
27. Nikawa H, Yamamoto H, Hamada T. Effect of components of resilient denture-lining materials on the growth, acid production and colonization of *Candida albicans*. *J Oral Rehabil* 1995; **22**: 817.
28. Deng DM, Liu MJ, ten Cate JM, Crielaard W. The VicRK system of *Streptococcus mutans* responds to oxidative stress. *J Dent Res* 2007; **86**: 606-610.
29. Li YH, Lau PC, Tang N, Svensater G, Ellen RP, Cvitkovitch DG. Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. *J Bacteriol* 2002; **184**: 6333-6342.
30. Koo H, Schobel BD, Scott-Anne K, Watson G, Bowen WH, Cury JA, Rosalen PL, Park YK. Apigenin and tt-farnesol with fluoride effects on *S. mutans* biofilms and dental caries. *J Dent Res* 2005; **84**: 1016-1020.
31. Aps JK, Martens LC. Review: The physiology of saliva and transfer of drugs into saliva. *Forensic Sci Int* 2005; **150**: 119-131.
32. Thein ZM, Samaranayake YH, Samaranayake LP. *In vitro* biofilm formation of *Candida albicans* and non-*albicans Candida* species under dynamic and anaerobic conditions. *Arch Oral Biol* 2007; **52**: 761-767.
33. Kuhn DM, Balkis M, Chandra J, Mukherjee PK, Ghannoum MA. Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. *J Clin Microbiol* 2003; **41**: 506-8.

34. Wood S R, Kirkham J, Marsh P D, Shore R C, Nattress B, Robinson C. Architecture of intact natural human plaque biofilms studied by confocal laser scanning microscopy. *J Dent Res* 2000; **79**: 21–27.
35. Lefebvre CA, Wataha JC, Cibirka RM, Schuster GS, Parr GR. Effects of triclosan on the cytotoxicity and fungal growth on a soft denture liner. *J Prosthet Dent* 2001; **85**: 352-356.
36. Wright PS, Clark P, Hardie JM. The prevalence and significance of yeasts in persons wearing complete dentures with soft-lining materials. *J Dent Res* 1985; **64**:122-125.
37. Graham BS, Jones DW, Burke J, Thompson JP. *In vivo* fungal presence and growth on two resilient denture liners. *J Prosthet Dent* 1991; **65**: 528-532.
38. Kulak Y, Kazazoglu E. *In vivo* and *in vitro* study of fungal presence and growth on three tissue conditioning materials on implant supported complete denture wearers. *J Oral Rehabil* 1998; **25**: 135-138.
39. Luo G, Samaranayake LP. *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. *APMIS* 2002; **110**: 601-10.
40. He XY, Meurman JH, Kari K, Rautemaa R, Samaranayake LP. *In vitro* adhesion of *Candida* species to denture base materials. *Mycoses* 2006; **49**: 80-4.
41. Samaranayake LP. Nutritional factors and oral candidosis. *J Oral Pathol* 1986; **15**: 61-65.
42. Ramage G, Vande Walle K, Wickes BL, Lopez-Ribot JL. Biofilm formation by *Candida dubliniensis*. *J Clin Microbiol* 2001; **39**: 3234-3240.
43. Gocke R, Gerath F, von Schwanewede H. Quantitative determination of salivary components in the pellicle on PMMA denture base material. *Clin Oral Investig* 2002; **6**: 227-235.
44. Hamada S Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev* 1980; **44**: 331-384.

45. El Azizi MA, Starks SE, Khardori N. Interactions of *Candida albicans* with other *Candida* spp. and bacteria in the biofilms. *J Appl Microbiol* 2004; **96**: 1067–1073.
46. Kirkpatrick R, Lopez-Ribot L, Mcatee K, Patterson F. Growth competition between *Candida dubliniensis* and *Candida albicans* under broth and biofilm growing conditions. *J Clin Microbiol* 2000; **38**: 902-904.

Figures legends

Figure 1 – SEM images showing the materials' surfaces. (A) Note the irregularities on the materials (x40); (B) Details of the samples, showing typical examples of maximum irregularities/holes (x500).

Figure 2 – CLSM images showing the structure of *C. albicans* (red)/ *S. mutans* (green) biofilms on SL. (A) Confocal image at the surface of the soft liner material; note the colonization of micro-organisms in the holes; (B) Confocal image right above the surface of the soft liner material, containing almost solely *S. mutans* (10µm from the surface); (C) Similar image taken at the water biofilm interface containing almost solely *C. albicans* (57µm from the surface).

Figure 3 – CLSM images showing the biofilms' structure under various conditions, after 24 h of growth. All images were taken at 50-60 µm from the surface. (A) *C. albicans* plus *S. mutans* on HA-discs after growth with sucrose, without saliva; (B) *C. albicans* plus *S. mutans* on HA after growth with glucose, without saliva; (C) *C. albicans* plus *S. mutans* on HA after growth with glucose, with saliva, displaying a less compact structure. (D) Single-species *C. albicans* biofilm on HA with glucose; (E) *C. albicans* plus *S. mutans* on PMMA after growth with sucrose. *C. albicans* and *S. mutans* are shown in red and green, respectively.

Figure 4 - CLSM images showing the biofilm structure (without saliva) of *C. albicans* plus *C. glabrata* on (A) SL with sucrose (80 µm from the surface); (B) HA with glucose (46 µm from the surface). Note the hyphal elements.

Table 1 – Average ± standard error of *Candida albicans* CFU enumeration (x 10^6).

		Glucose		Sucrose	
Material	Type of Biofilm	Uncoated	Saliva Coated	Uncoated	Saliva Coated
HA	*C. albicans*	210.71 ± 19.33 Ab	312.46 ± 38.74 Aa	115.21 ± 11.56 Aa	54.92 ± 4.12 Ab
	C. albicans + *S. mutans*	395.00 ± 33.03 Aa	273.00 ± 19.27 Aa	124.44 ± 5.13 Aa	173.33 ± 5.20 Aa
	C. albicans + *C. glabrata*	121.11 ± 10.29 Ac	127.78 ± 5.59 Ab	175.56 ± 14.68 Aa	61.33 ± 6.08 Ab
SL	*C. albicans*	29.5 ± 4.22 Cb	50.51 ± 5.05 Cb	2.00 ± 0.22 Cb	0.78 ± 0.05 Cc
	C. albicans + *S. mutans*	41.24 ± 4.97 Cab	78.67 ± 5.93 Ca	1.82 ± 0.09 Cb	3.42 ± 0.08 Ca
	C. albicans + *C. glabrata*	60.67 ± 11.26 Ba	28.13 ± 1.46 Cc	4.58 ± 0.45 Ca	2.00 ± 0.17 Cb
PMMA	*C. albicans*	101.50 ± 27.60 Bb	113.78 ± 11.13 Ba	32.17 ± 5.86 Ba	15.00 ± 1,91 Bb
	C. albicans + *S. mutans*	143.79 ± 23.83 Ba	94.28 ± 32.51 Ba	30.38 ± 1.71 Ba	46.50 ± 5.96 Ba
	C. albicans + *C. glabrata*	38.89 ± 3.02 Cc	47.22 ± 7.58 Bb	19.61 ± 3.74 Bb	11.78 ± 0.59 Bc

Distinct upper case letters represent statistically significant differences among materials. Distinct lower case letters represents differences among types of biofilms (microbial combination). No significant effects of saliva were observed among experimental groups. All groups were statistically different regarding the sugar used (ANOVA; p<0.05).

Table 2 – Average ± standard error of *Candida glabrata* CFU enumeration (x 10^6).

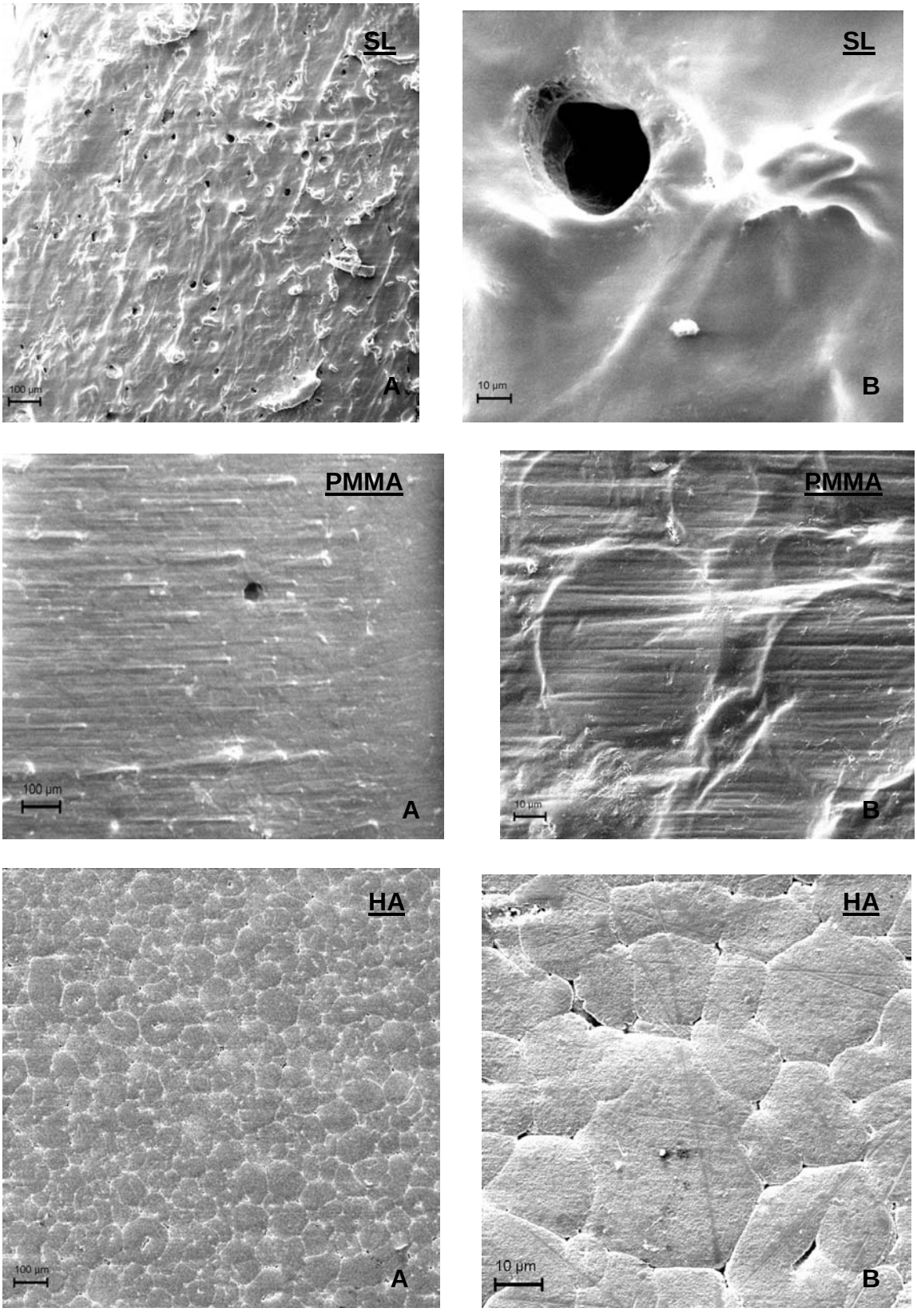
		Glucose		Sucrose	
Material	Type of Biofilm	Uncoated	Saliva Coated	Uncoated	Saliva Coated
HA	*C. glabrata*	339.58 ± 34.49 Ac	263.75 ± 16.12 Ab	251.33 ± 56.22 Ab	167.50 ± 7.08 Ab*
	C. glabrata + *S. mutans*	901.67 ± 75.49 Aa	487.62 ± 37.85 Aa*	304.76 ± 13.07 Aab	284.44 ± 11.25 Aa
	C. glabrata + *C. albicans*	533.33 ± 43.95 Bb	453.33 ± 22.80 Aa*	414.44 ± 37.88 Aa	176.67 ± 7.53 Ab*
SL	*C. glabrata*	94.88 ± 6.16 Bb	116.92 ± 7.92 Ba	5.53 ± 0.42 Cb	7.08 ± 0.27 Cb
	C. glabrata + *S. mutans*	109.76 ± 12.43 Cb	103.33 ± 11.09 Ca*	65.44 ± 9.25 Ca	5.80 ± 0.43 Cb*
	C. glabrata + *C. albicans*	411.73 ± 201.92 Aa	114.00 ± 7.98 Ba*	98.89 ± 9.19 Ba	23.56 ± 3.52 Ca*
PMMA	*C. glabrata*	304.67 ± 57.06 Aa	233.33 ± 51.37 Ab	64.17 ± 2.61 Bb	24.89 ± 0.56 Bc*
	C. glabrata + *S. mutans*	172.50 ± 32.40 Bb	130.11 ± 9.01 Bc*	81.25 ± 7.38 Ba	79.44 ± 6.70 Ba
	C. glabrata + *C. albicans*	232.91 ± 25.88 Cab	358.94 ± 176.94 Aa	69.04 ± 6.64 Bab	33.89 ± 2.78 Bb*

Distinct upper case letters represent statistically significant differences among materials. Distinct lower case letters represents differences among types of biofilms (microbial combination). (*) represents experimental groups that differed regarding saliva coating (p<0.001). All groups were statistically different regarding the sugar used (ANOVA; p<0.05).

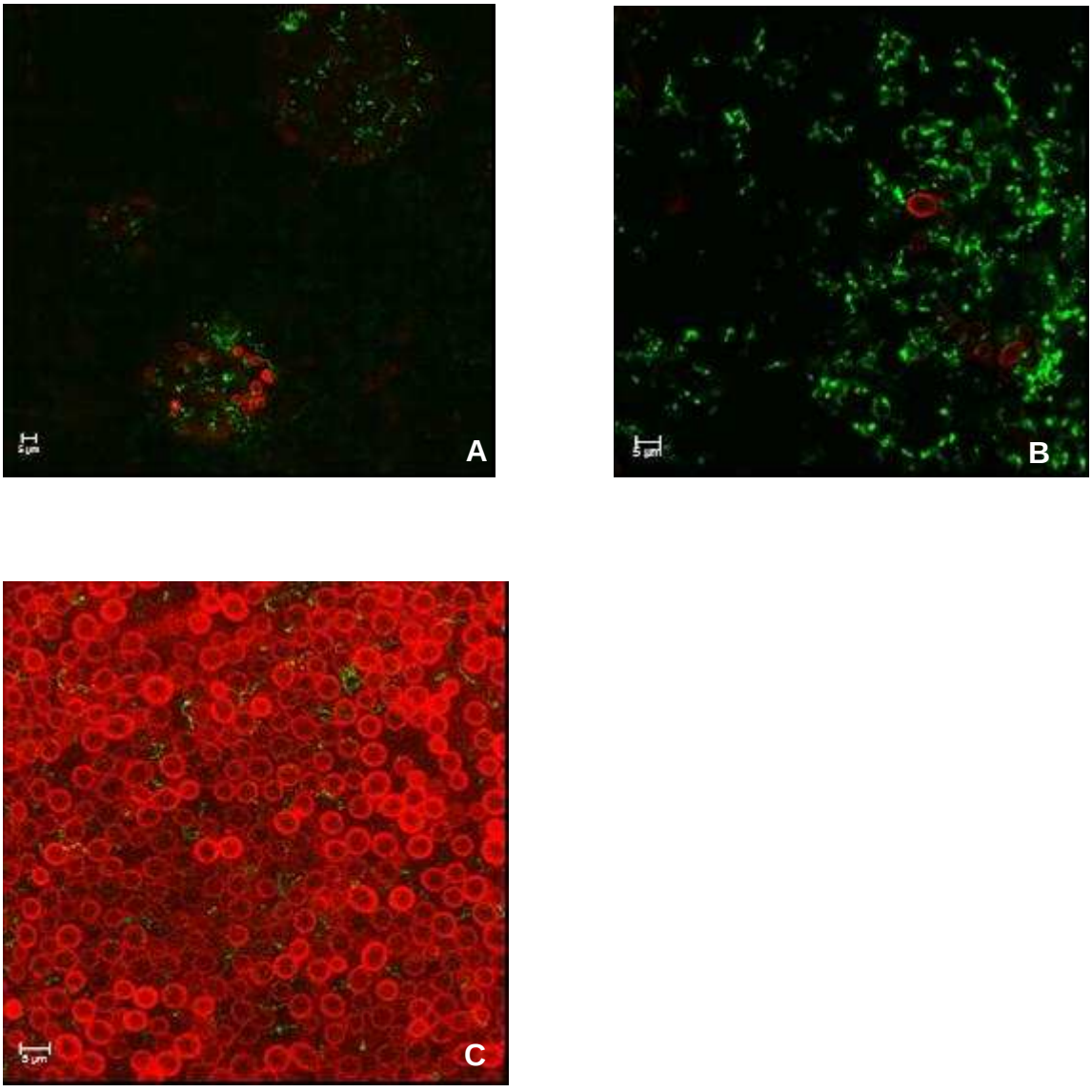
Table 3. Average ± SD of *Candida albicans* hyphae counts per field (mean value of 8-10 fields analyzed in each sample – 65 x 65 µm; z-step ranging from 0.5 to 2 µm).

		Glucose		Sucrose	
Material	Type of Biofilm	Uncoated	Saliva Coated	Uncoated	Saliva Coated
HA	*C. albicans*	15.50 ± 8.66Aa	14.25 ± 9.98Aa	13.00 ± 7.56Aa	9.80 ± 6.37Ab
	C. albicans + *S. mutans*	4.00 ± 2.91Ab	5.00 ± 2.00ABb	11.4 ± 6.29Aa§	11.33 ± 6.82Ba§
	C. albicans + *C. glabrata*	10.25 ± 6.13Aa	9.00 ± 6.68Aab	12.00 ± 4.24Aa	7.00 ± 2.83Aab
SL	*C. albicans*	13.80 ± 8.40Aa	15.00 ± 7.28Aa	10.60 ± 6.15Aa	12.2 ± 5.87Aa
	C. albicans + *S. mutans*	3.70 ± 3.16 Ab	2.40 ± 1.26Bb	8.40 ± 4.30Aa§	9.10 ± 3.98Aa§
	C. albicans + *C. glabrata*	6.50 ± 1.73Bb	9.25 ± 6.50Aa	8.00 ± 1.41Aa	12.00 ± 4 .24Ba
PMMA	*C. albicans*	4.00 ± 1.00Ba	13.40 ± 8.85Aa*	4.70 ± 4.19Ba	9.10 ± 6.85Aab*
	C. albicans + *S. mutans*	3.00 ± 2.36Aa	8.40 ± 7.75Aab*	5.40 ± 5.13Ba§	11.40 ± 6.29Aa*§
	C. albicans + *C. glabrata*	2.75 ± 1.50Ca	4.75 ± 1.50Bb*	4.50 ± 0.70Ba	6.00 ± 4 .24Ab*

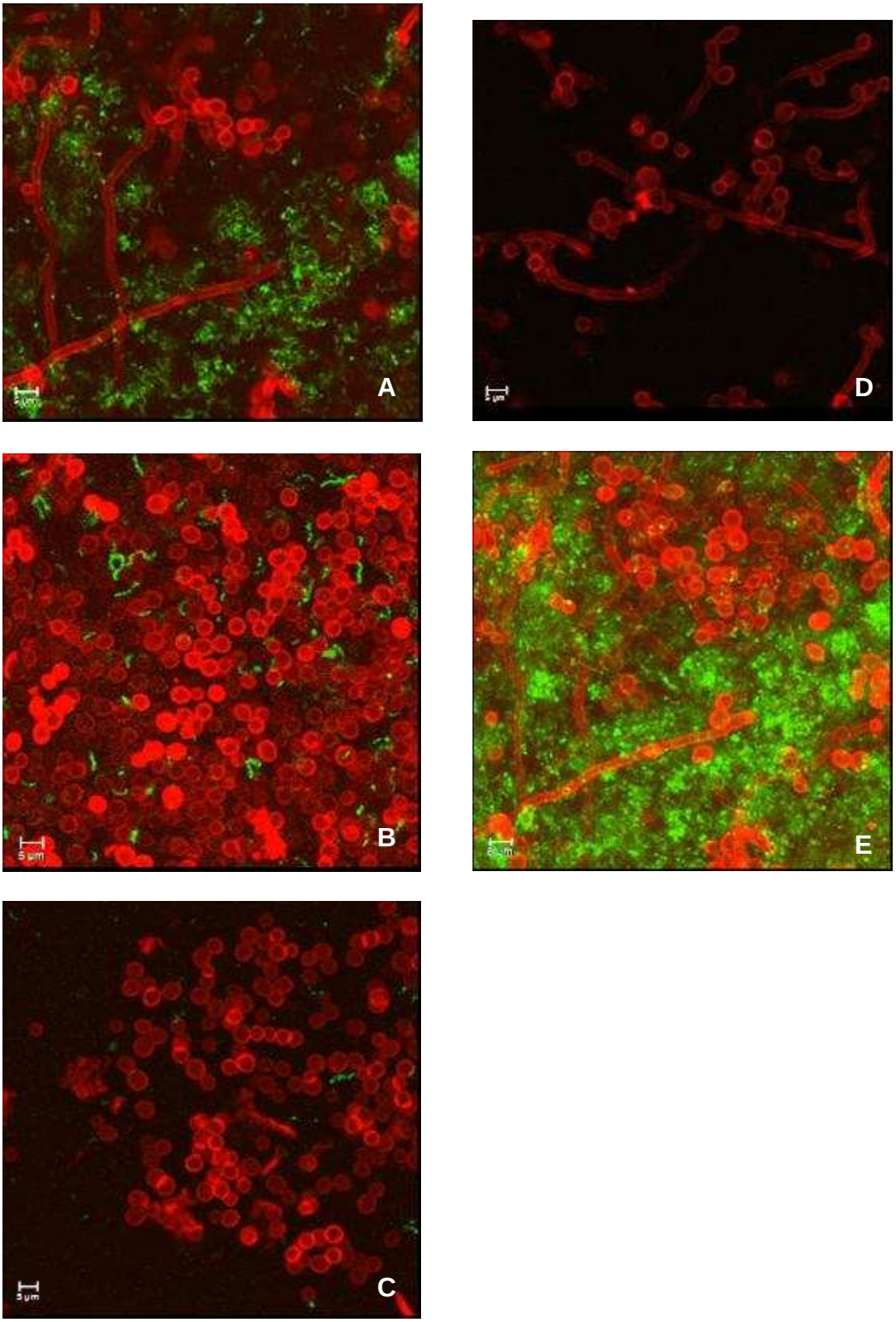
Distinct upper case letters represent statistically significant differences among materials. Distinct lower case letters represents differences among types of biofilms (microbial combination). ***** Indicates differences between saliva coating and uncoating; § Indicates differences between sugars (ANOVA; $p<0.05$).



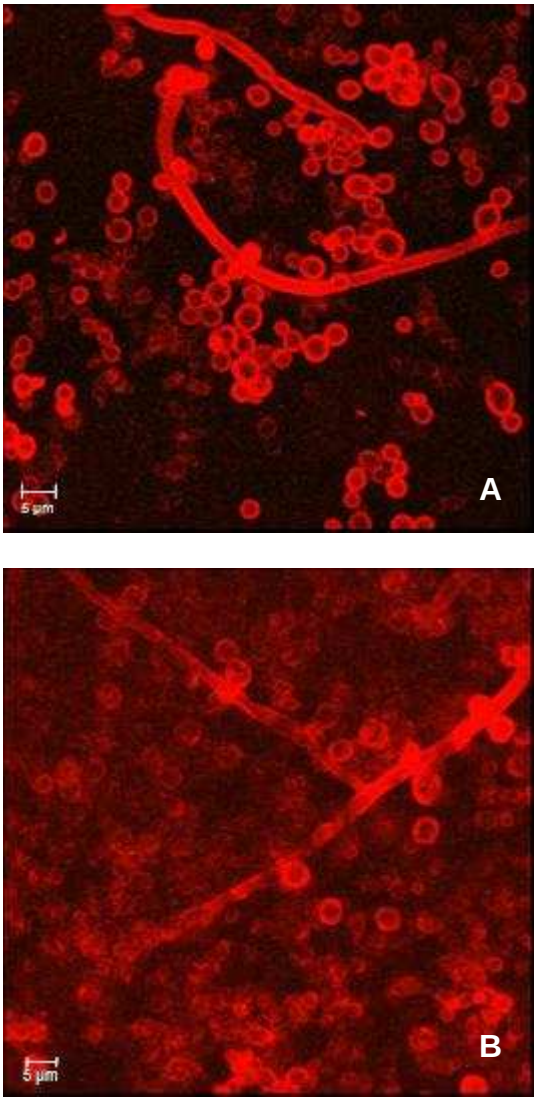
Pereira-Cenci *et al.* Figure 1



Pereira-Cenci *et al.* Figure 2



Pereira-Cenci *et al.* Figure 3



Pereira-Cenci *et al.* Figure 4

Temporal changes of different acrylic substrata and its relation to biofilm composition and development in complete denture wearers*

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Running title: Temporal changes in denture biofilms

Key words: biofilm, *Candida*, denture, *in situ*

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* Este artigo foi submetido ao periódico The International Journal of Prosthodontics

ABSTRACT

Temporal changes on denture biofilms are possibily affected by substratum types and surface properties, although this was not explored *in situ*. This study assessed how biofilm composition is affected in relation to various substrata and temporal changes in *Candida* colonisation up to 14 days in complete denture wearers. Twenty-one healthy volunteers but *Candida* carriers, wearing complete dentures participated in this study. Biofilm was formed on acrylic resin and denture liners (soft and hard) specimens mounted in the buccal surface of the volunteers' lower dentures in two phases of 14 days. Specimens were randomly removed on days 2, 7 and 14. Surface free energy (SFE) and roughness (Ra) of the materials were assessed before insertion and after removed in order to determine temporal changes on materials' SFE and Ra. Colony forming units/mg of biofilm of *Actinomyces*, total streptococci, mutans streptococci and *Candida* species were determined and expressed in absolute counts or percentages in relation to total micro-organisms. Substratum surfaces changed throughout the experiment Acrylic resin was smoother than the denture liners ($p<0.001$). In general, the soft liner showed the highest SFE values after biofilm collection at all time points ($p<0.05$). Percentages of total *Candida* species and *C. glabrata* recovered from the biofilm were higher after 7 and 14 days, respectively ($p<0.05$). Higher counts of total streptococci, *Actinomyces*, total micro-organisms and percentages of *Actinomyces*, were observed after 7 and 14 days ($p<0.05$). *Candida* species simultaneously colonised the biofilm, while *C. glabrata* was the only species evaluated to show progressively rising counts from the 2nd to the 14th day.

Introduction

Candida albicans is accepted as the main pathogen responsible for the development of denture stomatitis, which is one of the most common infections in the oral environment (Webb *et al*., 1998; Barbeau *et al*., 2003). Poorly fitting dentures and continuous denture wearing, the use of denture liners and poor oral hygiene facilitate denture plaque formation and therefore are the most frequent local causes of this opportunistic infection (Webb *et al*., 1998; Barbeau *et al*., 2003; Espinoza *et al*., 2003). Especially among the elderly, these predisposing factors are associated to systemic conditions as malignancies, broad-spectrum antibiotics, xerostomia, dietary factors, diabetes mellitus, iron and vitamin deficiencies (Bodey, 1984; Samaranayake, 1986; Scully and Cawson, 1998; Soysa *et al*., 2006), which often leads to severe candidal infections. In this context, it is particularly important to consider the factors governing *Candida* biofilm formation, especially in relation to substratum, interactions with other micro-organisms and host characteristics. Hence, it is clear that data on the role of these features related to the onset of the disease are still needed.

Although *C. albicans* is the predominant isolate in the elderly and denture stomatitis patients (Zaremba *et al*., 2006), other non-*albicans* species such as *Candida glabrata* (reported as the second most predominant species), *C. krusei* and *C. tropicalis* are also frequently isolated from acrylic denture surfaces and the palatal mucosa (Zaremba *et al*., 2006; Figueiral *et al*., 2007). Additionally, while *Candida* species are identified as the major cause of the disease (Webb *et al*., 1998), studies suggest a pathogenic association between bacteria and fungi in denture biofilm (Chandra *et al*., 2001; Espinoza *et al*., 2003; Barbeau *et al*., 2003).

On a given surface, the formation of multi-species biofilms improves the chances of survival for all the constituents in the oral environment and may be the first step for fungi colonisation leading to an infectious process (Cannon *et al*., 1999; Chandra *et al*., 2001; Ramage *et al*., 2004). As a result, *Candida* species may adhere directly or via a layer of denture plaque to the denture base materials (Samaranayake and MacFarlane, 1980; Branting *et al*., 1989; Edgerton *et al*., 1993). Nevertheless, little is known on the effect of different surfaces on

interactions among *Candida* species and other oral micro-organisms, including surfaces containing antimicrobials, such as several soft and hard denture liners. The use of denture liners is advantageous in many clinical situations and has increased in recent years. However, one of the problems directly associated to these materials is still the biofilm accumulation and *Candida* colonisation.

Despite extensive investigations on bacterial biofilms, the development of fungal-bacterial biofilms, and various factors affecting this process remain to be determined. Only limited attention has been paid to the important interactions between yeasts, substratum surfaces, oral bacteria and time (Holmes *et al*., 1985; Baena-Monroy *et al*., 2005; Yildirim *et al*., 2005; Pereira-Cenci *et al*., 2007). Prospective studies under *in vivo*-like conditions would bring significant contribution to the understanding of these interactions. Thus, this *in situ* study aimed to assess how biofilm composition is affected in relation to various substrata and temporal changes in *Candida* colonisation up to 14 days in complete denture wearers. Another aim was to assess if surface properties i.e., surface free energy and roughness of different substrata are affected by time.

Materials and methods

Experimental design

This *in situ*, double-blinded, crossover study was approved by the Local Research and Ethics Committee (Protocol 040/2006). The oral health of the volunteers was assessed, and all participants signed written informed consent before being accepted into the study. During 2 phases of 14 days each, 21 healthy adult volunteers wearing complete dentures had inserted in the buccal surface of their lower denture 6 acrylic resin specimens and 6 denture liner specimens (soft or hard denture liner, depending on the experimental phase). All specimens were previously assessed for their surface roughness and surface free energy. Specimens were placed 1 mm below the denture's acrylic level and covered by a plastic mesh to allow biofilm accumulation. Each complete denture received acrylic resin specimens in one side and one of the tested denture liners in the other side. In each phase, after 2, 7 and 14 days of biofilm accumulation, 2 specimens of each material were randomly chosen, removed and re-assessed for surface free energy

and surface roughness. The biofilm formed on the specimens was collected and processed for microbiological composition analysis, and the results were expressed in colony forming units (CFU)/mg biofilm and in percentage of *Actinomyces*, total streptococci, mutans streptococci and *Candida* species in relation to total micro-organisms.

Panellists and Ethical Aspects

Intra-oral examination was carried out for 48 subjects who were seeking for treatment (substitution of inadequate complete dentures) in the Faculty of Dentistry of Piracicaba, SP, Brazil. One examiner examined oral soft tissues and dental prostheses of all patients. These patients were screened for *Candida* species presence. This step allowed the inclusion of volunteers who had *Candida* species in their oral habitat, without however, having the clinical signs and symptoms of the disease it could cause (candidosis). The palatal mucosa, tongue, jugal mucosa and prostheses were swabbed and saliva was collected (also to determine salivary flow rate) at least 2 h after meals and oral hygiene procedures. Swabs were cultured in CHROMagar™ *Candida* (Difco, Sparks, MD, USA) at 37 ^{o}C for 48 h. Volunteers' whole saliva was collected during masticatory stimulation with Parafilm M (American Can Co., Greenwich. CT, USA) in an ice-chilled polypropylene tube and serially diluted in phosphate buffer saline (PBS). Samples (20 µl) were plated in CHROMagar™ *Candida* and incubated at 37℃ under aerobic conditions for 24-48 h. CFU were counted for swab and saliva samples using a stereomicroscope.

Forty-three patients were identified as *Candida* carriers and fulfilled inclusion criteria. These patients were invited to take part in this study and presented an average of *Candida* species of 69 CFU/mL of saliva. Twenty-one volunteers (mean age 65.5 ± 13.6; 16 female and 5 male) agreed to participate. Inclusion criteria included: adults (over 18 years old), of both genders, with complete dentures but who had not had a new or modified prosthesis within the previous 6 months, normal salivary flow rate (0.3 – 0.5 mL/min), good general and oral health, ability to comply with the experimental protocol, not having used antibiotics during the 2 months prior to the study, and not using any other type of

intraoral device. The exclusion criteria eliminated those who were taking any medication known to predispose them to oral candidosis, were taking antifungal agents or using antiseptic mouth-washes and had a medical history that revealed any disease or medical condition predisposing to oral candidosis (e.g. diabetes mellitus or iron and vitamin deficiencies).

Preparation of specimens

All materials were prepared by a single operator according to the manufacturers specifications at room temperature (25 ± 1.0ºC and 50 ± 5% relative humidity), under aseptic conditions. Microwave polymerized polymethylmetacrylate (Acron MC, GC America, Alsip, IL, USA) specimens were manufactured using a sheet of wax. Initially, squared patterns (4 x 4 x 2 mm) were cut of wax sheets and were invested in plastic flasks and subsequently boiled out. The acrylic resin was packed and once processed all flasks were allowed to bench cool for 150 min. Acrylic resin specimens were immersed in distilled water at 37^{o}C for 12 h for residual monomer release (Moura *et al.*, 2006).

Denture liner specimens (Coe Soft and Kooliner, GC America, Alsip, IL, USA) were prepared to be relined by the compression-mould technique using a glass mould with the same dimensions described for the acrylic resin preparation. Specimens of acrylic resin previously obtained were inserted into the glass mould and the denture liner was poured. An uniform surface was ensured by placing glass slides on both sides of the mould and firmly fixing both ends, then separating the glass slides after curing (Nikawa *et al.*, 1995).

Specimens were ground using progressively smoother aluminum oxide papers (320-, 400-, and 600-grit) in a horizontal polisher (APL-4; Arotec, Sao Paulo, Brazil). For mechanical polishing, a brush wheel (TMP-200; Equilam, Diadema, Brazil) with pumice slurry and a felt cone with chalk powder (Branco-Rio, OAB-ME, Sao Paulo, Brazil) were used. All specimens were polished by a single operator, except for the soft denture liner, where surface roughness was standardized by the contact with the glass slides (Pereira-Cenci *et al*., 2007). Specimens were prepared and immediately assessed for surface roughness (Ra) and surface free energy (SFE) prior to their insertion into the dentures (Fig. 1a).

Surface roughness

Surface roughness (Ra) of the specimens was measured using a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd, Kosaka, Japan) with a 0.01-mm resolution, calibrated with a cut-off value of 0.8 mm, 2.4-mm percussion of measure, and 0.5 mm/s. Three readings were made for each specimen, and a mean value was calculated (Verran and Maryan, 1997). Specimens were re-evaluated after each experimental phase (Fig. 1b).

Surface free energy

To characterize the wetting properties of the surfaces, contact angles were measured on each specimen. Water was chosen as the test liquid (Minagi *et al.*, 1985; Moura *et al.*, 2006). The experimental setup consisted of an adjustable stage where the samples were placed, and a droplet (5 µL) of deionized distilled water was dispensed on 0-degree tilt specimen surface by a micropipette (Moura *et al*., 2006). Photographs (Sony Cybershot F-717, SONY, Tokyo, Japan) of the droplets were taken immediately under standard conditions and contact angles were measured (AutoCAD 2005, Autodesk Inc., USA) from the left boundaries of the magnified photographs to the point of air-water-sample intersection (Fig. 1c). The mean value of three measurements for each surface was used to calculate substrata contact angle. Surface free energy (SFE) was calculated (Maple 9.5, Waterloo Maple Inc., Canada) using the cossine of the contact angles (Minagi *et al*., 1985).

After surface roughness and surface free energy measurements were completed, the specimens were randomly assigned to one of the experimental conditions. The contaminants were removed by sonication in sterilized deionized distilled water for 20 min previously to the adherence assay (Luo and Samaranayake, 2002).

Denture preparation and clinical phase

Each lower denture was prepared by manufacturing 6 recesses at each side of the buccal area of the denture. Each specimen was positioned and fixed with wax in the 5 x 5 x 3 mm recess created in the denture, leaving a 1 mm space for biofilm formation and accumulation (Figure 1d). This recessed space was

protected with a plastic mesh. The specimens were randomly distributed according to the phase the volunteer was designated. The volunteers received instructions to wear the dentures all the times, including at night. The subjects received oral and written information to refrain from using any antibacterial or antifungal product during the pre-experimental and experimental periods. Considering that the study followed a crossover design, with the volunteers participating in both steps, the subjects did not receive any instructions regarding their daily diet. During a 7-day pre-experimental period and the experimental period, the volunteers brushed their dentures with fluoride toothpaste, but the region containing the specimens protected by the plastic mesh were not brushed. A washout period of 7 days was allowed between the two phases to eliminate possible residual effects from the materials.

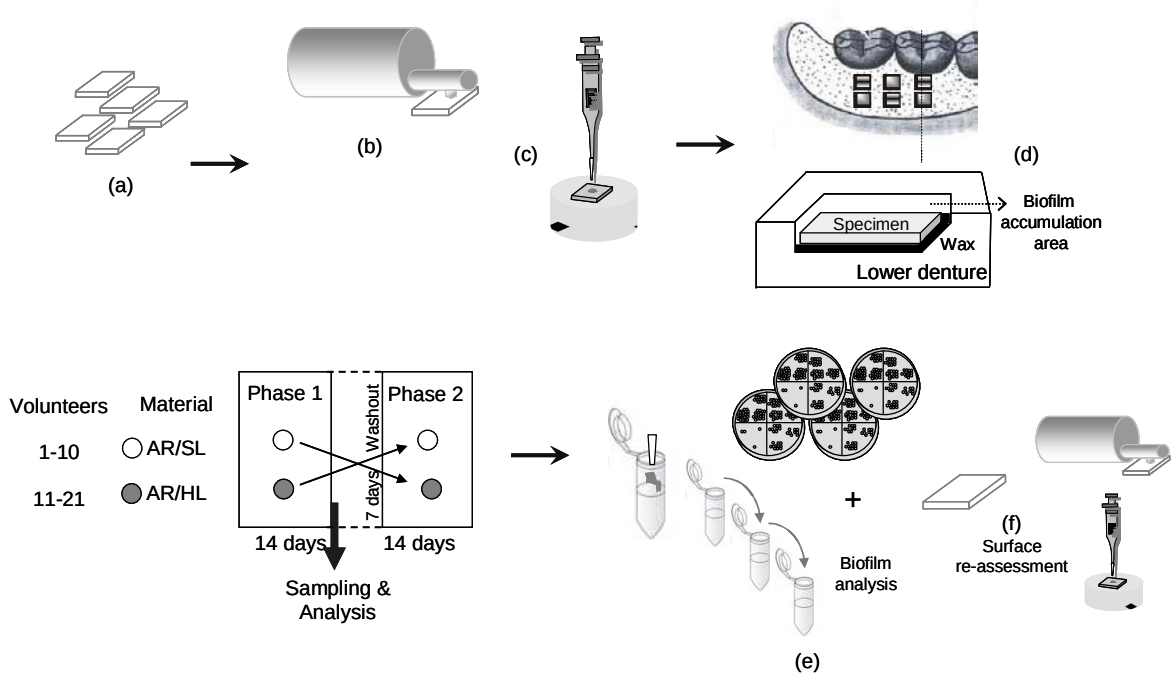

Figure 1. Illustration of the experimental design.

Microbiological analysis of the biofilm

The biofilm formed on the specimens was collected on the 2nd, 7th and 14th day of each experimental phase, in the morning and approximately 2 h after the last meal and hygiene procedures. Two specimens of each substratum

type (acrylic resin or denture liner) were randomly selected to be removed. Biofilm was collected with a plastic spatula by removing the acrylic mesh with a scalpel (Fig. 1e). The acrylic specimens were thoroughly clean with deionised distilled water and stored until re-assessment of Ra and SFE (Fig. 1f), and the recess in the denture cleaned and filled with wax. At the end of the second phase, all recesses were completed with acrylic resin, finished and polished until a new pair of dentures was manufactured.

Biofilm was weighed to ± 10 µg (Analytical Plus AP 250D, Ohaus Corp., Florham Park, N.J., USA) in sterile microcentrifuge tubes, suspended in PBS (phosphate buffer solution - 1 mL/mg biofilm, wet weight) and sonicated (Sonifier Vibra Cell, Sonics and Materials, Danbury, Conn., USA) at 40 W, 5% amplitude, 6 pulses of 9.9 s each. The suspensions were serially diluted in PBS and three drops of 20 µL were inoculated on blood agar (for enumeration of total micro-organisms), mitis salivarius agar (MSA, for total streptococci), mitis salivarius-bacitracin agar (MSB, for mutans streptococci), CFAT agar (*Actinomyces*) and CHROMagar™ *Candida* (*Candida* species). The plates were incubated at 37 ℃, in atmosphere of 10% CO_2 (MSB and MSA), in anaerobiosis (blood agar and CFAT) or aerobiosis (CHROMagar™ *Candida*) for 24–96 h. The CFU were counted using a stereomicroscope, and the results expressed in CFUs per milligram of denture biofilm. Different colony morphologies were identified by Gram staining and morphology and biochemical tests of sugar fermentation were used to confirm mutans streptococci and *Candida* species. *Candida* species that could not be differentiated by these tests were considered as “other *Candida* species”.

Statistical analysis

Statistical analyses were done using SAS software (SAS Institute Inc., version 9.0, Cary, NC, USA) employing a significance level fixed at 5%. The null hypotheses assumed no differences among substrata, micro-organisms or time point assessed. A randomized block design was used for the statistical analyses, considering the volunteers as statistical blocks, and time points and

substratum types as factors under study. For microbiological analysis, data that violated the assumptions of equality of variances and normal distribution of errors were transformed and analyzed by ANOVA, followed by Tukey test. Contact angles, surface free energy and surface roughness were assessed and compared before and after their insertion at each time point by paired t test or Wilcoxon signed rank test and by ANOVA on ranks to assess differences among materials in each evaluation time point. The null hypotheses were tested assuming no differences among materials or time points of biofilm formation.

Results

During the experiment, there was a withdrawal of 2 volunteers. One due to lack of compliance to the experiment, as the volunteer did not wear the denture during the trial. The other volunteer complained in the beginning of the trial about the plastic mesh covering the specimens and asked to stop participating in the study. Thus, these two volunteers were excluded and we considered the data of 19 subjects.

All volunteers presented at least two *Candida* species throughout the experiment. *C. albicans* was always present together with other species, except for one volunteer who did not have detectable *Candida* counts in any of the experimental phases. *Candida* species prevalence in the collected biofilm was assessed for each volunteer considering all experimental phases and the pooled prevalence results were as follows: *C. albicans* – 94.7%, *C. krusei* – 84.2%, *C. glabrata* – 79.0%, *C. tropicalis* – 26.3%, and other *Candida* species – 42.1%.

Table 1 shows the results for surface roughness (Ra) and surface free energy (SFE) for acrylic resin (AR), hard denture liner (HL) and soft denture liner (SL) at the three different time points. Acrylic resin was smoother than the hard and soft denture liner, before and after insertion in the denture and regardless of the time point considered ($p<0.001$). Acrylic resin was also smoother before than after the clinical experiment ($p<0.05$), while surface roughness for both HL and SL increased after the clinical trial only after 14 days of biofilm accumulation, in comparison to the baseline values ($p<0.05$). In the comparison among materials,

in general the SL showed the lowest SFE values before the specimens were subjected to the clinical trial ($p<0.05$), but SL also presented the highest SFE values for the same samples after the clinical trial and biofilm accumulation ($p<0.05$). The SL exhibited increased SFE values after the clinical trial compared to the baseline SFE determined before the clinical trial ($p<0.001$).

Table 2 shows the microbiological results for *Actinomyces* species, mutans streptococci, total streptococci and total micro-organisms recovered from biofilm, and the percentages of mutans streptococci in relation to total streptococci and total micro-organisms and percentage of *Actinomyces* in relation to total micro-organisms. There were statistical differences in total streptococci, *Actinomyces* and total micro-organisms counts and percentage of *Actinomyces* in relation to total micro-organisms counts considering the time point, where higher counts could be observed after 7 and 14 days ($p<0.05$). No statistical difference was found in the different time points for mutans streptococci and percentage of mutans streptococci in relation to total streptococci and total micro-organisms ($p>0.05$). Biofilm formed after 7 and 14 days was not statistically different for all bacteria and percentages tested, except for the percentage of *Actinomyces* in relation to total micro-organisms.

Table 1. Surface roughness (Ra – μm) and surface free energy (SFE – erg/cm^2) according to substratum type and time point of evaluation.

		2 Days		7 Days		14 Days	
		Before	After	Before	After	Before	After
AR	Ra	0.21 (0.18) ± 0.12a	0.41 (0.27) ± 0.33a*	0.21 (0.18) ± 0.11a	0.48 (0.29) ± 0.54a*	0.21 (0.19) ± 0.10a	0.40 (0.28) ± 0.38a*
	SFE	39.5 (39.6) ± 4.2c	46.6 (41.2) ± 24.8a*	39.9 (39.4) ± 3.8b	41.4 (38.9) ± 13.7a	40.7 (39.5) ± 12.9b	45.0 (39.8) ± 22.8a
HL	Ra	0.65 (0.55) ± 0.46b	0.71 (0.51) ± 0.57b	0.74 (0.67) ± 0.50b	0.83 (0.76) ± 0.62b	0.65 (0.39) ± 0.52b	1.7 (0.92) ± 3.7b*
	SFE	37.3 (37.1) ± 3.6b	44.8 (37.4) ± 25.5a	37.9 (37.7) ± 3.7b	39.1 (39.3) ± 6.1a	40.6 (37.4) ± 18.8b	41.6 (38.7) ± 18.9a
SL	Ra	1.3 (0.90) ± 1.4b	1.6 (0.72) ± 1.8b	1.3 (0.89) ± 1.1b	2.0 (1.2) ± 2.2b	1.5 (0.86) ± 1.5b	2.8 (1.9) ± 2.7b*
	SFE	32.9 (32.1) ± 3.9a	51.7 (43.0) ± 32.1a*	34.4 (34.2) ± 4.2a	59.2 (45.3) ± 37.4b*	34.2 (34.1) ± 3.8a	60.4 (46.3) ± 40.6b*

Values are mean (median) ± SD. Lower case letters show Ra, CA and SFE differences among materials before and after insertion at each time point (ANOVA on Ranks, p<0.05). * shows differences between Ra, CA and SFE "before" and "after" the specimens were subjected to the clinical trial, fixing the evaluation time points (Paired t test or Wilcoxon signed rank test, p<0.05).

Table 2. Microbiological results for bacteria in the biofilm according to the experimental conditions.

Time point	Material	Mutans streptococci (CFU x 10^4)	Total streptococci (CFU x 10^6)	*Actinomyces* (CFU x 10^6)	Total micro-organisms (CFU x 10^7)	% mutans streptococci/ Total streptococci	% mutans streptococci/ Total micro-organisms	% *Actinomyces*/ Total micro-organisms
2 days	AR	0.57 ± 1.21	3.03 ± 4.07	0.77 ± 1.94	0.64 ± 0.55	0.45 ± 0.88	0.12 ± 0.19	7.88 ± 14.75*
	HL	0.27 ± 0.57	2.17 ± 3.55	0.89 ± 1.80	1.12 ± 2.24	0.28 ± 0.55	0.18 ± 0.44	11.48 ± 19.61*
	AR	2.07 ± 6.64	9.08 ± 21.78	0.58 ± 1.63	1.04 ± 1.36	0.62 ± 1.90	0.21 ± 0.61	3.4 ± 8.13*
	SL	3.37 ± 11.02	4.4 ± 6.19	1.53 ± 2.84	2.53 ± 3.20	1.06 ± 2.49	0.37 ± 0.97	7.32 ± 14.82*
7 days	AR	0.33 ± 0.71	7.08 ± 8.47*	1.74 ± 6.22*	2.48 ± 3.39*	0.28 ± 0.86	0.03 ± 0.09	2.99 ± 7.42
	HL	0.24 ± 0.45	12.58 ± 14.92*	3.06 ± 7.28*	2.95 ± 3.49*	0.26 ± 0.82	0.03 ± 0.07	7.14 ± 14.86
	AR	2.74 ± 7.46	7.47 ± 13.22*	0.66 ± 1.73*	1.4 ± 1.39*	0.54 ± 1.11	0.51 ± 1.58	2.98 ± 5.56
	SL	4.02 ± 7.64	7.13 ± 5.74*	1.15 ± 2.46*	1.79 ± 1.62*	1.81 ± 5.36	0.37 ± 0.83	6.55 ± 12.35
14 days	AR	2.9 ± 6.53	17.57 ± 18.11*	5.73 ± 9.50*	4.23 ± 4.36*	0.39 ± 0.98	0.17 ± 0.53	18.4 ± 26.80*
	HL	8.35 ± 19.02	85.08 ± 268.67*	2.99 ± 5.42*	3.66 ± 3.83*	0.83 ± 2.32	0.33 ± 0.71	16.13 ± 30.45*
	AR	0.63 ± 1.69	9.59 ± 10.67*	3.68 ± 13.33*	1.73 ± 1.13*	0.62 ± 2.38	0.06 ± 0.18	28.65 ± 94.00*
	SL	0.49 ± 0.92	7.45 ± 4.97*	1.96 ± 3.16*	2.01 ± 1.60*	0.25 ± 0.84	0.09 ± 0.29	13.69 ± 22.33*

Values are mean ± SD (n=19). * Indicates differences considering the time point evaluated (p<0.05), for each response variable. No statistical difference was found in the different time points for *S. mutans* and percentage of *S. mutans* in relation to total streptococci and total micro-organisms (p>0.05).

Table 3 shows the microbiological results for *Candida* species and the percentage of *C. albicans* and all *Candida* species in relation to total micro-organisms. There was no difference in *C. albicans* counts and percentage of *C. albicans* in relation to total micro-organisms in all materials and time points studied ($p>0.05$). When considering the percentage of *Candida* species in relation to total micro-organisms, there was a statistical difference among time points of biofilm formation. Percentage of all *Candida* species in relation to total micro-organisms rose from day 2 to day 7, while *C. glabrata* counts showed a statistical difference in the biofilm when comparing day 14 with days 2 and 7. There was a larger proportion of *C. glabrata* in day 14.

Table 3. Microbiological analysis for *Candida* species in the biofilm according to the experimental conditions.

Time point	Material	*C. albicans* (CFU x 10^3)	*C. glabrata* (CFU x 10^3)	*C. tropicalis* (CFU x 10^3)	*C. krusei* (CFU x 10^3)	Other *Candida* species (CFU x 10^3)	% *C. albicans*/ Total micro-organisms	% *Candida* species/ Total micro-organisms
2 days	AR	1.06 ± 3.73	0.06 ± 0.26	2.69 ± 10.4	16.71 ± 48.51	4.07 ± 16.25	0.04 ± 0.12	0.65 ±1.99 A
	HL	0.82 ± 2.17	0.08 ± 0.29	3.24 ± 12.91	1.87 ± 7.06	5.32 ± 21.25	0.05 ± 0.12	0.30 ± 067 A
	AR	0.19 ± 0.43	1.74 ± 7.06	1.11 ± 4.71	1.8 ± 4.01	0 .001 ± 0.004	0.01 ± 0.01	0.07 ± 0.15 A
	SL	0.18 ± 0.33	0.08 ± 0.30	1.25 ± 5.00	0.82 ± 2.99	0.84 ± 3.33	0.01 ± 0.01	0.04 ± 0.10 A
7 days	AR	0.05 ± 0.18	3.14 ± 12.95	0.10 ± 0.40	49.99 ± 172 .88	1.20 ± 5.11	0.003 ± 0.01	0.13 ± 0.32 B
	HL	0.76 ± 2.18	19.49 ± 78.42	0.03 ± 0.08	14.27 ± 56 .38	0.20 ± 0.65	0.00001 ± 0.00001	1.35 ± 4.42 B
	AR	0.11 ± 0.23	12.22 ± 36.73	0.09 ± 0.39	14.76 ± 46 .08	6.49 ± 27.50	0.002 ± 0.01	0.36 ± 0.87 B
	SL	1.09 ± 3.30	17.59 ± 74.64	0.09 ± 0.39	40.72 ± 12 4.90	N/D	0.01 ± 0.03	0.50 ± 0.99 B
14 days	AR	8.03 ± 30.07	98.73 ± 229.56 §	N/D	4.77 ± 15.15	N/D	0.05 ± 0.19	0.79 ± 1.82 AB
	HL	10.01 ± 34.39	22.36 ± 13.12 §	N/D	17.16 ± 51.69	N/D	0.09 ± 0.32	0.46 ± 0.91 AB
	AR	0.38 ± 1.24	13.12 ± 53.74 §	2.94 ± 12.13	56.48 ± 146.50	6.18 ± 25.47	0.007 ± 0.02	0.58 ± 0.74 AB
	SL	0.58 ± 1.34	13.18 ± 55.78 §	0.83 ± 3.54	41.74 ± 126.19	2.04 ± 8.64	0.002 ± 0.01	0.43 ± 0.87 AB

Values are mean ± SD (n=19). N/D: Not detected; Upper case letters represent statistical differences among time points of biofilm formation regarding percentage of *Candida* species in relation to total micro-organisms. § represents differences among time points of biofilm formation (ANOVA; $p<0.05$).

Discussion

Our study is the first to show temporal changes on different substrata commonly used to fabricate and reline complete or partial dentures, and its association with biofilm formation and *Candida* colonisation. A recent study using acrylic resin samples of denture wearers has shown that different subjects present different biofilm formation rates, architecture and densities (Avon *et al*., 2007). However, the only substratum tested was acrylic resin and there was no attempt to characterize the surface properties, which might have resulted in a better understanding of the process. Clearly, understanding the biofilm behaviour of *Candida* species under various environmental conditions is the key to the development of effective preventive measures for *Candida* infections (Thein *et al.,* 2007).

Substratum surfaces changed throughout the experiment, namely after 14 days of biofilm accumulation. It is known that roughness is a crucial factor in the entrapment of micro-organisms and therefore protects from shear forces in the initial adherence (Quyrinen *et al*., 1990; Bollen *et al*., 1997; Verran and Maryan, 1997; Pereira-Cenci *et al*., 2007). *Candida* (or other micro-organism) is attached to the surface (e.g. dentures) via direct surface adhesion or co-aggregation. This is likely why studies on initial adherence of *Candida* species show direct correlation between surface roughness and *Candida* counts (Minagi *et al*., 1985; Verran and Maryan, 1997; Pereira-Cenci *et al*., 2007). Additionally, the nature of the substrata may influence the composition and the formation of the salivary pellicle, which may be more important for biofilm formation and *Candida* colonisation than the surface properties of the dental materials (Gocke *et al*., 2002). It has been shown that saliva decreases the surface roughness (Radford *et al*., 1998) and surface free energy (Sipahi *et al*., 2001) of acrylic resins, which may possibly explain the similar results for different micro-organisms counts in all materials tested in the present study, when considering the same time point evaluated.

It is important to highlight, however, that in stagnant areas of dentures, as we mimicked in our study, the denture plaque is likely to be more acidogenic and therefore favours streptococci and *Candida* species development

(Coulthwaite and Verran, 2007). Our results have shown that mutans streptococci varied from 0.03 to 0.51% of the total micro-organisms, but when considering total streptococci, this percentage raised to 58.4% (in average). In comparison to dental plaque, it is known that denture plaque exhibits high proportions of obligate anaerobes and *Actinomyces* species (Marsh and Martin, 1999). These findings corroborate our study where an *Actinomyces* species percentage of 10.55% (in average) was found in relation to total micro-organisms. It is important to emphasize that biofilm counts have changed for several of the studied micro-organisms, as happened with substratum surfaces throughout the experiment.

Denture plaque has a similar composition of dental plaque (Thelaide *et al*., 1983). In this study, the biofilm species recovered were *Streptococcus* species, gram-positive rods (*Actinomyces* spp.) and yeasts, which are known to be the predominant cultivable micro-organisms in denture plaque (Marsh and Martin, 1999). It is also important to assess the presence of mutans streptococci due to its importance in dental plaque, as it is aetiologically associated to dental caries. In removable partial denture wearers, the presence of mutans streptococci and its acid production may be related to dental caries of the remaining teeth (Nikawa *et al*., 1998). In addition, it has been shown that *S. mutans* may support growth of *C. albicans* (Pereira-Cenci *et al*., 2008).

Our results showed that *Candida* species constitutes less than 1% of the total micro-organisms found in the formed biofilm. This finding concurs with other studies where the same trend has occurred (Thelaide *et al*., 1983). The mere presence of *Candida* in the oral environment does not mean that the individual necessarily has or will develop *Candida*-related pathologies, as it depends on a complex fungi-bacteria-host interaction that modulates the host's response which may lead to inflammation. Nevertheless, if a slight inflammation is not controlled and plaque accumulation continues, this could have a detrimental impact on the patient's health. Moreover, they contribute as a significant mass to the biofilm as a result of their large size when compared with bacteria (Coulthwaite and Verran, 2007). When considering *Candida* species, our results support the idea that when compared to *C. albicans*, other species represent higher proportions on biofilm

formation. After 7 and 14 days, we have found that other species counts rose. This is important as a shift in disease-associated *Candida* species has been found from *C. albicans* towards non-*albicans* species (Samaranayake, 1997), supporting the idea that long time of biofilm accumulation due to lack of hygiene could be a predisposing factor to candidosis development. While *C. albicans* is the predominant isolate (Zaremba *et al*., 2006; Figueiral *et al*., 2007) other species as *C. glabrata* emerges as one of the most prevalent species isolated from acrylic resin surfaces and the palatal mucosa (Li *et al*., 2007). Besides this shift, increasing evidence confirms that more than one *Candida* species may simultaneously colonise oral habitats (Dronda *et al*., 1996; Schmidt-Westhausen *et al*., 2004), as also occurred in our study. *Candida* species simultaneously colonised the biofilm, while *C. glabrata* was the only species evaluated to show rising counts from the 2nd to the 14th day, progressively increasing in number in the biofilm. Pathogens as *C. glabrata* may exhibit higher denture surface adherence and acquired resistance against antifungal drugs (Li *et al*., 2007), which may explain our results. Our results also suggest that *C. glabrata* could be more competitive into the biofilm community and its complexity, since it was the most prevalent species found after 14 days of biofilm formation.

Oral anti-mycotic agents seem helpful, but recurrence is rapid and assured unless the denture is modified (Kulak *et al.*, 1994). Additionally, compliance with antifungal regimens can be hampered by patients' non-perception of the disease. Therefore, there has been a tendency toward the incorporation of antimicrobial agents into the denture liners or the resin itself (Etienne *et al*., 2005). Once the biofilm is formed there could be two reasons why the incorporation of antimicrobials may be ineffective: (i) the nutrient rich environment of the oral cavity might overpower any inhibitory effect present in the denture liners; (ii) when there are insufficient host defences, this will lead to proliferation of *Candida* in a multi-species biofilm embedded in a self-produced polymeric matrix with increased resistance to antimycotics (Graham *et al*., 1991).

Our study was designed to evaluate biofilm formation in order to verify on which parameters biofilm formation depends. We have simulated a niche

with lack of cleaning and constant plaque accumulation, as an ill-fitting or poorly cleaned denture. As a result, it would be possible to identify the stages when *Candida* and other emerging pathogenic species can be targeted in treatment and prevention. Obviously it would have been highly interesting to study this colonisation in denture stomatitis patients, but it would be unethical since patients with stomatitis would demand immediate treatment. One of the most interesting findings in our study was related to the other *Candida* species rather than *C. albicans*. It seems from our study that *C. albicans* is the first to attach as our results showed that they were more prevalent in the 2-day biofilm. However, in the days 7 and 14, higher absolute values and proportion for other species (mainly *C. glabrata* and *C. krusei*) were recovered in comparison to *C. albicans*. It is known, according to the ecological plaque hypothesis (Marsh, 1994) that the proportions of pathogenic micro-organisms will dictate the changes that will turn health to disease rather than the presence of any particular species. This highlights the need for effective physical removal of denture plaque, which may be associated to chemical cleansing in regular basis (e,g. NaOCl solution immersion).

Symptom-free oral carriage of *Candida* has been recognised for many years. As the elderly population is rapidly rising (Oeppen and Vaupel, 2002) and therefore their need for dental treatment, studies considering their oral hygiene are becoming increasingly important. It is important to point out that the clinical setting where this study was conducted has a dental practice focused on health promotion, with a preventive approach based on the control and prevention of denture stomatitis. Volunteers in this study not only received a new pair of dentures but education on, and maintenance of proper oral hygiene and health status, which is essential for denture wearers (Coulthwaite and Verran, 2007).

Acknowledgements

The authors thank the volunteers for their valuable participation. This work was based on a thesis in partial fulfilment of the requirements for the PhD degree of the first author (FAPESP 06/00396-8; 06/03043-9).

References

1. Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW. *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 2. Oral diseases caused by *Candida* species. Aust Dent J 1998; 43: 160-166.
2. Barbeau J, Séguin J, Goulet JP, de Koninck L, Avon SL, Lalonde B, Rompré P, Deslauriers N. Reassessing the presence of *Candida albicans* in denture-related stomatitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003; 95:51-59.
3. Espinoza I, Rojas R, Aranda W, Gamonal J. Prevalence of oral mucosal lesions in elderly people in Santiago, Chile. J Oral Pathol Med 2003; 32:571-575.
4. Bodey GP. Candidiasis in cancer patients. Am J Med 1984; 77:13-19.
5. Samaranayake LP. Nutritional factors and oral candidosis. J Oral Pathol 1986; 15:61–65.
6. Scully C, Cawson RA. Medical problems in dentistry. 4th ed. Boston: Butterworth-Heinemann, 1998; 408-437.
7. Soysa NS, Samaranayake LP, Ellepola AN. Diabetes mellitus as a contributory factor in oral candidosis. Diabet Med 2006; 23:455-459.
8. Zaremba ML, Daniluk T, Rozkiewicz D, Cylwik-Rokicka D, Kierklo A, Tokajuk G, Dabrowska E, Pawińska M, Klimiuk A, Stokowska W, Abdelrazek S. Incidence rate of *Candida* species in the oral cavity of middle-aged and elderly subjects. Adv Med Sci 2006; 51:233-236.
9. Figueiral MH, Azul A, Pinto E, Fonseca PA, Branco FM, Scully C. Denture-related stomatitis: identification of aetiological and predisposing factors - a large cohort. J Oral Rehabil 2007; 34:448-55.
10. Chandra J, Mukherjee PK, Leidich SD, Faddoul FF, Hoyer LL, Douglas LJ, Ghannoum MA. Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. J Dent Res 2001; 80:903-908.
11. Cannon RD, Chaffin WL. Oral colonization by *Candida albicans*. Crit Rev Oral Biol Med 1999; 10:359-383.

12. Ramage G, Tomsett K, Wickes BL, López-Ribot JL, Redding SW. Denture stomatitis: a role for *Candida* biofilms. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004; 98:53-59.
13. Samaranayake LP, MacFarlane TW. An *in-vitro* study of the adherence of *Candida albicans* to acrylic surfaces. Arch Oral Biol 1980; 25:603-609.
14. Branting C, Sund ML, Linder LE. The influence of *Streptococcus mutans* on adhesion of *Candida albicans* to acrylic surfaces *in vitro*. Arch Oral Biol 1989; 34: 347-353.
15. Edgerton M, Scannapieco FA, Reddy MS, Levine MJ. Human submandibular-sublingual saliva promotes adherence of *Candida* albicans to Polymethylmethacrylate. Infect Immun 1993; 61: 2644-2652.
16. Holmes AR, Gopal PK, Jenkinson HF. Adherence of *Candida albicans* to a cell surface polysaccharide receptor on *Streptococcus gordonii*. Infect Immun 1995; 63:1827-1834.
17. Baena-Monroy T, Moreno-Maldonado V, Franco-Martinez F, Aldape-Barrios B, Quindos G, Sanchez-Vargas LO. *Candida albicans*, *Staphylococcus aureus* and *Streptococcus mutans* colonization in patients wearing dental prosthesis. Med Oral Patol Oral Cir Bucal 2005; 1: E27-39.
18. Yildirim MS, Hasanreisoglu U, Hasirci N, Sultan N. Adherence of *Candida albicans* to glow-discharge modified acrylic denture base polymers. J Oral Rehabil 2005; 32:518-525.
19. Pereira-Cenci T, Del Bel Cury AA, Cenci MS, Rodrigues-Garcia RC. In vitro Candida colonization on acrylic resins and denture liners: influence of surface free energy, roughness, saliva and adhering bacteria. Int J Prosthodont 2007; 20: 308-310.
20. Moura JS, da Silva WJ, Pereira T, Del Bel Cury AA, Rodrigues Garcia RC. Influence of acrylic resin polymerization methods and saliva on the adherence of four *Candida* species. J Prosthet Dent 2006; 96: 205-211.

21. Nikawa H, Yamamoto H, Hamada T. Effect of components of resilient denture-lining materilas on the growth, acid production and colonization of *Candida albicans*. J Oral Rehabil 1995; 22:817.
22. Verran J, Maryan CJ. Retention of *Candida albicans* on acrylic resin and silicone of different surface topography. J Prosthet Dent 1997; 77:535-539.
23. Minagi S, Miyake S, Inagaki K, Tsuru H, Suginaka H. Hydrophobic interaction in *Candida albicans* and *Candida tropicalis* adherence to various denture base resin materials. Infect Immun 1985; 47:11-14.
24. Luo G, Samaranayake LP. *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. APMIS 2002; 110:601-610.
25. Avon SL, Goulet JP, Deslauriers N. Removable acrylic resin disk as a sampling system for the study of denture biofilms *in vivo*. J Prosthet Dent 2007; 97:32-38.
26. Thein ZM, Samaranayake YH, Samaranayake LP. *In vitro* biofilm formation of *Candida albicans* and non-*albicans Candida* species under dynamic and anaerobic conditions. Arch Oral Biol 2007; 52:761-767.
27. Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Darius PL, van Steenberghe D. The influence of surface free energy and surface roughness on early plaque formation. An *in vivo* study in man. J Clin Periodontol 1990; 17:138-144.
28. Bollen CM, Lambrechts P, Quirynen M. Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: a review of the literature. Dent Mater 1997;13: 258-269.
29. Gocke R, Gerath F, von Schwanewede H. Quantitative determination of salivary components in the pellicle on PMMA denture base material. Clin Oral Investig 2002; 6:227-235.

30. Radford DR, Sweet SP, Challacombe SJ, Walter JD. Adherence of *Candida albicans* to denture-base materials with different surface finishes. J Dent 1998; 26:577-583.

31. Sipahi C, Anil N, Bayramli E. The effect of acquired salivary pellicle on the surface free energy and wettability of different denture base materials. J Dent 2001; 29:197-204.

32. Coulthwaite L, Verran J. Potential pathogenic aspects of denture plaque. Br J Biomed Sci 2007; 64:180-189.

33. Marsh P, Martin MV. Oral microbiology. Oxford: Wright, 1999.

34. Theilade E, Budtz-Jørgensen E, Theilade J. Predominant cultivable microflora of plaque on removable dentures in patients with healthy oral mucosa. Arch Oral Biol 1983; 28:675-680.

35. Nikawa H, Hamada T, Yamamoto T. Denture plaque--past and recent concerns. J Dent 1998; 26:299-304.

36. Pereira-Cenci T, Deng DM, Kraneveld EA, Manders EMM, Del Bel Cury AA, ten Cate JM, Crielaard W. The effect of *Streptococcus mutans* and *Candida glabrata* on *Candida albicans* biofilms formed on different surfaces. Arch Oral Biol 2008; *in press*.

37. Samaranayake LP. *Candida krusei* infections and fluconazole therapy. Hong Kong Med J 1997; 3:312–314.

38. Li L, Redding S, Dongari-Batgtzoglou. *Candida glabrata*, an emerging oral opportunistic pathogen. J Dent Res 2007; 86:204-215.

39. Dronda F, Alonso-Sanz M, Laguna F, Chaves F, Martinez-Suarez JV, Rodriguez-Tudela JL, Gonzalez-Lopez A, Valencia E. Mixed oropharyngeal candidiasis due to *Candida albicans* and non-*albicans Candida* strains in HIV-infected patients. Eur J Clin Microbiol Infect Dis 1996;15: 446–452.

40. Schmidt-Westhausen AM, Bendick C, Reichart PA, Samaranayake LP. Oral candidosis and associated *Candida* species in HIV-infected Cambodians exposed to antimycotics. Mycoses 2004; 47:435–441.

41. Kulak Y, Arikan A, Delibalta N. Comparison of three different treatment methods for generalized denture stomatitis. J Prosthet Dent 1994; 72:283-288.

42. Etienne O, Gasnier C, Taddei C, Voegel JC, Aunis D, Schaaf P, Metz-Boutigue MH, Bolcato-Bellemin AL, Egles C. Antifungal coating by biofunctionalized polyelectrolyte multilayered films. Biomaterials 2005; 26:6704-6712.

43. Graham BS, Jones DW, Burke J, Thompson JP. *In vivo* fungal presence and growth on two resilient denture liners. J Prosthet Dent 1991; 65: 528-532.

44. Marsh PD. Microbial ecology of dental plaque and its significance in healthy and disease. Adv Dent Res 1994; 8: 263–271.

45. Oeppen J, Vaupel JW. Demography. Broken limits to life expectancy. Science 2002; 296:1029-31.

CONSIDERAÇÕES GERAIS

O interesse na candidose oral parece ter aumentada evidência pelo número de publicações no assunto. Durante a última década, a US National Library of Medicine (www.pubmed.com) publicou 240 artigos somente numa pesquisa contendo os termos *Candida* e *dentadura*. De fato, este é um número bastante expressivo quando comparado à década anterior, que resultou em 113 publicações. Existe um interesse recente nos fatores que governam o processo de adesão de espécies de *Candida*, especialmente em relação às interações entre hospedeiro e substrato. No entanto, poucos estudos avaliaram esses fatores relacionando-os ao início e progressão da doença.

Diferentes espécies de *Candida* são comumente encontradas na cavidade oral, com prevalências descritas entre 15 e 77,5% (Radford *et al*., 1999 Zaremba *et al*., 2006). Estas diferentes espécies são recuperadas de vários sítios da cavidade oral, como dentes, língua, mucosa jugal, palato e de todos os biomateriais utilizados para reparo e confecção de próteses. *Candida* também é encontrada associada a cáries de raiz e próxima ou dentro do sulco gengival (Zaremba *et al*., 2006b; Shen *et al*., 2002). Em indivíduos saudáveis e com dentição completa, a presença de *Candida* raramente provoca doença. A patologia induzida por *Candida* de maior prevalência está, portanto, associada a pacientes imunocomprometidos e que possuam outros fatores predisponentes à iniciação da doença. Como exemplo, podemos citar a candidose em pacientes HIV positivos (Sroussi e Epstein, 2007). Somado a isso, a hipossalivação e edentulismo são fatores de risco entre indivíduos. No caso de desdentados, espécies de *Candida* geralmente causam estomatite no tecido em contato com a prótese (Espinoza *et al*., 2003; Barbeau *et al*., 2003).

É sabido que a prevalência de espécies de *Candida* está entre 11 e 67% (Ramage, 2006), em pacientes saudáveis, sem qualquer prótese. Entretanto, a presença do fungo não significa que o indivíduo possui ou possuirá a doença. Tipicamente isto dependerá de interações complexas entre microrganismos e hospedeiro que modulam a resposta deste último levando à inflamação. Dependendo das condições locais, as bactérias podem fornecer aos fungos compostos que influenciam nos fatores de virulência. Dentre estas se destacam:

(i) na sobrevivência dos fungos e leveduras, com a produção de compostos antifúngicos, modificação do meio ambiente e promovendo morte ou sobrevivência e proteção contra antibióticos em biofilmes mistos; (ii) na morfologia dos fungos e leveduras, produzindo compostos específicos moduladores de morfologia bem como modificações no meio ambiente (pH, nutrientes, etc); (iii) direta ou indiretamente na produção de fatores de virulência e pela formação pelas bactérias de moléculas que podem ser precursoras na produção de metabólitos secundários fúngicos; (iv) no crescimento, pela alteração de níveis de nutrientes e fatores bacterianos que dificultam o crescimento fúngico; (v) na aderência através da coagregação e competitividade por sítios de adesão (Wargo e Hogan, 2006). Isto se torna importante não apenas nas infecções por *Candida*, mas no que concerne o porquê da *Candida* ser responsável por outras infecções induzidas pela microflora natural do indivíduo (Fridkin e Jarvis, 1996).

Enquanto a grande maioria dos estudos foca *C. albicans*, outras espécies de *Candida* também vêm sendo estudadas. Isto reflete a aumentada prevalência das espécies de *Candida* não-*albicans*, diretamente associadas às patologias das mucosas na cavidade oral (Rasool *et al*., 2005). Estes estudos vêm mostrando que outras espécies de *Candida* respondem diferentemente no que concerne a resistência aos fármacos da família dos azóis e ainda, que *C. albicans*, *C. glabrata* e *C. tropicalis* diferem em relação à histatina e β-defensina humanas (Sanglard *et al*., 1999; Joly *et al*., 2004; Feng *et al*., 2005; Helmerhorst *et al*., 2005; Thiele *et al*., 2008). Apesar de as espécies de *Candida* já estarem identificadas como causa da estomatite por dentadura desde 1936 (Cahn, 1936), grande progresso no entendimento da etiologia e patogênese da doença somente ocorreu recentemente. Sem dúvida, este é o resultado do emprego de diversas metodologias de biologia molecular e disponibilidade de dados genômicos. Adicionalmente, as espécies de *Candida* num ambiente oral residirão em forma de biofilmes mistos com interações entre fungos e bactérias ditando as propriedades e sobrevivência das espécies (Wargo e Hogan, 2006). A adesão inicial, crescimento e maturação são geneticamente controladas tanto em fungos quanto em bactérias. Da mesma forma, o crescimento e fisiologia dos microrganismos

são controlados por *quorum sensing*, peptídeos que sentem e respondem quando densidades máximas de microrganismos são atingidas (Blankenship e Mitchell, 2006). Adicionalmente, alguns fungos têm a modificação entre blastoporo e hifa como fator de virulência adicional, que faz com que haja diferente sensibilidade aos tratamentos antifúngicos. Para complicar este quadro, os fungos já aderidos no substrato ativam respostas genéticas levando às modificações morfológicas supracitadas. Esta cascata de eventos é decisiva na formação de biofilme e/ou penetração nos tecidos subjacentes (Nobile e Mitchell, 2006; Kumamoto e Vinces, 2005).

A formação de biofilme é o mecanismo que permite a sobrevivência de microrganismso dentro da cavidade bucal. Em biofilmes, bactérias e fungos se encontram encapsulados numa matriz de glicoproteínas segregadas pelos componentes microbianos, geralmente residindo em um estado de atividade metabólica dormente. Isto representa um fator protetor adicional aos fungos presentes em biofilmes complexos, que como as bactérias, estarão menos sensíveis aos tratamentos com antimicrobianos. Considerando o controle da doença, isto implica que as infecções originadas de infecções fúngicas somente poderão ser curadas substituindo ou modificando características do dispositivo médico, no caso da Odontologia, as próteses.

A aderência à superfície é crucial para que fungos e bactérias permaneçam e sobrevivam na cavidade oral. Entretanto, as espécies de *Candida* também aderem a outros dispositivos médicos como próteses de voz, cateteres sanguíneos e urinários e válvulas cardíacas. Desta maneira, o estudo da aderência de *Candida* à superfícies tem um significado muito maior do que somente a relevância oro-dental. A presença de *Candida* na cavidade oral serve de reservatório para inoculação e infecções em outros locais do corpo humano. Quando a *Candida* penetra o epitélio e invade os tecidos do hospedeiro, isto pode levar à disseminação para corrente sanguínea e candidemia. A candidemia tem difícil tratamento com antifúngicos e tem alta mortalidade (40%) (Lafleur *et al*., 2006; Pfaller e Diekema, 2007). Assim, isto explica e justifica a crescente atenção dada às espécies de *Candida* no meio bucal.

CONCLUSÃO GERAL

O presente estudo sugere haver influência da saliva, substrato e de várias espécies de microrganismos na formação de biofilme de *Candida*. Somado a isso, os resultados indicam que fatores relacionados ao substrato podem influenciar na formação de biofilme no que concerne às diversas espécies de microrganismos presentes na cavidade oral. Os resultados deste estudo suportam ainda que embora a *C. albicans* seja mais prevalente nos estágios iniciais de formação de biofilme, outras espécies de *Candida* tornam-se mais prevalentes em biofilmes com maior tempo de formação.

REFERÊNCIAS*

1. McMichael AJ, McKee M, Shkolnikov V, Valkonen T. Mortality trends and setbacks: global convergence or divergence? Lancet. 2004; 363(9415): 1155-9.
2. Nucci M, Marr KA. Emerging fungal diseases. Clin Infec Dis. 2005; 41(4) :521-6.
3. Cheng MF, Yang YL, Yao TJ, Lin CY, Liu JS, Tang RB, Yu KW, Fan YH, Hsieh KS, Ho M, Lo HJ. Risk factors for fatal candidemia caused by *Candida albicans* and non-*albicans Candida* species. BMC Infec Dis. 2005; 5(1): 22.
4. Wey SB, Mori M, Pffaler MA, Woolson RF Wenzel RP. Hospital acquired candidemia: the attributable mortality and excess length of stay. Arch Intern Med. 1988; 148(12): 2642-5.
5. Leleu G, Aegerter P, Gudet B, Collège des Utilisateurs de Base de Données en Réanimation. Systemic candidiasis in intensive care units: a multicenter, mached-cohort study. J Crit Care. 2002; 17(3): 168-75.
6. Muzyka BC. Oral fungal infections. Dent Clin North Am. 2005; 49(1): 49-65.
7. Budtz-Jørgensen E. Oral mucosal lesions associated with the wearing of removable dentures. J Oral Pathol. 1981; 10(2): 65-80.
8. Jeganathan S, Lin CC. Denture stomatitis - a review of the aetiology, diagnosis and management. Aust Dent J. 1992; 37(2): 107-14.
9. Emami E, Séguin J, Rompré PH, de Koninck L, de Grandmont P, Barbeau J. The relationship of myceliated colonies of *Candida albicans* with denture stomatitis: an *in vivo*/*in vitro* study. Int J Prosthodont. 2007; 20(5): 514-20.
10. Pires FR, Santos EB, Bonan PR, De Almeida OP, Lopes MA. Denture stomatitis and salivary *Candida* in Brazilian edentulous patients. J Oral Rehabil. 2002; 29(11): 1115-9.
11. Procops GW, Roberts GD. Emerging fungal diseases: the importance of the host. Clin Lab Med. 2004; 24(3): 691-719.

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

12. Sarifakioglu E, Gunduz C, Gorpelioglu C. Oral mucosa manifestations in 100 pregnant versus non-pregnant patients: an epidemiological observational study. Eur J Dermatol. 2006; 16(6): 674-6.
13. Kreher JM, Graser GN, Handelman SI, Eisenberg AD. Oral yeasts, mucosal health, and drug use in elderly denture-wearing population. Spec Care Dentist. 1991; 11(6): 222-6.
14. Budtz-Jörgensen E. Etiology, pathogenesis, therapy, and prophylaxis of oral yeast infections. Acta Odontol Scand. 1990; 48(1): 61-9.
15. Budtz-Jörgensen E. Clinical aspects of *Candida* infection in denture wearers. J Am Dent Assoc. 1978; 96(3): 474-9.
16. Moskona D, Kaplan I. Oral lesions in elderly denture wearers. Clin Prev Dent. 1992; 14(5): 11-4.
17. Zegarelli DJ. Fungal infections of the oral cavity. Otolaryngol Clin North Am. 1993; 26(6): 1069-1089.
18. Dorey JL, Blasberg B, MacEntee MI, Conklin RJ. Oral mucosal disorders in denture wearers. J Prosthet Dent. 1985; 53(2): 210-3.
19. Perezous LF, Flaitz CM, Goldschmidt ME, Engelmeier RL. Colonization of *Candida* species in denture wearers with emphasis on HIV infection: A literature review. J Prosthet Dent. 2005; 93(3): 288-93.
20. Baysan A, Whiley R, Wright PS. Use of microwave energy to disinfect a long-term soft lining material contamined with *Candida albicans* or *Sthapylococcus aureus*. J Prosthet Dent. 1998; 79(4): 445-54.
21. Egusa H, Ellepola ANB, Nikawa H, Hamada T, Samaranayake LP. Exposure to subtherapeutic concentrations of polyene antifungal of candida species to denture acrylic. Chemotherapy. 2000; 46(4): 267-74.
22. Quirynen M, Bollen CM. The influence of surface roughness and surface free energy on supra and subgingival plaque formation in man. A review of the literature. J Clin Periodontol. 1995; 22(1): 1-14.

23. de Jong HP, de Boer P, Busscher HJ, van Pelt AW, Arends J. Surface free energy changes of human enamel during pellicle formation. An *in vivo* study. Caries Res. 1984; 18(5): 408-15

24. Van Dijk J, Herkstroter F, Busscher H, Weerkamp A, Jansen H, Arends J. Surface-free energy and bacterial adhesion. An *in vivo* study in beagle dogs. J Clin Periodontol. 1987; 14(5): 300-4.

25. Razek MKA, Mohamed ZM. Influence of tissue conditioning materials on the oral bacteriologic status of complete denture wearers. J Prosthet Dent. 1980; 44(2): 137.

26. Wright PS, Clark P, Hardie PM. The prevalence and significance of yeasts in persons wearing complete dentures with soft-lining materials. J Dent Res. 1985; 64(2): 122-5.

27. Gusberti FA, Gada TG, Lang NP, Geering AH. Cultivable microflora of plaque from full denture bases and adjacent palatal mucosa. J Biol Buccale. 1985; 13(3): 227-36.

28. Sanglard D, Ischer F, Calabrese D, Majcherczyk PA, Bille J. The ATP binding cassette transporter gene CgCDR1 from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. Antimicrob Agents Chemother. 1999; 43(11): 2753-65.

29. Joly S, Maze C, McCray PB Jr, Guthmiller JM. Human beta-defensins 2 and 3 demonstrate strain-selective activity against oral microorganisms. J Clin Microbiol. 2004; 42(3): 1024-9.

30. Feng Z, Jiang B, Chandra J, Ghannoum M, Nelson S, Weinberg A. Human beta-defensins: differential activity against candidal species and regulation by *Candida albicans*. J Dent Res. 2005; 84(5): 445-50.

31. Helmerhorst EJ, Venuleo C, Beri A, Oppenheim FG. *Candida glabrata* is unusual with respect to its resistance to cationic antifungal proteins. Yeast. 2005; 22(9): 705-14.

32. Thiele MC, Carvalho AD, Gursky LC, Rosa RT, Samaranayake LP, Rosa EA. The role of candidal histolytic enzymes on denture-induced stomatitis in patients living in retirement homes. Gerodontology. 2008 *in press*.
33. Cahn LR. The denture sore mouth. Ann Dent. 1936; 3: 33-6.
34. Nobile CJ, Mitchell AP. Genetics and genomics of *Candida albicans* biofilm formation. Cell Microbiol. 2006; 8(9): 1382-91.
35. LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Chemother. 2006; 50(11): 3839-46.
36. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 2007; 20(1): 133-63.

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

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Adesão de Candida spp sobre a superfície de condicionadores teciduais e resina acrílica: estudo in situ e in vivo**", protocolo nº **040/2006**, dos pesquisadores **ALTAIR ANTONINHA DEL BEL CURY, TATIANA PEREIRA e WANDER JOSÉ DA SILVA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 12/04/2006.

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Candida spp adhesion on tissue conditioners and acrylic resin surface: an in situ and in vivo study**", register number **040/2006**, of **ALTAIR ANTONINHA DEL BEL CURY, TATIANA PEREIRA and WANDER JOSÉ DA SILVA**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 12/04/2006.

Profa. Cecília Gatti Guirado
Secretária
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.