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PIRACICABA

2013

UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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Orientadora: Profa. Dra. Altair Antoninha Del Bel Cury

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

Doctorate thesis presented to the Piracicaba Dental School of the University of Campinas to obtain the Ph.D grade in Dental Clinic, concentration area of Dental Prosthesis.

Este exemplar corresponde à versão final da tese
defendida pelo aluno Antônio Pedro Ricomini Filho e
orientado pela Profa. Dra. Altair Antoninha Del Bel Cury.

Assinatura do Orientador

PIRACICABA
2013

FICHA CATALOGRÁFICA ELABORADA POR
JOSIDELMA F COSTA DE SOUZA – CRB8/5894 - BIBLIOTECA DA
FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNICAMP

R426a

Ricomini Filho, Antônio Pedro, 1983-
Avaliação de biofilmes multiespécies formados em diferentes substratos e expostos a concentrações salivares de agentes antimicrobianos / Antônio Pedro Ricomini Filho. -- Piracicaba, SP : [s.n.], 2013.

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

1. Hidroxiapatita. 2. Titânio. 3. Polimetil metacrilato. 4. Antimicrobianos. 5. Antifúngicos. I. Del Bel Cury, Altair Antoninha, 1948- II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para a Biblioteca Digital

Título em Inglês: Evaluation of multispecies biofilms formed on different substrata and exposed to salivary concentrations of antimicrobial agents
Palavras-chave em Inglês:
Hydroxyapatite
Titanium
Polymethyl metacrylate
Antimicrobial Agent
Antifungal Agent
Área de concentração: Prótese Dental
Titulação: Doutor em Clínica Odontológica
Banca examinadora:
Altair Antoninha Del Bel Cury [Orientador]
Fernanda Faot
William Custódio
Renata Cunha Matheus Rodrigues Garcia
Livia Maria Andaló Tenuta
Data da defesa: 27-02-2013
Programa de Pós-Graduação: Clínica Odontológica

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 27 de Fevereiro de 2013, considerou o candidato ANTÔNIO PEDRO RICOMINI FILHO aprovado.

Profa. Dra. ALTAIR ANTONINHA DEL BEL CURY

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Profa. Dra. LÍVIA MARIA ANDALÓ TENUTA

A **Deus**, por continuar iluminando o meu caminho.

Aos meus pais **Antônio Pedro** e **Maria Cecília** e minha irmã **Carolina**, pessoas que admiro pelo caráter. Agradeço pelo amor e carinho, pelo apoio em minhas decisões e pelo incentivo ao longo desses anos. Obrigado seria muito pouco para retribuir tudo que me proporcionaram.

AGRADECIMENTO ESPECIAL

À minha orientadora **Profa. Dra. Altair Antoninha Del Bel Cury** pela oportunidade de conviver e aprender mais a cada dia. Agradeço pelas inúmeras oportunidades a mim concedidas e pela confiança depositada em meu trabalho. Exemplo de pesquisadora e educadora, sempre me estimulou a refletir sobre a ciência e pensar criticamente. A busca constante pelo conhecimento, a objetividade, a clareza de raciocínio, a agilidade na execução dos trabalhos e a rapidez na tomada de decisões são qualidades que muito admiro, e que busco incorporá-las em minha pessoa. Meu eterno muito obrigado por tudo que tem feito pelo meu crescimento pessoal e profissional ao longo desses anos.

AGRADECIMENTOS

À **Universidade Estadual de Campinas** por meio do seu Magnífico Reitor, Prof. Dr. Fernando Ferreira Costa.

À **Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas**, na pessoa de seu Diretor, Prof. Dr. Jacks Jorge Junior.

À Coordenadora dos Cursos de Pós-Graduação da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, **Profa. Dra. Profa. Dra. Renata Cunha Matheus Rodrigues Garcia**. Agradeço pela recepção sempre atenciosa e por todos os ensinamentos compartilhados, e ainda por toda ajuda e suporte.

Ao Coordenador do Programa de Pós-Graduação em Clínica Odontológica da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, **Prof. Dr. Márcio de Moraes.**

À **Fundação de Amparo à Pesquisa do Estado de São Paulo**, (**FAPESP)**, pela bolsa de doutorado concedida (2009/10698-0) e pelo auxílio à pesquisa (2010/07894-9), e à **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)**, pela bolsa do Programa de Doutorando no Brasil com Estágio no Exterior - PDEE (2335/11-5).

Ao **Prof. Dr. Jaime Aparecido Cury** do Departamento de Ciências Fisiológicas, pelas inúmeras oportunidades de aprendizado ao longo da minha vida científica. Agradeço pelos conhecimentos adquiridos e discussões. Agradeço também pela permissão de uso do laboratório de Bioquímica Oral.

À **Profa. Dra. Lívia Maria Andaló Tenuta** do Departamento de Ciências Fisiológicas, por ter sido importante alicerce na minha vida científica e por sempre ser solícita aos meus questionamentos.

À **Profa. Dra. Cínthia Pereira Machado Tabchoury** do Departamento de Ciências Fisiológicas, pela prontidão em ajudar e pelos ensinamentos compartilhados.

Ao **Prof. Dr. Wander José da Silva** do Departamento de Prótese e Periodontia pelas conversas, ensinamentos e conselhos durante a realização desse trabalho.

À **Profa. Dra. Celia Marisa Rizzatti Barbosa** do Departamento de Prótese e Periodontia, pela agradável convivência e pela prontidão em ajudar.

À **Profa. Dra. Fernanda Cristina Petersen**, pela orientação durante estágio realizado no Instituto de Biologia Oral da Universidade de Oslo. Agradeço por todo o conhecimento compartilhado e pelas oportunidades de novos aprendizados. Sempre me lembrarei do "quebrar o gelo" para enfrentar o desconhecido e avançar no conhecimento.

As minhas sobrinhas **Isabela** e **Giovana**, fontes inesgotáveis de amor. O sorriso e o brilho no olhar de cada uma de vocês sempre me estimularam a continuar.

Ao meu cunhado **Fábio** que sempre me incentivou nos estudos. Obrigado por todo suporte.

Aos meus avós **Alcides Ricomini†**, **Dolores Pagotto Ricomini†** e **Cília Balan†**, por todo afeto ao longo da minha vida. Obrigado pelos ensinamentos, conversas e risadas. Apesar de não estarem mais presentes, sei que estão sempre me apoiando.

Aos meus tios **Romualdo Ricomini** e **Édna Ricomini** e meus primos **Yara, Renan†** e **Taís**, pelo acolhimento, carinho e preocupação. Obrigado pelo incentivo e por tudo que fazem por mim.

Aos meus grandes e eternos amigos **Bruno Bet**, **Claudivan Cavalcante**, **Felipe Chiarion**, **Heloisa Gerez**, **Hugo Pazianotto**, **João Tonin Júnior**, **Leonei Pellegrini**, **Luciana Berto**, **Marcelo Rinaldi†**, **Mateus Cibin**,

Maurício da Silva, **Mauro Piva**, **Rafael Vitti**, **Renata Mareto**, **Tatiana Bruni**, **Tomas Bortoluzzi** e **Vinícius Cibin**, pessoas especiais que sempre me incentivaram nessa jornada, meu muito obrigado por todo apoio.

Aos meus amigos **Fabiana Straioto**, **Frederico Fernandes**, **Priscila Gomes** e **Simone Gomes** pelo carinho e pela companhia indispensáveis. Obrigado pelas conversas científicas, ou não, pelas risadas e pela prontidão em sempre me ajudar. Em momentos difíceis sempre me apoiaram, mesmo que fosse com um abraço, ou um simples sorriso.

À minha amiga **Sílvia Lucena**, pela amizade ao longo do doutorado e por todas as "aventuras" científicas. Obrigado por toda ajuda e paciência!

Aos antigos e atuais amigos e colegas da Pós-Graduação **Andréa Vasconcellos**, **Alfonso Ayala**, **Aloísio Spazzin**, **Ana Flávia Calvo**, **Arcelino Neto**, **Ataís Bacchi**, **Bruno Maior, Camila Lima, Camila Heitor, Carolina Aires**, **Cláudia Zamataro**, **Constanza Fernández**, **Cristiane Machado**, **Cindy Dodo**, **Dimorvan Bordin**, **Fernanda Signoretti**, **Francisco Montagner**, **Gisele Moi**, **Gláuber Vale**, **Germana Camargos**, **Indira Cavalcanti**, **Jéssica Takahashi**, **Juliana Botelho**, **Juliana Fernandes**, **Kelly Andrade**, **Jonas de Oliveira**, **Juliana Nuñez**, **Junior Ribeiro**, **Larissa Vilanova**, **Leonardo Luthi**, **Leonardo Panza**, **Lis Meirelles**, **Livia Forster**, **Luana Aquino**, **Marcele Pimentel**, **Marco Aurélio Carvalho**, **Marília Correia**, **Mateus Fernandes dos Santos**, **Maximiliano Cenci**, **Mirella Campos**, **Paula Furlan**, **Plínio Senna**, **Priscila Serrano**, **Regiane Amaral**, **Renzo Vasquez**, **Rodrigo Arthur**, **Sandro Kusano**, **Tales Candido**, **Tamires Munerato** e **Tatiana Pereira Cenci**, **Thaís Gonçalves** e **Yuri Cavalcanti**, pela convivência e por me proporcionarem momentos felizes. Muito obrigada a cada um de vocês.

Aos meus amigos **Carolina Meloto** e **William Custódio**, por todos os momentos vividos desde a época de graduação na FOP-UNICAMP. Obrigado pelas conversas, conselhos e risadas.

Aos meus amigos **Stian André Engen** e **Zahra Armingohar**, pela amizade durante o estágio em Oslo. Obrigado por toda ajuda e pelos momentos maravilhosos que me proporcionaram.

À **Anne Karin Kristoffersen**, **Cecilie Attramadal**, **Håkon Rukke**, **Ingun Witsø**, **Louise Bjerkan**, **Tor Henry Wold**, por compartilharem sabedoria e pelo agradável convívio em Oslo.

À Sra. **Joselena Casati Lodi**, antiga responsável técnica pelo Laboratório de Prótese Removível, pelo convívio agradável, paciência e incentivo.

À Sra. **Gislaine Piton**, responsável técnica pelo Laboratório de Prótese Removível, pela ajuda durante todo o Doutorado e por todo carinho, incentivo e compreensão.

Aos técnicos do Instituto de Biologia Oral **Ali-Oddin Naemi** e **Heidi Aarø Åmdal**, por todos os ensinamentos compartilhados e agradável convívio.

Aos técnicos do Laboratório de Bioquímica Oral **Waldomiro Vieira Filho** e **José Alfredo da Silva**, agradeço pela ajuda e agradável convivência.

À **Érica Alessandra Pinho Sinhoreti**, **Raquel Q. Marcondes Cesar Sacchi** e **Roberta Clares Morales dos Santos** secretárias da Coordenadoria Geral dos Programas de Pós-Graduação; à **Priscilla Zuzi Boldrin**, secretária do Programa de Pós-Graduação em Clínica Odontológica; à **Eliete A. Ferreira Lima Marim**, secretária do Departamento de Prótese e Periodontia; meu sincero agradecimento pela atenção e gentileza dispensada durante esses anos de convívio como aluno de pós-graduação.

À **ACECIL** (Campinas, SP, Brasil), pela valiosa contribuição com a esterilização dos espécimes.

A todos que indiretamente contribuíram para a realização deste trabalho, meu muito obrigado.

RESUMO

A colonização de diferentes substratos presentes na cavidade oral por micro-organismos e o desenvolvimento de biofilme são fatores etiológicos da maioria das doenças orais. Além dos dentes, materiais como titânio e polimetilmetacrilato são comumente encontradas neste ambiente e o papel que estes substratos desempenham na prevalência de populações bacteriana e fúngica em biofilmes orais são pouco compreendidas. Além disso, o comportamento da população microbiana de biofilmes orais multiespécies na presença de antimicrobianos liberados na saliva permanece desconhecido. Assim, o objetivo deste estudo foi (i) avaliar o efeito de diferentes substratos na prevalência de micro-organismos em biofilmes orais multiespécies e (ii) o efeito de antimicrobianos liberados na saliva na população microbiana de biofilmes multiespécies. Para o primeiro estudo, discos de hidroxiapatita, titânio e polimetilmetacrilato (PMMA) foram utilizados como substrato para o desenvolvimento do biofilme mimetizando esmalte dental, implantes dentários e base de prótese, respectivamente. O modelo de biofilme multiespécies foi composto por cinco bactérias (*Streptococcus oralis*, *Streptococcus mutans*, *Actinomyces naeslundii*, *Veillonella dispar* e *Fusobacterium nucleatum*) e um fungo (*Candida albicans*). Biofilmes maduros (64,5 h de desenvolvimento) foram removidos por ondas ultrassônicas, plaqueados em meio ágar e as contagens de UFC de cada micro-organismo foram calculadas. A microscopia eletrônica de varredura foi utilizada para visualizar a superfície dos materiais. Os dados foram analisados por ANOVA um critério. Para o segundo estudo o mesmo modelo de biofilme multiespécies foi utilizado. Dois antibióticos, azitromicina e metronidazol, e um antifúngico, fluconazol, foram avaliados. Biofilmes maduros (64,5 h de desenvolvimento) foram expostos a azitromicina, metronidazol ou fluconazol em concentrações encontrada na saliva de 2,12 µg/mL, 15,15 µg/mL e 2,56 µg/mL, respectivamente, por 24h. Após este período, o biofilme foi removido por ondas ultrassônicas, plaqueados em meio ágar e as contagens de UFC de cada micro-organismo foram calculadas. Microscópio eletrônico de varredura e microscópio a laser de varredura confocal com células

coradas por hibridização *in situ* por fluorescência (FISH) foram utilizados para avaliar a estrutura do biofilme. Os dados foram analisados por teste t para amostras independentes e testes não paramétricos de Mann-Whitney. O primeiro estudo não mostrou diferença na população para cada micro-organismo no biofilme entre os entre três materiais avaliados ($p>0,05$). No segundo estudo, todos os antimicrobianos avaliados foram capazes de alterar a população microbiana ($p<0,05$), no entanto nenhum dos agentes antimicrobianos foi capaz de eliminar completamente um micro-organismo específico do biofilme. Azitromicina reduziu as populações de *A. naeslundii* e *V. dispar* enquanto aumentou *C. albicans* ($p<0,05$). Metronidazol reduziu todos os micro-organismos avaliados, com uma grande redução para *V. dispar* e *F. nucleatum* ($p<0,001$). Fluconazol reduziu populações de *C. albicans* e *F. nucleatum* e aumentou as contagens de *S. oralis* e *V. dispar* ($p<0,05$). Pode concluir-se que os substratos não foram capazes de interferir na formação dos biofilmes multiespécies e que os antimicrobianos em concentrações semelhantes às liberadas na saliva alteraram a população microbiana.

Palavras-chave: Biofilme, Hidroxiapatita, Titânio, Polimetilmetacrilato, Azitromicina, Metronidazol, Fluconazol.

ABSTRACT

The colonization of different substrata present in the oral cavity by microorganisms and the biofilm development are the etiological factors of the majority of oral diseases. Besides the teeth, materials such as titanium and polymethylmetacrylate are commonly found in this environment and the role these substrata play on the prevalence of bacterial and fungal population in oral biofilms are poorly understood. In addition, the behavior of microbial population of multispecies oral biofilms in the presence of antimicrobials released in saliva remains unknown. Thus, the aim of this study was (i) to evaluate the effect of different substrata on the prevalence of microorganisms in an oral multispecies biofilms and (ii) the effect of antimicrobials released in saliva on the microbial population of a multispecies biofilms. For the first study hydroxyapatite, titanium and polymethylmetacrylate (PMMA) discs were used as substrata for biofilm development mimicking tooth enamel, dental implant and denture base, respectively. The multispecies biofilm model was composed by five bacteria (*Streptococcus oralis*, *Streptococcus mutans*, *Actinomyces naeslundii*, *Veillonella dispar* and *Fusobacterium nucleatum*) and one yeast (*Candida albicans*). Mature biofilms (64.5 h of development) were removed by ultrasonic waves, plated on agar media and CFU counts of each microorganism were calculated. Scanning electron microscopy was used to visualize the materials' surface. Data were analysed by one-way ANOVA. For the second study the same multispecies biofilm model was used. Two antibiotics, azithromycin and metronidazole, and one antifungal, fluconazole, were evaluated. Mature biofilms (64.5 h development) were exposed to azithromycin, metronidazole or fluconazole at concentrations found in saliva of 2.12 µg/mL, 15.15 µg/mL and 2.56 µg/ml, respectively, for 24h. After this period, the biofilm was removed by ultrasonic waves, plated on agar media and CFU counts of each microorganism were calculated. Scanning electron microscopy and confocal scanning laser microscopy with cells stained by fluorescent *in situ* hybridization (FISH) technique were used to assess the biofilm structure. Data were analysed by independent-samples t-test and Mann-Whitney nonparametric test. The first study showed no difference in the biofilm population for each

microorganism among the three materials evaluated ($p>0.05$). In the second study, all antimicrobials evaluated were able to change microbial population ($p<0.05$), however none of the antimicrobials was able to completely eliminate a specific microorganism from the biofilm. Azithromycin reduced *A. naeslundii* and *V. dispar* population while increased *C. albicans* ($p<0.05$). Metronidazole reduced all the microorganisms evaluated, with a great reduction for *V. dispar* and *F. nucleatum* ($p<0.001$). Fluconazole reduced *C. albicans* and *F. nucleatum* population and increased *S. oralis* and *V. dispar counts* ($p<0.05$). It can be concluded that the substrata were not able to interfere with the formation of multispecies biofilms and antimicrobials in concentrations similar to those released in the saliva changed microbial population, however they were not able to eliminate microorganisms.

Key Words: Biofilm, Hydroxyapatite, Titanium, Polymethylmetacrylate, Azithromycin, Metronidazole, Fluconazole.

SUMÁRIO

INTRODUÇÃO GERAL

O desenvolvimento de biofilme na cavidade oral em superfícies não descamativas é o principal responsável pelas doenças orais. Dentes, implantes osseointegrados e próteses removíveis são substratos passíveis de colonização por bactérias e fungos e, portanto, são superfícies susceptíveis ao acúmulo de biofilme (Marsh *et al.*, 2011; Samaranayake *et al.*, 2009; Teughels *et al.*, 2006). Os micro-organismos organizados em biofilme e os metabólitos produzidos por estes podem ocasionar danos no substrato ao qual estão aderidos e também podem causar danos aos tecidos adjacentes (Diaz, 2012; Marsh *et al.*, 2011; Williams *et al.*, 2011).

A cárie dental é um exemplo de doença que resulta em danos ao substrato dental. Na presença de carboidratos fermentáveis, bactérias presentes no biofilme produzem ácidos, os quais causam a desmineralização do tecido dental (Marsh, 2003). Diferentemente, na periodontite e peri-implantite, a presença dos metabólitos microbianos na região do sulco gengival e peri-implantar, respectivamente, desencadeiam reação imune inflamatória e destruição progressiva dos tecidos de suporte, podendo ocasionar na perda do elemento dental ou do implante osseointegrado (Diaz, 2012; Marsh *et al.*, 2011). De maneira semelhante, nos casos de estomatite protética a agressão ocorre no tecido mucoso em íntimo contato com a base da prótese na presença do biofilme (Samaranayake *et al.*, 2009; Williams *et al.*, 2011).

Embora estas doenças estejam associadas a biofilmes formados em um ambiente com condições físico-químicas semelhantes, a composição microbiológica difere nos distintos sítios orais acometidos. As diferentes composições dos materiais e suas características de superfícies têm sido apontadas como fatores que facilitariam a colonização dos materiais por determinadas espécies, a exemplo das bases de próteses removíveis, as quais seriam mais facilmente colonizadas pelo fungo *Candida albicans* (Busscher *et al.*, 2010; Verran and Maryan, 1997; Williams *et al.*, 2011).

Dentre os fatores relatados que poderiam interferir na adesão dos micro-organismos nos diferentes substratos estão a energia livre de superfície do material e a rugosidade de superfície. A energia livre de superfície está relacionada com a adesão dos micro-organismos aos diferentes substratos por interações inespecíficas como ligações iônicas e pontes de hidrogênio (Teughels *et al.*, 2006). Entretanto tem se observado que a formação da película adquirida sobre a superfície dos substratos tende a equalizar as forças eletrostáticas dos materiais (Hannig and Hannig, 2009; van der Mei *et al.*, 2012), não atuando como um forte fator na seleção microbiana. Entretanto ainda pouco se sabe sobre o papel do material na composição microbiológica em biofilmes orais.

Na cavidade oral os diferentes substratos são banhados por saliva e sobre eles há a formação de uma película de glicoproteínas salivares, a qual favorece a adesão de colonizadores iniciais (Hannig and Hannig, 2009; Marsh *et al.*, 2011). O acúmulo de micro-organismos, caso não seja interrompido, determina a formação de biofilme e as condições do microambiente em cada sítio oral influência a prevalência de determinadas espécies, constituindo o biofilme uma organização dinâmica de células (Aas *et al.*, 2007; Aas *et al.*, 2008; Busscher *et al.*, 2010; Diaz, 2012; Marsh *et al.*, 2011). Apesar de *Streptococcus oralis*, *Streptococcus mutans*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Veillonella dispar* e *Candida albicans* (Guggenheim *et al.*, 2001; Marsh *et al.*, 2011; Samaranayake *et al.*, 2009; Williams *et al.*, 2011) serem micro-organismos comensais na cavidade oral, a prevalência de cada uma dessas espécies pode diferir nos biofilmes relacionados às patologias em diferentes sítios orais (Guggenheim *et al.*, 2001; Fejerskov, 2004; Aas *et al.*, 2005; Teughels *et al.*, 2006; Baelum *et al.*, 2007; Busscher *et al.*, 2010; Marsh *et al.*, 2011).

A literatura é rica em estudos com biofilmes mono espécie avaliando resposta da espécie mais prevalente em biofilmes patogênicos, entretanto a interação entre as espécies não é contemplada (Ccahuana-Vasquez and Cury, 2010; Jang *et al.*, 2012; Koo *et al.*, 2003; Pereira-Cenci *et al.*, 2008). Bactérias e fungos interagem entre si no processo de formação de um biofilme. Os

metabólitos secretados e os sistemas de comunicação via moléculas sinalizadoras contribuem na colonização e formação de biofilme (De Sordi and Muhlschlegel, 2009; Jakubovics, 2010; Jang *et al.*, 2012; Jarosz *et al.*, 2009; Mashburn-Warren *et al.*, 2010; Senadheera and Cvitkovitch, 2008). Dessa maneira, estudos com modelos de biofilmes multiespécies são necessários, visto a complexidade desses biofilmes nas diferentes patologias (Aas *et al.*, 2005; Aas *et al.*, 2007; Aas *et al.*, 2008; Guggenheim *et al.*, 2001; Marsh *et al.*, 2011).

Embora algumas espécies não estejam associadas a biofilmes patogênicos, elas são importantes para a formação do biofilme, a exemplo dos *Streptococcus oralis*, que são bactérias importantes na colonização inicial e atuam como fatores adjuvantes para adesão de outros micro-organismos (Kolenbrander, 2011; Marsh *et al.*, 2011). Entretanto, outros micro-organismos são prevalentes em determinadas doenças, como por exemplo, *Streptococcus mutans*, que está relacionado à cárie dental (Burne, 1998; Marsh *et al.*, 2011); *Actinomyces naeslundii*, a cárie radicular (Brailsford *et al.*, 1999); *Fusobacterium nucleatum*, a doença periodontal (Aas *et al.*, 2007; Diaz, 2012; Zijnge *et al.*, 2012); e *Candida albicans*, a casos de estomatite protética (Pereira-Cenci *et al.*, 2008; Samaranayake *et al.*, 2009). Além dessas espécies patogênicas, outras podem estar presentes em grande quantidade em biofilmes orais e devem ser consideradas em estudos envolvendo biofilmes multiespécies, como a *Veillonella dispar* (Aas *et al.*, 2005; Arif *et al.*, 2008). Além da existência de diversos micro-organismos durante o processo de colonização e maturação do biofilme ser um fator relevante na estruturação do mesmo, outros fatores podem interferir na composição microbiológica do biofilme.

A presença de antimicrobianos liberados na saliva poderia alterar o microambiente do biofilme e modificar a organização dos biofilmes, bem como alterar a prevalência de determinadas espécies (Soriano and Rodriguez-Cerrato, 2002). Nos tratamentos de infecções bacterianas, alguns antibióticos administrados por via oral apresentam-se em elevadas concentrações salivares, como a azitromicina e o metronidazol com valores de 2,12 µg/mL e 15,15 µg/mL,

respectivamente (Van Oosten *et al.*, 1986; Blandizzi *et al.*, 1999; Pahkla *et al.*, 2005;). Entretanto a ação desses antibióticos liberados na saliva e a alteração que poderia ocorrer na organização e população das espécies presentes no biofilme são desconhecidas. Devido ao medicamento ser antibacteriano, poderia favorecer a proliferação de espécies fúngicas no biofilme. Diferentemente, a utilização de fluconazol via oral, o qual atinge concentrações salivares de 2,56 µg/mL (Force and Nahata, 1995), poderia favorecer a proliferação de espécies bacterianas, entretanto não há conhecimento dessas hipóteses em estudos que mimetizam biofilmes formados em condições semelhantes ao ambiente oral.

Tendo em vista o escasso conhecimento sobre a colonização e organização das espécies de micro-organismos constituintes do biofilme em diferentes substratos presentes na cavidade oral e também da ausência de estudos sobre as possíveis alterações que possam ocorrer no biofilme quando exposto a concentrações de antimicrobianos frequentemente utilizados o presente estudo se faz necessário. Dessa maneira, o objetivo do estudo foi avaliar o efeito de diferentes substratos na prevalência de micro-organismos em biofilmes orais multiespécies e também a ação de antimicrobianos em concentrações salivares na população microbiana de biofilmes multiespécies.

CAPÍTULO 1

Microbial population of oral multispecies biofilm formed on hydroxyapatite, titanium and polymethylmetacrylate

Short communication

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ABSTRACT

This study evaluated the effect of substrata on the prevalence of microorganisms in a multispecies biofilm. Hydroxyapatite, titanium and polymethylmetacrylate were used as substrata for the development of multispecies biofilm (*Streptococcus oralis*, *Streptococcus mutans*, *Actinomyces naeslundii*, *Veillonella dispar*, *Fusobacterium nucleatum* and *Candida albicans*). Mature biofilms (64.5 h) were collected, plated on agar media and CFU counts of each microorganism were calculated. Materials' surfaces were visualised by SEM before biofilm formation. The materials presented distinct surface irregularities observed in the SEM images and no difference in the biofilm population for each microorganism was found among the three materials evaluated ($p>0.05$). In conclusion, the substrata only were not able to interfere in the prevalence of microorganisms in biofilm.

INTRODUCTION

In the oral cavity hard surfaces other than teeth are present such as titanium, in oral implants and prosthetic components, and polymethylmetacrylate (PMMA), in denture bases. All these substrata are subjected to colonization by bacteria and fungi present in the mouth and consequently, biofilm accumulation (1).

In biofilms, the prevalence of different microbial species depends on the oral site where they are formed. The frequent isolation of *Streptococcus mutans* in dental caries-associated biofilms (2), gram-negative anaerobes in periodontitis and peri-implantitis (3), and *Candida albicans* in denture stomatitis (4), have been reported linking higher predominance of some species to specific-pathogens pathogenic biofilms (1).

Teeth, titanium and polymethylmetacrylate present different composition and they are associated with different biofilm diseases (1, 3, 5). It has been stated that the substratum is an important factor for microbial colonization (1, 4-6). Polymethylmetacrylate, used as denture base material, has shown to favour *Candida* spp. colonization (1, 4), however the role that different materials play on

microbial prevalence is poorly understood in oral biofilms in similar condition as found in the mouth.

The purpose of this study was to investigate the effect of different substrata on the prevalence of microorganisms in an oral multispecies biofilm.

MATERIAL AND METHODS

Preparation of hydroxyapatite, titanium and PMMA discs

Hydroxyapatite discs were purchased from Clarkson Chromatography Products Inc. (South Williamsport, PA). Titanium discs were fabricated from a cylindrical bar of titanium grade IV (10 mm diameter; Sandinox, São Paulo, Brazil) sliced by electrical discharge machining. Polymethylmetacrylate (PMMA) discs were prepared using acrylic resin polymerized by hot water bath (QC-20 PMMA, Dentsply Ltd., Weybridge, England). The acrylic resin was placed inside a stainless steel matrix and the polymerization cycle was performed according to the manufacturer's instructions. The disks of all the three substrata (10 mm diameter and 2 mm thickness) had the surface roughness standardized by grinding both sides with progressively smoother aluminium oxide papers (320, 400 and 600 grit) in a horizontal polisher (APL-4; Arotec, São Paulo, Brazil). Next, they were washed twice with sterile distilled water and then ultrasonicated for 20 min to remove any residues from the surface.

Surface roughness

The surface roughness was measured using a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd, Kosaka, Japan) with a 0.01mm 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 side of the specimen, and a mean value was calculated (7). Previously to the biofilm assay, the discs were placed in disc holders and sterilized by ethylene oxide.

Multispecies biofilm assay

The multispecies biofilm assay was performed as described by Guggenheim *et al.* (2001) with slight modifications (8-10). The microorganisms used in this study were *Streptococcus oralis* OMZ 607, *Streptococcus mutans* OMZ 918, *Actinomyces naeslundii* OMZ 745, *Veillonella dispar* OMZ 493, *Fusobacterium nucleatum* OMZ 596 and *Candida albicans* OMZ 110 (8). First of all, the sterilized discs were placed in 24-well tissue culture plate in vertical position using disc holders, covered with 2.0 mL of saliva, and incubated for 4 h at 37 ºC to form the salivary pellicle (9). Each disc was removed and placed in another well containing 1.8 mL of a medium mixture composed of 70% saliva + 30% mFUM 0.3% glucose (fluid universal medium supplemented with 67 mmol/L Sorensen's buffer, pH 7.2) and 225 µL of the 6-species, which were prepared by mixing equal volumes of each density-adjusted culture at 1.0 ± 0.05 (OD500) (10). The culture plate was incubated anaerobically at 37°C for 16.5 h. After that, the discs were washed by three consecutive dips in 0.9% NaCl solution and inserted in fresh medium mixture composed of 70% saliva + 30% mFUM with 0.15% glucose and 0.15% sucrose. This change to fresh medium mixture was performed at each 24 h (16.5 h, 40.5 h and 64.5 h). In addition to this step, the discs were dipped twice a day (4 h and 8 h after the change of the medium mixture) in 2 mL of 0.9% NaCl solution. At the last medium change (64.5 h), the biofilm formed on the discs were collected and evaluated. The multispecies biofilm assay was performed in triplicate in three independent experiments on different days (n = 9).

Biofilm analyses

After the biofilm development (64.5 h), each disc was washed three times in 2 mL of 0.9% NaCl solution and aseptically inserted into a cryogenic tube containing 3 mL of 0.9% NaCl solution. Biofilm was removed from the disc by ultrasonic waves at 7 W for 30 s (2) and this suspension was serially diluted (10^{-1} to 10^{-6}) in 0.9% NaCl solution. Aliquots of 50 µL were plated on Columbia blood agar (Difco, Sparks, MD, USA) supplemented with 5% defibrinated sheep blood

(CBA), Mitis Salivarius Agar (MSA; Difco, Sparks, MD, USA), CBA supplemented with erythromycin (1 mg/L; Sigma-Aldrich), norfloxacin (1 mg/L; Sigma-Aldrich), and vancomycin (4 mg/L; Sigma-Aldrich) (CBA+), and Biggy Agar (BBL, BD, Franklin Lakes, NJ, USA). CBA and CBA+ plates were incubated anaerobically at 37 ºC for 72 h. MSA plates were incubated at 37 ºC and 10% CO_2 for 48 h and Biggy Agar plates incubated aerobically at 37 ºC for 24 h. Colony-forming units (CFU) were counted using a stereomicroscope. CBA plates were used to count total microorganisms*, Actinomyces naeslundii* and *Veillonella dispar*; MSA, *S. oralis* and *S. mutans*; CBA+, *F. nucleatum*; and Biggy Agar, *C. albicans*. Biofilm dry weight was also estimated with 400 µl of the biofilm suspension (2). The results were expressed in CFU per mg of dry weight of biofilm.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed to visualize the surface of the three different materials before biofilm formation. The discs were mounted on stubs, sputter-coated with gold and examined with a scanning electron microscope (JEOL JSM-5600LV; Peabody, MA, USA) at an accelerating voltage of 15 kV.

Statistical analysis

The statistical analyses were done using SAS software (SAS Institute Inc., version 8.01, Cary, N.C., USA) employing a significance level fixed at 5%. The null hypothesis assumed no difference among the three materials evaluated for microorganism counts. Data that violated the assumptions of equality of variances and normal distribution of errors were transformed to $\log_{10}$ before they were analyzed by one-way ANOVA.

RESULTS

Assessment of the various materials with SEM revealed microporosities on the hydroxyapatite discs, while titanium and PMMA presented a smooth surface

with grooves due to the grinding process (Fig. 1). Despite the differences of surface irregularities, all materials showed similar surface roughness of 0.32 (± 0.01), 0.32 (± 0.01) and 0.34 ± (0.02) µm obtained for hydroxyapatite, titanium and PMMA discs, respectively. No difference was found for the counts of each microorganism and total microorganisms in biofilms collected from the three materials evaluated (p>0.05) (Table 1).

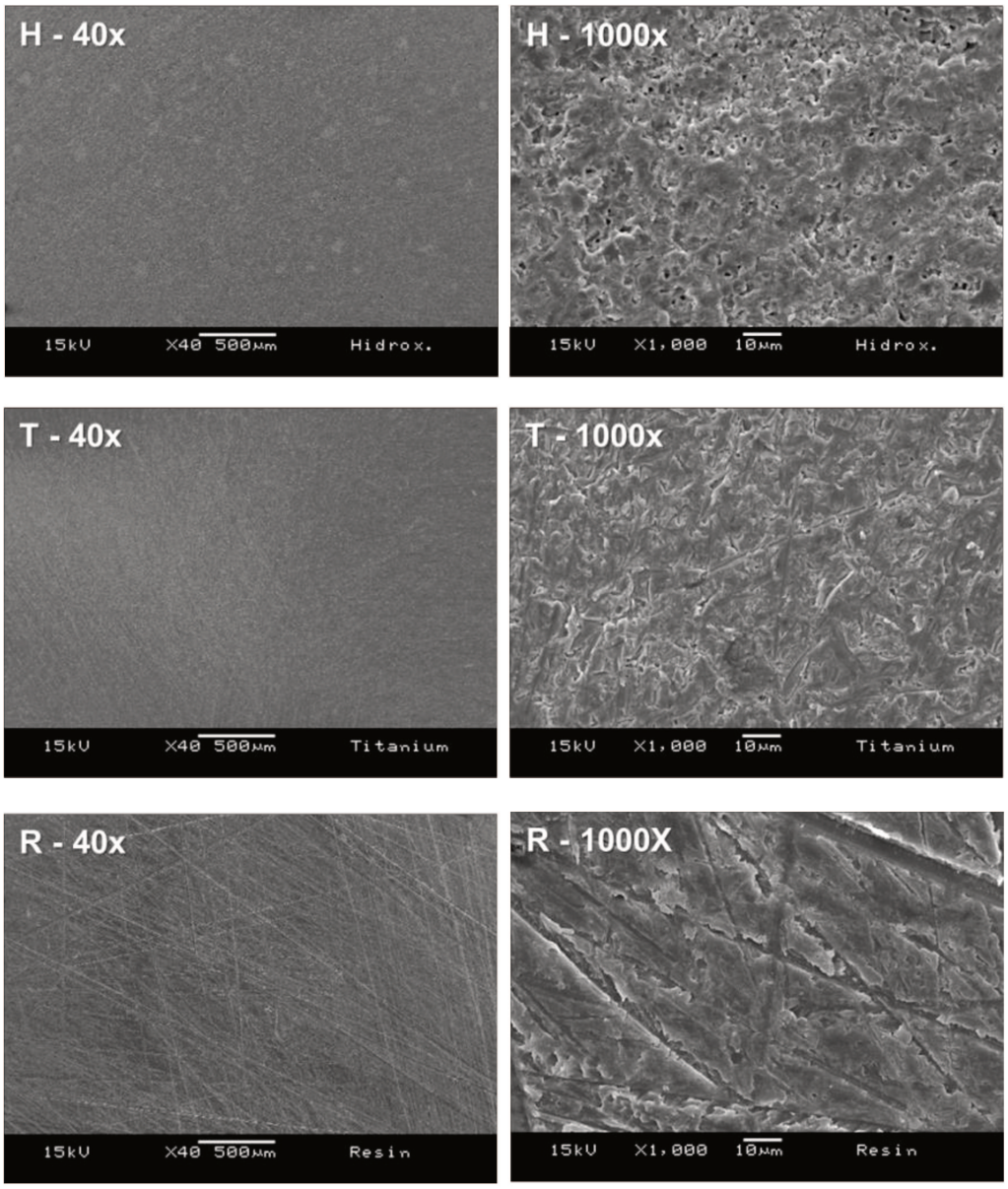

Figure 1. SEM images showing the materials' surfaces: H- hydroxyapatite, T- titanium and R- PMMA.

Table 1. Counts (CFU/dry weight) of each microorganism and total microorganisms in the biofilms formed on hydroxyapatite, titanium and PMMA (mean ± SD).

	S. oralis ($x10^8$)	*S. mutans* ($x10^7$)	*A. naeslundii* ($x10^8$)	*V. dispar* ($x10^7$)	*F. nucleatum* ($x10^8$)	*C. albicans* ($x10^4$)	*Total Microorganisms* ($x10^8$)
Hydroxyapatite	2.8 ± 1.0	3.6 ± 2.5	3.2 ± 2.2	0.5 ± 0.6	1.0 ± 0.4	1.4 ± 0.6	5.8 ± 3.1
Titanium	2.7 ± 2.0	2.2 ± 2.0	2.4 ± 1.2	1.0 ± 2.1	1.1 ± 0.7	1.1 ± 0.7	4.8 ± 2.9
PMMA	3.9 ± 1.3	3.9 ± 2.8	4.0 ± 1.0	0.7 ± 1.5	1.3 ± 0.5	1.1 ± 0.7	6.3 ± 2.3

No significant differences were found for microbial counts among the different substrata.

DISCUSSION

Our study has shown that the substrata were not able to interfere in the prevalence of microorganisms in an established biofilm. This finding help us to better understand the role that materials play on the biofilms formed in the oral cavity. Although the various substrata in mouth possess peculiar surface properties, which are implied to be an important factor in the colonization process (1, 5), they did not affect the bacterial and fungal population in the mature biofilms.

Surface roughness is a surface property well known to favour microbial adhesion and, consequently, the amount of biofilm formed (1, 4, 11). The surface irregularities and porosities contribute as sites for microbial colonization, protecting the microorganisms from removal by shear forces. In this study we submit each substratum to a double-side grinding process in order to provide surfaces with similar smoothness to avoid bias among the three materials.

Another surface property that could interfere in microbial colonization is related to the surface physicochemical characteristic, such as surface free energy, which is inherent to material composition. It has been reported that polymethylmetacrylate, used as denture base material, could favour higher colonization by *Candida albicans* due the hydrophobicity of the substratum (1, 4). The higher prevalence of yeasts in the biofilm would let denture wearers more prone to develop denture stomatitis. However this role was not observed in our

study, since the prevalence of *C. albicans* was similar among the substrata. Likewise, there was no difference on counts of bacterial species in the biofilms formed on the different materials.

The lack of difference in microbial prevalence can be explained by two factors. The first one is related to the stage when the biofilm was collected. Our aim was to assess the microorganisms organized as biofilm on the materials. A great number of studies evaluate only adherent cells on the surface (1, 7, 12), and not microorganisms organized in biofilms as it occurs in the disease process in the mouth (5). The second factor is concerned to the presence of the acquired pellicle that probably levelled off the different properties of the substrata (1, 6). The glycoproteins adsorbed on the substratum surface favour a similar condition for colonization by microorganisms and biofilm growth (6).

The multispecies biofilm model was used to mimic the oral environment during biofilm formation. Besides is composed of different species commonly found in oral biofilms, the model relies on the constant presence of high amount of saliva as in the mouth (8, 9). However, the oral microenvironment in dental caries, periodontitis, peri-implantitis and denture stomatitis are very distinct. In an attempt to extrapolate our data to the clinical reality we could infer that the different microenvironments present in the mouth are the main responsibles for species selection in the biofilm. Oxygen tension, pH, inflammatory exudates, host-derived macromolecules and presence of fermentable carbohydrates are examples of factors that contribute to the shift in prevalence of species in oral biofilms (2, 3). Further studies with other restorative materials are needed to further increase our understanding on the role of substrata on oral biofilms. In addition, studies with multispecies biofilms growing in environments that mimic the different oral disease sites would be helpful to explain the process of different microbial species prevalence.

CONCLUSION

Based on the results, it can be concluded that different substrata present in the oral cavity are not able to interfere in the prevalence of species in mature biofilms.

ACKNOWLEDGMENTS

The authors thank Prof. Dr. Bernhard Guggenheim for providing the microorganisms used in the multispecies biofilm model (OMZ strains). We are grateful to São Paulo Research Foundation (FAPESP) for the scholarship received by the first author during his graduation course (# 2009/10698-0) and for the financial support provided by the research grant (# 2010/07894-9).

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CAPÍTULO 2

Effect of salivary concentrations of antimicrobials on microbial population of oral biofilms

Original article

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ABSTRACT

Oral biofilms are responsible for the majority of oral diseases and the effect of antimicrobials released in saliva on bacterial and fungal population is poorly understood. The aim of this study was to investigate the effect of salivary concentration of antimicrobials on microbial population of a multispecies biofilm. Two antibiotics, azithromycin and metronidazole, and one antifungal were evaluated. Hydroxyapatite, titanium and polymethylmetacrylate (PMMA) discs were used as substrata for biofilm development mimicking tooth enamel, dental implant and denture base, respectively. The multispecies biofilm model were composed by five bacteria (*Streptococcus oralis*, *Streptococcus mutans*, *Actinomyces naeslundii*, *Veillonella dispar* and *Fusobacterium nucleatum*) and one yeast (*Candida albicans*). Mature biofilms (64.5 h of development) were exposed to one of each antimicrobial (azithromycin, metronidazole or fluconazole) at salivary concentrations for 24 h. After this period, the biofilm was removed by ultrasonic waves, plated on agar media and CFU counts of each microorganism were calculated. Scanning electron microscopy and confocal scanning laser microscopy with cells stained by fluorescent *in situ* hybridization (FISH) technique were used to assess the biofilm structure. All antimicrobials evaluated were able to change microbial population in biofilms ($p<0.05$), however none of them were able to completely eliminate a specific microorganism from the biofilm. Azithromycin reduced *A. naeslundii* and *V. dispar* population while increased *C. albicans* ($p<0.05$). Metronidazole reduced all the microorganisms evaluated, with a great reduction for *V. dispar* and *F. nucleatum* ($p<0.001$). Fluconazole reduced *C. albicans* and *F. nucleatum* population and increased *S. oralis* and *V. dispar* counts ($p<0.05$). It was possible to conclude that antimicrobials at concentrations released in saliva alter the biofilm microbial population.

INTRODUCTION

Biofilms on hard and soft tissues are the main cause of diseases in the oral cavity (1-3). Teeth, dental implants and dentures are substrata amenable to

colonization by bacteria and fungi present in the mouth, and therefore, they are prone to biofilm accumulation. The microorganisms organized in biofilms and the metabolites produced by them can cause damage on the substratum to which they are attached to, as in dental caries (2, 4), or in the surrounding tissues, as in periodontitis, peri-implantitis (1, 5) and denture stomatitis (3, 6).

The biofilms are formed by early colonisers, mainly streptococci and actinomyces (2), which bind to acquired pellicle-coated surfaces, and then other species bind to the already-adhered cells (7). Biofilm development is a dynamic temporal process and its structure and microbial composition are affected by environmental factors (1-3). In different oral sites, higher prevalence of some microorganisms in pathogenic biofilms has been reported, such as *Streptococcus mutans*, in dental caries (2, 8); *Actinomyces naeslundii*, in root caries (9); gram-negative anaerobes, in periodontitis and peri-implantitis (1, 5, 10), and *Candida albicans*, in cases of denture stomatitis (3, 6).

A common feature for oral biofilms is that all of them are surrounded by saliva. Besides being a nutritional source and providing glycoproteins to form the acquired pellicle (7), saliva can deliver high concentration of drugs in the oral cavity (11-14), which could affect structure and population of oral biofilms. Some antimicrobials used to treat bacterial and fungal infection via systemic therapy reach high concentrations in saliva, due the chemical properties of the drug in the absorption and distribution pharmacokinetic steps (14). The bioavailability of antimicrobial in saliva can be steady for a long period of time in orally administered drugs, either in short- or long-term treatments.

Azithromycin, metronidazole and fluconazole are drugs commonly used not only in the treatment of oral infections, but also in the treatment of a wide range of infections in non-oral sites. Azithromycin has a wide spectrum of action towards aerobic and facultative gram-positive microorganisms, mainly staphylococci and streptococci, and some anaerobic bacteria as well (11, 15). Metronidazole, which also has a wide spectrum of action, is used to treat obligate anaerobes (13, 16, 17). Fluconazole, a fungistatic drug, is used to treat *Candida* spp. infections (12, 18).

These antimicrobials are bioavailable in high concentration in saliva, which could lead to changes in oral biofilms (14). Azithromycin and metronidazole provide high concentration of antibiotics in saliva with values of 2.12 µg/mL and 15.15 µg/mL, respectively (11, 13, 17). However their action in saliva to change the organization and population of the species present in the biofilm are poorly understood. As antibacterial drugs, they also could favour the proliferation of fungal species in the biofilm. In contrast, the use of fluconazole, which reaches salivary concentrations of 2.56 µg/ mL (12), could favour the proliferation of bacterial species; however these hypotheses in controlled studies of biofilms formed on conditions similar to the oral environment remain to be tested.

Biofilms have been extensively studied mainly as mono and duo-species (19-21), however few studies have evaluated more complex biofilms regarding bacterial and fungal interactions in multispecies models mimicking the oral cavity (22-24). Since oral biofilms accumulated on different substrata serve as a reservoir of bacterial and fungal species and the effect of antibacterial and antifungal agents present in saliva on biofilms are poorly understood, the purpose in our study was to evaluate the effect of antimicrobials released in saliva on microbial population of a multispecies biofilm model.

MATERIAL AND METHODS

Experimental design

This *in vitro* study had a completely randomised and blinded design (regarding CFU counts) with antimicrobials (azithromycin, metronidazole or fluconazole) as factors. CFU counts of the microorganisms in the multispecies biofilm (*Streptococcus oralis* OMZ 607, *Streptococcus mutans* OMZ 918, *Actinomyces naeslundii* OMZ 745, *Veillonella dispar* OMZ 493, *Fusobacterium nucleatum* OMZ 596, *Candida albicans* OMZ 110 and total microorganisms) were dependent variables. Hydroxyapatite, titanium and polymethylmetacrylate (PMMA) discs were used as substrata for biofilm development mimicking tooth enamel,

dental implant and denture base, respectively. Biofilms grown on hydroxyapatite were exposed to azithromycin, on titanium to metronidazole and on PMMA to fluconazole. Biofilms formed on the different substrata without drug exposure was used as control for each treatment. Discs were placed in vertical position by disc holders in 24-well tissue culture plates. Mature multispecies biofilms (64.5 h of development) were exposed to antimicrobial (azithromycin, metronidazole or fluconazole) at salivary concentrations for 24 h. After this period, the biofilm was removed from the discs by ultrasonic waves, plated on agar media and CFU counts of each microorganism were calculated. Scanning electron microscopy (SEM) was used to visualise the biofilm structure and confocal scanning laser microscopy (CLSM) was used to assess the spatial arrangement of the cells stained by fluorescent *in situ* hybridization (FISH) technique.

Preparation of hydroxyapatite, titanium and PMMA discs

Hydroxyapatite discs were purchased from Clarkson Chromatography Products Inc. (South Williamsport, PA). Titanium discs were fabricated from a cylindrical bar of titanium grade IV (10 mm diameter; Sandinox, São Paulo, Brazil) sliced by electrical discharge machining. Polymethylmetacrylate (PMMA) discs were prepared using acrylic resin polymerized by hot water bath (QC-20 PMMA, Dentsply Ltd., Weybridge, England). The acrylic resin was placed inside a stainless steel matrix and the polymerization cycle was performed according to the manufacturer's instructions. The PMMA discs were immersed in distilled water at 37 ºC for 12 h for residual monomer release (25). The disks of all the three substrata (10 mm diameter and 2 mm thickness) had the surface roughness standardized by grinding both sides with progressively smoother aluminium oxide papers (320, 400 and 600 grit) in a horizontal polisher (model APL-4; Arotec, São Paulo, Brazil). Next, they were washed twice with sterile distilled water and then ultrasonicated for 20 min to remove any residues from the surface. The surface roughness was measured using a profilometer (Surfcorder SE 1700; Kosaka Laboratory Ltd, Kosaka, Japan) (26). The average surface roughness obtained for

hydroxyapatite, titanium and PMMA discs were 0.32 (± 0.02), 0.33 (± 0.01) and 0.34 ± (0.02) µm, respectively. Previously to the biofilm assay, the discs were placed in disc holders and sterilized by ethylene oxide (27).

Inoculum and media

The microorganisms used in this study were *Streptococcus oralis* OMZ 607, *Streptococcus mutans* OMZ 918, *Actinomyces naeslundii* OMZ 745, *Veillonella dispar* OMZ 493, *Fusobacterium nucleatum* OMZ 596 and *Candida albicans* OMZ 110 (22, 28). The microorganisms were first grown on Columbia blood agar (Difco, Sparks, MD, USA) supplemented with 5% defibrinated sheep blood (CBA), and then in fluid universal medium (29) supplemented with 67 mmol/L Sorensen's buffer, pH 7.2 (modified fluid universal medium, mFUM). *V. dispar* was cultivated in mFUM containing 0.10% (w/v) Na lactate. The five bacteria were incubated in anaerobic atmosphere at 37 ºC, while the yeast was incubated in aerobic atmosphere at 37 ºC. The cultures were adjusted independently to an optical density of 1.0 ± 0.05 (OD_{500}) by dilution with fresh mFUM or by centrifugation of the cells and resuspension with fresh mFUM. The inoculum was composed of aliquots of 1 mL of each density-adjusted culture that were mixed and stored on ice until the onset of the biofilm assay.

Human saliva collection

Human whole unstimulated saliva was collected from healthy volunteers who had not used antimicrobials, mouth rinses or any other medication known to affect salivary composition and flow in the past 3 months and who provided written informed consent previously approved by the local Ethics Committee. Unstimulated saliva was collected in the morning before any meal for 1 h using 50 mL polypropylene tubes immersed in ice and frozen at -20 ºC. After several days of collection, the total amount was pooled and centrifuged (10,000x rpm, 30 min, 4 ºC). The supernatant was pasteurized (30 min, 60 ºC) and centrifuged again in sterile bottles. The supernatant saliva was stored in 50 mL polypropylene tubes at -

20 ºC. To verify contamination, samples of the processed saliva were plated on CBA and incubated aerobically and anaerobically for 72 h (22). Saliva was used to form the salivary pellicle on the discs and with the medium mFUM during the biofilm cultivation.

Antimicrobial drugs

Two antibacterial drugs, azithromycin ($C_{38}H_{72}N_2O_{12}$; MW 748.98) and metronidazole ($C_6H_9N_3O_3$; MW 171.15), and one antifungal drug, fluconazole ($C_{13}H_{12}F_2N_6O$; MW 306.27), were evaluated. The antimicrobials were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used to prepare stock solutions at a final concentration of 1,000 µg/mL. Metronidazole and fluconazole were ressuspended in distilled water, while azithromycin was ressuspended in ethanol. The stock solutions were sterilized by filtration (0.22 µm pore size membrane filter) and stored in freezer at -20 ºC until the onset of the biofilm assay.

Multispecies biofilm assay

The multispecies biofilm assay was performed as described by Guggenheim *et al.* (2001) with slight modifications (22, 30, 31). First of all, the sterilized discs were placed in 24-well tissue culture plate in vertical position using disc holders, covered with 2.0 mL of processed whole unstimulated saliva, and incubated for 4 h at 37 ºC to form the salivary pellicle. Each disc was removed and placed in another well containing 1.8 mL of a medium mixture composed of 70% saliva + 30% mFUM with 0.3% glucose and 225 µL of the inoculum previously described. The culture plate was incubated anaerobically at 37°C for 16.5 h. After that, the discs were washed by three consecutive dips in 0.9% NaCl solution and inserted in fresh medium mixture composed of 70% saliva + 30% mFUM with 0.15% glucose and 0.15% sucrose. This change to fresh medium mixture was performed at each 24 h (16.5 h, 40.5 h and 64.5 h). In addition to this step, the discs were dipped twice a day (4 h and 8 h after the change of the medium mixture) in 2 mL of 0.9% NaCl solution. At the last medium change (64.5 h), the biofilm formed on the

discs were exposed to one of each antimicrobial evaluated for 24 h. Biofilms used as control were immersed only in the medium mixture without drug exposure. It was used the concentration of the antimicrobial reported in saliva as a normal release of drug. The biofilm formed on hydroxyapatite were exposed to azithromycin concentration of 2.12 µg/mL (11), on titanium to metronidazole concentration of 15.15 µg/mL (13), and on PMMA to fluconazole concentration of 2.56 µg/mL (12). The multispecies biofilm assay for each antimicrobial was performed in triplicate in three independent experiments on different days (n = 9).

Biofilm analyses

After the biofilm development phase and drug exposure (88.5 h), each disc was washed three times in 2 mL of 0.9% NaCl solution and aseptically inserted into a cryogenic tube containing 3 mL of 0.9% NaCl solution. Biofilm was removed from the disc by ultrasonic waves at 7 W for 30 s (32) and this suspension was serially diluted (10^{-1} to 10^{-6}) in 0.9% NaCl solution. Aliquots of 50 µL were plated on Columbia blood agar (Difco, Sparks, MD, USA) supplemented with 5% defibrinated sheep blood (CBA), Mitis Salivarius Agar (MSA; Difco, Sparks, MD, USA), Cadmium Sulfate Fluoride Acridine Trypticase Agar (CFAT) (33), Veillonella Agar (34), CBA supplemented with erythromycin (1 mg/L; Sigma-Aldrich), norfloxacin (1 mg/L; Sigma-Aldrich), and vancomycin (4 mg/L; Sigma-Aldrich) (CBA+), and Biggy Agar (BBL, BD, Franklin Lakes, NJ, USA) (35). CBA, Veillonella agar, CFAT and CBA+ plates were incubated anaerobically at 37 ºC for 72 h. MSA plates were incubated at 37 ºC and 10% CO_2 for 48 h and Biggy Agar plates incubated aerobically at 37 ºC for 24 h. Colony-forming units (CFU) were counted using a stereomicroscope. CBA plates were used to count total microorganisms; MSA, *S. oralis* and *S. mutans*; CFAT, *A. naeslundii*; Veillonella Agar, *V. dispar*; CBA+ *F. nucleatum*; and Biggy Agar, *C. albicans*. Biofilm dry weight was also estimated with 400 µL of the biofilm suspension (36). The results were expressed in CFU per mg of dry weight of biofilm.

Scanning electron microscopy and confocal scanning laser microscopy

Scanning electron microscopy (SEM) was performed to examine the surface of the biofilm formed after exposure to antimicrobial. After biofilm development phase and drug exposure (88.5 h) all discs were washed three times in 0.9% NaCl solution and fixed in 2% glutaraldehyde for 2 h. The discs with the biofilm was dehydrated by rinsing in ethanol, air-dried, mounted on stubs, sputter-coated with gold and examined with a scanning electron microscope (JEOL JSM-5600LV; Peabody, MA, USA) at an accelerating voltage of 15 kV.

Confocal scanning laser microscopy (CSLM) was used to visualize *C. albicans* in the biofilm formed on PMMA discs exposed to fluconazole. The oligonucleotide probes to specific 16S rRNA and 18S rRNA sequences labelled with different fluorophore at the 5'-end were purchased from Invitrogen (Eugene, OR, USA). The eubacteria probe EUB 388 (5´- *Alexa Fluor 488* - GCT GCC TCC CGT AGG AGT-3´) (37) was used to stain all bacteria and the eukaryotic probe EUK 516 (5´- *Alexa Fluor 647* - ACC AGA CTT GCC CTC C-3´) (38) to stain *C. albicans*. The biofilm cells were stained by fluorescent *in situ* hybridization (FISH) as described by Thurnheer *et al.* (2004) with slight modifications. Briefly, the discs with biofilm (control and experimental) were fixed in 4% paraformaldehyde/PBS for 1 h, permeabilized by treatment with lysozyme (70,000 U/mL in Tris-HCl pH 7.5; Sigma-Aldrich) for 10 min at 37 ºC, and washed in 0.9% NaCl solution. The discs were removed from the disc holders and the biofilms were pre-incubated in hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, 30% formamide and 0.01% SDS) at 46 ºC for 15 min. After that, the discs were immersed in hybridization buffer containing the probes (5 µg/mL) and incubated at 46 ºC for 3 h. The discs were transferred to washing buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 112 mM NaCl and 0.01% SDS) and were incubated for 15 min at 48 ºC. The excess of saline was removed and the discs were embedded upside-down in 10 µl of Mowiol (39). Discs were stored protected from the light at room temperature for 6 h before examination. Biofilms were evaluated using DMI 6000 CS inverted microscope (Leica Microsystems CMS, Mannheim, Germany) coupled to TCS SP5 computer-

operated confocal laser scanning system (Leica Microsystems CMS, Mannheim, Germany). Ar-ion laser tuned at 488 nm and a He-Ne laser at 633 nm were used for excitation of EUB 388 (*Alexa Fluor 488*) and EUK 516 (*Alexa Fluor 647*), respectively. Filters were set to 500 - 540 nm for detection of "Alexa Fluor 488" and 660 - 710 nm for "Alexa Fluor 647". To assess the structure of the biofilms, a series of optical sections was taken throughout the full depth of the biofilm by acquisition with Z-step. Confocal images were obtained using 40x oil immersion objective (numeric aperture 1.25). Each biofilm was scanned at randomly selected positions. Image acquisition was done in 8x line average mode.

Statistical analysis

The statistical analyses were done using SAS software (SAS Institute Inc., version 8.01, Cary, N.C., USA) employing a significance level fixed at 5%. The null hypothesis assumed no difference for microorganisms counts in the biofilms exposed to antimicrobials. Data that violated the assumptions of equality of variances and normal distribution of errors were transformed to $\log_{10}(40)$ before they were analyzed by independent-samples *t* test. When no transformation was adequate to normalize data (*V. dispar* and *F. nucleatum* counts in the biofilm exposed to metronidazole; and *V. dispar* counts in the biofilm exposed to fluconazole), they were analyzed by the Wilcoxon-Mann-Whitney nonparametric test.

RESULTS

All evaluated antimicrobials were able to change microbial population in biofilms ($p<0.05$), however none of the antimicrobials were able to completely eradicate a specific microorganism from the biofilm. The exposure to azithromycin reduced *A. naeslundii* and *V. dispar* population while increased *C. albicans* in the biofilm, as shown in Fig. 1. Although changes were observed in microbial population, no difference was found for total microorganism counts. The exposure to metronidazole reduced significantly the counts of all microorganisms evaluated,

which reflected reduction for total microorganism counts (p<0.05), as shown in Fig. 2. A great reduction was observed mainly for the two anaerobic gram-negative bacteria, *V. dispar* (3-log decrease) and *F. nucleatum* (5-log decrease) (p<0.001). The microbial counts for the biofilms exposed to fluconazole are shown in Fig. 3. Fluconazole reduced *C. albicans* population, however it was not enough to eliminate the yeast. A reduction was also found for *F. nucleatum*. Microbial population increases were observed for *S. oralis*, but mainly for *V. dispar*. No difference was found for total microorganism counts.

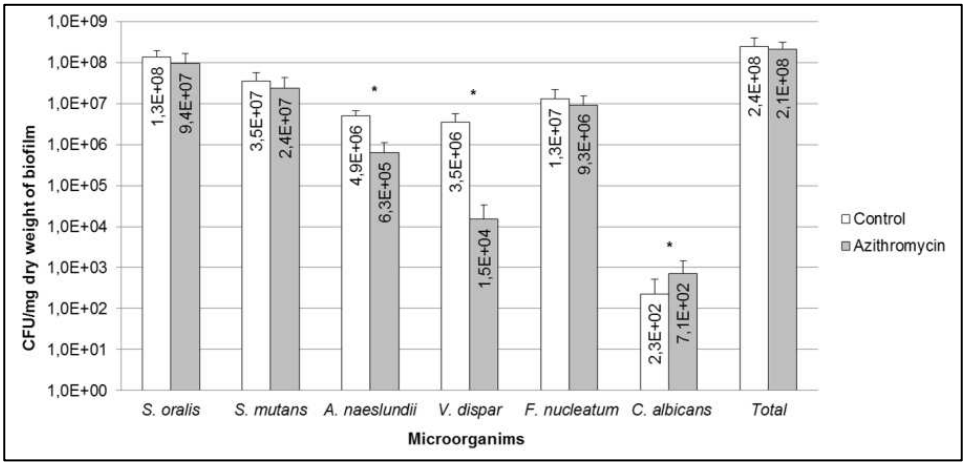

Figure 1. Multispecies biofilm formed on hydroxyapatite and exposed to salivary concentration of azithromycin (2.12 µg/mL) for 24h. Asterisk represents statistically significant difference between control (white bar) and experimental (grey bar) groups for each microorganism (p<0.05). Data are presented in a $\log_{10}$ scale.

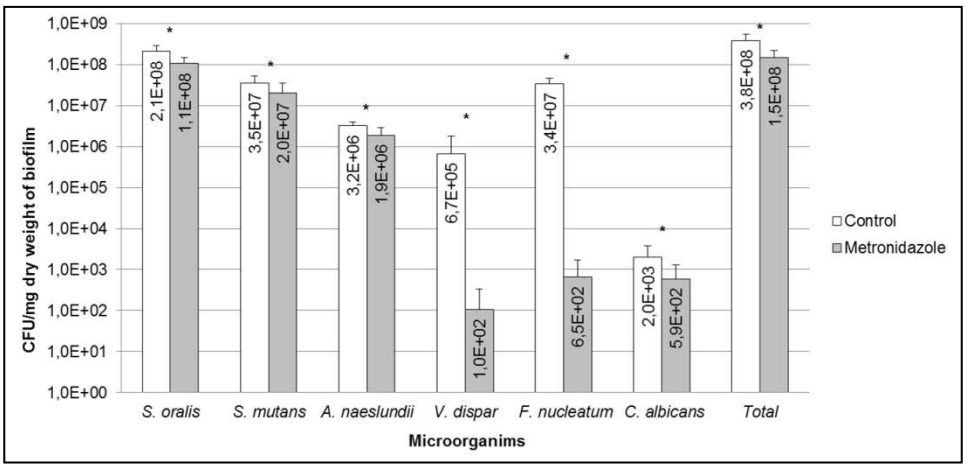

Figure 2. Multispecies biofilm formed on titanium and exposed to salivary concentration of metronidazole (15.15 µg/mL) for 24h. Asterisk represents statistically significant difference between control (white bar) and experimental (grey bar) groups for each microorganism (p<0.05). Data are presented in a $\log_{10}$ scale.

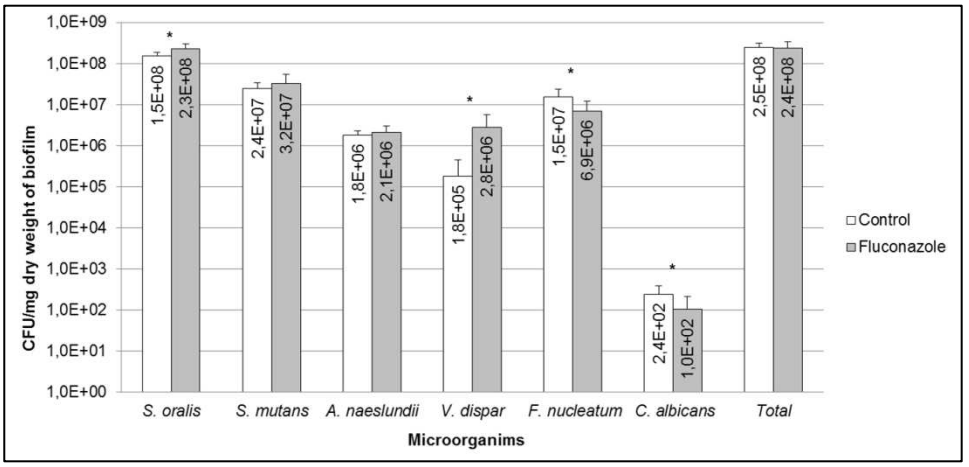

Figure 3. Multispecies biofilm formed on PMMA and exposed to salivary concentration of fluconazole (2.56 µg/mL) for 24h. Asterisk represents statistically significant difference between control (white bar) and experimental (grey bar) groups for each microorganism (p<0.05). Data are presented in a $\log_{10}$ scale.

Scanning electron microscopy (SEM) images showed that the antimicrobials in salivary concentration were able to alter the multispecies biofilm structural organization (Fig. 4). In the biofilms evaluated, *C. albicans* was often seen not on the superficial layers, the yeast was localized mainly on the deepest layers, close to the substratum. However, in the biofilm exposed to azithromycin it was possible to see *C. albicans* even on the superficial layers, probably due the decrease of some bacteria and increase of *C. albicans* population. It was also observed the growth of *F. nucleatum* on yeast surface. The biofilm exposed to metronidazole showed high amounts of empty spaces, probably due to the considerable loss of *F. nucleatum*, and it was possible to see mainly *streptococci* and *actinomyces*. The SEM images of the biofilm exposed to fluconazole showed to be very similar to the control group, however, when visualized by FISH, it was possible to see the presence of pseudohyphae and hyphae cells in the experimental group (Fig. 5).

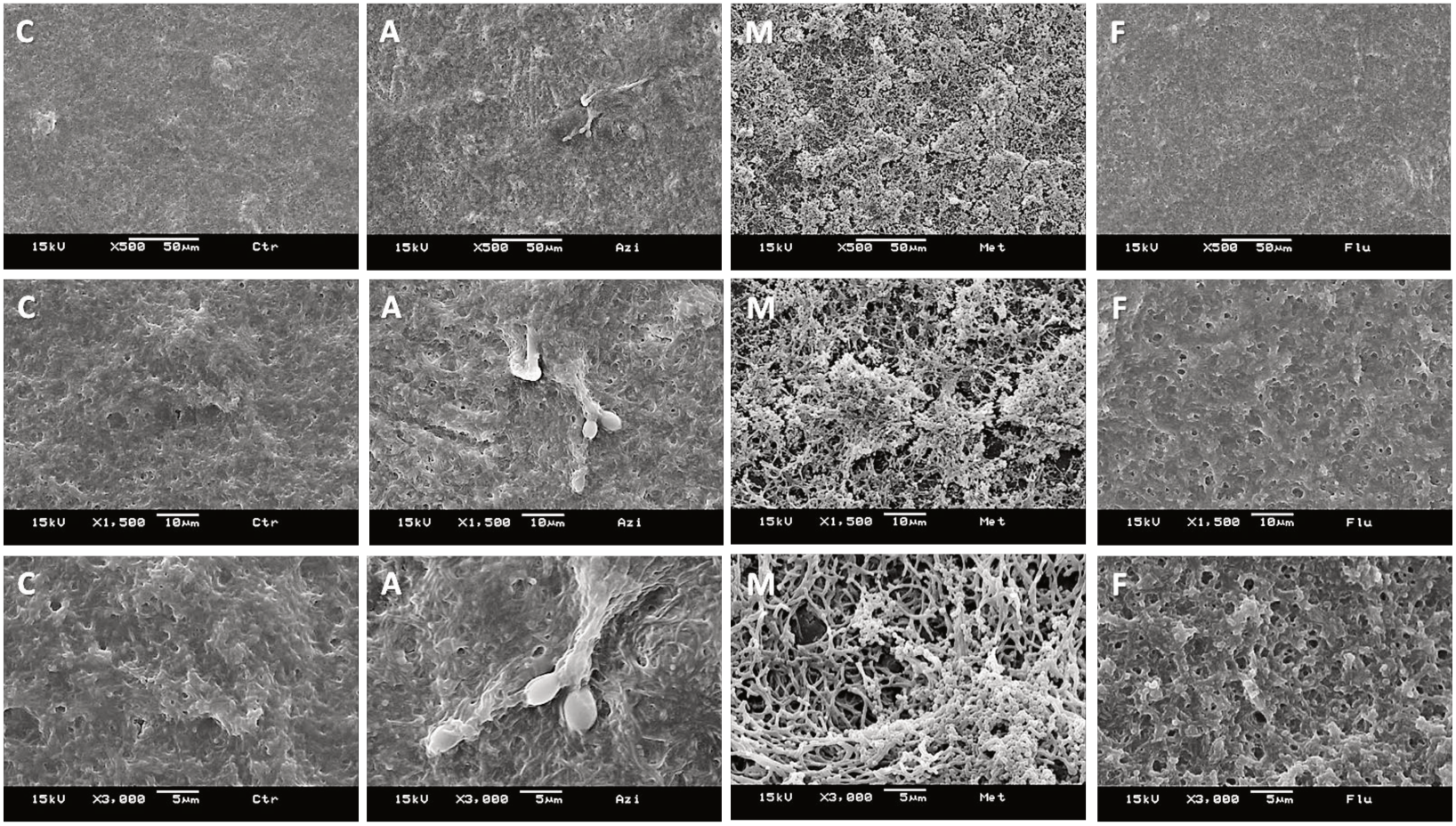

Figure 4. SEM images showing the multispecies biofilm organization. Multispecies biofilms were grown for 64.5 h and then were exposed to salivary concentration of antimicrobials for more 24 h or not (control). C – control group (no antimicrobial exposure; biofilm formed on PMMA); A - exposure to azithromycin (2.12 µg/mL); M - exposure to metronidazole (15.15 µg/mL); and F - exposure to fluconazole (2.56 µg/mL).

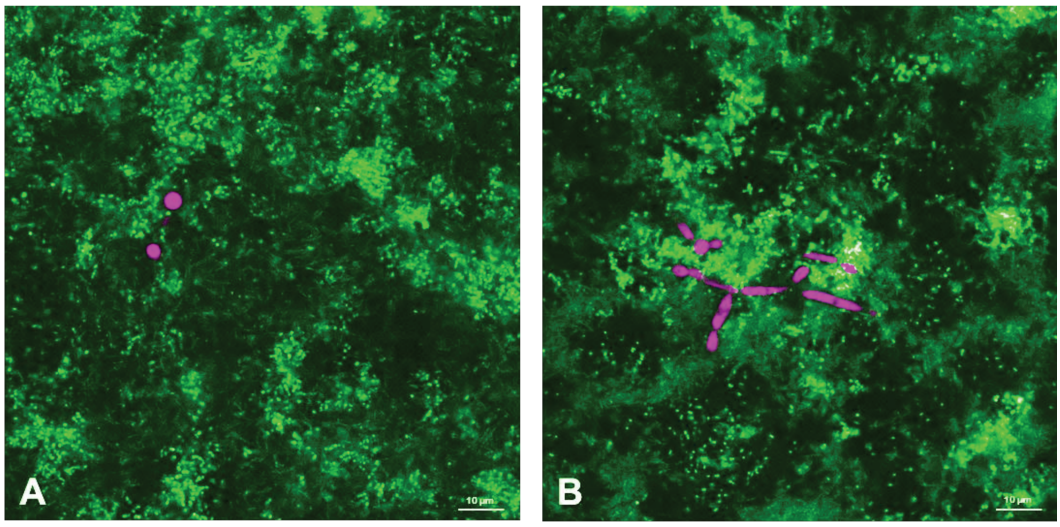

Figure 5. CSLM images showing *C. albicans* in the multispecies biofilm. Multispecies biofilms were grown for 64.5 h and then were exposed to salivary concentration of fluconazole (2.12 µg/mL) for more 24 h or not (control). The microorganisms were stained by FISH technique: total bacteria in green (probe EUB 388 coupled to Alexa Fluor 488) and *C. albicans* in purple (EUK 516 coupled to Alexa Fluor 647). Control group (A) presented *C. albicans* as yeast cells, while the experimental group (B) showed predominance of *C. albicans* as pseudohyphal and hyphal forms. (40x oil immersion objective; zoom 3x).

DISCUSSION

In our study we evaluated how antimicrobials present in saliva can affect the microbial population of a mature oral biofilm. Considering that oral biofilms act as a reservoir of microorganisms that can cause disease in oral and non-oral sites, compromising even the systemic health, it is important to investigate if microbial population can be affected by antimicrobials. We evaluated three antimicrobials, two antibiotics and one antifungal agent, which are highly bioavailable in saliva and are prescribed for the treatment of a wide range of infections via systemic drug administration. In view of our results, we could observe that the antimicrobials not only altered bacteria and yeast population, but also affected the biofilm structural organization.

Oral biofilms are complex and dynamic communities composed by different species of microorganisms which interact themselves since the initial adhesion step on substratum until growth and maturation. Although studies use mainly single-species (19, 36) or even duo- or three-species biofilm models (20), they point out the need for studies in multispecies biofilms that could represent in a most faithful way the oral cavity. In this study we used a validated multispecies biofilm model (22), which has been used and refined throughout the last eleven years (23, 24, 28, 30, 31, 41, 42). This model was composed by five bacteria that represent the main population in supragingival biofilms and one yeast, a microorganism also found in this environment. The advantage of using an *in vitro* model was the possibility to standardize species and amount of microorganisms, which are very difficult in studies with clinical samples, in view of the great variability of the microbial composition of oral biofilms. However, the great advantage of using this model was the possibility to evaluate the exposure of oral biofilms to antimicrobials without the need to use animals or humans for research purposes.

The antimicrobials evaluated are used for the treatment of bacterial or fungal infections and the reason for their choice was based on their high bioavailability, being well distributed into most body tissues and fluids, including saliva. These drugs are naturally released in saliva, which also favours the drug redistribution by saliva swallowing. The reported bioavailability after oral administration of azithromycin, metronidazole and fluconazole is around 37%, 80% and 90%, respectively (11, 13, 15-18), which can be high when compared with other antimicrobials (14, 43). Our aim was not to assess the antimicrobials for biofilm control, but to evaluate if microbial population could be affected by the concentration of antimicrobials commonly released in saliva.

Azithromycin was one of the antibiotics evaluated. It is a semi-synthetic macrolide that penetrates the cell wall and binds to the 50S ribosomal subunit of the 70S ribosome of susceptible organisms, thereby inhibiting RNA-dependent protein synthesis. Azithromycin is as a bacteriostatic antibiotic, however it can be

bactericidal depending on antibiotic concentration and bacterial sensitivity. The biofilm exposed to azithromycin showed reduced levels of *A. naeslundii*, a facultative gram-positive bacterium, and *V. dispar*, an anaerobic gram-negative bacterium. The reduction of these microorganisms is in agreement of the drug spectrum of action, which includes action towards aerobic and facultative gram-positive microorganisms and some anaerobic bacteria as well. Though this drug is widely used for treatment of *streptococci* infections, such as *S. pyogenes* and *S. pneumonia* (44), no significant reduction was observed in the counts of the two streptococci used in this model, *S. oralis* and *S. mutans*. There was a decrease tendency in the counts, but they were not statically significant.

The lack of drug action in *S. oralis* and *S. mutans* could be due to distinct factors. First, it depends on how these streptococci are susceptible to this drug. Second, the supplied azithromycin concentration of 2.12 µg/mL, similar as found in mouth, could not be sufficient to affect these bacteria. An interesting observation was the increase of *C. albicans* levels, probably due to the reduction of bacteria on the biofilm. In addition, only the biofilm exposed to azithromycin showed *C. albicans* cells on its surface, the yeast was not visualized on the surface of the other biofilms, control and treated with other antimicrobials (Fig. 4). It has been reported that the use of wide spectrum antibiotics prone denture wearers to develop *Candida*-associate diseases (6), this is the first study to show this relation in a multispecies biofilm. The higher counts and the yeast presence on the biofilm surface could favour *C. albicans* to penetrate the mucosa through the fitting denture surface.

Multispecies biofilm exposed to metronidazole showed reduced population for all microorganisms, but mainly for the anaerobes *F. nucleatum* and *V. dispar*. Metronidazole is a selective drug for anaerobic bacteria, because although it is able to penetrate all cells, only sensitive anaerobes have the electron transport proteins necessary to reduce the drug and produce metabolites that are responsible to cause DNA damage, and consequently cell death, being a bactericidal drug (16). *F. nucleatum* is one of the most prevalent microorganisms in

this multispecies biofilm model, and its reduction, affected the tridimensional structure of the biofilm. It was possible to observe by the SEM images (Fig. 4; 3a to 3c) that the exposure to metronidazole left a scaffold of cells, composed mainly by *actinomyces* and *streptococci*, with empty spaces through the biofilm. Considering that a mature biofilm was used, probably a great number of *F. nucleatum* and *V. dispar* cells was killed and they detached from the biofilm taking together other microbial cells, which would explain the reduction for all of the other microorganisms (Fig 2). Despite the great reduction in anaerobes caused by metronidazole, it was not able to completely eliminate these microorganisms. Removal of the antibiotic probably would favour the growth of the anaerobes to initial levels.

Fluconazole was the only antifungal evaluated. The drug is mainly fungistatic and acts inhibiting the cytochrome P450 enzyme Erg11, which is required for ergosterol synthesis, the main sterol in the fungal cell membrane. The replacement of ergosterol by methylated sterols affects membrane packing, which alters membrane permeability and functions (18, 45). The biofilm exposed to fluconazole showed reduced counts for *C. albicans*, probably due the antifungal presence. The decrease observed for *F. nucleatum* could be associated with *C. albicans* reduction. It was observed in SEM images of the group exposed to azithromycin (Fig. 4; 2c) that *F. nucleatum* was present on *C. albicans* surface. Interactions between facultative/anaerobe bacteria and *C. albicans* have been reported. The yeast acts reducing the oxygen tension, which provides a better micro environment for *F. nucleatum* growth (46). The higher counts of *V. dispar* and the slight increase for *S. oralis* are probably due the reduction of the other microorganisms.

The biofilm exposed to fluconazole presented similar structural organization when compared with the control, as seen by SEM images (Fig 4), however it was not possible to visualize *C. albicans*, the main target of the antifungal used. Therefore we used FISH technique and CLSM to gain understanding of the yeast localization. It was interesting to visualize the

morphological differentiation for *C. albicans*, with the presence of pseudohyphae and hyphae cells in the biofilm exposed to fluconazole. Although the drug could act inhibiting hyphal differentiation (18), it was reported that the exposure to lower concentrations of the drug favoured the cells to stay in the hyphal form (45). It is important to emphasize that hyphae is considered as a virulence factor for *C. albicans* and probably acts as a dormant state for the yeast during drug exposure in low concentration.

Despite the fact that the antimicrobials can be released in high concentration in the oral environment, they are not able to completely eliminate microbial species. Probably the drug concentration is not sufficient to kill the sensitive microorganisms, and in addition, the intercellular material among the microorganisms, which is composed mainly by extracellular polysaccharides, can also provide protection for cells in the lowest layers against antimicrobial agents. The aim of using antimicrobials is to aid the immunological system to control infection, however in the oral cavity the immune response is restricted. The oral biofilms are mostly present in non-vascularized areas, bathed mainly by saliva, and the action of immune system cells is very limited. Therefore, the mechanical disturbance and removal of biofilms remain important in order to control oral infectious diseases.

Our null hypothesis tested was rejected since the results showed that antimicrobials were able to change microbial population of oral biofilms. The clinical importance of our study is that antimicrobials released in saliva can modulate bacterial and fungal population in biofilms. The use of antibiotics can favour *C. albicans* growth and, on the other hand, the use of antifungals can alter bacterial population. In our study we evaluated a six multi-species model, however the oral biofilms are composed by larger amount of microorganisms, which has to be evaluated in more complex biofilms. Moreover, the evaluation of other antimicrobials is needed in order to assess the role of different agents on oral biofilms. Further studies on how virulently the microorganisms behave during and after the antimicrobial exposure and the relationship with immune system control

has also to be evaluated. Oral biofilms, exposure to drugs and immune system is a wide field of study to be investigated.

CONCLUSION

It was possible to conclude that multispecies biofilms exposed to antimicrobials in concentration commonly released in saliva alter the bacterial and fungal population, and also affect the biofilm structural organization.

ACKNOWLEDGMENTS

The authors thank Prof. Dr. Bernhard Guggenheim for providing the microorganisms used in the multispecies biofilm model (OMZ strains). We are grateful to São Paulo Research Foundation (FAPESP) for the scholarship received by the first author during his graduation course (# 2009/10698-0) and for the financial support provided by the research grant (# 2010/07894-9). We also thank Eliene A. Orsini Narvaes and Adriano L. Martins for the technical assistance in the scanning electron and confocal scanning laser microscopy.

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CONCLUSÃO GERAL

Os resultados do presente trabalho demonstraram que os diferentes substratos presentes na cavidade oral não favorecem a prevalência de espécies de micro-organismos no biofilme e também que antimicrobianos de uso sistêmico em concentrações encontradas na saliva são capazes de promover alterações nas populações de bactérias e fungos dos biofilmes orais.

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

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APÊNDICE 1 - Ilustrações de Materiais e Métodos

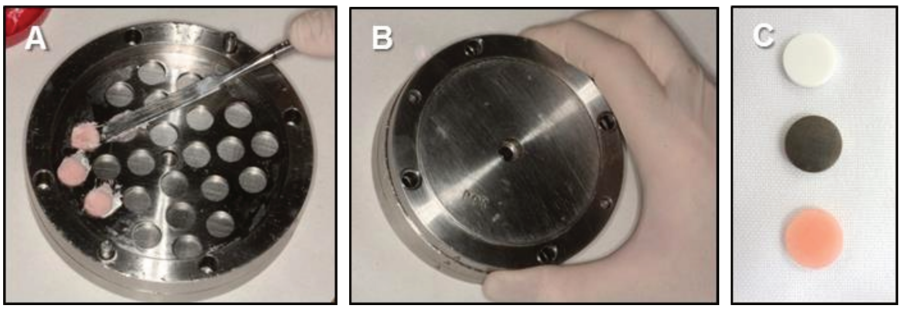


Figura 1: Confecção de espécimes de resina acrílica (PMMA) nas mesmas dimensões dos espécimes de hidroxiapatita e titânio. A - Inserção da resina dentro da matriz metálica, B - fechamento da matriz para posterior polimerização da resina e C - espécimes de hidroxiapatita, titânio e resina acrílica nas dimensões de Ø 10 mm x 2 mm.

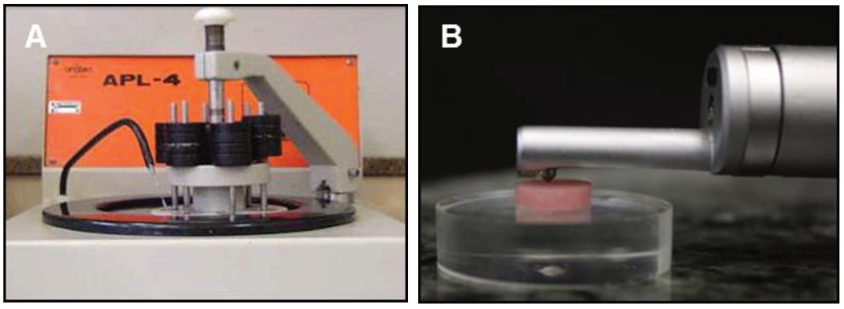


Figura 2: A - Polimento dos espécimes em politriz horizontal (APL-4; Arotec, São Paulo, Brazil) e B - mensuração da rugosidade de superfície dos espécimes por meio de rugosímetro (Surfcorder SE 1700 Kozaka Industry, Kozaja, Japão).

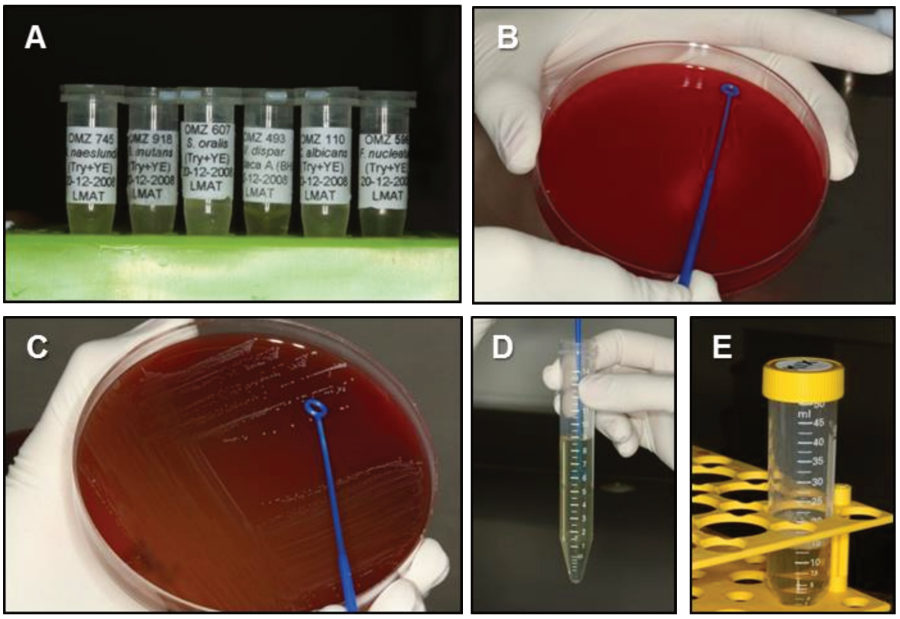


Figura 3: Reativação dos micro-organismos para preparo do biofilme multiespécies. A - culturas de estoque de cada micro-organismo armazenadas a -80 ºC, B - semeadura de cada micro-organismo em meio agar sangue Columbia (CBA) para reativação inicial, C - coleta de colônias crescidas no meio CBA, D - transferências das colônias para meio FUM 0,3% glicose e E - mistura dos micro-organismos crescidos em meio FUM (OD500=1.0 ± 0.05) para formar o inóculo .

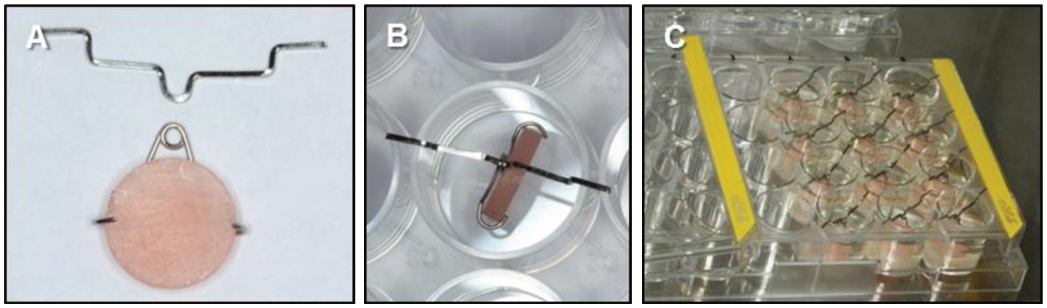


Figura 4: Formação do biofilme multiespécies. A - acoplamento dos espécimes a suportes metálicos, B - posicionamento dos espécimes dentro dos poços da placa de cultura de 24 poços e C - inóculo adicionado aos poços para inicio da formação do biofilme.

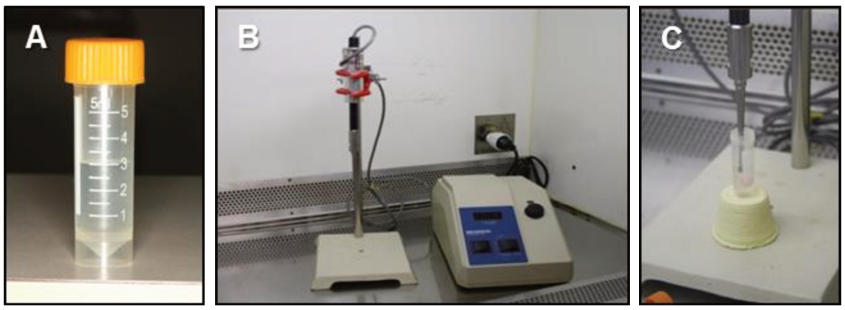
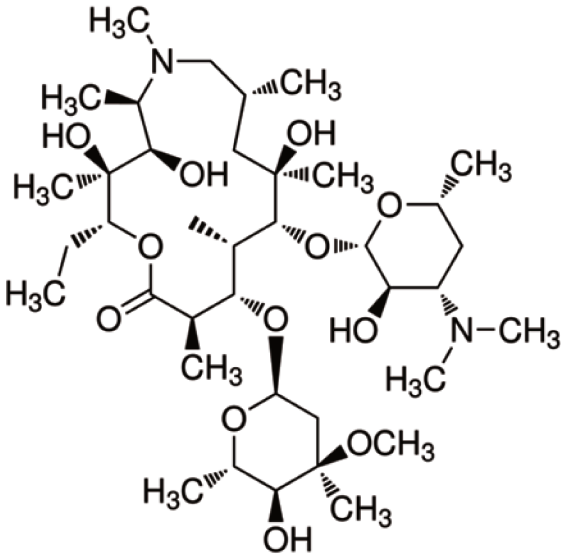
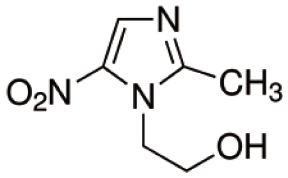


Figura 5: Coleta do biofilme para posterior plaqueamento e contagem. A - tubo criogênico contendo 3 mL de solução salina, B - sonicador utilizado (Sonifier 150™, Branson Ultrasonics Corporation, Danbury, CT, USA) e C - tubo criogênico com o espécime em seu interior para remoção do biofilme aderido ao substrato e separação das células aderidas entre si.

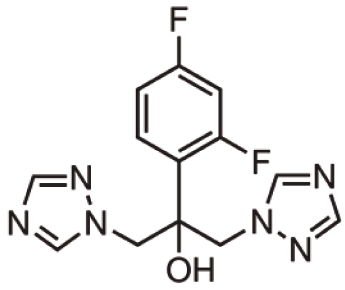
APÊNDICE 2 - Estrutura química dos antimicrobianos



Azitromicina ($C_{38}H_{72}N_2O_{12}$)
Peso molecular: 748,98 g/mol



Metronidazol ($C_6H_9N_3O_3$)
Peso molecular: 171,15 g/mol



Fluconazol ($C_{13}H_{12}F_2N_6O$)
Peso molecular: 306,27 g/mol

ANEXO 1 - Certificado 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 **"Avaliação de biofilmes multiespécie formados em diferentes substratos e expostos a concentrações salivares de agentes antimicrobianos: Quantificação de microrganismos e morfologia tridimensional do biofilme"**, protocolo nº 102/2009, dos pesquisadores Altair Antoninha Del Bel Cury e Antonio Pedro Ricomini Filho, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 12/08/2009.

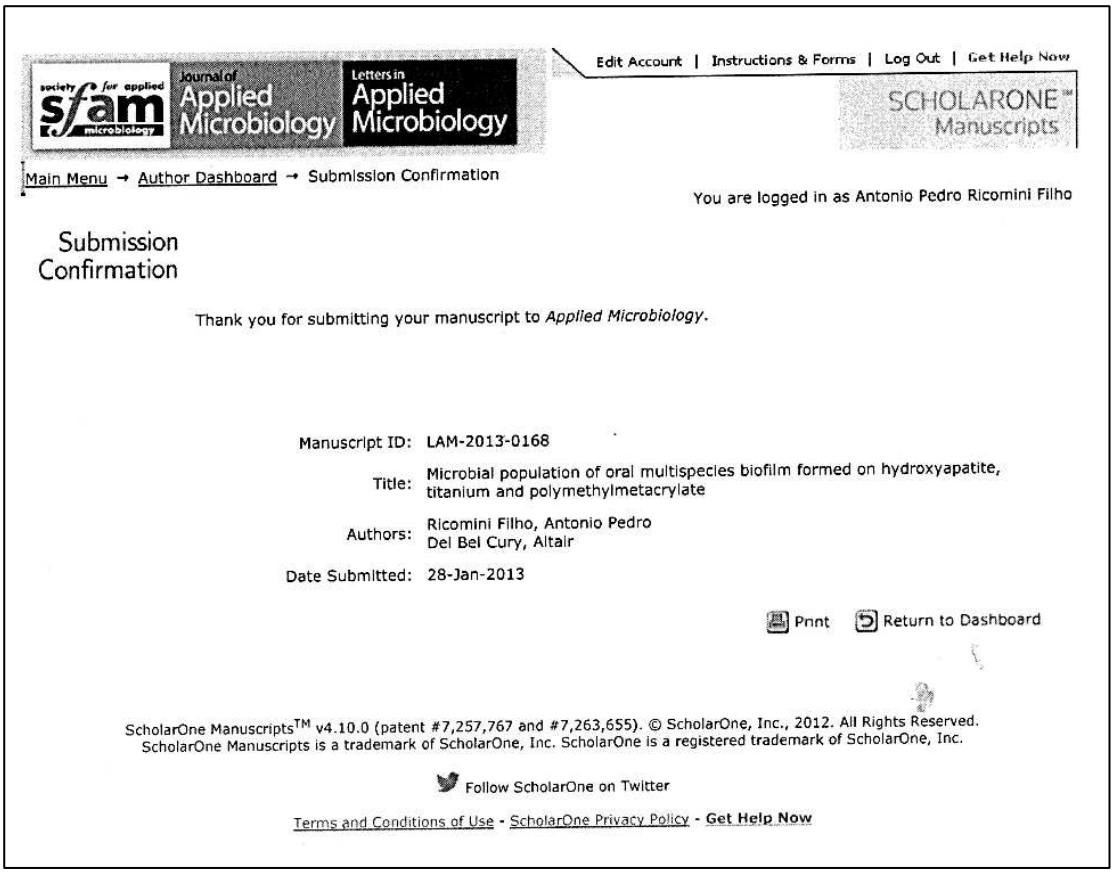
The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Evaluation of multi-species biofilms formed on different substrata and exposed to salivary concentrations of antimicrobial agents: Microorganisms quantification and biofilm tridimensional morphology"**, register number 102/2009, of Altair Antoninha Del Bel Cury and Antonio Pedro Ricomini Filho, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at .

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

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

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

ANEXO 2 - Confirmação de submissão de atigo ao periódico Letters in Applied Microbiology



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