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Dissertação de Mestrado

Formação de biofilme em reembasadores temporários para prótese dentária

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FORMAÇÃO DE BIOFILME EM REEMBASADORES TEMPORÁRIOS PARA PRÓTESE DENTÁRIA

Dissertação de Mestrado apresentada à Faculdade de Odontologia da Universidade Federal de Pelotas obtenção do título de Mestre em Odontologia (área do conhecimento: Prótese Dentária).

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Augusto Curry

NOTAS PRELIMINARES

A presente dissertação foi redigida segundo o Manual de Normas para Dissertações, Teses e Trabalhos Científicos da Universidade Federal de Pelotas de 2006, adotando o Nível de Descrição 4 – estruturas em Artigos, que consta no Apêndice D do referido manual. Disponível no endereço eletrônico:

(http://www.ufpel.tche.br/prg/sisbi/documentos/Manual_normas_UFPel_2006.pdf).

Resumo

VALENTINI, Fernanda. **Formação de biofilme em reembasadores temporários para prótese dentária**. 2012. 97f. Dissertação (Mestrado) – Programa de Pós-Graduação em Odontologia. Universidade Federal de Pelotas, Pelotas.

Os fungos oportunistas são responsáveis por doenças infecciosas na cavidade bucal que aumentaram em prevalência nos últimos anos, especialmente em usuários de prótese total. Assim, a colonização e o crescimento de espécies de *Candida* e outros microrganismos em próteses tem fundamental importância clínica. Este estudo teve por objetivo (i) fazer uma revisão sistemática para determinar se existe um protocolo de prevenção ou tratamento da colonização por *Candida* em reembasadores de prótese e (ii) avaliar clinicamente como a composição do biofilme é afetada por diferentes materiais, tempo e a presença ou não de candidíase, em usuários de prótese total. Estudos clínicos e in vitro foram avaliados quanto ao tratamento e / ou prevenção da colonização por *Candida* e formação de biofilme em reembasadores de prótese. Seis bases de dados eletrônicas foram pesquisadas (Lilacs, Scopus, Pubmed / Medline, Scielo e Cochrane Database of Systematic Reviews) de 1950 a 2012 usando as palavras-chaves “denture liner”, “*Candida”*, “tissue conditioner*”*, “denture stomatitis” e “antifungal agents”. Para o estudo *in situ* vinte e oito voluntários usuários de prótese total, quinze portadores de estomatite por dentadura, e doze pacientes com alguma espécie de *Candida* avaliados por *screening* inicial foram selecionados. Foi quantificada a formação de biofilme sobre espécimes de resina acrílica e reembasadores temporários (a base de resina acrílica ou silicone) inseridos na parte interna da prótese total superior em duas fases de 21 dias. Os espécimes foram removidos aleatoriamente no 7º, 14º e 21º dia. Amostras representativas foram analisadas em MEV nos diferentes períodos de avaliação. Unidades formadoras de colônia/mm^2 de biofilme de estreptococos do grupo mutans, lactobacilos, microrganismos totais e espécies de *Candida* foram determinados. Através da revisão sistemática foi possível observar que a incorporação de nistatina para prevenir o aparecimento da doença e a imersão em hipoclorito de sódio para desinfectar reembasadores de tecidos são o tratamento mais frequentemente encontrado. No entanto, os dados encontrados foram quase que exclusivamente baseados em estudos in vitro, o que gera alto risco de viés. Para o estudo *in situ*, a contagem de *Candida* não-*albicans* para reembasadores a base de silicone foi maior

em pacientes com candidíase (p=0,01). Pacientes com candidíase apresentaram maiores contagens de estreptococos do grupo mutans após 7 dias (p=0,0041), mas essa diferença desapareceu após 14-21 dias de formação de biofilme. Com isso, reembasadores a base de silicone devem ser evitados em pacientes com candidíase já que estes materiais apresentaram aumento da contagem de espécies de Candida não-*albicans*, as quais são mais virulentas e resisitentes às terapias convencionais.

Palavras-chave: Condicionadores de Tecido. Biofilme. Prótese Total. *Candida*

Abstract

VALENTINI, Fernanda. **Biofilm formation on temporary denture liners.** 2012. 97f. Dissertação (Mestrado) – Programa de Pós-Graduação em Odontologia. Universidade Federal de Pelotas, Pelotas

Opportunistic fungi are responsible for infectious diseases in oral cavity that rose in prevalence in the last years, especially in complete denture wearers. Thus, colonization and growth of *Candida* species and other microorganisms are of clinical importance. The aims of this study were (i) systematically review the literature to determine if there is a protocol of prevention or treatment of *Candida* colonization in denture liners and (ii) clinically assess how biofilm composition is affected by different materials, time and the presence of candidiasis in denture wearers. In vitro and in vivo studies were evaluated with regard to treatment and/or prevention of *Candida* colonization and biofilm formation in denture liners. Six databases were searched (Lilacs, Scopus, Pubmed / Medline, Scielo and Cochrane Database of Systematic Reviews) from 1950 to 2012 using the keywords “denture liner”, “*Candida”*, “tissue conditioner*”*, “denture stomatitis” and “antifungal agents”. For the in situ study, twenty-eight volunteers, half with candidiasis, half healthy but *Candida* carriers wearing complete dentures were selected to participate in this study. Biofilm formed on acrylic resin and temporary denture liners (silicone based and acrylic resin based) specimens mounted in the internal surface of the volunteers’ upper dentures were collected in two phases of 21 days. Specimens were randomly removed on days 7, 14 and 21. Representative samples of the specimens were analyzed by SEM in the various periods under evaluation. Colony forming units/mm^2 of biofilm of mutans streptococci, lactobacilli, total microorganisms and *Candida* species were determined. Through the systematic review it was possible to observe that the incorporation of nystatin to prevent the disease and the immersion in sodium hypoclorite to disinfect denture liners was the most frequently found treatment. However, as the data was in general derived from in vitro studies, there is a high risk of bias. For the in situ study, non-*albicans Candida species* showed higher counts in the silicone-based denture liner in diseased patients (p=0,01). Patients with candidiasis showed higher counts of mutans streptococci after 7 days (p=0.0041), but this difference disappeared after 14-21 days of biofilm formation. Thus, silicone-based denture liners should be avoided in diseased patients, as they have shown

higher nonn-*albicans* *Candida* species, which are known to be more virulent and resistant to conventional therapies.

Keywords: Tissue Conditioner. Biofilm. Complete Denture. *Candida*

Sumário

1 Projeto de Pesquisa

1.1 Introdução

A cavidade bucal é colonizada por diversos microrganismos, os quais se apresentam em número limitado, o que é determinado pelas condições que seletivamente os favorecem, em condições de saúde (SAN MILLÁN et al., 2000). No entanto, de acordo com a teoria da placa ecológica, sabe-se que a presença e especialmente a proporção de algumas espécies propicia modificações que transformam um estado de saúde em doença, muito mais do que a presença de alguma espécie específica (MARSH, 1994).

Alguns dos microrganismos residentes na cavidade bucal são patógenos oportunistas, dos quais destacam-se as espécies de *Candida.* Este microrganismo eucariótico causa a candidíase bucal, comumente diagnosticada em humanos (MUZYKA, 2005). O crescimento desse fungo sobre superfícies é natural no ciclo de vida das espécies de *Candida* (KUMAMOTO; VINCES, 2005), o que pode explicar a ocorrência comum da colonização fúngica nos usuários de próteses.

As lesões da mucosa bucal relacionadas às próteses removíveis são reações agudas ou crônicas decorrentes da presença de biofilme dental, de leveduras, de constituintes do material utilizado para a confecção das próteses e da pouca retenção ou injúrias mecânicas oriundas do uso de próteses mal adaptadas (BUDTZ-JORGENSEN, 1978; BUDTZ-JORGENSEN 1981; DOREY et al., 1985). Entretanto, de todas as lesões que podem ocorrer, conforme supracitado, aquelas ocasionadas pela candidíase podem interferir com o tratamento e principalmente ser

uma barreira para a saúde do paciente (PEREZOUS, 2005), uma vez que as próteses podem servir como fonte de microrganismos para novas infecções (MUZYKA, 2005), sendo a prevalência de até 67% nos usuários de próteses removíveis (ARENDORF; WALKER, 1987; SPIECHOWICZ et al., 1991; RADFORD et al., 1999).

Esta inflamação também é denominada estomatite induzida por prótese, estomatite por dentaduras ou candidíase atrófica crônica, sendo que a *Candida albicans* foi e continua sendo fortemente associada como o principal agente etiológico desta patologia (WEBB et al., 1998; BARBEAU et al., 2003; ZAREMBA et al., 2006). Entretanto, hoje é sabido que espécies de *Candida* não-*albicans* (*C. tropicalis, C. parapsilosis*, *C. glabrata*, *C. krusei* e *C. dubliniensis*) podem ser isoladas e responsáveis por mais de 50% dos casos de infecção (SAMARANAYAKE; SAMARANAYAKE, 1994; COLEMAN et al., 1997; ELLEPOLA; SAMARANAYAKE, 2001; ZAREMBA et al., 2006; FIGUEIRAL et al., 2007).

A candidíase bucal pode ser classificada a partir da presença de grandes placas brancas pseudomembranosas na mucosa, língua e boca, lesões palatais eritematosas características da candidíase atrófica crônica e queilite angular nas comissuras labiais (SAMARANAYAKE, 1990; SCULLY et al., 1994; SHAY et al., 1997). A candidíase pode ser classificada de acordo com Newton em Tipo (I) lesões inflamatórias, eritematosas; Tipo (II) Eritema difuso, simples ou generalizado em mucosa coberta por prótese e Tipo (III) lesões granulares ou papilares comumente envolvendo a parte central do palato duro e rebordo alveolar.

A adesão de microrganismos em superfícies de biomateriais depende da estrutura e composição de sua superfície e das propriedades físico-químicas da superfície das células microbianas (BELLON-FONTAINE at al., 1990; BUSSCHER;

COWAN; VAN DER MEI, 1992), as quais vão aderir via formação de um biofilme. Biofilme pode ser definido como uma película não calcificada, fortemente aderida às superfícies dentais, resistindo a presença do fluxo salivar. O termo biofilme é usado para denotar uma comunidade microbiana encapsulada em polímero que se acumula em uma superfície, que também protege contra colonização de patógenos exógenos (WILSON, 2001). O biofilme constitui-se de depósitos bacterianos e constituintes salivares, com um crescimento contínuo, sendo considerada a principal causa das doenças infecciosas e estomatites (ROSAN; LAMONT, 2000).

A formação de biofilmes multi-espécie, envolto por uma matriz extracelular, protege o biofilme da ação de patógenos exógenos, da ação de alguns medicamentos e da ação da própria saliva, isso aumenta a chance de sobrevivência de todos os constituintes do ambiente bucal e é considerado o primeiro passo para a colonização fúngica, levando a um processo infeccioso (CHANDRA et al., 2001; CANNON; CHAFFIN, 1999; RAMAGE et al., 2004). Dessa forma, as espécies de *Candida* podem aderir diretamente ou via uma camada de “placa de dentadura” às bases de próteses (SAMARANAYAKE; MACFARLANE, 1980; BRANTING et al.,1989; EDGERTON et al., 1993; COULTHWAITE; VERRAN , 2007).

Entretanto, pouco se sabe sobre o efeito de diferentes superfícies na interação entre espécies de *Candida* e outros microrganismos, incluindo a superfície de materiais que contem antifúngicos, como os reembasadores e condicionadores de tecido (PEREIRA-CENCI et al., 2010). A utilização destes materiais é vantajosa em diversas situações clínicas e tem aumentado nos últimos anos. Porém, um dos problemas diretamente relacionados a estes materiais ainda é o acúmulo de biofilme (BOSCATO et al., 2009) e a colonização por *Candida.*

Reembasadores de prótese são materiais macios temporários, usados em prótese mucossuportadas totais e/ou parciais, com o intuito de realizar o forramento desses aparelhos protéticos, em áreas submetidas a cirurgias ou que apresentam inflamação na fibromucosa de revestimento, com o propósito de se conseguir distribuição de forças mastigatórias mais homogêneas, reduzindo dessa forma pressões localizadas sobre a mucosa e tecidos ósseos, originando mais conforto ao paciente e facilitando a cicatrização (LOVATO et al., 2002) além de auxiliar no restabelecimento da saúde da mucosa da área de suporte da prótese (HARRISON, 1981). São também utilizados em trauma mecânico por próteses mal adaptadas, na instalação de próteses imediatas, como adjuvante em tratamento de estomatite protética (HARRISON, 1981) em moldagens funcionais (BRADEN; CAUSTON, 1971; MURATA, 2005), após a colocação de implantes osseointegrados (KULAK; KAZAZOGLU, 1998) e para estabilizar a prótese total durante o registro das relações maxilomandibulares.

Mesmo com o crescente aprimoramento, esses materiais resilientes apresentam problemas de ordem físico-biológica que comprometem sua utilização clínica por longos períodos de tempo. Segundo Qudah et al (1990), as limitações são decorrentes do elevado índice de absorção dos fluidos bucais, levando a perda da estabilidade dimensional, a má adaptação da prótese e a descoloração por alguns agentes de limpeza impróprios, tais como o hipoclorito de sódio (NaOCl), que causa a ruptura na adesão entre os materiais. A perda de água, plastificante e etanol leva os materiais resilientes ao aumento de sua dureza e consequentemente a uma superfície mais porosa, rugosa e áspera que facilita a contaminação por biofilme e colonização por *Candida albicans* (NIKAWA et al*.,* 2003; CRAIG, 2004).

Os reembasadores de prótese podem ser divididos em dois grupos principais conforme o material que os compõem: resinas acrílicas ou silicones (polímero dimetil siloxano). Ambos estão disponíveis nas formas térmicas e quimicamente ativada (NIKAWA et al., 2000; RAZEK; MOHAMED,1980). Os materiais constituídos de resina acrílica apresentam-se, geralmente, na forma de pó e líquido. O pó é basicamente o poli (metacrilato de metila ou etila) e o líquido contém monômero acrílico e plastificante (álcool etílico e/ou acetato de etila) (DOUGLAS, 1987; VERRAN; MARYAN,1997). Os reembasadores de prótese à base de silicone quimicamente ativado são fornecidos como um sistema de dois componentes que polimerizam via reação por condensação (ANUSAVICE, 1996).

Até o presente momento, poucos estudos clínicos dedicaram-se a estudar materiais diretamente inseridos nas bases das próteses dos pacientes. Além disso, também existem poucos estudos clínicos comparando diferentes materiais e tempo de formação de biofilme (PEREIRA-CENCI et al., 2008; BOSCATO et al., 2009; 2010).

1. 2 Objetivos

1.2.1 Geral

Os objetivos deste estudo serão avaliar a composição do biofilme formado sobre a superfície de condicionadores de tecido temporários *in situ*, bem como a influência da rugosidade de superfície nos padrões de colonização.

1.2.2 Específicos

1. Avaliar o percentual de diferentes espécies de *Candida* em relação a microrganismos totais em pacientes usuários de prótese total superior;
2. Avaliar a possível alteração da rugosidade de superfície de condicionadores de tecido temporários nos diferentes tempos de avaliação 1°, 7° e 14° dias;
3. Avaliar quantitativamente (através da contagem de UFC) e qualitativamente (através de MEV) a influência do tempo de uso do reembasador temporário inserido na base da prótese através de microscopia eletrônica de varredura.

A hipótese testada é que haverá influência do tempo sobre os reembasadores de prótese neste ensaio clínico, bem como as condição de saúde do paciente irão influenciar na formação de biofilme *in situ*.

1.3 Justificativa

A alta prevalência de infecções causadas por *Candida* em usuários de próteses removíveis é um problema para a saúde do indivíduo seja pela dificuldade de diagnóstico ou de tratamento, sendo a remoção da chamada “placa de dentadura” (*denture plaque*) (COULTHWAITE; VERRAN, 2007). essencial para manutenção da saúde bucal. Adicionalmente, se um dado material perpetua a condição de doença, justifica-se o esclarecimento das interações adesivas e a longevidade efetiva de diferentes condicionadores de tecidos temporários.

1.4 Materiais e Métodos

Os materiais e equipamentos a serem utilizados neste trabalho estão detalhados nas Tabelas 1 e 2.

Tabela 1 - Materiais

Produto	Fabricante
Resina acrílica termopolimerizada	CLASSICO
Dentes artificiais BiotoneResmbasador de prótese	DENTSPLY
temporário a base de resina acrílica	DENCRIL
Reembasador de prótese temporário a base de silicone	ZHERMACK GMBH
Tubos Falcon 15 e 50 mL	
Meio de Cultura CHROMagar	DIFCO
Placa de Petri descartável	
Alça digalssica de plástico estéril e descartável (0,01 mL) para semeadura dos microrganismos	Newprov
Ponteiras para pipeta 0 – 200	Eppendorf
Ponteiras 100 -1000	Eppendorf
Tubos para microcentrífuga	Eppendorf
Lixas d’água número 320, 400 e 600	
Swab	
Meio de cultura Blood Agar base	DIFCO
Meio de cultura MSB	DIFCO
Meio de cultura Agar Rogosa	DIFCO

Tabela 2 - Equipamentos

Equipamento	Fabricante
Mufla Metálica	Dental Campineira
Vibrador para Gesso	Dental Campineira
Espatulador de Gesso a vácuo	Dental Campineira
Agitador Orbital TE-420	Tecnal
Estufa para Esterilização	Fanem
Gerador de anaerobiose	
Jarras para anaerobiose	Jouan
Rugosímetro Surf Corder SE 1700	Kozakalab
Agitador de tubos AP 56	Phoenix
Sonicador	Bausch & Lomb
Pipeta 0-200	Gilson
Pipeta 100-1000	Gilson
Câmera Fotográfica Cybershot 707	Sony

1.4.1 Delineamento Experimental

Este estudo terá uma avaliação clínica *in situ*, com duas fases de formação de biofilme, onde cada fase terá 14 dias, aqueles voluntários que participarem da primeira fase do estudo serão os mesmos à participar da segunda fase de mais 14 dias. Serão convidados a fazer parte do estudo 20 voluntários usuários de prótese total superior, com indicação para substituição, sendo portadores do microrganismo *Candida*, avaliado por um *screening* inicial, através da coletando de biofilme do palato com o auxílio de *swab* e posteriormente semeando em placas CHROMagar *Candida* e incubadas em aerofilia a 37 ±1º C durante 24 horas para verificar a presença do microrganismo O cálculo do número de pacientes a serem incluídos no estudo foi baseado em publicações prévias (PEREIRA-CENCI et al., 2010), considerando perda de 10%. O estudo clínico envolveu um desenho experimental cruzado, duplo-cego, com duas fases de acúmulo de biofilme. Cada fase terá 14 dias, sendo os voluntários aleatoriamente designados a uma condição

experimental, de acordo com o tipo de reembasador de prótese temporário (a base de resina acrílica ou a base de silicone).

Serão inserido na prótese superior antiga dos voluntários de forma randomizada através do programa Microsoft Office Excel, 6 espécimes, de resina acrílica termopolimerizável (controle) e 6 de reembasadores de prótese a base de silicone ou a base de resina acrílica, dependendo da fase. Os recessos receberão numeração de 1 a 6 de cada lado da prótese (Figura 1), onde os espécimes serão alocados seguindo essa sequência de numeração. No 1º, 7º e 14º dia da fase, tanto o biofilme formado como os espécimes serão removidos, dois a dois, sendo o biofilme usado para análise microbiológica e os espécimes para reavaliação de sua rugosidade de superfície (Ra) e também para análise em microscopia eletrônica de varredura (MEV). Após a primeira fase haverá um intervalo mínimo de 07 dias (*washout*). Terminado o estudo *in situ*, os voluntários receberam próteses novas superiores e inferiores.

Figura 1 - Esquema da randomização da alocação dos espécimes.

1.4.2 Seleção dos Voluntários

Serão selecionados 20 voluntários que atendam os critérios de inclusão:

1. Adultos saudáveis portadores de prótese total superior com indicação para substituição;

2. Que não apresentem histórico de uso de antifúngicos, antibióticos, medicamentos para xerostomia ou anti-sépticos bucais nos últimos 03 meses;

3. Vinte pacientes portadores do fungo *Candida* residente;

5. Tenham disponibilidade para comparecerem a FO/UFPel nos dias pré-determinados;

6. Concordem com o termo de consentimento livre e esclarecido, aprovado pelo comitê de ética em pesquisa da FO/UFPel.

Critérios de exclusão:

1. Pacientes com doenças sistêmicas não controladas, portadores de diabetes mellitus ou que façam uso de antibióticos ou medicação que sabidamente diminuam o fluxo salivar;
2. Pacientes que não forem portadores do fungo *Candida* residente;
3. Pacientes que não forem usuários de prótese total superior.

1.4.3 *Screening* para a Presença de *Candida*

Para se certificar da presença do microrganismo *Candida* na cavidade bucal será feito um *screening* inicial coletando biofilme do palato com o auxílio de *swab* e

semeado em placas CHROMagar *Candida* e incubadas em aerofilia a 37 ±1º C durante 24 horas para verificar a presença do microrganismo. Os voluntários que não apresentarem resultados positivos para a presença do microrganismo *Candida* serão excluídos do estudo mas serão encaminhados para clínicas de referência para confecção de novas próteses.

1.4.4 Parte I – Estudo *In Situ*

1.4.4.1 Preparo dos Espécimes

Reembasadores de prótese temporário a base de resina acrílica Soft Confort- SC (Dencril, Pirassununga, Brazil) e a base de silicone Elite® Super Soft Reling ESSR (Zhermack GmbH, Alemanha) serão proporcionados e manipulados de acordo com as instruções dos fabricantes para a confecção de espécimes nas dimensões de 0,5 x 0,5 x 0,2 cm. Para mimetizar as condições de reembasamento, os espécimes de condicionadores serão fixados sobre bases de resina acrílica, os quais serão reembasados contra uma placa de vidro. Os espécimes de resina acrílica (controle) (Acron MC, GC America, Alsip, IL, Estados Unidos) receberão acabamento com lixa d'água (320, 400 e 600). Já os confeccionados com o reembasadores de prótese receberão somente acabamento. O acabamento para os reembasadores e o polimento para a resina acrílica será realizados pois se fossem reembasados diretamente na cavidade bucal de cada paciente, cada um dos espécimes teria rugosidade variável e sabe-se que este fator influenciaria diretamente na adesão microbiana.

1.4.4.2 Rugosidade de Superfície

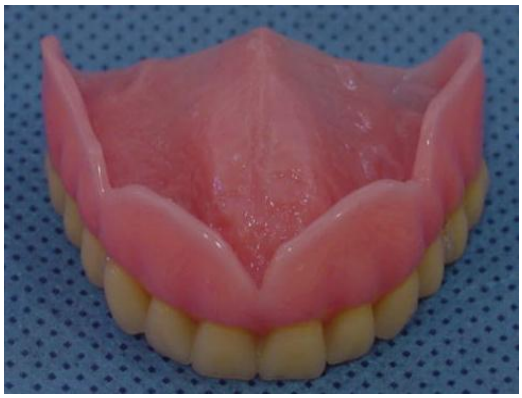
A rugosidade de superfície de cada espécime será mensurada com rugosímetro Surf Corder SE 1700 de resolução 0,01 µm, em temperatura ambiente. Três mensurações em diferentes locais de cada espécime serão realizadas e a média aritmética será o valor de rugosidade de superfície para o referido espécime. Após a mensuração da rugosidade de superfície, os espécimes serão submetidos à desinfecção em banho ultra-sônico durante 20 minutos (LUO; SAMARANAYAKE, 2002).

1.4.4.3 Inserção dos Espécimes na Prótese

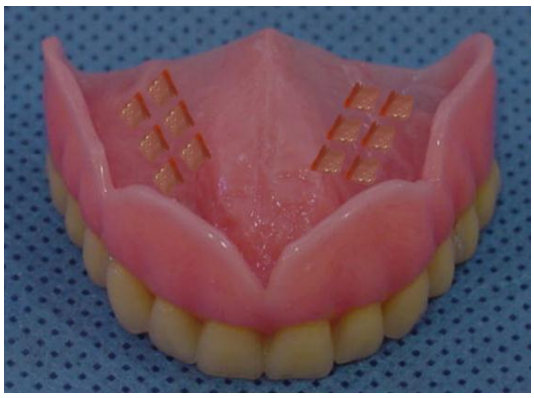
A prótese total antiga de cada voluntário (as usadas pelo paciente quando o mesmo será selecionado) será limpa com jato de óxido de alumínio para remoção de cálculo e biofilme aderidos à superfície da prótese. Posteriormente a prótese serão polida com escova e pedra-pomes , disco de feltro e branco de espanha, nesta ordem. Desta forma, todos os aparelhos protéticos apresentarão as mesmas condições superficiais de lisura e limpeza, para que este aspecto não interfira nos resultados deste estudo. Após acabamento e polimento dos espécimes, estes serão imediatamente colocados nas próteses antigas dos pacientes para simular o uso clínico dos reembasadores.

Seis espécimes de cada lado da prótese (seis de um dos reembasadores e seis de resina acrílica) serão fixados com cera pegajosa em um recesso medindo 0,6 x 0,6 x 0,3 cm previamente preparado na região palatina, correspondente a localização dos pré-molares e molares da prótese (vertente palatina do rebordo alveolar). O recesso incialmente desenhado com lápis na região padronizada da base da prótese onde posteriormente com o micromotor, peça reta e fresa apropriada, realizamos uma cavidade na delimitação do desenho. Estes recessos

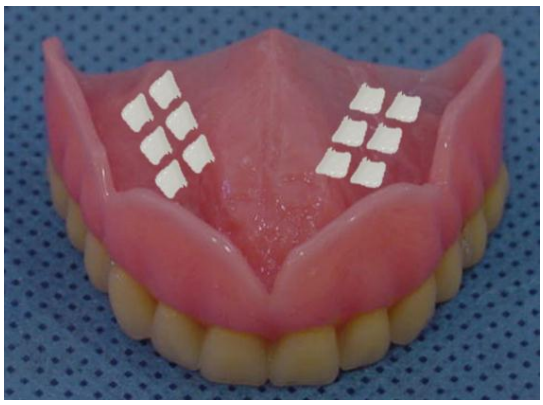
estarão na parte interna da prótese total, diretamente em contato com o palato. Este local será escolhido porque esta região de palato e prótese superior são os locais de maior prevalência destes fungos (VANDEN ABBEELE et al., 2008; LUND et al., 2010). Após realizados os procedimentos acima descritos, as próteses foram devolvidas aos voluntários, e estes utilizaram as suas próteses normalmente.



(a)



(b)



(c)

Figura 2 - Adequação das bases da prótese (a); esquema dos recessos para colocação dos espécimes (b); espécimes inseridos nos recessos na região palatina de uma prótese total superior (c).

Os voluntários utilizarão normalmente as suas próteses, sendo, em todos os momentos, acompanhados pelos pesquisadores. Os mesmos se alimentaram normalmente e dormiram com as próteses, removendo as mesmas 3x/dia para higienização. O local onde serão fixados os espécimes será instruído a receber somente a espuma feita com o dentifrício durante a escovação. A utilização apenas da espuma é justificada pelo fato de que em reembasadores resilientes, está contra-indicada escovação, uma vez que esta pode danificar a superfície do reembasador. Não foi permitido autilização de quaisquer enxaguatórios ou medicamentos durante as duas fases do estudo. Eventualmente se algum voluntário relatasse tal uso, o mesmo será automaticamente excluído do estudo.

Todos os voluntários receberão instruções impressas de higiene oral, cuidados com as próteses e esclarecimento da pesquisa.

1.4.4.4 Coleta do Material

Decorridos os tempos de 1, 7 e 14 dias, dois espécimes (de reembasadores de prótese e de resina acrílica) coletaremos com auxílio de espátula estéril e depositaremos em tubos para microcentrífuga previamente esterilizados; os tubos serão mantidos em banho de gelo até o processamento. Os recessos serão limpos e preenchidos com cera utilidade.

Decorridos 07 dias de intervalo (*washout*), os mesmos voluntários participarão a segunda fase do experimento. Os espécimes serão coletados e avaliados conforme já descritos para a fase 1. Ao final da segunda fase, os recessos das próteses serão preenchidos com resina acrílica auto polimerizável que passará por acabamento e polimento. O voluntário em momento algum ficará sem usar sua

prótese. Após finalizadas as duas fases, prosseguiremos a sequência clínica para confecção de nova prótese total superior e inferiores.

1.4.5 Parte II – Análise Microbiológica

Os espécimes coletados serão acondicionados em tubo para microcentrifuga, onde será adicionado 1mL de solução de NaCl a 0,9% esterilizada e este conjunto será sonicado. A seguir, as amostras serão normalizadas por peso seco. O peso seco, consiste no peso inicial do eppendorf, antes da colocação da solução e do espécieme, subtraído pelo peso final do biofilme, após centrifugação.

A parte da suspensão restante será diluída serialmente até a proporção de 1:100.000.000 (10^{-7}) em solução salina. As diluições serão semeadas em placas de petri contendo os meios de cultura: a) mitis salivarius bacitracina (MSB), contendo 0,2 unidades de bacitracina/mL e 0,001% de telurito de potássio, para determinação de **estreptococos do grupo mutans**; b) Meio Agar sangue, para determinação das **microrganismos totais**; c) Meio CHROMagar *Candida* para determinação de espécies de ***Candida***; d) Meio Agar Rogosa, para determinação de **lactobacilos**.

Para o meio MSB será utilizado a diluição de 10^{-0} até 10^{-5}; para o meio Agar Sangue será utilizado a diluição de 10^{-3} até 10^{-7}; para o meio CHROMagar *Candida* será utilizado a diluição de 10^{-0} até 10^{-4}; para o ,meio Agar Rogosa será utilizado a diluição de 10^{-2} até 10^{-4}.

A semeadura será realizada pela deposição de alíquotas (20 µL) destas diluições em duplicata nas placas. As placas de CHROMagar *Candida* serão incubadas em estufa a 37 ±1 o C por 48h. As placas de MSB, Rogosa, e Ágar sangue serão incubadas em estufa a 37 ±1 ^{o}C por 72 h, em atmosfera anaerobiose. As unidades formadoras de colônia (UFC) serão contadas, e os resultados serão

expressos em UFC/mg biofilme. Além disso, a porcentagem de estreptococos do grupo mutans, lactobacilos e cada espécie de *Candida* em relação aos microorganismos totais viáveis do biofilme serão calculadas. Para a contagem das colônias, um microscópio estereoscópico será utilizado; colônias atípicas serão identificadas através de coloração de Gram e bacterioscopia.

Os espécimes serão limpos com água destilada deionizada estéril, secos e acondicionados em frascos plásticos até a segunda avaliação da rugosidade de superfície e MEV.

Após a obtenção dos resultados, os mesmos serão tabulados e submetidos à análise exploratória dos dados. A escolha do teste estatístico a ser utilizado dependerá da homogeneidade dos resultados. O nível de significância de 5% será utilizado nas análises.

1.4.6 Parte III – Análise Microscópica

Será realizada análise em microscópio eletrônico de varredura (MEV) com a finalidade de ilustração da condição de superfície e da formação de biofilme nos três tempos de formação de biofilme avaliados, a análise será feita com um espécime para cada material e para cada tempo de avaliações (1°, 7° e 14° dias), totalizando três espécimes de reembasadore de prótese a base de silicone, três espécimes de reembasador de prótese a base de resina acrílica e três espécimes do grupo controle de resina acrílica termopolimerizável. Os espécimes não serão avaliados qualitativamente porque precisaríamos um numero muito grande de espécimes e este não será nosso objetivo.

Os nove espécimes serão montados em um stub, secos com ar por pulverização catódica revestido com ouro (Balzers Union MED 010 evaporador) e

examinadas com um microscópio eletrônico de varredura (SSX-550; Shimadzu) em uma voltagem de aceleração de 15 kV para a superfície. Para esta análise, dois voluntários, um de cada grupo serão selecionado conforme disponibilidade de inserir um maior número de espécimes, ou seja, voluntários com próteses maiores para que não sejam perdidos os espécimes de coleta de biofilme. Nesse item os espécimes serão avaliados em todos os tempos, porém não em todos os voluntários.

1.5 Cronograma de Execução

As etapas de execução do presente estudo serão:

1. Levantamento bibliográfico inicial;
2. Seleção e *screening* de pacientes;
3. Definição da metodologia e teste de equipamentos;
4. Execução dos testes experimentais;
5. Recolhimento e análise estatística dos resultados obtidos;
6. Levantamento bibliográfico adicional;
7. Redação de relatórios e artigo para publicação;
8. Divulgação em congressos e/ou seminários;
9. Defesa de Dissertação.

O cronograma de execução das etapas está detalhado na Tabela 3.

Tabela 3 - Cronograma de execução das etapas

2010											
Jan	Fev	Mar	Abr	Mai	Jun	Jul	Ago	Set	Out	Nov	Dez
										1	1

2011											
Jan	Fev	Mar	Abr	Mai	Jun	Jul	Ago	Set	Out	Nov	Dez
2	2;3	4	3	4	4	5	5	6;7	6;7	7;	7;

2012											
Jan	Fev	Mar	Abr	Mai	Jun	Jul	Ago	Set	Out	Nov	Dez
7	7	7;8	8;9								

2 Relatório de Trabalho de Campo

2.1 Aspectos éticos

O projeto qualificado foi submetido e aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia da Universidade Federal de Pelotas (FO-UFPel/ RS) sob parecer nº191/2011 (Anexo A). Os voluntários assinaram um termo de consentimento livre e esclarecido, a fim de autorizar sua participação no estudo (Apêndice A).

2.2 Condições gerais

Para a revisão sistemática, dois avaliadores fizeram toda a busca e análise de dados (JAS e TPC), conforme critérios de inclusão e exclusão, baseando-se nas normas do PRISMA Statement. O estudo *in situ* foi completamente cego (quanto a análise microbiológica) e aleatorizado. Os espécimes foram alocados na base das próteses superiores respeitando uma sequência de alocação dos recessos enumerada de 1 a 6 e removidos de dois em dois de forma aleatorizada. O estudo foi dividido em dois grupos, totalizando trinta pacientes usuários de prótese total, quinze portadores de estomatite por dentadura, clinicamente diagnosticada e quinze pacientes com alguma espécie de *Candida* avaliados por *screening* inicial. Destes, três voluntários foram perdidos devido ao uso de antibiótico e necessidades cirúrgicas, resultando em doze pacientes com o microrganismo *Candida.*

2.3 Rotinas laboratoriais

2.3.1 Coleta e processamento

Decorridos os tempos de 7, 14 e 21 dias, dois espécimes (de reembasadores de prótese e de resina acrílica) foram coletados com auxílio de espátula estéril e depositados em tubos para microcentrífuga previamente esterilizados; os tubos forma mantidos em banho de gelo até o processamento. Os recessos forma limpos e preenchidos com cera. Decorridos 07 dias de intervalo (*washout*), os mesmos voluntários participaram da segunda fase do experimento. Os espécimes coletados foram acondicionados em tubo para microcentrifuga, onde foi adicionado 1mL de solução de NaCl a 0,9% estéril e este conjunto foi sonicado e diluído para o plaqueamento nos meios de cultura.

2.3.2 Protocolo de obtenção do biofilme

Os espécimes de resina acrílica (controle) e reembasadores (a base de silicone e a base de resina) eram mantidos em contato com a cavidade bucal até a remoção para avaliação microbiológica nos dias 7, 14 e 21 dias. Os espécimes removidos eram colocados em tubo para microcentrífuga contendo 1mL de salina estéril e então sonicados (Sonicador UNIQUE, Indaiatuba, SP, Brasil) com potência de 30W, amplitude de 5%, com 3 pulsos de 10s cada, para obtenção do biofilme em suspensão homogênea. Em seguida, as suspensões de biofilme era diluídas serialmente e plaqueadas em meios de cultura para contagem de estreptococos do grupo mutans, lactobacilos, espécies de *Candida* e microrganismos totais (CENCI, 2008.; TENUTA et al., 2006).

2.4 Alterações no projeto original

2.4.1 Dificuldades encontradas

Após sugestão da banca e aceite do comitê de ética em pesquisa, foi acrescentado no projeto o grupo de voluntários portadores de estomatite por dentadura, clinicamente diagnosticados. Desta forma, o número de voluntários passou de 20 para 30, sendo divididos em dois grupos, 15 portadores de estomatite por dentadura e 15 pacientes com alguma espécie de *Candida.* A fase de avaliação teve alteração, passou de 14 dias de avaliação, para 21 dias de avaliação, uma vez que a indicação do fabricante para utilização de reembasadores temporários pode variar de 15 a 30 dias. Sendo assim, talvez 14 dias não fossem suficientes para mostrar diferenças entre os materiais.

Em virtude do tempo dispendido na adequação da metodologia, preparo dos espécimes e seleção de voluntários, o cronograma previsto para início dos experimentos foi alterado. Adicionalmente, o rugosímetro de nossa escola quebrou e levou 12 meses para ser consertado, o que também alterou o cronograma de início dos experimentos. Adicionalmente, modificamos o protocolo de análise de biofilme de UFC/mg de biofilme para UFC/mm^2, já que não foi possível realizar a análise de peso seco.

Em decorrência de problemas com o laboratório de prótese na confecção das próteses, tivemos atraso para iniciarmos a 2° fase do estudo. Embora a confecção das novas próteses fosse a partir da 1° fase do estudo, o primeiro laboratório com o qual trabalhamos não cumpriu prazos e houve grande taxa de repetição dos trabalhos, além de termos que lidar com a ansiedade dos pacientes em obter suas novas próteses.

Aqueles com a doença, após o térmico do estudo, receberam terapia antifúngica com Fluconazol 150 mg dose única e Nistatina creme 3 vezes ao dia (SAMARANAYAKE et al., 2009; CANNON; FIRTH, 2006; NININ et al., 2010). Além

de instrução de higiene com escova de dente macia e pasta de dente, foi instruída a desinfecção através da imersão em solução hipoclorito de sódio a 0,5% durante 10 minutos a cada 4 dias (FERREIRA et al., 2009) Todos os voluntários foram acompanhada a cada 3 meses para avaliar a remissão totasl dos sinais clínicos da infamação.

Acrescentamos uma revisão sistemática da literatura no projeto inicial, uma vez que sentimos necessidade de pesquisar se existia na literatura um protocolo de prevenção, tratamento ou desinfecção de reembasadores de prótese, baseado em evidências científicas.

ARTIGO 1

Prevention and treatment of *Candida* colonization on denture liners: a systematic review[§]

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Abstract

Statement of Problem: Denture liners are well known for their poor physical properties that favour the accumulation of plaque and colonization by *Candida* species, leading to irritation of the oral tissues and therefore resulting in denture stomatitis.

Purpose: A systematic review was conducted to determine if there is a prevention protocol for *Candida* colonization in denture liners and an effective treatment after the fungi has colonized the material.

Material and Methods: Clinical and in vitro investigations that assessed the treatment and/or prevention of *Candida* colonization and biofilm formation in denture liners were selected according to PRISMA statement. Seven electronic databases were searched from 1950 to April 2012 using the key words "denture liner" OR relin* OR "tissue conditioner" AND *Candida"* OR "denture stomatitis" OR "antifungal agents" OR denture clean*. There was no language restriction.

Results: Incorporation of nystatin into denture liners or tissue conditioners to prevent the onset of the disease and immersion in sodium hypochlorite for disinfection were the most often found in this systematic review and both were able to prevent or inhibit *Candida* colonization depending on their concentrations. Due to a lack of standardized results (especially considering the way microbial count was done), a meta-analysis could not be performed.

Conclusion: It seems from the literature that the use of 0.5% sodium hypochlorite could be of help to disinfect denture liners and tissue conditioners; however, to reach more consistent results, randomized controlled trials are mandatory, as most of the studies were in vitro, which could lead to overestimated results.

Key words: *Candida;* review; denture liners; tissue conditioning; denture stomatitis; antifungal agents.

INTRODUCTION

Oral candidosis is a type of denture-related stomatitis strongly associated with the presence of *Candida* species.[1,2,3] Although primarily related to the presence of these fungi, it is important to identify other reasons related to the onset and development of this oral disease as age, gender, income, general health, oral hygiene, daily period of use of prosthesis, alcohol consumption, trauma and diet.[2,4,5] *Candida albicans* is the primary microbiological factor in denture stomatitis.[6,7]

The adherence of microorganisms over the surface of denture materials is necessary to initiate the process. Factors such as structure and composition of the surface of these materials and chemical/physical properties of microbial cells surfaces can influence the adhesion process.[8,9] Thus, the colonization depends on numerous factors related to substrate characteristics, which play an important role in the adhesion of microorganisms.[10,11,12] In this context, lining materials are frequently used in dentures, although they present some deleterious characteristics as their leaching process,[13] which makes efficient mechanical cleaning difficult. Toothbrushing can deteriorate the surface, although chemical cleansers must also be evaluated regarding their effectiveness in preventing yeast infections , without damaging the surface of the materials.

Epidemiological studies report denture stomatitis prevalence among denture wearers to range from 15% to over 70%.[14] There is strong evidence showing that specific factors are directly related to the clinical manifestation of oral candidiasis,[15,16] but methods to prevent the onset of the disease remain unclear. Guidelines to avoid the colonization of microorganisms, especially in dentures were liners were used are of upmost importance, especially considering that there is a lack of protocol for its prevention, once it can result in future health care strategies for patients at risk. Hence, the aim of this study was to systematically review the literature to find out whether there is a strong evidence-based protocol for the prevention of

Candida colonization in denture liners or at least disinfect these materials and if there is a protocol to treat patients that use denture liners and had these materials colonized by *Candida*.

MATERIAL AND METHODS

Systematic literature search

This systematic review was performed according to PRISMA statement. Seven databases were searched (Trip, Lilacs, Scopus, Pubmed/Medline, Scielo, Web of Science and Cochrane Database of Systematic Reviews) using the following keywords: "denture liner" OR relin* OR "tissue conditioner" AND "*Candida"* OR *"*denture stomatitis" OR "antifungal agents" OR denture clean*. All papers found were evaluated and selected, following the inclusion criteria, which was any in vitro, in situ or in vivo study, with protocols for treatment, disinfection, cleaning or prevention of *Candida* colonization for denture liners or tissue conditioners only. No restriction to language was made. The literature search was carried out by two independent researchers (JAS and TPC) from November 2011 to April 2012, and all articles from 1950 to April 2012 were included. The references of papers included in the review were carefully searched for additional papers that could be included, including handsearch.

Selection criteria

According to the PRISMA statement,[17] all abstracts were analyzed. A total of 152 articles were found. Abstracts were independently reviewed by two researchers. After the screening and eligibility criteria had been individually accomplished, if a consensus was reached, the article was included, if not, a third author was invited to discuss about the article.

Studies without protocol for treatment or prevention of *Candida* colonization were excluded. The protocol should be tested in denture liners or tissue conditioners; if the methodology was performed only using acrylic resin, the study was excluded. Studies could also be related to other microorganisms involved in denture plaque development, dual or

multi species biofilm, but all studies had to contain *Candida albicans* as the main pathogen related to denture stomatitis.

Three of the fifty two papers selected to have studies extracted could not be found despite several attempts to contact the authors, the journals and libraries.[18,19,20] Among all studies included in the search strategy, 104 articles were included in the review. After carefully reading the abstracts, 52 articles were selected to a full-text evaluation. Figure 1 indicates the step-by-step throughout the articles' selection. Thirteen articles were excluded after assessed for eligibility due the reasons explained in Table 1.

Data collection and analysis

The study design, the type of microorganism and material found on articles were recorded. The main findings of the studies, as results and conclusion were extracted.

Duo to a large variability of data, a meta-analysis was discarded. Different tools were used to measure the antimicrobial activity, which resulted in distinct ways to quantify *Candida* colonization, such as rate of pH decrease, colony forming unit count, weighing of biofilm formation and the use of a kit to measure bioluminescence adenosine triphosphate (ATP). Thus, only a qualitative investigation was possible and different comparisons were made among studies trying to find out the best protocol to prevent, treat or eliminate *Candida* colonization. Data were grouped in order to describe the main methods for prevention and treatment.

RESULTS

Considering the 39 articles included in the study, articles were separated into two categories, according to the strategy performed by the authors to achieve (1) a prevention protocol or (2) a cleaning, treatment or disinfection strategy for denture liners. In the first category (20 articles), all studies that had incorporation of any antimicrobial into a denture liner were included (Table 2). Nystatin was often incorporated as antimicrobial agent in denture liners (40%). Silver-based antimicrobials and fluconazole were present in four and three studies respectively (20% and 15%), and chlorhexidine, amphotericin B and miconazole were found to be incorporated in two studies (10%). Other antimicrobials were incorporated in one study only such as zinc peroxide, clotrimazole, itraconazole, ketonazole, *Melaleuca alternifolia*, human lactoferrin, magnesium oxide and triazine.

Category two included studies (19 articles) that had any protocol for cleaning, treating, preventing or disinfecting denture liners that did not include the addition of an antifungal/antibacterial agent (Table 3). The most frequent protocol tested (47.4%) was the immersion of the liner into a sodium hypochlorite solution in various concentrations (0.5, 1, 2, and 5.25%), with 0.5% already showing good results. Microwave irradiation and medical tabs for dentures were also commonly used to prevent microbial colonization or disinfect denture liners (26.3% and 31.6% respectively).

DISCUSSION

This systematic review has shown that attempts have been made to prevent denture stomatitis or treat /disinfect denture liners commonly used for denture wearers. The incorporation of fungicidal compounds into the denture liners or immersion of the denture containing the denture liner in cleansing solutions was often performed to reach this objective.

The first idea to use readily available tools combining with other available agents is pretty interesting to prevent the fungal infection. The addition of fungicidal compounds directly to denture liners can be low cost, successful and especially attractive because it does not require patient cooperation. In addition, denture cleansers may cause significant deterioration of denture liners e.g. sodium hypochlorite.[49] Tooth brushing may also cause surface modifications, thereby facilitating colonization of microorganisms. The addition of these compounds would lead to less detrimental effects when compared to the use of denture cleansers. On the other hand, the amount of antifungal agents should be carefully planned as they could be harmful to older people.[69] In addition, the addition of antifungal to these liners to prevent colonization by *Candida* should be carefully considered only for those patients at high risk i.e., patients with xerostomia, previous history of denture stomatitis and motor disabilities.

Despite the fact that there are differences between tissue conditioners and denture liners, especially concerning viscosity no division on the results section was made particularly because after the colonization of microorganisms, both seems to perform in a similar way (and may also be used to treat denture stomatitis), providing no differentiation once a biofilm is formed.[70] In addition, once the aim of this review was to present an overview regarding methods to decrease microorganisms counts, no comparisons among the materials concerning their temporary or permanent use was performed once it is expected that temporary liners will perform poorer than permanent ones.

Regarding the incorporation of antimicrobial agents into denture liner, nystatin seems to be the gold standard in terms of prevention/treatment. All studies that used nystatin presented at least a decrease in yeast levels. In a general way, the concentration of nystatin is directly related to the inhibition of *Candida* growth. It is important to emphasize however, that the concentration cannot be increased indiscriminately, because it can cause changes in mechanical and chemical properties of these materials.[49] Silver-based antimicrobial also presented good results. All studies that mixed silver particles with denture liner showed a fungicidal effect. Due to the few studies available, it was not possible to conclude if using some specific agents may present beneficial antimicrobial effects or not. Once itraconozole and miconazole were tested in only one study and showed good performance, or zinc peroxide and triclosan (Microban), which demonstrated a poor result and again, were tested in only one study, it is not possible to conclude the real potential of these agents. This is also true for the use of *Melaleuca alternifolia*, which was effective in treating denture stomatitis in an in vivo study.[40]

Several protocols were found to eliminate *Candida* colonization. Some denture cleansers and irradiation (mainly immersed in water) with microwave presented decreased yeasts counts, but immersion in sodium hypochlorite still remained the most effective cleaning agent. Concentrations of 0.5, 1, 2 and 5.25% were tested and all presented a decrease in *Candida* levels or complete elimination of these microorganisms. The ideal sodium hypochlorite concentration must be studied once the immersion in this solution could jeopardize the surface roughness over time, making yeast (re)colonization easier in long-term analyses.[60] Yet, it seems from the studies found that 0.5% sodium hypochlorite solution is able to clean or disinfect denture liners, i.e. the lowest concentration could both clean and still prevent surface deterioration caused by higher concentrations of NaOCl.

Strategies to prevent *Candida* colonization were also found. Olan-Rodriguez *et al.*[54] tested in vivo the influence of sealers on denture liners, showing that this strategy could decrease the colonization by yeasts and bacteria. Other strategy used was to compare immersion technique vs. spraying technique through the use of chlorine dioxide as a denture liner disinfectant. Although the immersion showed better results than the spray, both had poor results,[52] contrasting with Uludamar *et al.*,[68] which demonstrated good results obtained with the use of chlorine dioxide to treat palatal inflammation. Clinical studies have reported that disrupting the biofilm may be more important than the use of antifungals or antimicrobials in the prevention and treatment of denture stomatitis.[40,41,59] As the etiology of the disease is multifactorial, a set of attitudes together with treatment is necessary for the total elimination of *Candida*.

The need of in vivo, prospective randomized clinical studies was evident. Only eight studies were performed in a clinical scenario and in two of them, in vivo and in vitro studies were performed together, thus demonstrating the lack of trials, prospective and retrospective studies, which provide a better level of evidence. The results obtained in vitro do not necessarily agree with the experience in vivo, because the oral cavity is an extremely rich environment in saliva and nutrients, which could somewhat cancel the inhibitory effect produced by the antimicrobials released from the liners in vitro. A possible reason why the release of the antifungal agent included in the denture liner does not clinically stand is the constant bathing in saliva in the mouth.[68] In addition, in vitro the specimens are usually smoother and have standardized surfaces, which gives a better picture for the antifungal tests; in vivo, denture liners lose their plasticizers, becoming hardened and rough. Another important reason why in vitro studies show good results that could not be real in clinical practice is that usually in vitro studies are performed with planctonic cells or single species biofilm. This means that the antifungal more easily penetrates into the biofilm when

compared to multi-species biofilms, which are complex communities with a matrix and therefore improves the chances of survival for these microorganisms as they are more protected.[71] The only study found that tried to mimic the oral cavity used a microcosm biofilm model and failed to show antifungal effect of triazine directly inserted in denture liners to prevent *Candida* colonization.[49]

Due to these differences between in vitro and in vivo studies, a separation in the results section depending on the design of the study was an option, but several protocols tested have been tested only in vitro; thus it seemed more interesting to carry out an overview about all the possibilities that could still be tested in vivo and what was not necessary to test in laboratory studies based on our findings.

Unfortunately, due to the heterogeneous data, a meta-analysis could not be performed. This does not mean that this review has no evidence, but increases the necessity to investigate more protocols to in a near future, establish a definitive protocol, with the best material, concentration or form of use of antifungals to achieve a good prognosis of preventing denture related stomatitis when tissue conditioners or denture liners are being used. Still, it is possible to state that based on in vitro results, nystatin could be of use mixed with denture liners, while the best way to disinfect these materials is through immersion in a sodium hypochlorite solution.

CONCLUSION

The addition of antifungal agents to denture liners appears to have some beneficial effect to prevent *Candida* colonization, but a definitive concentration remains uncertain, as the protocols found in literature are completely different. The use of 0.5% sodium hypochlorite could be of help to disinfect denture liners. However, there is insufficient reliable evidence to truly provide recommendations on which is the ideal cleaning method, or whether the addition of antifungal agents is beneficial or not. Well designed randomized controlled trials are needed to provide answers to these questions.

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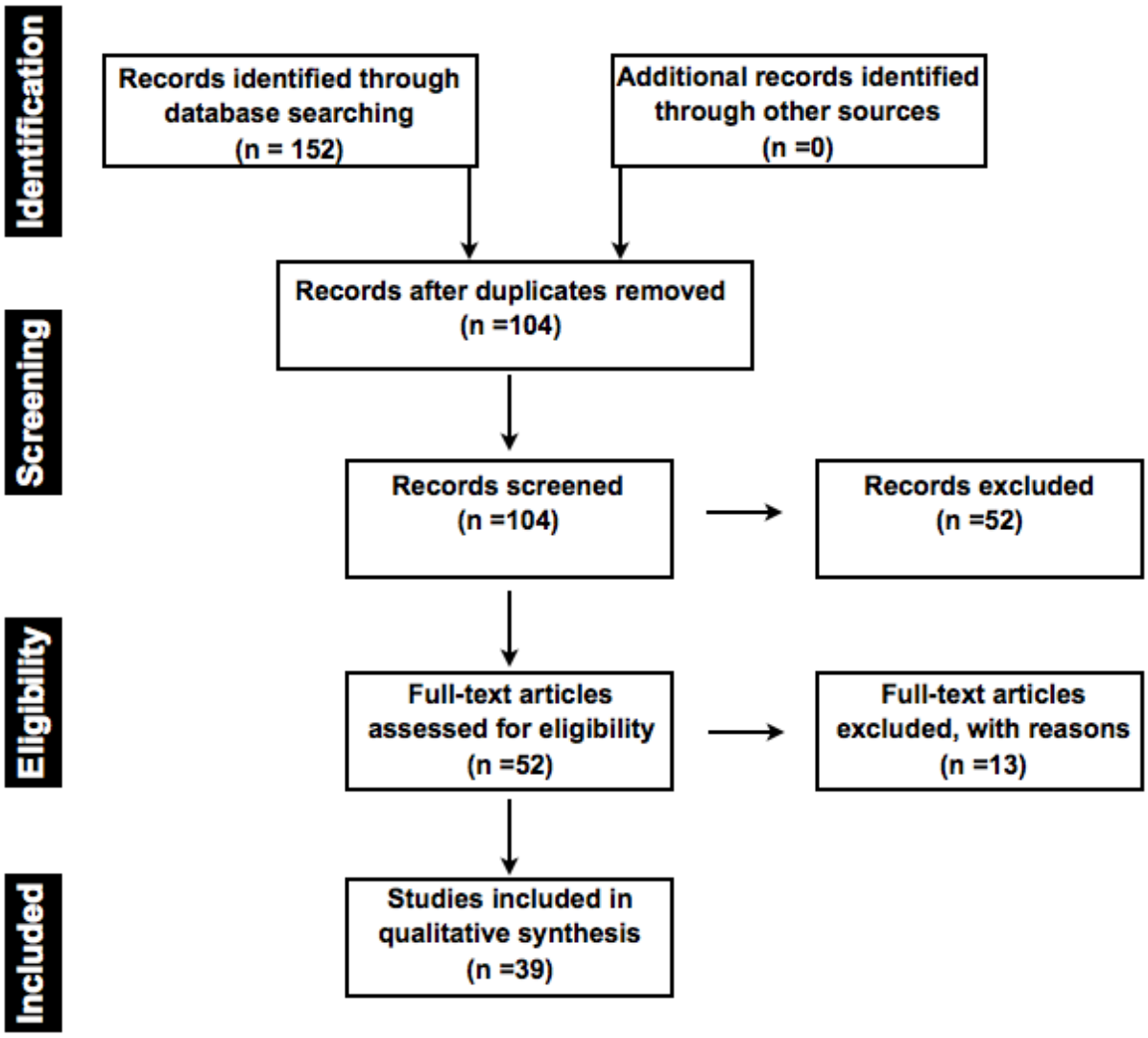

Figure 1 – Selection criteria according PRISMA statement.

Table 1 - Reasons for exclusion of papers selected to assess the full-text.

Author	**Reason**
Burns *et al*., 1987[21]	*Candida* growth was analyzed in new denture liners; no treatment/prevention protocol was tested.
Tanaka, 1988[18]	Article not found.
Granata and Staffanou, 1991[22]	The influence of immersion of denture liners in denture solutions on surface hardness was studied.
el-Charkawi *et al*., 1994 [19]	Article not found.
Nikawa *et al*., 1995[23]	Denture liners had their surface porosity tested after immersion in denture cleansers.
Kulak and Kazazoglu, 1998[24]	No protocol for prevention, cleaning or treatment was studied.
Radford *et al*., 1998[25]	The surface roughness and its implication on *Candida* adhesion were tested.
McLain *et al*., 2000[26]	Only the effect of tissue conditioners on fungal growth was studied.
Nevzatoğlu *et al*., 2007[27]	The influence of different surface finishes on *Candida albicans* adherence was studied.
Pereira-Cenci *et al*., 2007[11]	No prevention protocol was tested, only *Candida* adhesion to denture materials.
Pereira-Cenci *et al*., 2010[28]	The influence of time, liner and roughness in the composition of biofilm was analyzed; no treatment/prevention protocol was tested.
Vural *et al*., 2010[29]	The influence of polymerization process was tested on *Candida albicans* adhesion.
Zhou *et al.*, 2011[20]	Article not found.

Table 2. Studies that had incorporation of antifungal agent to denture liners.

Manuscript	**Study Design**	**Microorganism**	**Material**	**Results**
Douglas and Walker, 1973[30]	In vitro and In vivo	*Candida albicans.*	Tempo; Coe Confort; Nystan/Tempo (400000 and 800000 units).	In vitro: Tempo and Coe confort presented some fungicidal effect, but Tempo was better. Tempo/Nystan (400000 units): effective until day 20 Tempo/Nystan (800000 units): activity for at least 50 days. In vivo: There was no difference between patients treated with Tempo + Nystatin Tablet or Tempo/Nystatin mixture.
Tromas and Nutt, 1978[31]	In vitro	*Candida albicans; Candida krusei; Candida tropicalis.*	Visco-Gel; Nystatin (500000 and 1000000 units); Amphotericin B (10mg and 20mg).	Visco-gel alone and mixed with amphotericin B was not effective, only visco-gel/nystatin was efficient and in higher concentrations the results were better.
Gettleman *et al.*, 1983[32]	In vitro	*Candida albicans; Staphylococcus aureus.*	Super-soft; Molloplast-B; Lucitone 199; Syloid 244 silica and alpha-cellulose fiber treated with zinc peroxide (10%) into Molloplast-B and Lucitone 199; Zinc peroxide (5 and 20%) into Molloplast-B and Super-soft).	*C. albicans* presented more susceptibility to growth inhibition. Silica filler showed worse results than cellulose filler. Zinc peroxide (5 e 20%) was not effective as antimicrobial.
Quinn, 1985[33]	In vitro	*Candida albicans.*	Ivoseal; Viscogel; Fitt; Nystatin (500000 Units); Amphotericin B (10,	For amphotericin, no differences were found between the concentrations and inhibitory effect was time and

			20 mg); Miconazole (250 mg); Ketonazole (200 mg).	material dependent. The best results were obtained with nystatin, miconazole and ketonazole, while all combinations presented inhibitory effect even after 15 days.
Schneid, 1992[34]	In vitro	*Candida albicans.*	Lynal Tissue Conditioner; Temporary Reliner; Chlorhexidine (250, 500, 1000 mg/unit); Clotrimazole (250, 500, 1000 mg/unit); Fluconazole (250, 500, 1000 mg/unit); Nystatin (125, 250, 500 mg/unit).	Nystatin presented the best results. All groups showed growth inhibition; however, they were time and concentration dependent.
Matsuura *et al*., 1997[35]	In vitro	*Candida albicans; Staphylococcus aureus; Pseudomonas aeruginosa.*	Visco-gel; GC Soft-Liner; Fitt; SR-Ivoseal; Shofu Tissue Conditioner; Zeomic (Zeolite) - 2 (wt/wt)%.	All tissue conditioners mixed with zeomic presented antimicrobial effects with or without immersion in saliva for 4 weeks.
Nikawa *et al*., 1997[36]	In vitro	*Candida albicans.*	GC Soft Liner; Coe Confort; Zeomic (Zeolite) - 1, 2, 3, 4 and 5 (wt/wt)% or 1, 2, 3, 4 and 5 (vol/vol)%	There was a dose-dependent effect. Zeolite specimens showed higher antifungal effect than Coe Confort, followed by Soft Liner. The antifungal effect of the Zeomic was significantly decreased by the presence of saliva.
Chow *et al*., 1999[37]	In vitro	*Candida albicans.*	Coe Soft; Viscogel; Fitt; Nystatin (1, 3, 5, 7, 9, 11%	In the presence of saliva, all groups had higher fungicidal effect. The concentration of 5% wt/wt had

			wt/wt); Fluconazole (1, 3, 5, 7, 9, 11% wt/wt); Itraconazole (1, 3, 5, 7, 9, 11% wt/wt);	the higher fungicidal activity for all combinations. The best result was obtained with 5% wt/wt itraconazole mixed with Coe Soft.
Lefebvre *et al*., 2001[38]	In vitro	*Candida albicans.*	PermaSoft; Microban (1 part for 80);	No difference was found between groups with or without microban.
Akiba *et al*., 2005[39]	In vitro	*Candida albicans.*	Fictioner; Top Coat; TiO_2 – 0, 2 and 3g.	The viability significantly decreased with increased concentration of TiO_2 in the coating agents. The viability also significantly decreased with increased radiation time.
Catalan *et al*., 2008[40]	In vitro and In vivo	*Candida albicans.*	In vitro: Fitt; Lynal; Coe-Comfort; *Melaleuca alternifolia* (0.5, 1, 2 and 4 ml); Nystatin (1ml). In vivo: Coe-Comfort + *M. alternifolia* (1ml); Coe-Comfort + Nystatin (2ml); Coe-Comfort.	In vitro: Nystatin was effective in all tissue conditioner. *M. alternifolia* was efficient mixed with Fitt and Coe Confort at concentration of least 1 ml. Lynal was ineffective in all concentratios. In vivo: Coe-Confort mixed with nystatin and *M. alternifolia* presented better results than control.
Geerts *et al*., 2008[41]	In vivo	Patients with denture-related stomatitis	Visco gel; Mycostatin (500000 U).	The treatment with liner and nystatin-incorporated only showed a decrease in yeast counts until day 4. After that, the control group significantly increased and the test group remained with low counts until day 7, increasing until day 14, but still showed lower yeast counts than day 0.
Yamamoto *et al*., 2009[42]	In vitro	*Candida albicans.*	Shofu Tissue Conditioner II Antimicrobial protein solution,	Fungal viability was significantly lower than the control in both concentrations, and between 4 and 8

			human lactoferrin; Cation exchange resin, Toyopearl CM650 4 an 8 wt%.	wt% was not significantly different.
Falah-Tafti *et al*., 2010[43]	In vitro	*Candida albicans.*	Acropars; Nystatin (1, 3, 5, 10% wt/wt); Fluconazole (1, 3, 5, 10% wt/wt).	Nystatin in all concentrations completely inhibited the attachment and colonization of *C. albicans*, but in the case of fluconazole, only the concentration of 10% showed complete inhibition of *Candida* colonization.
Radnai *et al*., 2010[44]	In vitro	*Candida albicans.*	Visco Gel; Chlorhexidine (5, 10, 15, 20 and 25% v/v); Miconazole (5, 10, 15, 20 and 25% v/v).	Miconazole inhibits the growth of *C. albicans* and the higher the concentration, the higher the inhibition.
Kanathila *et al*., 2011[45]	In vitro	*Candida albicans.*	Visco Gel; GC Soft liner; Magnesium oxide 1, 3, 5 and 7%.	GC presented best results. The inhibition of *Candida* growth increased in mixtures with higher concentrations of magnesium oxide.
Uchimaru *et al*., 2011[46]	In vitro	*E. coli*; *S. mutans*; *S. aureus; C. albicans.*	Shofu Tissue Conditioner II; Photohap; Concentration of 0, 10, 15 and 20%.	The colony-forming units were lower in irradiated specimens. *Candida albicans* decrease was not long-time effective.
Chladek *et al*., 2011[47]	In vitro	*Candida albicans.*	Ufi Gel; AgNPs (10, 20, 40, 80,120 and 200 ppm).	The highest concentration of AgNPs leade to the best antifungal efficacy.
Nam, 2011[48]	In vitro	*S. aureus;* *S. mutans;* *Candida albicans.*	GC Soft-Liner; Silver nanoparticles (0, 0.1, 0.5, 1.0, 2.0 and 3.0% v/v).	Silver nanoparticle at 1.0% concentration was able to eliminate *S. aureus* and *S. mutans* and 2.0% eliminated *C. albicans.*
De Moraes, *et al.*, 2012[49]	In vitro	Streptococci and *Candida* species	CoeSoft; SoftConfort; Kooliner; Triazine (0, 2.5, 5 and 10%)	CoeSoft showed higher counts of *Candida* and *Streptococci* and the addition of triazine did not result in decreased counts of total microorganisms.

Table 3. Studies on cleaning or prevention protocols for denture liners without the incorporation of antifungals directly to the materials.

Manuscript	**Study Design**	**Microorganism**	**Material**	**Results**
Masella *et al*., 1975[50]	Prevention / treatment – In vitro	*C. albicans; C. glabrata; C. tropicalis; C. parapsilosis.*	Silastic 390; Pro-Kem; Zephiran; Listerine; Mersene; Cidex; Water 60^{o} C.; Zinc undecylenate.	All denture cleaners were effective at an appropriate concentration, except for Zinc undecylenate (5%) which was not totally effective.
Baysan *et al.*, 1998[51]	Disinfection – In vitro	*Candida albicans; Staphylococcus aureus.*	Molloplast-B; Sodium Hypochlorite 2%.	The best method for disinfection was immersion in sodium hypochlorite, followed by microwave irradiation.
Furukawa *et al. 1998*[52]	Disinfection – In vitro	*Candida albicans; Eschericia coli; Staphylococcus aureus.*	Coe Soft; Coe Comfort; Chlorine Dioxide;	Immersion technique was more effective than spray technique. However, chlorine dioxide was not able to achieve the minimal disinfection standard for both denture liners tested.
Dixon *et al*., 1999[53]	Disinfection – In vitro	*Candida albicans.*	Molloplast-B; Permaflex; Lucitone 199.	*C. albicans* was killed by 5-minute irradiation, with specimens immersed in water. Dry irradiation did not effectively sterilize any of the materials.
Olan-Rodriguez *et al.*, 2000[54]	Prevention – In vivo	Healthy patients.	Coe-Soft; Palaseal; Mono-Poly.	The soft-denture liner sealed presented less colonization by yeasts and bacteria. Among sealers, no difference was found.
Price *et al*., 2002[55]	Prevention – In vitro	*Candida albicans.*	Silicone material; Silane;	Modification of surface through plasma increased *Candida* adherence while silane treatment significantly reduced it.
Glass *et al*.,	Cleaning – In	*Staphylococcus aureus;*	Methylmethacrylate dentures	The use of Medical tabs for dentures eliminated *C.*

2004[56]	vitro	*Pseudomas aeniginosa; Bacillus cereus; Candida albicans; Herpes simplex virus 1;*	without soft liners (hard dentures); Methylmethacrjlate dentures containing processed soft liners (soft-liner dentures); Medical tabs for dentures.	*albicans, S. aureus, P. aeruginosa* and HSV-1 target virus in soft denture liner. Hard denture eradicated *C. albicans* and HSV-1 target virus and substantially reduced populations of *S. aureus* and *P. aeruginosa. B cereus* appeared to be the least affected by the one-time use of Medical tabs.
Yilmaz *et al*., 2005[57]	Disinfection – In vitro	*Staphylococcus aureus; Streptococcus sobrinus; Candida albicans.*	Tempo; Immediate; Flexacryl Soft; Ufi Gel P; Deconex 5%; Savlex 3.5%; Sodium Hypochlorite 2%; Sodium Hypochlorite 5.25%.	The best result was obtained by immersion in 5.25% sodium hypochlorite for all liners, but in all groups the disinfectant solution significantly reduced microorganisms.
Meşe and Meşe, 2007[58]	Disinfection – In vitro	*Candida albicans.*	Vertex Soft; Sodium hypochlorite 2%; Alkaline peroxide solutions.	Sodium hypochlorite showed the best results, followed by exposition to microwave energy (650 W for 2.5 minutes per side), but both were extremely effective. The worst result was obtained with alkaline peroxide solutions.
Zuluaga *et al*., 2007[59]	Treatment– In vivo	Patients with denture-related stomatitis	Coe Confort; Kool Liner;	The treatment with both tissue conditioners was effective.
Buergers *et al*., 2008[60]	Disinfection – In vitro	*Candida albicans.*	Mucopren E; Hydrogen peroxide 3%; Sodium hypochlorite 1%; Glutaraldehyde 2%; Household vinegar; Listerine coolmint; Plax (triclosan 0.3%); Blend-a-	The immersion in sodium hypochlorite (1%; 10 min), microwave irradiation immersed in water (800 W; 6 min), and the immersion of effervescent cleansing tabs (Blend-a-dent tabs; 10 min) proved to be effective against *C. albicans* colonization.

			dent 2 Phasen tabs.	
Mima *et al*., 2008[61]	Disinfection – In vitro	*P. aeruginosa; S. aureus; C. albicans; Bacillus subtilis*	Tokuso Rebase Fast Set.	Irradiation for 3 minutes or more at 650 w showed consistent sterilization to all microorganisms.
Boscato *et al*., 2009[62]	Prevention – In vivo	Biofilm formation	QuickLine; Colgate Triple Action toothpaste; Sodium hypochlorite 0.5%.	Irrespective of time, daily prosthetic hygiene with soft toothbrush and toothpaste presented the lowest biolfilm formation.
Ferreira *et al*., 2009[63]	Cleaning – In vitro	*Candida albicans; Candida glabrata.*	CoeSoft; Kooliner; Ufi Gel P; Polident; Efferdent; Sodium hypochlorite 0.5%.	0.5% NaOCl was effective for both species. *C. glabrata* showed higher number of remaining cells in all treatments, except for NaOCl.
Gedik and Ozkan, 2009[64]	Disinfection – In vitro	*Candida albicans,*	Ufi Gel P; Ufi Gel C; Mollosil; Soft-Liner; Moloplast B; Luci Soft; Efferdent; Polident; Steradent; Corega; Denclen; Klorhex; Axion (5.25% NaOCl);	Cleaning with NaOCl led to significantly lower counts compared to the other disinfectants tested.
Boscato *et al*., 2010[65]	Prevention – In vivo	Biofilm formation	Coe Confort; Varnish;	The use of varnish was not recommended, as it provided higher biofilm formation.
Vieira *et al*,, 2010[66]	Cleaning – In vitro	*Candida albicans; Candida glabrata.*	Lucitone 550; Kooliner; Polident; Efferdent; Sodium hypochlorite 0.5%.	The only effective treatment to clean the liner surfaces was the use of sodium hypochlorite. Both denture cleansers were responsible to decrease *Candida* counts. *C. glabrata* showed significantly higher cell counts in comparison to *C. albicans* when treated with both alkaline denture cleansers.

Hahnel *et al*., 2011[67]	Cleaning – In vitro	*Candida albicans.*	Ufi Gel SC; GC Reline soft; Silagum comfort; Mucopren soft; Blend-A-Dent; 2% NaOCl; Toothbrush Professional Care 9900 Braun Oral B and Sensodyne.	The best result was achieved by immersion in NaOCl. For long-term *Candida albicans* biofilm analysis, NaOCl also showed better results; however, mechanical cleaning was statistically superior to chemical denture cleaner Blend-A-Dent.
Uludamar *et al*., 2011[68]	Treatment – In vivo	*Candida albicans.*	Visco-gel; chlorine dioxide (0.8%); Corsodyl mouthrinse (0.2%)	The three treatments decreased palatal inflammation, but 0.8% chlorine dioxide and 0.2% chlorhexidine gluconate resulted in better results.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4,5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5,6
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	8

Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	8
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	N/A
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	8
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	N/A

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	N/A
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Table 1 and figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 2 and 3
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	n/a
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Table 2 and 3

Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	Not applicable
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	n/a
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	9,10,11,12
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	13
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Not applicable

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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ARTIGO 2

Biofilm formation on denture liners in a randomised, controlled in situ trial[§]

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Running Title: Biofilm in denture liners in a controlled trial

Keywords: denture liners; biofilms; *Candida;* randomized trial; in situ; fungal adherence

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Biofilm formation on denture liners in a randomised, controlled in situ trial

ABSTRACT

Objective: This randomized, in situ clinical trial assessed how biofilm composition is affected by time and denture material type in denture wearers with and without denture stomatitis. The randomized clinical trial design was performed using CONSORT statement.

Design: Twelve specimens of acrylic resin (control) and two denture liners (silicone-based or acrylic resin based, depending on the experimental phase) were manufactured and inserted into the surface intaglio of 28 denture wearers. Biofilm was formed in two phases of 21 days, and counts of viable micro-organisms in the accumulating biofilm were determined and converted to colony forming units per unit surface area after 7, 14 and 21days of biofilm formation. The surface structure was analyzed by scanning electron microscopy (SEM). Data were analyzed by three-way ANOVA followed by Tukey test to assess differences among health condition (healthy or candidiasis), materials and time point.

Results: Non-*albicans Candida* species counts were higher in candidiasis patients with silicone-based denture liners (p=0.01). Candidiasis patients showed higher mutans streptococci counts after 7 days (p=0.0041), but this difference disappeared after 14-21 days of biofilm formation. Lactobacilli and total micro-organisms counts were higher in denture liners, irrespective of the health condition or aging. SEM analysis showed that denture liners presented rougher surfaces while aging increases micro-organisms adhesion irrespective of the material tested.

Conclusion: Silicone-based denture liners should be avoided in candidiasis patients as these materials showed increased non-*albicans* species counts. Clinically, aging provided increased roughness and therefore higher micro-organisms adhesion.

INTRODUCTION

Biofilm formation and the presence of *Candida* species is strongly associated with high prevalence of denture stomatitis in denture wearers.[1,2] Fungi colonization can interfere with dental treatment and be a barrier to the patient's health, [3,2] since dentures can serve as a reservoir of micro-organisms for new infections.[4-6] Epidemiological studies report denture stomatitis prevalence from 15% to over 70% among denture wearers.[2] The adhesion of micro-organisms on the surface of acrylic resin and denture liners depends on the surface topography and the composition of these biomaterials.[7-9] In this context denture liners have been found to be more prone to microbial adhesion than acrylic resin used as denture base materials.[10]

Currently, denture liners are available as silicone-based and acrylic resin-based. The adhesion on these materials depends on the physicochemical properties of the surface of the microbial cells,[11-13] which will adhere and form biofilm forming a complex three-dimensional architecture.[14] One of the problems directly related to these materials is still the accumulation of biofilm[15] while there is no consensus on how long these materials last considering longer clinical service. *C. albicans and non-albicans species* are often found on the dentures and oral mucosa of individuals without any signs of denture stomatitis,[16] but a quantitative presence of *Candida* has been found to be associated with the onset of denture stomatitis. It is possible that the etiological role in denture stomatitis occurs in combination with other factors.[17] However, the interaction among substratum surfaces, oral bacteria, and the differences between healthy and diseased patients is yet poorly understood, especially considering differences between healthy and diseased patients,[18] with few clinical studies evaluating materials directly inserted into the denture base.[18-20] Therefore, this randomized in situ clinical trial evaluated the effect of time, substratum surface and health condition on biofilm

composition and surface characteristics of acrylic resin and denture liners. The hypothesis tested was that there is influence of time, denture liner and health condition in the biofilm formed in situ in this clinical trial.

MATERIALS & METHODS

Experimental design

This in situ, crossover, double-blinded (patient and biofilm analysis) study had a completely randomized design with substratum type (acrylic resin or denture liner), biofilm aging (7, 14 and 21 days) and health condition (healthy or candidiasis) as factors. The study was approved by the Local Research and Ethics Committee (protocol 191/2011). The oral health of the volunteers was assessed, and all participants signed written informed consent before being accepted into the study. The randomized clinical trial design was performed using CONSORT statement. Sixty- six patients wearing complete dentures were evaluated. After explaining the study, thirty-six patients accepted to participate into the study had their mouths and dentures swabbed for *Candida* species, but six patients could not be inclusion criteria included, result in fifteen were identified as *Candida* carriers, and fifteen diagnosed with denture stomatitis (candidiasis).

During 2 phases had inserted in recesses created in their palatal denture's flange 6 acrylic resin specimens and 6 temporary denture liner specimens (silicone or acrylic resin, depending on the randomly assigned experimental phase). Respectively temporary denture liners specimes the base of acrylic resin Soft Confort- SC (Dencril, Pirassununga, Brazil) and a base the silicone Elite® Super Soft Reling ESSR (Zhermack GmbH, Alemanha).

Inclusion criteria included adults 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 3 months prior to the study, and not using any other type of intraoral device. For the candidiasis patients, good general and oral health did not apply, as they presented denture stomatitis. The exclusion criteria eliminated those 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) that could insert a bias in the study (Fig 1).

Three patients withdrew the experiment (one had a surgery and the other had antibiotics). In each phase, after 7, 14 and 21 days of clinical service, 2 specimens of each material were randomly chosen and removed. The biofilm formed on the specimens was processed for microbiological composition analysis, and the results were expressed in colony forming units (CFU)/mm^2. Specimens were analyzed by scanning electron microscopy (SEM).

Panellists and Ethical Aspects

One examiner carried out intra-oral examination of 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 candidiasis, while the other group was classified according to Newton's [21]classification: the clinical appearance of the inflamed mucosa was considered with diffuse hyperemia and micropapules, inflammation and widespread, the mucosa was smooth and swollen, covering the entire region covered by the prosthesis. Swabs were cultured in CHROMagar™ *Candida* (Difco, Sparks, MD, USA) at 37^{o}C for 48 h.

Patients were instructed to wear the dentures at all times and to brush their dentures 3x/day after the main mealtimes with a soft toothbrush and toothpaste (provided by the

researchers) except for the area containing the specimens, where only the slurry from the toothpaste was spread during a 7-day pre-experimental period and the experimental period.

Preparation of specimens

All materials were prepared by a single operator at room temperature (25 ± 1.0ºC and 50 ± 5% relative humidity), under aseptic conditions. Specimens (5 x 5 x 2 mm) were prepared according to manufacturers' recommendations acrylic resin (Acron MC, GC America, Alsip, IL, USA), Elite® Super Soft Reling (Zhermack GmbH, Germany), and Soft Confort (Dencril, Pirassununga, Brazil). The acrylic resin was processed as previously described[7] and were ground using progressively smoother aluminum oxide papers (320-, 400-, and 600-grit) in a horizontal polisher. For the soft denture liners, surface roughness was standardized by the contact with the glass slides.

Were prepared of twelve control specimes (acrylic resin), six denture liners silicone based and six denture liners acrylic resin based for each peciente and for each experimental phase. So, were inserted into the surface intaglio of 28 denture weares.

Denture preparation and clinical phase

Initially, the original patients prostheses received a standardised mechanic polishing with a lathe, a brush wheel with pumice slurry and a felt cone with chalk powder were used so that all the surfaces presented the same smooth baseline condition. Six recesses of 6 x 6 x 3 mm depth were made at each side of the intaglio surface of the maxillary denture in contact with either normal or inflamed mucosa. Each specimen was positioned and fixed with wax in the recess created. The specimens were randomly distributed according to the phase the patient was designated. Considering that the study followed a crossover design, with the patients participating in both phases, the subjects did not receive any instructions regarding

their daily diet. A washout period of 7 days was allowed between the two phases to eliminate possible residual effects from the materials. Specimens were not reinserted and the recess was cleaned and filled with wax.

Microbiological analysis of the biofilm

The biofilm formed and the specimens were collected on the 7^{th}, 14^{th} and 21^{th} 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. Specimens containing the biofilm were sonicated at 40 W and 5% amplitude with three pulses of 10 seconds each, serially diluted and inoculated on specific media, and incubated at 37 °C in (anaerobiosis - blood agar, rogosa agar and mitis salivarius bacitracin; aerobiosis - CHROMagar *Candida*) for 24 to 96 hours. The CFU were counted using a stereomicroscope, and the results expressed in CFU/mm^2. Different colony morphologies were identified by Gram staining and morphology and biochemical tests of sugar fermentation were used to confirm mutans streptococci and *C. albicans* and non-*albicans* species. 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.

SEM Analysis

In order to observe the surface characteristics of all materials extra specimens were also added to the dentures in the same way as previously described for each time point and type material. Nine specimens were mounted on a stub, air-dried, sputter-coated with gold (Balzers Union MED 010 evaporator) and examined with a scanning electron microscope (SSX-550; Shimadzu) at an accelerating voltage of 15 kV for surface characterization after

the biofilm formation focusing on surface morphology and biofilm at each time point. This methodology was merely illustrative did not have to qualifitu the surface.

Statistical analysis

Statistical analyses were done using SAS software (SAS InstituteInc., version 9.0, Cary, NC, USA) employing a significance level fixed at 5%. The null hypotheses assumed differences among substrata, time point and health condition assessed. A randomized block design was used for the statistical analyses, considering the patients as statistical blocks, and time points, substratum types and health condition as factors under study. For microbiological analysis, data that violated the assumptions of equality of variances and normal distribution of errors were transformed by rank and analyzed by ANOVA, followed by Tukey test.

RESULTS

Assessment of the three materials with SEM showed different degrees of surface irregularities. Remarkably, large amounts of porosities and irregularities were observed in the denture liner samples, with micro-organisms clusters on the surfaces. In general, *C. albicans* adherence was observed in cluster forms and whole attached cells were viewed in blastospore morphology (Figure 2).

Table 1 shows the microbiological results for *C. albicans* and non-*albicans* species. There was no difference in *C. albicans* counts in all materials and time points studied ($p>0.05$). Also, healthy or diseased patients did not show differences in *C. albicans* counts ($p>0.05$). However, non-*albicans Candida* species counts showed statistically significant differences in the silicone-based liner, with higher proportions of these species; candidiasis

patients showed highest counts of non-*albicans* species in the silicone based denture liner (p=0.0111).

For mutans streptococci counts, there were statistically significant differences between healthy and diseased patients only in the beginning of the experiment, i.e. 7 days, where mutans streptococci counts were higher in candidiasis patients (p=0.0041); however, when the biofilm matured for 14-21 days, this difference was no longer observed, irrespective of the material tested.

For lactobacilli counts, the silicone-based liner showed higher counts when compared to the other denture liner, in both healthy and diseased patients and for all time points assessed (p=0.032). when considering total micro-organisms, the resin based denture liner showed higher counts, irrespective of the time point assessed or the health condition of the patient (p=0.0404).

DISCUSSION

This clinical study has shown that non-*albicans Candida* species are responsible for higher counts in candidiasis patients. In addition, it seems from our study that liners will always present higher counts compared to acrylic resin regularly used to fabricate dentures, however, the time elapsed since the commencement of biofilm formation does not seem to change biofilm composition. The present study evaluated denture wearers with and without denture stomatitis, to understand how biofilm composition could be affected by time and denture material type in healthy and diseased subjects. The biofilm was grown up to 21 days to better understand if time would be responsible for changes in biofilm composition especially in diseased subjects, as manufacturers usually indicate the use of these liners for very short periods of time. Therefore, our hypothesis was accepted since there was difference

among time for mutans streptococci counts, differences between liners and between health condition in the biofilm formed in situ in this clinical trial.

These new results are important since *in vitro* studies had already shown that denture liners are easily colonised and deeply infected by *Candida species*,[23,24] but no attempt to evaluate mature biofilms or to compare the differences between subjects has been made. Furthermore, intraorally a denture is rapidly coated with a salivary pellicle, modifying the properties of the exposed surfaces, which is the reason why in vitro studies fail to show this trend, as they rarely account for all the factors which likely play a role during biofilm infection.[25]

In this study, we chose to analyze the surface structure by SEM, because it allows the surface characterization after the biofilm formation focusing on surface morphology and biofilm at each time point. The morphology of the materials' surface was examined and the analysis revealed that the surface topography could affect microbial adhesion, with higher numbers of cell clusters retained on the rougher surfaces (denture liners). The aging process probably increases the surface irregularities and the likelihood of micro-organisms on the surface (Figure 2). After 21 days, the biofilm will keep maturing and, with an increase of the surface irregularities of the denture liner, the cells will be entrapped in the denture liners´ prorosities, thus making it more and more difficult to remove biofilm either mechanically or chemically. However, the SEM images in this study are merely illustrative.

Biofilm formation is an important virulence factor for a number of Candida species, as it confers significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and protecting cells from host immune responses.[26,27] Moreover, biofilms formed by *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* isolates have been associated with higher morbidity and mortality rates compared with isolates unable to form biofilms.[28] Although the mechanisms of biofilm drug resistance are not fully understood, the

current consensus is that biofilm tolerance is a complex multifactorial phenomenon involving different molecular mechanisms, restricted penetration of the drug through the matrix and the presence of so-called 'persister' cells within the biofilm, which survive exposure to the agent.[29,26,30]

Although our study has shown no differences in *C. albicans* counts in any of the conditions tested, *Candida albicans* is recognized as a contributing factor in the cause of denture stomatitis since these fungi are capable of proliferating in healthy hosts by surviving immune factors, demonstrating increased resistance to commonly used antifungal drug therapies.[27,31-33] Moreover, in this study, for mutans streptococci counts, there were differences between healthy and diseased patients only in the beginning of the experiment, i.e. where mutans streptococci counts were higher in candidiasis patients. These results are important since mutans streptococci appear in the initial phases of biofilm development and are known to have synergism with *Candida* species.[22]

For lactobacilli counts, the silicone-based liner showed higher counts when compared with the other denture liner, in both healthy and diseased patients and for all time points assessed. When considering total micro-organisms, the resin based denture liner showed higher counts, irrespective of the time point assessed or the health condition of the patient. Although these findings seem contradictory, the substratum may influence the composition and the formation of the pellicle, together with host characteristics, which may be less important than the surface properties of the dental materials.[34] In addition, most studies showing these differences are in vitro and again, may not account for the numerous factors involved in vivo in biofilm formation, while antimicrobial properties of saliva may contribute to the tissue/patient factors influence biofilme formation, not the substrate.[18]

A change in a key environmental factor (or factors) will trigger a shift in the balance of the resident plaque microflora, and this might predispose a site to disease will trigger a

shift in the balance of the resident plaque microflora, and this might predispose a site to disease.[35] Microbial specificity in disease would be due to the fact that only certain species are competitive under the new (changed) environmental conditions as it happened with non-*albicans Candida* species and mutans streptococci.

In our study, denture hygiene was standardized with the same toothbrush and toothpaste for all individuals, which had the same hygiene instructions. However, poor denture hygiene is clearly accepted as a critical risk factor for denture stomatitis. Thus, it is mandatory to carry out studies comparing different hygiene methods and the effect they will promote in denture liners. While access to dental care is improving and teeth are still present in the elderly patients, there is still a high incidence of individuals with complete dentures, correlating the disease with other factors, ultimately preventing the disease that is still a sizable at-risk population for denture stomatitis.[36] Further studies are needed to increase our understanding of the oral ecosystem and the clinically important micro-organisms/materials interactions. Moreover, it is important to enphasize that the results obtained in this study should be interpreted with caution, since individual factors may influence the findings, according to age, gender, income, general health, oral hygiene, daily period of use of prosthesis, time of use of the prosthesis alcohol consumption, trauma, diet and salivary components.

CONCLUSIONS

The use of the silicone liners should be avoided in patients with denture stomatitis due to an increase in non-*albicans Candida* species, known to be difficult to treat. In general, denture liners evaluated in this study accumulate greater amount of biofilm, and therefore their use should be cautious.

ACKNOWLEDGMENT

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CONSORT 2010 Flow Diagram

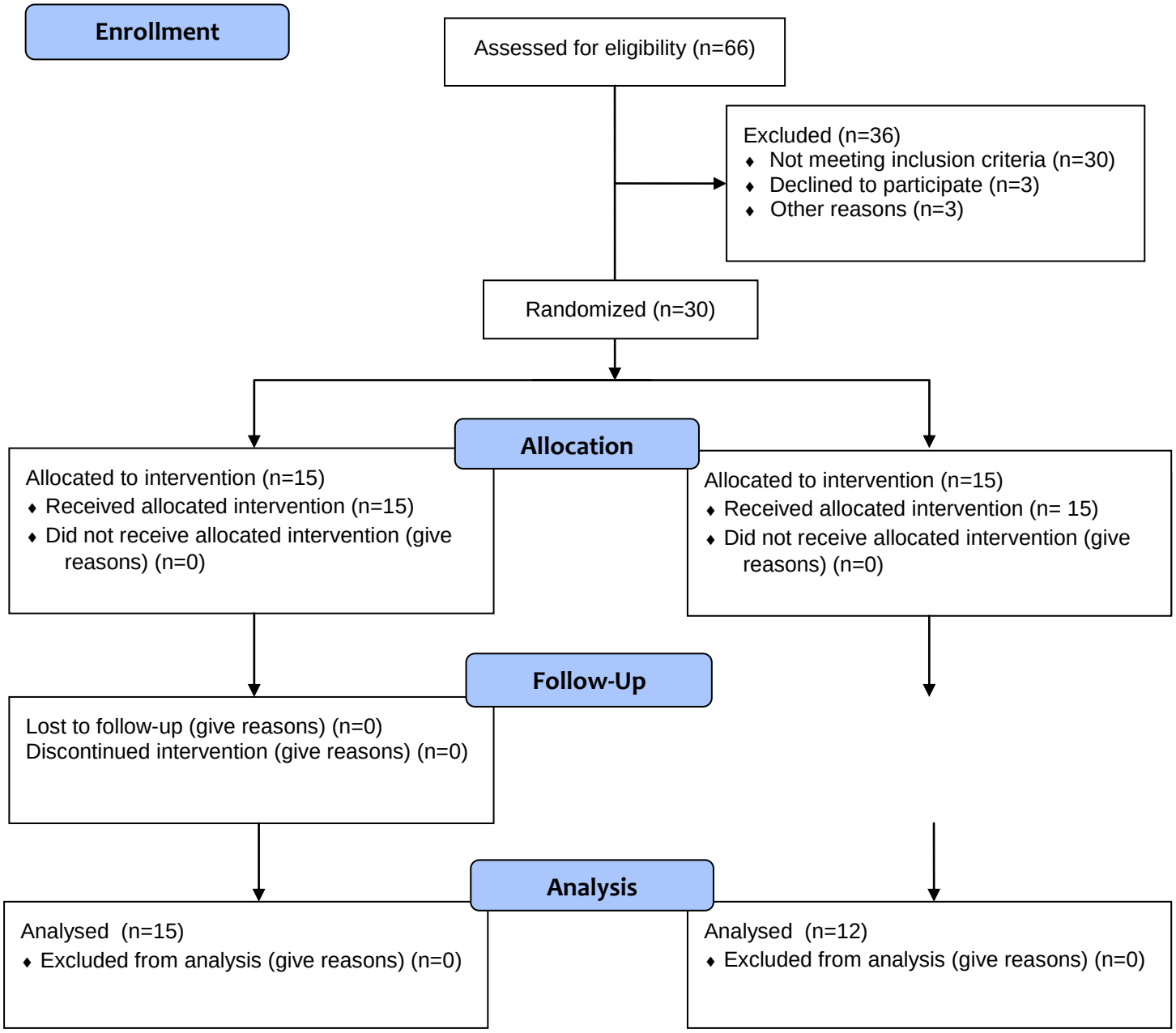

Figure 1. Selection criteria according CONSORT Statement.

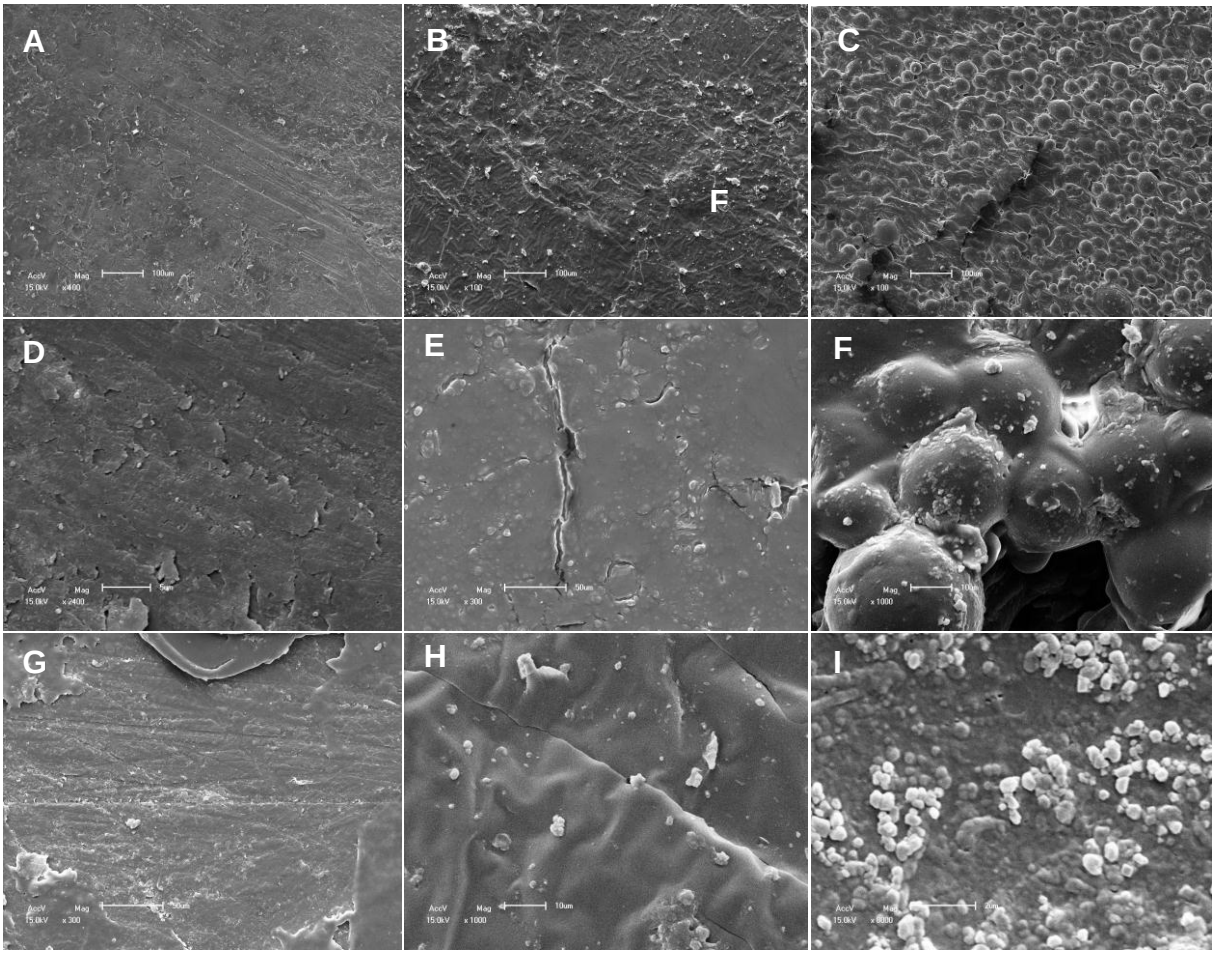

Figure 2. Representative SEM images of surface characteristics for each time point and type of material. **7 days**: **A,** acrylic resin; **B**, silicone-based denture liner; **C**, acrylic-based denture liner (original magnification ×400, 100, 100, respectively); **14 days: D**, acrylic resin; **E**, silicone-based denture liner; **F**, acrylic-based denture liner (original magnification ×2400, 300, 1000, respectively); **21 days: G,** acrylic resin; **H**, silicone-based denture liner; **I**, acrylic-based denture liner (original magnification ×300, 1000, 8000, respectively).

Table 1 – *C. albicans* and non-*albicans* species counts in the biofilm according to experimental conditions (average±SD).

		C. albicans (x 10^2)			*C.* non-*albicans* (x 10^2)		
Candidiasis		Control	acrylic liner	silicone liner	control	acrylic liner	silicone liner
	7	2.1±11.0a	0.9±2.7a	0.4±1.0a	4.6±23.7a	4.2±13.4a	2.2±6.4b
	14	12.2±78.1a	5.2±14.3a	4.7±16.9a	9.8±50.1a	2.1±6.2a	11.5±36.0b
	21	7.7±27.6a	1.1±4.9a	2.6±8.5a	10.3±37.7a	0.1±0.2a	12.4±26.8b
Healthy							
	7	1.7±6.4a	1.8±6.1a	0.4±1.1a	1.1±4.9a	4.2±17.5a	0.6±2.4a
	14	4.8±18.5a	3.4±9.7a	3.6±9.8a	0.9±4.2a	1.3±4.0a	1.8±6.4a
	21	6.9±31.9a	6.7±21.0a	7.7±22.0a	0.6±2.4a	3.4±12.0a	1.3±4.2a

There were no statistically significant differences for *Candida albicans* counts considering all variables tested. Lower case letters represents statistically significant differences among materials and disease for non-*albicans Candida* species (three-way ANOVA followed by Tukey test, $p<0.05$)

Table 2 – Microbiological results for bacteria in the biofilm according to the experimental conditions (average±SD)

	mutans Streptococci (x 10^3)			Lactobacilli (x 10^5)			Total micro-organisms (x 10^6)		
Candidiasis	control	acrylic liner	silicone liner	control	acrylic liner	silicone liner	control	acrylic liner	silicone liner
7	40.2±278.4b	6.5±15.6b	4.3±7.6b	2.2±5.2a	2.1±2.9a	3.4±5.1b	1.9±3.5a	2.9±3.8b	3.3±5.7a
14	4.1±9.3a	2.0±5.1a	5.9±25.6a	3.5±8.7a	2.9±5.0a	6.2±15.4b	3.7±6.8a	2.0±3.3b	2.8±3.8a
21	3.5±10.4a	24.4±102.5a	4.5±10.4a	2.6±4.6a	5.5±16.1a	2.8±2.7b	1.9±4.3a	1.9±2.2b	1.5±1.6a
Healthy									
7	1.2±3.3a	1.9±4.6a	0.6±1.9a	4.7±11.6a	2.1±3.3a	4.8±7.5b	2.0±3.1a	2.7±2.4b	1.5±2.0a
14	4.7±13.1a	0.3±0.7a	6.6±31.0a	4.0±12.1a	3.5±8.0a	3.0±6.1b	2.5±3.2a	2.2±3.3b	2.6±4.7a
21	5.0±11.4a	2.3±4.4a	4.5±11.0a	2.3±3.9a	7.4±14.2a	3.0±3.6b	1.8±2.6a	3.4±5.8b	1.7±2.7a

Lower case letters represents statistically significant differences among materials for lactobacilli fixing time and health condition ($p=0.0302$) and total micro-organisms ($p=0.0404$); (Three-way ANOVA followed by Tukey test, $p<0.05$)

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	1
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	2,3
	2b	Specific objectives or hypotheses	3
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	3
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	N/A
Participants	4a	Eligibility criteria for participants	4
	4b	Settings and locations where the data were collected	3
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	3,4
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	6,7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	N/A
Sample size	7a	How sample size was determined	4
	7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	6
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	6
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to	4,6

		interventions	
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	3
	11b	If relevant, description of the similarity of interventions	N/A
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	7
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	N/A
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	3
	13b	For each group, losses and exclusions after randomisation, together with reasons	3, 7
Recruitment	14a	Dates defining the periods of recruitment and follow-up	N/A
	14b	Why the trial ended or was stopped	N/A
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	N/A
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	3,7
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	7
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	N/A
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	8
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	N/A
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	5
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	11,12
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	11
Other information			
Registration	23	Registration number and name of trial registry	N/A
Protocol	24	Where the full trial protocol can be accessed, if available	N/A
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	12

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

3 Conclusões

Embora a adição de um agente antifúngico em reembasadores pareça ter algum efeito benéfico para prevenir a colonização por *Candida* e a utilização de hipoclorito de sódio pareça ajudar na sua desinfecção, não há evidências científicas para fornecer recomendações definitivas sobre qual protocolo de desinfecção é o ideal para reembasadores a base de prótese. No entanto, parece de acordo com os estudos in vitro utilizados pela nossa revisão sistemática, que 0,5% de Hipoclorito de Sódio é suficiente para limpar e desinfectar reembasadores de prótese sem danificar a superfície dos materias.

A utilização de reembasadores a base de silicone, testados nesse estudo, está contra indicada em pacientes com estomatite por dentadura devido a uma maior colonização de *C. não albicans*, a qual é mais virulenta e resistente às terapias convencionais. Em geral, os reembasadores de prótese, testados neste estudo acumulam maior quantidade de biofilme e, portanto, seu uso deve ser cauteloso.

4 Referências

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APÊNDICE A – Termo de Consentimento Livre e Esclarecido

UNIVERSIDADE FEDERAL DE PELOTAS
FACULDADE DE ODONTOLOGIA

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está convidado a participar, como voluntário, em uma pesquisa. Após ser esclarecido sobre as informações a seguir, no caso de aceitar fazer parte do estudo, assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é das pesquisadoras responsáveis. Alertamos que não existem riscos envolvidos neste estudo e em caso de recusa você não será penalizado de forma alguma. Esclarecemos que a participação é decorrente de sua livre decisão, após receber todas as informações que julgar necessárias, e que poderá ser a qualquer tempo, retirada.

INFORMAÇÕES SOBRE A PESQUISA:

Título do Projeto: Avaliação clínica da formação de biofilme sobre condicionadores de tecido temporários

Pesquisadora participante: Fernanda Valentini

Pesquisadora responsável: Profa. Dra. Tatiana Pereira Cenci e Profa. Dra. Noéli Boscato

Prezado paciente, a nossa pesquisa tem como objetivo principal, avaliar a composição da placa bacteriana que se forma sobre a superfície de diferentes matérias usados na parte de dentro da dentadura para evitar que ela machuque sua gengiva , bem como avaliar a rugosidade dessa superfície ao longo do tempo e avaliar a colonização de *Candida*, que é um fungo comum na cavidade bucal e que causa doença em alguns casos específicos, como por exemplo quando a prótese fica muito tempo sem ser trocada ou sem adequadamente limpeza. Para isso será realizado um estudo clínico em sua prótese total (dentadura) superior antiga ao mesmo tempo em que se realizam as etapas clínicas para confecção de uma nova prótese total. Os materiais estudados serão colocados na parte de dentro de sua dentadura antiga sem provocar qualquer desconforto. Você terá que retornar à Faculdade de Odontologia da UFPel após passados 1, 7 e 14 dias para que possamos remover os matérias testados. Depois, haverá um intervalo de 07 dias e colocaremos um segundo material para análise que ocorrerá da mesma maneira que para a primeira fase , sendo necessário mais 14 dias, e você deverá retornar para as consultas no 1, 7 e 14 dia da segunda fase, igual feito para primeira fase. Ao final da segunda fase, seguirá a sequência clínica para a finalização da confecção de nova prótese total superior.

CONSENTIMENTO DA PARTICIPAÇÃO DA PESSOA COMO SUJEITO E RESPONSÁVEL LEGAL

Eu, _____, RG/ CI _____, abaixo assinado, concordo em participar do estudo sobre a avaliação clínica da formação de biofilme em condicionadores de tecidos temporários realizado na clínica de prótese dentária do curso de Odontologia da Universidade Federal de Pelotas. Fui devidamente informado e esclarecido sobre a minha participação. Foi-me garantido que posso retirar meu consentimento a qualquer momento, sem que isto leve a qualquer penalidade ou interrupção do acompanhamento/ assistência/ tratamento.

Pelotas, _____de _____ de 2010.

Assinatura

ANEXO A. Parece do Comitê de Ética em Pesquisa

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE PELOTAS
FACULDADE DE ODONTOLOGIA
COMITÊ DE ÉTICA EM PESQUISA

PELOTAS, 12 de abril de 2011.

PARECER Nº 191/2011

O projeto de pesquisa intitulado "**Avaliação clínica da formação de biofilme e da influência da rugosidade superficial sobre condicionadores de tecidos temporários**" está constituído de forma adequada, cumprindo, na suas plenitudes preceitos éticos estabelecidos por este Comitê e pela legislação vigente, recebendo, portanto, **PARECER FAVORÁVEL** à sua execução.

Prof º. Marcos Antonio Torriani
Coordenador do CEP/FO/UFPel

Prof. Marcos A. Torriani
Coordenador
Comitê de Ética e Pesquisa