UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

ALINE SILVA BRAGA

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# Avaliação do efeito de diferentes enxaguatórios bucais comerciais sobre a viabilidade e atividade de biofilme microcosmo e a desmineralização do esmalte

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2017

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Dissertation presented to the Bauru School of Dentistry of the University of São Paulo to obtain the degree of Master in Science in the Applied Dental Science Program, Stomatology and Oral Biology concentration area.

Supervisor: Prof. Drª Ana Carolina Magalhães

Dissertação apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, área de concentração Estomatologia e Biologia Oral.

Orientadora: Prof. Drª Ana Carolina Magalhães

**Versão Corrigida**

BAURU

2017

Braga, Aline Silva

B73e Evaluation of the effect of different commercial mouthrinses on the viability and activity of microcosm biofilm and on enamel demineralization / Aline Silva Braga – Bauru, 2017.

107 p. : il. ; 31cm.

Dissertação (Mestrado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientadora: Prof. Drª Ana Carolina Magalhães

**Nota:** A versão original desta dissertação encontra-se disponível no Serviço de Biblioteca da Faculdade de Odontologia de Bauru – FOB/USP



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Protocolo nº CAAE 48100315.3.0000.5417
Registro nº CEPA 005/2016

Data: 22/10/2015 e 20/12/2016

FOLHA DE APROVAÇÃO

## *DEDICATÓRIA*

*À **Deus,***

*Por me manter firme em toda a minha trajetória, pois sei que nada sou sem o Senhor, minha luz, meu respirar, meu refúgio, me consolou nos momentos mais difíceis, conduziu da melhor forma minha vida, me mostrou a direção correta, pois ele é perfeito em todos os sentidos.*

***"Se tiveres de atravessar a água, estarei contigo. E os rios não te submergirão; se caminhares pelo fogo, não te queimarás, e a chama não te consumirá".***

***Isaías 43,2***

*À minha irmã **Alessandra Cristina da Silva** (in memorian),*

*Por ter me dado os melhores conselhos, por me incentivar a buscar sempre o meu melhor, por me fazer acreditar que aquilo que parecia ser impossível para alguns, para o nosso Deus não. Pois quando trilhamos nosso caminho com amor, dedicação e respeito ao próximo, alcançamos nossos objetivos. Por me mostrar o caminho da fé, pois a melhor maneira de viver é na presença do Pai.*

***"Mas nós, vibramos em outra frequência***

***Sabemos que não é bem assim***

***Se fosse fácil achar o caminho das pedras,***

***Tantas pedras no caminho não seriam ruim"***

***Outras frequências - Humberto Gessinger***

*Aos meus queridos pais* ***Alaor*** *e* ***Bia,***

*Por sempre aceitarem minhas escolhas, por acreditarem que cada passo era importante para o meu crescimento, por me mostrarem que a vida não foi e não será fácil, mas que independente do caminho vocês sempre estarão comigo, por me mostrarem que depois da tempestade sempre haverá a calmaria. Como é bom ter um porto seguro, vocês são fundamentais em minha vida. Agradeço a Deus todos os dias por ter me dado vocês de presente, o bem mais precioso que tenho.*

*"Seus olhos meu clarão*
*Me guiam dentro da escuridão*
*Seus pés me abrem o caminho*
*Eu sigo e nunca me sinto só..."*
*Velha infância - Marisa Monte e Arnaldo Antunes*

# AGRADECIMENTOS

**A Deus**,

Por me proporcionar a vida, por me permitir nascer em uma família maravilhosa sustentada pela fé, por ter me tirado dos caminhos perigosos, por ter me amparado nos momentos difíceis que percorri, por me permitir ter tantas pessoas que me amam ao meu lado, por ter amigos (irmãos) que sei que posso contar, por ter tantas pessoas abençoadas ao meu lado, por acordar todos dos dias com a certeza de que é o Senhor quem conduz cada passo em minha vida. Tu és meu refúgio, meu consolo, a ti toda honra e toda glória.

Aos meus pais **Alaor** e **Bia,**

Há dias venho pensando como expressar em palavras o que sinto por vocês, difícil começar sem me emocionar, pois essa vitória também é de vocês; sou grata por vocês existirem, por vocês sofrerem junto comigo, percorrerem cada passo ao meu lado; vocês me encorajaram em cada momento, dando força para nunca desistir; por entenderem minha vida, por respeitarem minhas escolhas, por me aguentarem quando já não tinham muita paciência. Quando penso onde tudo começou, vejo como Deus foi perfeito em tudo e que sem vocês nada seria possível; era apenas uma tentativa prestando o primeiro vestibular em outra cidade, o medo não nos fez desistir, pensávamos não ser a minha hora de sair das asas de vocês, parecia tudo impossível, mas a força e a coragem de vocês me motivaram a buscar meus sonhos. Fecho os olhos e consigo lembrar onde tudo começou, quando os pais da Thais, seu Marcus e Maria do Carmo, foram me buscar em casa para irmos para Bandeirantes/PR e a tia Maria do Carmo brincando perguntou a você mãe, “e se elas passarem, Bia?” você com os olhos lacrimejando respondeu “aí elas irão se mudar!” por mais que não fosse à hora nem o momento mãe, você nunca mediu esforços para me fazer feliz, nunca disse não, então, o resultado do vestibular foi positivo e vibramos juntas; depois da mudança veio saudade e você, mãe, durante as intermináveis ligações me acalmou e nunca me deixou voltar atrás, este foi o primeiro passo importante para o meu amadurecimento, agradeço muito a Deus e a

você por ter me proporcionado esses quatro anos de um enorme aprendizado pessoal e acadêmico. Os anos se passaram e a vontade pela docência foi crescendo, após voltar a Bauru e trabalhar durante três anos em diferentes escolas estaduais, mas o sonho de realizar o mestrado nunca foi esquecido e, então, vocês mais uma vez me deram suporte para mais essa conquista. Vocês sem dúvida são os maiores explemos de fé e coragem. Obrigada por existirem!

À minha querida irmã **Alessandra Cristina da Silva** (*in memorian*),
Deus permitiu-me ter ela como irmã, conviver ao lado de um anjo durante quinze anos, a qual eu carinhosamente reconheço por Tata, sem dúvida ela passou por nossas vidas para despertar o melhor de cada um, me ensinou dentre tantas coisas a ter caráter, educou, me mostrou o caminho certo a percorrer, preparou para a vida, mostrou que sem estudo e preparo, nenhum sonho seria alcançado. Com todas as dificuldades que enfrentou, nunca desistiu de viver. Deixou a todos nós uma lição de superação, força de vontade, garra, determinação e honestidade. O seu jeito carinhoso de agir com todos me faz acreditar que vale a pena sim, dar sempre o nosso melhor ao próximo, pois é dando que se recebe. Infelizmente nos deixou muito cedo, com uma imensa saudade, o que me conforta é saber que está ao lado do Pai, zelando por nossas vidas todos os dias. Obrigada pelos conselhos e por ter nos deixado a maior herança de todas – o significado da palavra perdão e sua importância, pois os humanos falham, e não cai uma folha da árvore sem a vontade de Deus.

À minha querida e amada avó **Sebastiana**,
Vó fico muito feliz em saber que tenho a senhora ao meu lado, obrigada por ensinar tantas coisas, por ser um exemplo de esposa e mãe. Obrigada por ter me dado carinho e atenção durante os anos que moramos juntas. Obrigada por me colocar todos os dias em suas orações, que o nosso amor e companheirismo só aumentem.

À minha tia e amada madrinha **Maria**,
Tia, sei que nem mesmo a distância pode afastar o amor que existe entre nós, a senhora com o seu jeito carinhoso e um coração gigantesco me mostrou a importância da família e a certeza de que sempre teremos para onde correr nos momentos de aflição. Obrigada por me incentivar e me acompanhar em todas as minhas conquistas. Além de tia é a melhor madrinha do mundo, uma segunda mãe.

As minhas queridas primas **Viviane, Flávia e Luciana**,
Obrigada por todo apoio de vocês, por estarem sempre presentes dando suporte e conselhos, ter vocês sempre ao meu lado me confortando e consolando. Deus sempre nos uniu nos momentos mais difíceis, pois é na dor que precisamos de amor e carinho familiar; me sinto muito orgulhosa em saber que posso contar sempre com vocês. Com os anos vocês se tornaram fundamentais em minha vida. Com toda a certeza não é só o sangue que nos une.

À toda minha **Família**,
Que sempre se orgulhou de mim, aos meus tios e minhas tias, de longe ou perto, obrigado por todo o carinho, respeito e atenção comigo. Uma família enorme em tamanho e coração. Espero ter vocês sempre ao meu lado, dividindo cada conquista!

As minhas queridas amigas **Francine Galhardo, Lívia Romani e Thais Lemos,**
Sempre que penso em vocês, me pergunto “O que seria da minha vida sem elas ao meu lado?!”, não encontro resposta, pois tudo seria bem mais difícil. No mesmo mês em que perdi minha amada irmã, Deus me deu vocês de presente. Isso me faz ter a certeza, de que irmãos não são apenas de sangue, e sim aqueles que você pode confiar para tudo na vida, e vocês são assim para mim. Obrigada por todos esses anos de amizade, por todos os conselhos, por todas as lágrimas e principalmente pelos risos.

A minha amiga **Sara Dentello,**

Sarinha impossível pensar em você e não sorrir, obrigada por me dar inúmeras oportunidades de sorrir ao seu lado, por toda atenção e carinho comigo, por diversas vezes em que eu achei que tudo estava perdido, você me levantou, me fez enxergar minhas qualidades. Devo muito a você; que um dia eu possa retribuir tudo o que você faz por mim.

Ao meu querido amigo **Anderson Pires,**
Pires uma vez me perguntaram sobre nossa amizade, se era possível amizade verdadeira entre um homem e uma mulher, e nós provamos que sim, é possível e verdadeiro. Já são doze anos de amizade e companheirismo; obrigada por ser mais que um amigo, um irmão.

Aos meus amigos da **Missão Resgate,**
Amados, como fico fortalecida ao lado de vocês, vocês me mostraram como é bom servir ao nosso senhor e quantas coisas Deus têm nos dado; durante esses anos de mestrado precisei muito de vocês, e vocês estavam sempre me dando todo o suporte necessário. Muito obrigada **Wilson, Vitor, Simone, Eliane, Lígia, Vinicius, Camila, Léo, Bruna, Laura, Karla, Amanda** e a todos do nosso **MJ**. Muito obrigada, que Deus abençoe todos vocês.

Aos queridos amigos de mestrado **Even Taira, Tatiana Martini e Vinícius Taioqui.**
Quando cheguei na FOB, no meu primeiro dia de mestrado, eu estava assustada e perdida com tanta novidade, foi quando encontrei vocês no anfiteatro, e vocês me receberam com um sorriso acolhedor; foi maravilhoso. Durante esses anos compartilhamos alegrias e aflições do mestrado; dizer que esta etapa foi fácil é mentira, mas com vocês tudo foi mais leve e tranquilo. Obrigada por fazerem parte da minha história. Para sempre “Tanquinho”.

Ao meu amigo e companheiro de expedição **Vinícius Taioqui Pelá,**
Dificil explicar nossa relação, que começou do nada e ficou tão intensa, o modo com que você me trata, conversa e respeita, me tornou muito mais feliz, você veio para somar, ter você ao meu lado me acalma e me faz acreditar que nossa amizade é para a eternidade, independente de onde a vida nos leve, você estará comigo e eu com você. Sei que nossa amizade se fortaleceu em Rondônia, onde passamos momentos difíceis longe da família e amigos. Fomos com a certeza de que tudo sairia muito bem, pois estávamos juntos, um cuidando do outro, dando suporte, foi então que voltamos com nossa amizade fortalecida na fé. Obrigada por tantos dias de sorrisos.

À minha amiga **Juliana Pires,**
Ju, você sabe que essa vitória também é sua! Obrigada por todo empenho e paciência comigo. Quando nos conhecemos não tive dúvida que daria muito certo, você com sua calma e atenção, me ensinou boa parte do que eu sei hoje. Obrigada por cada noite no laboratório, pela ajuda aos eternos finais de semanas onde passamos no fluxo, foi ali que nossa amizade ficou marcada, com muitas conversas, músicas da nossa rádio "Rádio Fluxo FM" rsrs, piadas e brincadeiras, muito obrigada por sua companhia nas missas do centrinho às quartas-feiras, nossos passeios no cinema e nas baladinhas. Por me passar os episódios da série favorita do nosso time! Obrigada por cada momento de alegria e também por me acalmar nos momentos onde achamos que não encontraríamos uma saída, mas a gente não desistiu né?! Pois com estudo e dedicação você me mostrou que conseguiríamos. E aqui vencemos mais uma etapa... é só o começo Ju!

À minha amiga e vizinha **Daiana Moreli,**
Daia você veio para somar a nossa equipe, chegou devagar e logo tomou meu coração, como seu jeito de ser, às vezes um pouco brava rsrs, mas com um coração enorme! Obrigada por sua amizade, pelos jantares no apê, os filmes e séries. Fico muito feliz por ter pessoas como você ao meu redor! Que Deus te ilumine muito! Muito obrigada e sucesso sempre!

À minha amiga **Talita Ventura,**
Ta, quantas vezes eu tentei ligar ou mandei mensagem deseperada precisando da sua ajuda e do seu conhecimento, às vezes era uma dúvida no meu hemograma rsrs, outras inúmeras sobre protocolos e tantas outras para conselhos. Obrigada por me ajudar com todo amor e carinho. Sei que sempre posso contar com você. Sinto o amor de Maria em seus olhos. Obrigada.

À minha amiga **Natália Mello,**
Nati, obrigada por todas as palavras de conforto, todas as ligações durante esses anos, os jantares onde nos reunimos para colocar nossas conversas em dia; você com esse jeito meigo de tratar o próximo conquistou a todos da bioquímica; pode ter certeza que você irá muito longe. Muito Obrigada.

À minha amiga técnica do laboratório **Thelma Silva,**
Thel, obrigada por ter transmitido um pouco do seu conhecimento, sei que tenho muito a aprender com você ainda, você foi essencial durante esse período de formação. Obrigada por me atender sempre com prontidão e carinho.

À minha amiga **Priscila Salomão,**
Pri, que benção a minha trabalhar com você, obrigada por ter me ajudado sempre que eu precisei, pelas conversas e por todo ensinamento. Que um dia eu possa ter a graça de ser uma mãe como você é para Gi, que Deus te ilumine muito!

À minha amiga **Aline Dionizio,**
Li, obrigada por sua ajuda; são pessoas como você que fazem a diferença por onde passam, ensinando com amor e dedicação. Todo sucesso sempre. Obrigada.

Às minhas amigas **Juliana Kniss, Melina, Cleu Meneghetti, Regina e Cristina,**
Obrigada pelo apoio e amizade de vocês, por sempre me incentivarem a buscar meus objetivos. Cada uma de vocês é muito especial para mim.

Às minhas amigas professoras **Cristina Sasso e Terezinha Minorello,**
Que um dia eu possa ser uma docente como vocês são, tive o prazer em lecionar ao lado de vocês, aprender com vocês e percebi que vocês ensinam por amor à educação, e é isso que nosso Brasil precisa! Obrigada por tudo.

Aos meus professores e amigos da **Universidade Estadual do Norte do Paraná – UENP,**
Obrigada aos queridos professores, que transmitiram seus conhecimentos e também as difíceis situações que iríamos encontrar após nossa formação, pelo amor a Universidade e ao ensino de qualidade. Foi na UENP que a semente da pesquisa foi semeada em meu coração, aprendi que com disciplina e determinação alcançamos nossos objetivos. Às amigas de república, Mayara, Thaina, Thayele, Carla e Mariana, foi com vocês que compartilhei uma das melhores fases da vida. Minha eterna gratidão.

Aos meus **queridos colegas** e **amigos do Laboratório de Bioquímica**,
Vinicius Taioqui, Juliana Pires, Daiana Moreli, Even Taira, Tatiana Martini, Talita Ventura, Priscila Salomão, Beatriz Souza, Sara Salustiano, Giovana Degand, Flávia Levy, Cíntia Tokuhara, Mariana Santesso, Isabela Tomazini, João Paulo Domezi, Adriana Matos, Flávia Amadeu, David Dezidério, Maria Aparecida, Juliana Trevizol, Mileni Fernandez, Cíntia Souza, Carlos, Zohaib, Polliana Scaffa, Heloisa Pereira, Tamara Araújo e Gabriela Neubern. Com cada um de vocês tenho uma história a contar, obrigada por alegrarem nosso laboratório, pela torcida durante os experimentos e por compartilharem vivências acadêmias. Com vocês nessa caminhada tudo se tornou mais tranquilo. Família Bioquímica, obrigada!

Aos **amigos** e **funcionários do Centro Integrado de Pesquisa - CIP**,

Dani, Nara, Márcia, Rafa e Marcelo, obrigada por toda ajuda nos experimentos e por transmitirem o conhecimento. Obrigada por me ajudarem a crescer como profissional.

Às técnicas e especialistas do Laboratório de Bioquímica, **Larissa, Thelma e Aline**,

Por todo conhecimento, atenção e dedicação. Por estarem sempre solucionando nossos problemas, por compartilharem seus conhecimentos durante este período no laboratório. Transmitirei cada conhecimento que aprendi com cada uma de vocês. Obrigada!

Aos professores da Disciplina de Bioquímica, **Professor Dr. Rodrigo Cardoso de Oliveira** e **Professora Dra. Marília Afonso Rabelo Buzalaf**,

Professores, muito obrigada por compartilharem o conhecimento com tanto amor, por nos ajudarem durante a disciplina até mesmo fora da sala de aula, atendendo com muito carinho, pelos conselhos repassados a nós, por se mostrarem tão competentes e atenciosos. Cada momento ao lado de vocês foi importante para meu crescimento e levarei cada um por toda a vida.

À secretária do Departamento **Dalva Ribeiro de Oliveira,**

Sempre que procuramos, você nos atende com um sorriso e carinho. Obrigada por toda a dedicação ao nosso departamento, por toda a atenção aos alunos; você é imprescindível para nós. Obrigada por toda atenção e paciência dedicada a nós alunos.

Às secretárias da Pós-graduação **Fátima, Leila, Letícia, Meg e Maristela**,

Pela atenção em responder todas as dúvidas, pela disposição em responder a e-mails, telefonemas e ajudar com assuntos relacionados à documentação, prazos e relatórios. Muito obrigada pela presteza de sempre!

Aos **voluntários** desta pesquisa,

Vocês foram fundamentais para que esse trabalho fosse realizado. Sem vocês nada seria possível. Obrigada por terem aceitado fazer parte da pesquisa. Muito obrigada!

À **Faculdade de Odontologia de Bauru- FOB/USP**, na pessoa da diretora **Prof.ª Drª Maria Aparecida Moreira Machado** e vice-diretor **Carlos Ferreira dos Santos,**
É uma honra realizar minha pós-graduação, em nível de Mestrado, nesta grande instituição, que nos dá total suporte e estrutura indiscutível. Obrigada FOB-USP por tudo que me proporcionou até hoje.

À todos os **funcionários da FOB-USP** que colaboraram de forma direta ou indireta para o desenvolvimento deste trabalho, que contribuíram para que a minha trajetória pelo mestrado se tornasse mais alegre e leve.

À **Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP),**
Pela concessão da minha bolsa de Mestrado, importante para meu aprimoramento profissional e pessoal (Processo 2015/11635-2)

*"A medida do amor é amar sem medida".*

*Santo Agostinho*

## AGRADECIMENTO ESPECIAL

Agradeço à minha orientadora **Profª Drª Ana Carolina Magalhães,**

Tudo começou com um e-mail em setembro de 2013, você como sempre respondeu imediatamente, marcamos para conversarmos na mesma semana. Ainda me lembro com muita clareza quando entrei na sua sala com tamanha timidez para discutirmos sobre um possível projeto de mestrado. Pensei que fossemos ter apenas uma entrevista, porém foi muito mais, em pouco tempo você me deu uma verdadeira aula sobre cárie e erosão dentária, e eu com olhos arregalados com tanta informação, fiquei me perguntando: “Nossa como ela é inteligente, falando tudo isso sem olhar nenhum papel” (risos). Fui embora pensativa e querendo muito voltar, mas quando peguei o edital da prova fiquei com muito medo e desisti, aguardei para prestar só no outro ano. Eu já amava lecionar, mas queria algo mais, sempre quis seguir a área acadêmica, mas não tive oportunidade, você me deu uma oportunidade e eu não poderia perder. Estudei durante as minhas férias escolares e graças a Deus passei!

Carol, eu demorei três meses para escrever meu projeto?! Hoje vejo que foi tempo demais, mas você com toda a paciência do mundo ensinou e esperou. Então, o projeto foi aceito pela FAPESP na nossa primeira tentativa e eu fiquei muito feliz, pois já havia deixado a escola com a certeza de que daria tudo certo.

Você me proporcionou o aprendizado, amadurecimento profissional e pessoal, sou muito grata por isso. Se me perguntarem hoje quem é a Drª Ana Carolina Magalhães, irei responder com toda a sinceridade, que você é uma excelente pesquisadora e uma maravilhosa docente, como pesquisadora gosta de desafios, adora o laboratório e como docente sabe transmitir seus conhecimentos, e que se for necessário repetir mil vezes, você não se incomoda, isso é ser docente, ter paciência e vontade para que o outro aprenda. Como pessoa tem um coração enorme, gosta sempre de ajudar o próximo, pois acredita e confia no potencial de cada um, sua bondade me encanta, uma mãe dedicada, paciente e amorosa.

Obrigada por todas as oportunidades, por confiar no meu trabalho e por sempre acreditar na minha competência. Obrigada por ser tão especial com seus alunos, por nos atender a qualquer momento, nos acalmar e nos incentivar nos momentos difíceis. Obrigada por cada conhecimento transmitido. Obrigada por entender as dificuldades pessoais de cada uma de suas alunas. Não tenha dúvida

de que você é muito especial, são raras as Universidades que possuem docentes como você. Que Jesus continue iluminando seu caminho e sua família. Muito obrigada por tudo!

*"Não é o que você faz, mas quanto amor você dedica no que faz que realmente importa".*

*Madre Teresa de Calcutá*

# ABSTRACT

**Evaluation of the effect of different commercial mouthrinses on the viability and activity of microcosm biofilm and on enamel demineralization**

The aim of this study was to evaluate the antimicrobial and anticaries effects of different commercial mouthrinses. The first chapter is about a review of literature whose the aim was to discuss the antimicrobial potential of different mouthrinses in respect to the control of dental caries and periodontal disease. The search of papers was conducted using PubMed and the keywords: "antimicrobial agent" or "antiplaque agent," "dental biofilm" and "dental caries" or "periodontal disease" or "gingivitis". We found a total of 22 papers (2011-2015). The main active agents tested were: CHX-Chlorhexidine, CPC-cetylpyridinium chloride and EO-Essential oils (alcohol/or alcohol-free). CHX was compared to EO in 6 studies, showing superiority in 3 studies, similarity in 1 study and inferiority in 2 studies. CPC has shown lower effect in plaque reduction compared to CHX and EO. More clinical studies are needed for better understanding the mechanism of action and the differences in performance among the antiplaque agents. The second chapter has as aim to compare the antimicrobial and anticaries effects of six commercial mouthrinses (PerioGard®, Noplak® Max, Oral-B® Complete, Listerine® Zero, Malvatricin® Plus and Cepacol® Plus Advanced) under a microcosm biofilm model formed on enamel. A microcosm biofilm was produced on bovine enamel, using inoculum from pooled human saliva mixture with McBain saliva (with 0.2% sucrose), for 14 days. The biofilm was treated with the mouthrinses daily (1 min). The bacterial viability (% death), lactic acid production (mmol/l), the colony-forming unit (CFU) counting for total microorganisms, lactobacilli, total streptococci and mutans streptococci ($log_{10}$ CFU/mL) and the extracellular polysaccharides production (EPS, mg/g) were quantified in the biofilm. The degree of enamel demineralization was analyzed using transverse microradiography-TMR (%min vol. µm). Oral-B® Complete, Listerine® Zero and Malvatricin® Plus had the greatest effect on the reduction of biofilm viability (69-75% dead cells vs. 13% in the control, $p<0.0001$). On the other hand, the lactic acid production was significantly reduced by PerioGard®, Noplak® Max and Listerine®

Zero compared to control (69% reduction, $p<0.0001$). There were no significant differences among the mouthrinses in respect to the CFU counting and EPS production. The enamel demineralization was significantly reduced by PerioGard®, Noplak® Max and Malvatricin® Plus compared to control (74% reduction, $p<0.0001$). Therefore, the commercial mouthrinses have different antimicrobial and anticaries effects. The mouthrinses containing chlorexidine or *Malva sylvestris* (with F, triclosan and xylitol) had the best anticaries effect under this model.

**Keywords:** Antimicrobial agents; dental biofilm; enamel caries; microcosm biofilm; oral disease

# RESUMO

**Avaliação do efeito de diferentes enxaguatórios bucais comerciais sobre a viabilidade e atividade de biofilme microcosmo e a desmineralização do esmalte**

O objetivo deste estudo foi avaliar os efeitos antimicrobiano e anticárie de diferentes enxaguatórios bucais comerciais. O primeiro capítulo se refere a uma revisão da literatura cujo objetivo foi discutir o potencial antimicrobiano de diferentes enxaguatórios em relação ao controle de cárie dentária e doença periodontal. A pesquisa dos artigos foi realizada usando o PubMed e as palavras-chave: "agente antimicrobiano" ou "agente antiplaca", "biofilme dental" e "cárie dentária" ou "doença periodontal" ou "gengivite". Encontramos um total de 22 artigos (2011-2015). Os principais agentes ativos testados foram: Clorexidina-CHX, cloreto de cetilpiridínio-CPC e óleos essenciais OE (álcool / ou sem álcool). A CHX foi comparada ao OE em 6 estudos, mostrando superioridade em 3 estudos, similaridade em 1 estudo e inferioridade em 2 estudos. CPC mostrou menor efeito na redução da placa em comparação com CHX e OE. Mais estudos clínicos são necessários para uma melhor compreensão do mecanismo de ação e as diferenças no desempenho entre os agentes antiplaca. O segundo capítulo teve como objetivo avaliar os efeitos antimicrobiano e anticárie de seis enxaguatórios comerciais (PerioGard®, Noplak® Max, Oral-B® Complete, Listerine® Zero, Malvatricin® Plus e Cepacol® Plus Advanced) sobre um modelo de biofilme de microcosmo formado no esmalte. O biofilme de microcosmo foi produzido em esmalte bovino, utilizando o inóculo da mistura de saliva humana com saliva de McBain (com 0,2% de sacarose), durante 14 dias. O biofilme foi tratado com enxaguatórios diariamente (1 min). A viabilidade bacteriana (% de morte), a produção de ácido láctico (mmol/l), as unidades formadoras de colônias (UFC) foram contadas para microrganismos totais, lactobacilos, estreptococos totais e *Streptococcus mutans* ($log_{10}$ UFC/mL) e a produção de polissacarídeos extracelulares (PEC, mg/g) foram quantificados no biofilme. O grau de desmineralização do esmalte foi analisado utilizando a microrradiografia transversal-TMR (%min vol. µm). Oral-B® Complete, Listerine® Zero e Malvatricin® Plus tiveram o maior efeito na redução da viabilidade do biofilme (69-

75% de morte celular vs 13% no controle, p<0,0001). Por outro lado, a produção de ácido lático foi significativamente reduzida por PerioGard®, Noplak® Max e Listerine® Zero comparado ao controle (redução de 69%, p<0,0001). Não houve diferenças significativas entre os enxaguatórios em relação à contagem de UFC e à produção de PEC. A desmineralização do esmalte foi significativamente reduzida pelo PerioGard®, Noplak® Max e Malvatricin® Plus em comparação ao controle (redução de 74%, p<0,0001). Portanto, os enxaguatórios bucais comerciais têm diferentes efeitos antimicrobiano e anticárie. Os enxaguatórios contendo clorexidina ou *Malva sylvestris* (com F, triclosan e xilitol) tiveram o melhor efeito anticárie neste modelo.

**Palavras-chave:** Agentes antimicrobianos; Biofilme dental; Biofilme microcosmo; Cárie no esmalte; Doenças orais.

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# 1-Introduction

# 1 INTRODUCTION

Dental caries is one of the most important chronic oral diseases caused by different acidogenic and aciduric microorganisms species organized in a dental biofilm under frequent exposure to sugar. These microorganisms metabolize sugar, especially sucrose from the diet, producing extracellular polysaccharides (EPS) and acids that alter the biofilm pH, inducing tooth demineralization (KEYES, 1960; MARSH; MOTER; DEVINE, 2011; PITTS et al., 2017). The main known cariogenic microorganisms are *Streptococcus mutans*, lactobacilli, bifidobacteria and fungi. In particular, *S. mutans* produce insoluble EPS from the sucrose in the functional matrix, increasing metabolic efficiency and protection against the host defense mechanisms (MARSH, 2004; KLEIN et al., 2010; KOO; FALSETTA; KLEIN, 2013).

White-spot lesions are the first sign involving the caries development in enamel, which may progress to cavitation and reach dentin according to the severity of the acid challenges (CAVALCANTI et al., 2014; FERNANDEZ; TENUTA; CURY, 2016). The epidemiological surveys have shown a decrease in the prevalence of dental caries due to the widespread use of fluoride and the oral health education programs (PETERSEN, 2005; BOWEN, 2016). The last epidemiological survey done in Brazil, (2010) has shown an increase of the percentage of caries-free, which varied according to the age (46.6% 5-years old, 43.5% 12-years old, 23.9% 15-years old and 0.9% 35-44-years old) (MINISTRY OF HEATH BRAZIL, 2011). On the other hand, the polarization phenomena has been evident, in which the worst oral conditions are seen in small populations under social and economic disadvantage (COLAK et al., 2013). When caries reach dentin, it can cause significant negative impact in quality of life, causing pain, lack of appetite, weight loss and high cost for treatment (ABANTO et al., 2011; RAMOS-JORGE et al., 2015;).

The rationale sugar consume and the mechanical disorganization of the biofilm in the presence of fluoride are associated with the control of the disease (RUGG-GUNN, 2013). However, in some situations, as for example xerostomic patients (FURNESS et al., 2011; ZERO et al., 2016), the classical preventive strategies may be failed, seeking for alternative approaches.

There are several commercial mouthrinses containing antiplaque agents as chlorhexidine digluconate, cetylpyridinium chloride, triclosan, essential oils or natural agents (CARVALHO et al., 2004). These antiplaque agents have been used by the patients mainly for the control of halitosis (FEDOROWICZ et al., 2008; TOLENTINO; CHINELLATO; TARZIA, 2011). A recent review pointed out that few studies have been done to elucidate the antiplaque effect of these agents (BRAGA; PIRES; MAGALHÃES, 2017). In general, the studies have shown that the essential oil has comparable effect than chlorexidine and both are better than cetylpyridinium chloride on the cariogenic biofilm (GUGGENHEIM; MEIER, 2011; MARCHETTI et al., 2011; OYANAGI; TAGAMI; MATIN, 2012; SREENIVASAN; HARASZTHY; ZAMBON, 2013; WAKAMATSU et al., 2013; SUN et al, 2014; FREIRES et al., 2015; QUINTAS et al., 2015). However, most studies have been done *in vitro*, using monospecies or multispecies biofilm models, whose the main response variable was the antimicrobial effect and not the anticaries effect. There is a consensus that the antimicrobial effect may not reflect the anticaries potential (BRAGA; PIRES; MAGALHÃES, 2017). Therefore, there is a need of further studies on this field to better elucidate the differences among the antiplaque mouthrinses using more realist models and incluing as response variable the effect on the tooth as well. The use of a microcosm biofilm model, produced from microorganisms present in human saliva, can bring advantages over studies with monospecies or multispecies biofilms, allowing the presence of high number of microorganisms and the interactions between them in the presence of the antimicrobial agents (TANG et al., 2003).

Accordingly, this study was divided in two parts. The first chapter is reffered to a review of literature whose the aim was to discuss the antimicrobial potential of different mouthrinses in respect to the control of dental caries and periodontal disease. The second chapter has as aim to compare the antimicrobial and anticaries effects of six commercial mouthrinses (PerioGard®, Noplak® Max, Oral-B® Complete, Listerine® Zero, Malvatricin® Plus and Cepacol® Plus Advanced) under a microcosm biofilm model formed on enamel.

# 2-Article I

## 2 ARTICLE I – Review of literature

Article under review in Brazilian Dental Science.

**Commercial antimicrobials mouthrinses on caries and periodontitis-related biofilm control - a review of literature**

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**ABSTRACT**

This review aims to discuss the antimicrobial potential of different mouthrinses in respect to the control of dental caries and periodontal disease. The survey was conducted using PubMed and the following keywords: "antimicrobial agent" or "antiplaque agent", "dental biofilm" and "dental caries" or "periodontal disease" or "gingivitis". Only studies published in English, from 2011 to 2015, in journals with impact factor greater than 0.8, were selected. We found a total of 22 papers, 13 related to dental caries and 9 related to periodontal disease. Among the 13 studies involving cariogenic bacteria and/or biofilm, 6 were conducted *in vitro,* 3 *in situ* and 4 *in vivo*. Among 9 studies involving periodontal disease, 2 were *in vitro* and 7 *in vivo*. The main active agents tested were: CHX-Chlorhexidine, CPC-cetylpyridinium chloride and EO-Essential oils (alcohol/or alcohol-free). CHX was compared to EO in 6 studies, showing superiority in 3 studies, similarity in 1 study and inferiority in 2 studies. CPC has shown lower effect in plaque reduction compared to CHX and EO. There is still controversy about the effect of alcohol, but some studies have shown superiority for EO and CHX with alcohol on cariogenic and periodontopathogenic biofilms, respectively, when compared to alcohol-free version; for CPC, no difference was found. More clinical studies are needed for better understanding the mechanism of action and the differences in performance among the antiplaque agents.

**Keywords:** Antimicrobial agents; Dental biofilm; Dental caries; Oral diseases; Periodontitis.

**Enxaguatórios comerciais antimicrobianos sobre o controle do biofilme relacionado à cárie dentária e à doença periodontal– uma revisão de literatura**

Esta revisão tem como objetivo discutir o potencial antimicrobiano de diferentes enxaguatórios bucais em relação ao controle da cárie dentária e doença periodontal. A pesquisa foi realizada usando PubMed e as seguintes palavras-chave: "agente antimicrobiano" ou "agente antiplaca", "biofilme dental" e "cárie dentária" ou "doença periodontal" ou "gengivite". Foram selecionados os estudos publicados em inglês, de 2011 a 2015, em revistas com fator de impacto maior que 0,8. Foram encontrados no total 22 artigos, 13 relacionados à cárie dentária e 9 relacionados à doença periodontal. Entre os 13 estudos envolvendo bactérias e/ou biofilme cariogênicos, 6 foram realizados *in vitro*, 3 *in situ* e 4 *in vivo*. Entre os 9 estudos envolvendo doença periodontal, 2 foram *in vitro* e 7 *in vivo*. Os principais agentes ativos testados foram: CHX-Clorexidina, CPC-cloreto de cetilpiridínio e OE-óleos essenciais (com álcool ou sem álcool). A CHX foi comparada ao OE em 6 estudos, mostrando superioridade em 3 estudos, similaridade em 1 estudo e inferioridade em 2 estudos. CPC mostrou menor efeito na redução da placa em comparação à CHX e ao OE. Ainda há controvérsias sobre o efeito do álcool, mas alguns estudos têm mostrado superioridade no caso de OE e CHX com álcool sobre biofilmes cariogênicos e periodontopatogênicos, respectivamente, quando comparados à versão sem álcool; para o CPC, não foi encontrada diferença. Mais estudos clínicos são necessários para melhor compreensão sobre mecanismo de ação e as diferenças de desempenho entre os agentes antiplaca.

**Palavras-chave:** Agentes antimicrobianos; Biofilme dental; Cárie dentária; Doenças orais; Periodontite.

## INTRODUCTION

The oral cavity is directly in contact with microorganisms [1]. Saliva, gingival fluid and our diet supply nutrients for them, making the environment propitious to microbiota development [2,3]. Microbiota can be organized as biofilm that potentially can cause oral diseases as dental caries and periodontitis [4].

Dental caries is one of the most relevant oral chronic diseases caused by microorganisms from different species organized in a supragingival biofilm. The cariogenic microorganisms metabolize sugar, especially sucrose derived from the diet, producing acids that reduce the biofilm pH and cause tooth decay [4,5]. The main cariogenic microorganisms present in biofilm are *S. mutans*, *Lactobacillus*, bifidobacteria and fungi. *S. mutans*, in particular, produce insoluble extracellular polysaccharides from sucrose in the biofilm matrix, increasing metabolic efficiency and protecting themselves against host defenses mechanisms [4,6].

On the other hand, subgingival biofilm rich in anaerobic and gram-negative bacteria (*A. actinomycetemcomitans, T. forsythia, Campylobacter spp., Capnocytophaga spp., E. corrodens, F. nucleatum, P. gingivalis, P. intermedia*) is related to periodontal disease, a common cause of tooth loss in adults [7].

The mechanical disorganization of dental biofilm by toothbrushing is extremely important to prevent dental caries [8] and gingivitis [9], but sometimes insufficient for patients who have unfavorable conditions as, for example, xerostomia [10] and using fixed orthodontic appliances. The use of antimicrobials agents may be an alternative for those patients at high risk of dental caries [10] and periodontal disease [11,12].

Among the active agents, chlorhexidine digluconate (CHX), Cetylpyridinium Chloride (CPC) and essential oils (EO) [13] are the most used by the population, who applied them for halitosis control [14]. CHX is considered a gold-standard

antimicrobial agent applied in patients with periodontal diseases. It has been indicated as a temporary coadjutant to regular oral hygiene procedures, as a preoperative and/or postoperative rinse either [15].

Despite the popularity of the antimicrobial agents often found in supermarkets, there is sparse information about their efficacy on the control of oral diseases. Therefore, the aim of this review was to compile information about the efficacy of the main commercial antimicrobial agents applied to prevent tooth decay and periodontal disease.

## REVIEW OF LITERATURE

The survey was conducted using PubMed and the following keywords: "antimicrobial agent" or "antiplaque agent", "dental biofilm" and "dental caries" or "periodontal disease" or "gingivitis". Only studies published in English, from 2011 to 2015, in journals with impact factor greater than 0.8, were selected. We found a total of 22 papers, 13 related to dental caries and 9 related to periodontal disease, involving *in vitro*, *in situ* and *in vivo* models.

### Commercial Agents and Biofilm/Dental Caries

Chlorhexidine (CHX) is considered a gold standard antimicrobial agent applied in dentistry. Accordingly, most studies testing new antimicrobial agents have included CHX as a positive control. Cetylpyridinium chloride (CPC), also a cationic agent as chlorhexidine, is indicated to combat dental plaque and halitosis [16]. Essential oil (EO: eucalyptol, thymol, salicylate and menthol), a non-ionic agent, is other agent popularly applied to control dental plaque [17]. The agents are mostly available as mouthrinses.

Different commercial mouthrinses containing CHX, in concentrations ranged from 0.05 to 0.2%, were compared to EO (formulae with alcohol) and water (negative control) using multispecies biofilms (*A. naeslundii, V. dispar, F. nucleatum, S. mutans, S. oralis* and *C. albicans)*. The total CFU were determined using Columbia blood agar, and the *S. mutans* and *S. oralis* CFUs were counted using Mitis-Salivarius agar. The treatments were done after 16.5, 24.5, 40.5, and 48.5 h of biofilm formation. After a total time of 64.5 h, CFUs were determined for total microorganisms (A*. naeslundii, V. dispar, F. nucleatum, S. mutans, S. oralis* and *C. albicans*), *S. mutans* and *S. oralis.* The total CFU numbers were not significant different among the mouthrinses. Biofilm formation was reduced in 7 $\log^{10}$ steps by 0.2% CHX (formulae with alcohol), and in 3 $\log^{10}$ steps by EO, 0.05% CHX (formulae with alcohol), and 0.12 and 0.2% CHX (without alcohol) solutions compared to water [18].

The effect of three commercial mouthrinses (1- 0.12% CHX plus 0.05% CPC; 2- 0.12% CHX; 3- 0.2% zinc chloride with 1.5% hydrogen peroxide-Zn), on bacteria adherence (*S. mutans, S. faecalis, S. gordonii, A. viscosus* and Mixed culture) to hydroxyapatite surfaces was evaluated. The % of viable bacteria adhered to hydroxyapatite ranged from 8-19% for CHX plus CPC; 11-17% for CHX and 79-89% for Zn. Therefore, both CHX and CHX plus CPC were effective against the tested bacteria, while Zn was not [19].

Wakamatsu et al. [20] compared the penetration kinetics of four mouthrinses (CHX, EO, CPC and isopropylmethylphenol- IPMP) into *S. mutans* biofilm. The penetration velocities were determined by monitoring fluorescence loss between 30 s and 5 min exposure. EO showed the best penetration, but within 30 s no mouthrinse

had any antiplaque effect. After 30 s, EO induced the highest reduction in CFU number, but not in bacteria detachment compared to the other mouthrinses.

Nanoemulsions prepared with 25% soybean oil, 1% CPC and 10% Triton X-100-TRI (NE) were applied on *S. mutans, L. casei, C. albicans* and *A. viscosus* both isolated and in a mixed-culture. *S. mutans* and *L. casei* biofilms were stained using a live/dead kit. MIC and MBC were determined for all microorganisms isolated and in a mixed-culture. The time kinetics was also analyzed for all microorganisms (1, 5, 15, 30 and 60 min) using optical density. The adherence of microorganism on glass plates (24h) and the growth of biofilm (72h) were analyzed after fixing and staining, using optical density. NE has shown reduced in 83% the viability of *S. mutans* and *L. casei* biofilm compared to negative control. MIC and MBC of NE were 9- to 27- fold smaller than those from CHX (positive control). In respect to killing curves, NE had a faster and powerful effect compared to CHX. The level of adhesion on glass surface was reduced by 94.2 to 99.5% in NE treated groups compared to positive (CHX) and negative controls. The anti-adherence and anti-biofilm effects of NE suggest a promising anticaries action [21].

Commercial rinses containing 0.05% CPC, alcohol or free-alcohol, were compared with 0.05% fluoride mouthwash (F) and 0.12% chlorhexidine (positive control-CHX). MIC was firstly determined for each mouthrinse considering 25 microorganism species associated with oral diseases. The second part of the study evaluated the antimicrobial activity using supragingival biofilm collected from 15 subjects, which was exposed ex vivo to the mouthrinses for 5-7 days in anaerobic environment. MIC values were significantly lower for both CPC rinses compared to fluoride rinse especially against gram-negative bacteria (most involved in halitosis etiology), showing a broad-spectrum activity. CHX had the greatest antimicrobial

effect. This ex vivo model showed no difference between CPC rinses formulated with alcohol or without alcohol. Both CPC (>90% killing) and CHX (98% killing) showed higher antimicrobial activity compared to F [22].

The effect of antimicrobial mouthrinses was also studied in adult patients under orthodontic treatment. The patients were treated with 0.1% CHX alcohol-free, essential oil (alcohol/alcohol-free) or negative control (1% hydroalcoholic solution) for 4 days (1x30s/day). Supragingival biofilm and microorganism on tongue were collected and analyzed for UFC counting (*S. mutans*). All mouthrinses were similarly able to significantly reduce the number of *S. mutans* colonies compared to control for both samples (tongue and biofilm) [23].

The antiplaque effect of EO with or without alcohol was compared *in vivo*. Thirty subjects were divided into two groups (EO with and without alcohol). They rinsed twice a day for 3 days. EO with alcohol showed better plaque inhibitory effect (plaque index of 2.18 in whole mouth) than alcohol-free solution (plaque index of 2.46) [24].

Oyanagi [25] compared 0.05% CHX, 0.2% benzethonium chloride, EO (0.09% 1.8-cineol, 0.06% thymol, 0.05% Methyl salicylate, 0.04% l-Menthol and 27% Ethanol), 7% povidone iodine (PVP-I) and PBS (negative control) using planktonic cariogenic bacteria (*S. mutans/ S. sobrinus*) and biofilm models. Additionally, two mouthrinses (CHX and EO) were evaluated using biofilm-induced caries and a secondary caries model. EO and PVP-I were the best treatments in reducing the cells viability and CFU counts in planktonic culture and biofilm (especially in top and middle layer). EO further had the best inhibitory effect on the progression of demineralization, showing potential to prevent dental caries.

Albertsson et al. [26] evaluated the antimicrobial effect of EO and CHX alcohol-free mouthrinses on *S. mutans* and *Lactobacillus* in saliva. Twenty healthy volunteers applied the mouthrinses twice times during 16 days after the regular mechanical oral hygiene. Saliva was collected and analyzed for CFU/mL. Only CHX rinse showed a significant reduction in *S. mutans* and *Lactobacillus* counting, while EO did not have antimicrobial effect.

Two mouthrinses, EO (0.092% of eucalyptol, 0.042% menthol, 0 .060% of methyl salicylate and 0.064% of thymol) and 0.2% CHX, were tested on biofilm *in situ*. Bacterial viability, thickness and covering grade were evaluated after 4 days of applying each of the mouthrinses (2 times x 30s/day). CHX showed 13.2% and EO 14.7% of live bacteria. CHX was better in reducing biofilm thickness compared to EO (CHX 6.5 μm vs. EO 10.0 μm) and covering grade (CHX 20.0% vs. EO 54.3%). CHX showed better antiplaque effect compared to EO [27].

The effect of CPC (concentrations of 0, 0.025%, 0.05%, 0.075%, and 0.1%), applied twice day for 1 minute, during early (0h to 50h) and mature (48h to 98h) *S. mutans* biofilm formation, was determined. All CPC concentrations showed complete anti-biofilm activity during early biofilm formation. For old biofilm, the highest CPC concentrations had effect on dry weight, viability and acidogenicity, but they had no effect on water-insoluble extracellular polysaccharides production. Therefore, CPC has inhibitory effect on young *S. mutans* biofilm only [28].

Hannig et al. [29] compared the effect of fluoride solution (100 ppm F as AmF and 150 ppm F as NaF) to 0.2% CHX (positive control) on biofilm adherence to enamel and dentin *in situ* after 8 h of 1 min-rinse. The bacterial viability and CFU for total microorganism were determined. In the control group, significantly higher amounts of adherent bacteria were detected on dentin ($4.8 \times 10^6 \pm 5.4 \times 10^6$

bacteria/cm$^2$) than on enamel (1.2 x $10^6$ ± 1.5 x $10^6$ bacteria/cm$^2$). Chlorhexidine significantly reduced the amount of adherent bacteria (dentin: 2.8 x $10^5$ ± 3.4 x $10^5$ bacteria/cm$^2$; enamel: 4.2 x$10^5$ ± 8.7 x $10^5$ bacteria/cm$^2$). Rinses with the fluoride solution also significantly reduced bacterial adherence to dentin (8.1 x $10^5$ ± 1.5 x $10^6$ bacteria/cm$^2$). The viability was reduced by both chlorhexidine and fluoride. While a significant reduction of bacterial adherence on enamel and dentin was seen for chlorhexidine, F reduced the bacterial adherence on dentin only.

Rabe et al. [30] compared the antimicrobial effect of 0.1% CHX with 0.2% NaF. Enamel discs were mounted on healing abutments in the pre-molar region of three subjects for 7 days. After this period, the treatment was done for 1 min. Then, the architecture, bacterial viability and total biomass of the biofilm were evaluated using fluorescence methods. The biofilm architecture was similar for both groups, however CHX had effect on the biofilm surface, while F caused cell damage in the middle and deep biofilm layers. Both rinses were able to significantly reduce the bacterial vitality (63% vs. 95% in control) and the total biomass (6.5 x $10^6$ arbitrary units/mm$^2$ for control, 0.82 x $10^6$ arbitrary units/mm$^2$ for CHX and 0.87 x $10^6$ arbitrary units/mm$^2$ for F). Rinse with CHX has antimicrobial effect in the cell/liquid interface at the top of biofilm. NaF, however, is able to penetrate and exert effect in the middle and deep levels of the biofilm.

Some *in vitro* studies have shown that fluoride reduces the production of lactate and the biomass of *S. mutans* biofilm when applied in high concentrations [31,32], however, other studies have shown no differences in lactate production, CFU number and pH drop by the application of fluoride compared to control in S. mutans biofilm [33,34]. The antimicrobial effect of fluoride is still not a consensus. Table I summarizes the results found in the above-cited studies. Generally, CHX and EO

seem to be the best antimicrobial agents against cariogenic bacteria. Both CHX and EO showed better antimicrobial effect than CPC, while F has limited antimicrobial effect. Few studies have analyzed the impact of these mouthrinses on the prevention of tooth demineralization, which should be the most relevant question to be answered.

**Table I- Effect of commercial antimicrobial mouthrinses on dental caries or bacteria caries-related**

| Authors | Active component | Type of microorganism and model/ treatment | Response variable | Results |
|---|---|---|---|---|
| Guggenheim & Meier [18] | 1. 0.1% CHX (alcohol)<br>2. 0.2% CHX (alcohol)<br>3. 0.12% CHX (alcohol-free)<br>4. 0.2% CHX (alcohol-free)<br>5. 0.05 CHX (alcohol)<br>6. EO (alcohol)<br>7. 0.15% CHX (alcohol-free) | Multispecies biofilm/ treatment of 1 min each 12h, during 64.5-h *in vitro* | Total microorganisms (*A. naeslundii, V. dispar, F. nucleatum, S. mutans, S. oralis* and *C. albicans*), *S. mutans* and *S. oralis* CFU numbers. | The total CFU numbers did not differ among the mouthrinses.<br><br>0.12% CHX (alcohol-free), 0.2% CHX (alcohol), 0.05% CHX (alcohol) and Listerine reduced the number of CFU of *S. mutans* in 7 $log^{10}$ steps compared to control (water).<br><br>0.1% CHX (alcohol), 0.2% CHX (alcohol), 0.15% CHX (alcohol-free) reduced the number of CFU of *S. orallis* in 3 $log^{10}$ steps compared to control (water). |
| Marchetti et al. [24] | 1. EO (alcohol-free)<br>2. EO (alcohol) | Cariogenic biofilm/ treatment twice a day during 3 days *in vivo* | Plaque index-PI in whole mouth | EO (alcohol) showed better inhibitory effect compared EO (alcohol-free) |
| Sreenivasan et al. [22] | 1. 0.05% CPC+ (alcohol)<br>2. 0.05% CPC- (alcohol-free)<br>3. 0.05% Sodium Fluoride (alcohol)<br>4. 0.12% CHX (alcohol) | (A) 25 Species associated with dental caries in planktonic phase.<br>(B) Supragingival biofilm collected from 15 subjects and treated with agar media containing 1% of each mouthrinse during 5–7 days | MIC (1) and CFU (2) of *S. gordonii, S. mutans*, *C. albicans*, *A. meyeri, A. Viscous.* | The cariogenic bacteria were inhibited by <6% CPC+ solution, <3% CPC- solution and <50% F solution. Both CPCs had similar effect and were better than F.<br>For CFU, CPC mouthrinses (>90% killing) and the chlorhexidine rinse (>98% killing) were better compared to F. |
| Ramalingam et al. [21] | 1. 1% CPC and 10% Triton X-100-TRI (NE)<br>2. 0.12% CHX (alcohol-free) | *S. mutans, L. casei*, *A. viscosus*, *C. albicans* and mixed culture biofilm (72h). | Level of microorganisms adhesion on glass surface and MIC/MBC values | MIC and MBC of NE were 9- to 27- fold smaller than those from CHX (positive control). NE has shown reduced in 83% the viability of *S. mutans* and *L. casei* biofilm compared to negative control. The level of adhesion on glass surface was reduced by 94.2 to 99.5% in NE treated groups compared to positive (CHX) and negative controls, respectively. |
| Oyanagi et al. | 1. 0.05% CHX | (A) *S. mutans* and *S.* | CFU counting of cariogenic | EO and PVP-I killed more planktonic cariogenic |

| | | | | |
|---|---|---|---|---|
| [25] | (alcohol-free)<br>2. 0.2% benzethonium chloride<br>3. EO (alcohol)<br>4. 7% povidone iodine (PVP-I) | *sobrinus* in planktonic phase (48-hour incubation).<br>(B) Multispecies Biofilm (*S. mutans*, *S. sobrinus and S. gordonii*)/ treatment (1x60s) for 7 days *in vitro* | bacteria and enamel lesions analysis. | bacteria and bacteria embedded in biofilms compared to PBS, CHX and benzethonium.<br>EO presented the smallest lesions among the three groups (PBS, CHX and PVP-I) |
| Albertsson et al. [26] | 1. EO and F<br>2. 0.12% CHX (alcohol-free) | Inhibition on *S. mutans* and *Lactobacillus* in saliva/ treatment twice a day for 16 days *in vivo* | CFU counting | CHX (alcohol-free) showed significant reduction in *S. mutans* and *Lactobacillus,* while the EO rinse did not. |
| Hannig et al. [29] | 1. 0.2% CHX (alcohol-free)<br>2. 100 ppm AmF and 150 ppm NaF | Biofilm formation (8h) on enamel and dentin/ treatment for 1 min *in situ* | Viability and Biofilm adherence | The viability was reduced by both solutions, while CHX was the only one showing inhibition of bacterial adherence on both enamel and dentin. |
| Ulkur et al. [23] | 1. EO (alcohol)<br>2. EO (alcohol-free)<br>3. 0.1% CHX (alcohol□free) | Treatment for 4 days *in vivo.* Groups 1 and 3 rinsed 2x30s/day, while Group 2 rinsed 3x30s/day | CFUs counting for *S. mutans* on the teeth and tongue surfaces | All mouthrinses were similarly able to significantly reduce the number of *S. mutans.* |
| Babu and Garcia-Godoy [19] | 1. 0.12% CHX plus 0.05% CPC (alcohol-free)<br>2. 0.12% CHX (alcohol-free)<br>3. 0.2% zinc chloride plus 1.5% hydrogen peroxide (alcohol-free) | *S. mutans*, *S. faecalis*, *S.gordonii*, *A. viscosus* in a mixed culture biofilm (48h)/ treatment during 1 min *In vitro* | Bacterial adhesion, viability and CFU counts | Both CHX and CHX plus CPC were effective against the tested bacteria in all assay, while Zn was not. |

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|---|---|---|---|---|
| Wakamatsu et al. [20] | 1. 0.12 % CHX (alcohol-free)<br>2. EO (alcohol)<br>3. CPC (alcohol-free)<br>4. Isopropyl methyl phenol (alcohol-free) | *S. mutans* biofilm/ treatment during 30s *in vitro* | Penetration velocities and antimicrobial effect by monitoring fluorescence loss of calcein AM-stained biofilms with time-lapse confocal laser scanning microscopy. | EO showed the best penetration in biofilm. None of the mouthrinses have antimicrobial effect on *S. mutans*. |
| Pandit et al. [28] | 1. 0.025% CPC<br>2. 0.05% CPC<br>3. 0.075% CPC<br>4. 0.1% CPC<br>5. Negative control (water). | *S. mutans* biofilm/ treatment each 12h, during 98 h *in vitro* | Weight, viability and acidogenicity on Early (0h to 50h) and mature (48h to 98h*) S. mutans* biofilm. | In early biofilm all concentrations had antimicrobial effect. In mature biofilm, only the highest concentrations of CPC had effect on dry weight, viability and acidogenicity. |
| Quintas et al. [27] | 1. EO (alcohol)<br>2. 0.2% CHX (alcohol) | Antiplaque effect *in situ*/ treatment twice a day during 4 days | Bacterial viability, thickness and covering grade of dental plaque | CHX and EO presented 13.2% and 14.7% of live bacteria (ns).<br>CHX was more efficient in reducing plaque thickness and covering grade compared to EO. |
| Rabe et al. [30] | 1. 0.1% CHX (alcohol-free)<br>2. 0.2% NaF (alcohol-free) | Cariogenic biofilm *in situ*/ treatment *for* 1 min a day during 7 days | Architecture, bacterial viability and total biofilm biomass | CHX and NaF caused a similar effect. |

Abbreviations: CHX – chlorhexidine; CFU – colony forming units; EO – essential oil; PI – plaque index; CPC - cetylpyridinium chloride; MIC – minimum inhibitory concentration; MBC - minimum bactericidal concentration; NE – Nanoemulsion; PVP-I – Povidone iodine; PBS – Phosphate Buffered Saline; ppm – parts per million; Zn – Zinc chloride; PCA – Pyrrolidone Carboxylic Acid; AM – Acetoxymethyl; NaF – Sodium fluoride.

**Commercial Agents and Biofilm/Periodontal Disease**

CHX, as previously shown, has been widely tested as antiplaque agent. In this *in vivo* study, the authors tested two formulations. The volunteers rinsed twice a day 0.12% CHX (alcohol) or 0.1% CHX (alcohol-free) with 0.1% of Formaldehyde (CHX-F) during 7 days. After the treatment, plaque indexes were recorded. The mean plaque of first group (0.76±0.38) was significantly lower compared to the second group (1.43 ± 0.56), showing that alcohol might have some influence on the antiplaque effect of CHX [35].

A crossover study was done with ten volunteers using an experimental gingivitis model. The volunteers applied 0.2% chlorhexidine mouthrinses with alcohol ($CHX_A$) or alcohol-free ($CHX_{NA}$) for 21 days (2x1min/day). The plaque index (PI), gingival inflammation (GI) and discoloration teeth (DI) were evaluated. Both solutions presented similar PI ($CHX_A$ initial 0.55 ± 0.23 and final 0.69 ±0.23 vs. $CHX_{NA}$ initial 0.52 ± 0.15 and final 0.75 ± 0.19), GI ($CHX_A$ initial 0.64±0.32 and final 0.73 ± 0.11 vs. $CHX_{NA}$ initial 0.61±0.24 and final 0.77±0.33), and DI ($CHX_A$ initial 0.0 ± 0.0 and final 0.20 ± 0.30 vs. $CHX_{NA}$ initial 0.0 ± 0.0 and final 0.06 ± 0.06). Therefore, both formulations presented comparable levels of action [36].

Three mouthrinses (0.12% chlorhexidine plus 0.05% CPC; 0.12% CHX pure and 0.12% CHX plus NaF) were tested for the inhibition of oral bacteria related with periodontal diseases (*S. oralis, A. naeslundii, V. parvula, F. nucleatum, A. actinomycetemcomitans, P. gingivalis*) *a*nd on biofilm formed *in vitro*. The effect of the mouthrinses was analyzed in bacteria under planktonic phase, which were treated for 1 min using the short interval-killing test. The antimicrobial effect was measured as CFU/mL and no difference was found among the rinses. For biofilm formation, the bacteria were grown on sterile ceramic calcium hydroxyapatite (HAP)

discs for 12h at 37ºC using a bioreactor. The discs were immersed in the mouthwash for 2 min and the biofilm cultivated for 5 days. The viable cells were analyzed using culture methods, scanning electron microscopy (SEM), Live/Dead staining and fluorescence *in-situ* hybridization (CLSM). SEM showed a typical biofilm structure. The fluorescence *in situ* hybridization technique confirmed the presence of the six bacterial species in biofilms older than 3 days. The live/dead ratio revealed that the majority of cells were alive in 3-, 4- and 5-d biofilms. Cells in biofilms showed more tolerance compared with planktonic cells. In 4-d biofilm, CHX+CPC showed more antimicrobial effect than CHX+NaF and CHX [37].

Both CPC and EO were also compared in an *in vivo* study, in which 142 subjects wore the mouthrinses for 2 weeks (2x30s/day). The Modified Gingival Index (MGI), Plaque Index (PI) and bleeding Index (BI) were analyzed. EO demonstrated significant reduction in MGI (9.4%), PI (6.6%) and BI (29%) compared to CPC. EO presented clinical superiority compared to CPC in the short-term management of plaque and gingivitis [38].

Cortelli et al. [11] compared the antiplaque/antigingivitis effect of EO (0.092% eucalyptol, 0.042% menthol, 0.060% methyl salicylate and 0.064% thymol), 0.07% CPC and 5% hydroalcohol solution (control) in 354 healthy volunteers. They were instructed to rinse twice daily (30s each) for 6 consecutive months. The Modified Gingival Index (MGI), Plaque Index (PI) and bleeding Index (BI) were quantified at baseline, after 1, 3 and 6 months. After six months, EO (42.0%) and CPC (13.9%) demonstrated significant reduction in PI compared to negative control. EO (42.6%) and CPC (17.1%) also demonstrated significant reduction in MGI compared to negative control. EO (74.5%) and CPC (22.8%) demonstrated significant reduction in

BI compared to negative control either. EO presented clinical superiority compared to CPC in the long-term management of plaque and gingivitis.

EO (with $ZnCl_2$ and NaF) was compared to 0.05% CPC (NaF) solutions. The subjects applied mouthrinses twice a day (30s) for 6 months. The PI and MGI were analyzed at baseline, after 3 and 6 months. EO mouthrinse showed significant superiority in reducing PI (23.6%) and MIG (19.5%) compared to CPC (4.2% and 1.7%) mouthrinses after 6 months. In respect to gingivitis, CPC was not different from control after 6 months. EO presented clinical superiority compared to CPC in the long-term management of plaque and gingivitis [39].

Sánchez et al. [40] evaluated the antimicrobial effect of three commercial mouthrinses (0.12% CHX plus 0.05% CPC, EO, and fluoride/stannous fluoride - AFSF) on mixed periodontopathogenic biofilm (*S. oralis, V. parvula, A. naeslundii, F. nucleatum, A. actinomycetemcomitans* and *P. gingivalis*) *in vitro* using ATP bioluminescence and CFU methods. 72h-biofilm was exposed to mouthrinses or control for 1 min. ATP bioluminescence showed antibacterial effect for all mouthrinses compared to PBS control. The lowest cell viability values were found for CHX plus CPC ($1.38x10^8 \pm 8.54x10^7$ CFU/mL), followed by AFSF ($1.42x10^8 \pm 9.03 x10^7$ CFU/mL), EO ($1.67x10^8 \pm 1.17x10^8$ CFU/mL) and the negative control ($2.55x10^8 \pm 1.63x10^8$ CFU/mL). Both CHX/CPC and F were similarly and more effective against biofilm compared to EO.

Commercial mouthrinse with 0.075% CPC (fluoride-free/alcohol-free) was compared with EO (fluoride-free/alcohol) in respect to antiplaque and antigingivitis effects *in vivo*. Fluoride-free/alcohol-free mouthwash was used as negative control. After 6 weeks, the subjects from CPC, EO and NC groups exhibited reductions in GI of 28.6%, 22.6% and 1.70%, respectively; while PI was reduced in 31%, 28% and

1.4% for CPC, EO and NC, respectively. Both mouthrinses provide a significant reduction in dental plaque and gingivitis [41].

A new rinse with CPC (alcohol-free) was tested to control plaque and gingivitis in 67 adults with moderate gingivitis during 6 months (3x30s/day). PI, bleeding on marginal probing (BOMP) and stain (S) indexes were applied. The presence of *A. actinomycetemcomitans, P. gingivalis, P. intermedia/nigrescens, T. forsythia, P. micra, Capnocytophaga spp., E. corrodens, Eubacterium spp.* and *F. nucleatum* were determined in the biofilm. Significant reduction of the clinical parameters was observed for the tested CPC solution compared to placebo. Among the periodontopathogenic bacteria, P. intermedia showed a clear reduction after 3 and 6 months of CPC treatment. CPC shows ability to reduce biofilm accumulation after 3 and 6 months of use [12].

Based mainly on clinical trials as shown in Table II, EO has a superior antiplaque and antigingivitis effects compared to CPC, while EO has a similar efficacy compared to CHX. On the other hand, CHX has often been responsible for inducing undesired effects as tooth discoloration.

**Table II - Effect of commercial antimicrobial mouthrinses on periodontitis/gingivitis or bacteria perio-related.**

| Authors | Active component | Type of microorganism and model/ treatment | Response variable | Results |
|---|---|---|---|---|
| Charles et al. [38] | 1. EO (alcohol)<br>2. 0.07% CPC (alcohol) | Antiplaque and antigingivitis effects *in vivo*. The subjects rinsed with 20 mL for 30 s twice daily during 2 weeks | Gingival Index-GI, Plaque Index-PI and bleeding Index-BI | EO demonstrated significant reduction in GI, PI and BI compared to CPC. |
| Cortelli et al. [39] | 1. EO with zinc chloride and 0.02% sodium fluoride (alcohol)<br>2. 0.05% CPC + F | Antiplaque and antigingivitis effects *in vivo*. The subjects rinsed with 20 mL for 30 s twice daily during 6 months | Gingival and Plaque Index | EO presented clinical superiority compared to CPC. |
| Costa et al. [12] | 1. 0.07% CPC (alcohol-free)<br>2.Saline-based solution (alcohol-free) | Treatment was done three times a day, for 6 months *in vivo*. | PI, BI and CFU counts (*A. actinomycetemcomitans, P. gingivalis, P.intermedia/nigrescens*, *T. forsythia*, *P. micra*, *Capnocytophaga* spp., *E. corrodens, Eubacterium* spp. and *F. nucleatum*) | Significant reduction of the clinical parameters was observed for the tested CPC solution.<br>*P. intermedia* CFU showed a clear reduction after 3 and 6 months of CPC treatment compared to control. |
| Ennibi et al. [35] | 1. 0.12% CHX (alcohol)<br>2. 0.1 % CHX (alcohol-free) containing 0.1% formaldehyde | Antiplaque and antigingivitis effect for 7 days *in vivo/* The treatment was done twice daily | Plaque Indexes | CHX with alcohol showed better inhibition of plaque growth than CHX with formaldehyde. |
| Sánchez et al. [40] | 1. 0.12% CHX and 0.05% CPC (alcohol-free)<br>2. EO (alcohol)<br>3. 125 ppm of Amine fluoride and 125 ppm of stannous fluoride (alcohol-free) - AFSF | *S. oralis*, *V. parvula*, *A. naeslundii*, *F. nucleatum*, *A. actinomycetemcomitans* in *a* mixed biofilm (72 hours)/ treatment was done for 1 min | CFU counting | CHX/CPC and AFSF containing mouthrinses demonstrated superior antimicrobial activity compared to EO rinse. |

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| Blanc et al. [37] | 1. 0.12% CHX plus 0.05% CPC;<br>2. 0.12% CHX<br>3. 0.12% CHX plus NaF | Bacteria related with periodontal disease in planktonic phase and multispecies Biofilm *in vitro/* treatment was done during 1 min (planktonic) and 2 min during 7 days (biofilm) | CFU counting for periodontal bacteria | In planktonic phase *(S. oralis F. nucleatum, P. gingivalis and A. actinomycetemcomitans)* no differences between the tested mouthrinses were found. For biofilm, CHX+CPC showed more inhibition of viable cells compared CHX and CHX+NaF. |
| Cortelli et al. [11] | 1. EO (alcohol)<br>2. 0.07% CPC (alcohol) | Antiplaque and antigingivitis effect *in vivo* for 6 months/ treatment was done twice daily | The MGI, PI and BI were quantified after 1, 3 and 6 months. | EO presented clinical superiority compared to CPC. |
| Elias-Boneta et al., [41] | 1. 0.075% CPC (alcohol-free and fluoride-free)<br>2. EO (alcohol) | The antiplaque and antigingivitis effect *in vivo* after 6 weeks/ treatment was done twice daily | PI and GI | Both mouthrinses proved a significant reduction in dental plaque and gingivitis. |
| Papaionnou et al. [36] | 1. 0.2% CHX (alcohol-free)<br>2. 0.2% CHX (alcohol) | The antiplaque and antigingivitis effect *in vivo* after 21 days/ treatment was done daily during 1 min | PI, GI and Discoloration Index | Both formulations presented comparable levels of action. |

Abbreviations: CHX – chlorhexidine; EO – essential oil; CPC - cetylpyridinium chloride; GI – gingival index; PI – plaque index; BI – bleeding index; F – fluoride; CFU – colony forming units; ppm – parts per million; AFSF - amine fluoride/stannous fluoride; NaF – Sodium fluoride; MGI – Modified Gingival Index.

## CONCLUSION

There are important differences in the antimicrobial performance among the commercial mouthrinses especially considering the bacteria specie (cariogenic vs. periodontopatogenic bacteria). More clinical studies are needed for better understanding the differences in their performance and side effects in short- and long-term studies.

## ACKNOWLEDGEMENTS

The authors thank FAPESP for the concession of scholarship to the first author (2015/11635-2).

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# 3-Article II

## 3 ARTICLE II

Article formatted according to the guideline of Biofouling

**Effect of different commercial mouthrinses on the viability and activity of microcosm biofilm and on enamel demineralization**

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**Short title:** Effect of different commercial mouthrinses on a microcosm biofilm

**Text:** 3811 words

**Figures:** 136 words

**Tables:** 466 words

**References:** 1233 words

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**Abstract**

This study evaluated the antimicrobial and anticaries effects of six commercial mouthrinses. Microcosm biofilm was produced on bovine enamel, using inoculum from pooled human saliva mixture with McBain saliva, under 0.2% sucrose exposure for 14 days. The biofilm was treated with the mouthrinses 1 min/day. Oral-B® Complete, Listerine® Zero and Malvatricin® Plus had the greatest effect on the reduction of biofilm viability (69-75% dead cells vs. 13% for control, $p<0.0001$). On the other hand, the lactic acid production was significantly reduced by PerioGard®, Noplak® Max and Listerine® Zero compared to control (69% reduction, $p<0.0001$). There were no significant differences among the mouthrinses in respect to the CFU counting and EPS production. Enamel demineralization was significantly reduced by PerioGard®, Noplak® Max and Malvatricin® Plus compared to control (74% reduction, $p<0.0001$). The mouthrinses containing chlorexidine or *Malva sylvestris* (with F, triclosan and xylitol) had the best anticaries effect under this model.

**Key words:** Antimicrobial agents; dental caries; microcosm biofilm.

## Introduction

Dental caries is one of the most important chronic oral diseases caused by different acidogenic and aciduric microorganisms species organized in a dental biofilm. These microorganisms metabolize sugar, especially sucrose from the diet, producing acids that alter the biofilm pH, inducing tooth demineralization (Marsh et al. 2011). The main known cariogenic microorganisms are *Streptococcus mutans*, lactobacilli, *Veillonella*, *Actinomyces*, bifidobacteria and fungi. In particular, *S. mutans* produce insoluble extracellular polysaccharides (EPS) from the sucrose in the

functional matrix, protection the biofilm against the host defense mechanisms as saliva neutralization and antimicrobial proteins action (Koo et al. 2013).

White-spot lesions, microscopically known as subsurface lesion, are the first sign involving caries development in enamel, which may progress to cavitation and reach dentin according to the severity of the acid challenges. When caries reach dentin, it can cause significant negative impact in quality of life (Ramos-Jorge et al. 2015), leading to pain, lack of appetite, weight loss and high cost for treatment.

The reduction in sugar consume and the mechanical disorganization of the biofilm by toothbrushing with fluoride toothpaste are good strategies to control the disease (Rugg-Gunn 2013). However, in some situations, as for example xerostomic patients (Vozza et al. 2015), these preventive strategies may be not enough, seeking for complementary approaches.

There are several commercial mouthrinses containing antiplaque agents as chlorhexidine digluconate, cetylpyridinium chloride, triclosan, essential oils or natural agents (Carvalho et al. 2004). The antiplaque agents have been used by the patients primarily for the control of halitosis (Fedorowicz et al. 2008). Few studies have been done to elucidate the antiplaque performance of these agents (Braga et al. 2017). In general, the studies have shown that the essential oil has comparable effect to chlorexidine and both are better than cetylpyridinium chloride on the cariogenic biofilm (Oyanagi et al. 2012, Sreenivasan et al. 2013, Pandit et al. 2015). However, most studies have been done *in vitro*, using monospecies or multispecies biofilm models, whose the main response variable was the antimicrobial and not the anticaries effect.

There is a consensus that the antimicrobial effect may not reflect the anticaries potential (Braga et al. 2017). Therefore, there is a need of further studies on this field

to better elucidate the differences among the antiplaque mouthrinses using more realist models as microcosm biofilm (Tang et al. 2003, Azevedo et al. 2014; Maske et al. 2016) and including the analysis of the tooth as well. Accordingly, this study compared the antimicrobial and anticaries effects of six commercial mouthrinses (PerioGard®, Noplak® Max, Oral-B® Complete, Listerine® Zero, Malvatricin® Plus and Cepacol® Plus Advanced) under a microcosm biofilm model formed on enamel.

## Material and methods

### *Saliva collection*

This study was firstly approved by the local Ethical Committee (CEEA 48100315.3.0000.5417). After sign the informed consent, saliva was collected from 2 healthy donors, who have followed the inclusion criteria: 1) normal salivary flow (stimulated saliva flow > 1 ml/min and non-stimulated saliva flow > 0.3 ml/min), 2) with previous history of caries, but no caries active (no active white spot and/or cavitated lesions), 3) without gingivitis/periodontitis (gum bleeding or tooth mobility) and, 4) without ingestion of antibiotics 3 months prior the experiment. Prior to the day of collection, the donors did not brush their teeth. Furthermore, they were not allowed to ingest food or drinks in the last 2 h before saliva collection. The saliva was collected under stimulation by chewing a rubber material for 10 min during the morning. After collection, saliva was diluted in glycerol (70% saliva and 30% glycerol). Aliquots of 1 ml were stored at -80° C (Pratten et al. 2003).

### *Tooth sample preparation and treatment groups*

A hundred sixty eight enamel samples (4 mm x 4 mm) were prepared from bovine teeth, using a semi-precision cutting machine (Buehler, Enfield, USA). The samples were fixed in acrylic discs with wax and polished in a metallographic

polishing machine (Arotec, Cotia, Brazil) using water-cooled silicon-carbide discs (600-grade papers ANSI grit; Buehler, Enfield, USA) to remove grooves and to standardize the surface roughness of approximately 0.153 ± 0.037 μm. The average surface roughness (Ra) was assessed using a contact profilometer and Mahr Surf XCR 20 software (Mahr, Göttingen, Germany). Two thirds of the enamel surfaces were protected with nail polish to obtain control areas for the TMR analysis. The samples were sterilized with ethylene oxide [Gas exposure time (30% ETO/70%$CO_2$) for 4 h under a pressure of 0.5 ± 0.1 kgF/$cm^2$].

From one hundred sixty eight samples, sixty-three were applied for viability analysis, forty-two for lactic acid production analysis and sixty-three for colony-forming unit counting and quantification of extracellular polysaccharides. Enamel samples were randomly divided in the treatments according to the Ra values: A) PBS – Negative control; B) PerioGard® (Colgate, São Bernardo do Campo, Brazil); C) Noplak® Max (Daudt, Rio de Janeiro, Brazil); D) Oral-B® Complete (Procter & Gamble, Rio de Janeiro, Brazil); E) Listerine® Zero (Johnson & Johnson, São José dos Campos, Brazil); F) Malvatricin® Plus (Daudt, Rio de Janeiro, Brazil); G) Cepacol® Plus Advanced (Sanofi, Suzano, Brazil). All mouthrinses were alcohol-free and their compositions are displayed in Table 1.

***Microcosm biofilm formation and treatments***

The human saliva was defrosted and mixture with McBain artificial saliva (McBain 2009) in a proportion of 1:50. The McBain saliva contained 2.5 g/l mucin from porcine stomach (type II), 2.0 g/l bacteriological peptone, 2.0 g/l tryptone, 1.0 g/l yeast extract, 0.35 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $CaCl_2$, 0.1 g/l cysteine hydrochloride, 0.001 g/l hemin, 0.0002 g/l vitamin K1, at pH 7.0. All reagents were from Sigma-Aldrich. The solution of human saliva and McBain saliva was added to each well

containing an enamel sample (v=1.5 ml/well) in a 24-well plate, which was incubated at 5% $CO_2$ and 37˚C. After 8h, the medium was removed, the enamel samples were washed using phosphate-buffered saline (PBS, 5 s) and fresh McBain saliva containing now 0.2% sucrose was added into the wells (v=1.5 ml/well). The microplates were incubated at 5% $CO_2$ and 37˚C for further 16h, completing the first day. From the 2$^{nd}$ to the 14$^{th}$ day, the samples were treated once a day for 1 min with the mouthrinses (1 ml/well). The treatment was removed, the samples washed using PBS (5 s) and fresh McBain saliva containing 0.2% sucrose was added. The microplates were incubated at the same conditions described above (Zhang et al. 2013). Figure 1 summarizes the experimental protocol. The analysis were done 24 h after the last treatment.

***Bacterial viability analysis***

The biofilm was stained using the nucleic acid markers diluted in PBS (1ml PBS + 1µl SYTO9 + 1µl propidium iodide, 10 µl/well) (Kit Live & Dead® cells viability assay, Thermo Fisher Scientific, Waltham, USA) for 15 min in a dark environment. Live bacteria were stained with SYTO9 producing a green fluorescence and dead lysed bacteria were stained with propidium iodide/SYTO9 producing a red fluorescence (Hannig et al. 2013). The biofilm was examined using confocal laser scanning microscope-CLSM (Leica TCS SPE, Mannheim, Germany) and Leica Application Suite-Advanced Fluorescence software (LAS AF, Mannheim, Germany). Three images (275 µm$^2$) were captured from each sample surface and analyzed using BioImage L 2.0 software, to quantify the live and dead bacteria (%).

***Lactic acid production analysis***

Biofilm samples were incubated in buffered peptone water (BPW) (Synth, Diadema, Brazil) supplemented with 0.2% sucrose (v = 1 ml/sample) for 3 h,

anaerobically, to allow the biofilm produces lactic acid. The anaerobic conditions were obtained using the Whitley A35 Anaerobic Workstation (Don Whitley Scientific, Shipley, UK), maintaining the environment at 80% $N_2$, 10% $CO_2$ and 10% $H_2$ and 37°C.

Lactate concentrations were evidenced by enzymatic method (lactic dehydrogenase method, Boehringer Mannheim, Germany) in the BPW solution according to the manufacture instruction. The absorbance was measured at 340 nm using a microplate reader (Fluorstar Optima- BMG Labtech, Ortenberg, Germany). The values were expressed as mmol lactate/ L (BPW).

***Quantification of extracellular polysaccharides -EPS***

The samples were transferred to microtubes previously weighted containing 1 ml of saline solution (0.89% NaCl) and sonicated for 30 s at 20W (Unique, Indaiatuba, Brazil). The cleaned tooth was removed, the tubes were weighted again and the biofilm weight was calculated from the weight differences.

For soluble EPS, 500 µl of the saline solution obtained above were centrifuged at 10.000 g and 4 °C for 5 min. The supernatants were transferred to other microtube. The microtubes with the sediments were stored for the insoluble EPS analysis. Three volumes of 95% ice-cold ethanol were added into the microtube containing supernatant and stored at -20 ºC for 30 min. After precipitation, the microtubes were centrifuged at 10.000 g and 4ºC for 10 min and the supernatant was completely removed. The pellets were resuspended in 1 M NaOH (v = 200 µl) and the total carbohydrate was measured using the phenol-sulphuric acid colorimetric assay and glucose (mg) curve was done. The absorbance was measured at 490 nm using a microplate reader (Fluorstar Optima- BMG Labtech, Ortenberg, Germany).

For the insoluble EPS analysis, the sediments were resuspended in 400 µl of 1 M NaOH, vortexed for 15 s and agitated using a shaker table for 15 min at environment temperature. The samples were then centrifuged at 10.000 g and 4 ºC, for 5 min. The supernatants were transferred to new microtubes and 3 volumes of 95% ice-cold ethanol were added. The microtubes were stored at -20 °C for 30 min. After precipitation, the microtubes were centrifuged at 10.000g and 4 ºC for 10 min and the supernatant was completely removed. The remaining pellet in the microtube was resuspended in 1 M NaOH (v = 200 µl) and the total carbohydrate was measured using the phenol-sulphuric acid colorimetric assay and glucose (mg) curve was done. The absorbance was measured at 490 nm using a microplate reader (Fluorstar Optima- BMG Labtech, Ortenberg, Germany) (Aires et al. 2008). The values were expressed as mg EPS/g (biofilm).

***Colony-forming unit (CFU) counting***

For CFU counting, 100 µl of the bacterial suspension obtained for EPS analysis was then diluted to $10^{-4}$ and spread on petri dishes (25 µl/dish) containing four different types of agar for CFU counting: 1) Brain Heart Infusion agar (BHI, Difco, Detroit, USA) for total microorganisms and 2) Mitis Salivarius Agar (MSA, Neogen, Indaiatuba, Brazil) containing 20% sucrose and 1% potassium tellurite for total streptococci (Lima et al. 2009); 3) SB-20M (Saravia et al. 2011) containing 15 g bacto-casitone (Difco, Detroit, USA); 5 g yeast extract (Kasvi, Curitiba, Brazil); 0.2 g L-Cysteine hydro-chloride (Sigma, Steinheim, Germany); 0.1 g sodium sulfite (Sigma, Steinheim, Germany); 20.0 g sodium acetate (Synth, Diadema, Brazil); 200.0 g coarse granular cane sugar; 15.0 g agar (Kasvi, Curitiba, Brazil); and 1 l distilled water. After autoclaving for 20 min at 120º C, 0.2 U/ml bacitracin (Sigma, Steinheim, Germany) was added for determination of mutans streptococci (*S. mutans* and *S.*

*sobrinus*); and 4) Rogosa (Kasvi, Curitiba, Brazil) supplemented with 0.13% glacial acetic acid to assess the number of lactobacilli (Lima et al. 2009).

The plates were then incubated at 5% $CO_2$ and 37 ºC, except the plate for total microorganisms that was incubated in an oven at 37ºC. After 48h (all except lactobacilli) and 60h (lactobacilli), the CFU numbers were counted and transformed in $log_{10}$ CFU/ml (Cheng et al. 2012).

***Transverse microradiography (TMR)***

After cleaning, all enamel samples (except those from lactic acid assay) were transversally sectioned and polished to obtain slices with 80-100 µm of thickness. The enamel slices were fixed in a sample-holder together with an aluminum calibration step wedge with 14 steps. A microradiograph was taken using an x-ray generator (Softex, Tokyo, Japan) on the glass plate at 20 kV and 20 mA (at a distance of 42 cm) for 13 min. The glass plates were developed for 7 min, rinsed in deionized water, fixed for 7 min in a dark environment, and then rinsed in running water for 10 min and air-dried (all procedures were done at 20°C). The developed plate was analyzed using a transmitted light microscope fitted with a 20x objective (Zeiss, Oberkochen, Germany), a CCD camera (Canon, Tokyo, Japan), and a computer. Two images per sample were taken using data-acquisition (version 2012) and interpreted using calculation (version 2006) softwares from Inspektor Research System bv (Amsterdam, The Netherlands). The mineral content was calculated based on the work of Angmar et al. (1963), assuming the density of the mineral to be 3.15 kg $l^{-1}$ and 87 vol% of mineral content for the sound enamel. The lesion depth (LD, µm), the integrated mineral loss ($\Delta Z$, %vol. µm) and the average mineral loss over the lesion depth (R, %vol) were calculated.

***Statistical Analysis***

All experiments were performed in triplicate (except the lactate assay, in duplicate) with three data points for each replicate. Data were statistically analyzed using software Graph Pad Instat for Windows (GraphPad Software, San Diego, USA). The normal distribution and homogeneity were checked using Kolmogorov & Smirnov and Bartlett's tests, respectively. The % live and dead microorganisms and CFU counting (total microorganism and mutans streptococci) from the different mouthrinses groups were compared using ANOVA and Tukey-Kramer test. For the other analysis (lactic acid and EPS production, CFU counting for total streptococci and lactobacilli and TMR data), we applied Kruskall-Wallis followed by Dunn test. The level of significance was set at 5%.

**Results**

In respect to the biofilm viability, all mouthrinses were able to increase the number of dead microorganisms. Oral-B® Complete, Listerine® Zero and Malvatricin® Plus had the greatest effect on the reduction of biofilm viability, differing from PerioGard® significantly either ($p<0.0001$, Table 2 and Figure 2). On the other hand, the lactic acid production was significantly reduced by PerioGard®, Noplak® Max and Listerine® Zero compared to control (69% reduction, $p<0.0001$). The other mouthrinses were not significantly different from the control. Furthermore, Listerine® Zero was also able to significantly reduce lactic acid production compared to Malvatricin® Plus and Cepacol® Plus Advanced (Table 3).

There were no significant differences among the mouthrinses in respect to the CFU counting (Table 4) and EPS production (Table 5). The integrated mineral loss was significantly reduced by PerioGard®, Noplak® Max and Malvatricin® Plus compared to control (74% reduction, $p<0.0001$). Furthermore, these three

mouthrinses significantly reduced the integrated mineral loss compared to Oral-B® Complete.

PerioGard®, Noplak® Max and Malvatricin® Plus were able to significantly reduce the average mineral loss and the lesion depth compared to control ($p<0.0001$); however, no treatment significantly differed from each other. Table 6 shows the TMR data and Figure 3 shows a representative image of the enamel lesion produced in each group.

**Discussion**

The short-term use of antimicrobial mouthrinses has been considered an additional approach to control dental caries in patient with poor oral hygiene or a systemic condition that compromise the oral health (Vozza et al. 2015). To compare the mouthrinses, we chose microcosm biofilm model, since it is an appropriate *in vitro* model to induce biofilm formation using inoculum from pooled human saliva or *in vivo* biofilm mixture with nutrients. It is able to mimic the heterogeneity and variability of the supragingival biofilms (Tang et al. 2003, Azevedo et al. 2014; Maske et al. 2016).

Some of the tested mouthrinses presented the antimicrobial agent combined with fluoride (Oral-B® Complete, Malvatricin Plus® and Cepacol® Plus Advanced). Latimer et al. (2015) showed that the presence of fluoride did not influence the antimicrobial effect of CPC, as well as Zhang et al. (2004) showed that fluoride did not have any further antimicrobial effect when combined with EO. However, none of the studies evaluated the anticaries effect by analyzing tooth demineralization.

Our results showed that all mouthrinses were able to reduce bacteria viability in accordance with Hannig et al. (2013), Babu and Garcia-Godoy (2014) and Pandit et al. (2015). However, the lactic acid production was reduced by PerioGard®,

Noplak® Max and Listerine® Zero only. Furthermore, the enamel demineralization was reduced by PerioGard®, Noplak® Max and Malvatricin® Plus only. Therefore, the antimicrobial and anticaries performance of the tested mouthrinses are different.

In the present study, Cepacol® Plus Advanced, which contains CPC and fluoride, presented the least beneficial effect. Also a limited effect was seen for Oral-B® Complete (CPC and fluoride), which reduced the bacteria viability, but not the development of dental caries. It is likely that CPC acts mostly against non-cariogenic bacteria. This result is in agreement with previous works that compared 0.05% CPC with 0.12% CHX and/or 0.042% menthol, 0.092% eucalyptol, 0.064% thymol, 0.06% methyl salicylate (EO) against cariogenic bacteria (planktonic phase and supragingival biofilm) (Babu and Garcia-Godoy 2014, Oyanagi et al. 2012).

On the other hand, Listerine® Zero (containing EO) has antimicrobial effect similar or higher than PerioGard® (containing CHX), in agreement with other studies (Marchetti et al. 2011, Oyanagi et al. 2012, Wakamatsu et al. 2013). Listerine® Zero was able to reduce both viability and lactic acid production in the present study, which might be explained by the presence of essential oil and its low pH. Zhang et al. (2004) observed 36% less lactate, 36% less acetate and 44% less propynoate in supragingival biofilm from patients who rinsed Listerine® compared to control. However, the previous studies did not evaluate the incidence of caries lesion over time.

An intriguing result was seen for Listerine® Zero in our study, its protection against demineralization was not as good as its effect on the lactic acid production. We suppose that other types of acid could have been produced by the Listerine-treated biofilm or that lactate consumer species (as *Veillonella* spp.) (Janus et al. 2016) might be absent in this biofilm. The hypothesis need to be further tested. Other

reason could be its low pH, however, we would not expect any relevant demineralization due to this fact, as the time of application per day was low. It is necessary to check if the daily application of Listerine® Zero would induce any relevant surface hardness loss in sound enamel after 14 days of experiment. There is only one study that observed anticaries effect of EO (Oyanagi et al. 2012); however, the authors tested a version containing alcohol and compared it with a low concentrated CHX solution (0.05%).

Among the tested mouthrinses, Malvatricin® Plus was the only one containing a natural agent (*Malva sylvestris*) combined with fluoride and xylitol. *Malva sylvestris* is native from Europe, North Africa, and South-west Asia especially Iran (Elsagh et al. 2015). It is worldwide applied as an alternative antiseptic, antifungal and anti-inflammatory agent. *Malva sylvestris* also has antimicrobial effect against *S. aureus*, *S. agalactiae, E. faecalis* and *C. albicans* (Razavi et al. 2011). It contains malvone and different known monoterpenes, aromatic compounds, and a tetrahydroxylated acyclic diterpense (Veshkurova et al. 2006). Malvone has been associated with the antimicrobial effect of *Malva sylvestris* (Razavi et al. 2011), which has been tested only on strains of *S. mutans*, *S. salivarius*, *S. oralis* and *L. casei* (Da Silva et al. 2012).

In the present study, Malvatricin® Plus reduced the bacteria viability, but not the acid production. As found in case of Oral B, it is likely that Malvatricin® Plus acts against non-cariogenic bacteria, which are mostly the first colonizers. With this hypothesis, it would be very interesting to analyze the biofilm development overtime, to understand if this effect would retard biofilm formation. Based on this finding, we would expect no anticaries effect by using this mouthrinse. Surprisingly, Malvatricin® Plus was able to reduce enamel caries lesion development.

We justified the anticaries effect of Malvatricin® Plus by the presence of fluoride, which has negligible antimicrobial effect, but it is known to be able to reduce demineralization (Buzalaf et al. 2011). Furthermore, the presence of xylitol could also have contributed to the protective effect. Xylitol is known to have antimicrobial effect on *S. mutans* biofilm (Decker et al. 2008) as well as positive effect on enamel remineralization under abiotic models (Cardoso et al. 2014). This is the first time that the antimicrobial effect on biofilm and the anticaries effect on enamel of Malvatricin® Plus have been demonstrated.

Finally, both Periogard® and Noplak® Max (containing CHX) reduced the bacteria viability and lactic acid production, as well as they reduced the enamel caries lesion development. CHX is one of the most tested antimicrobial agents and the present result is in agreement with the literature (Shapiro et al. 2002; Haerian-Ardakani et al. 2015). Interesting that the effect of CHX compared to other agents is more pronounced *in vitro* than *in vivo* (Haerian-Ardakani et al. 2015). Furthermore, no benefits of the application of chlorexidine varnish in the prevention of caries in children and adolescents have been stated in systematic reviews (Twetman, 2004; James et al. 2010,). CHX is also known to induce some side effects as tooth discoloration and astringent taste under uninterrupted use (Santos et al. 2017).

Our model was unable to show differences among the mouthrinses and the control in respect to CFU counting and EPS production. Despite that we applied sucrose to change the bacterial virulent properties and favor mutans and lactobacillus species (Diaz-Garrido et al. 2016), it might be expected other types of bacteria in the biofilm, which were not taken into account in the CFU analysis and could have influenced the EPS production. Microbial species such as *Scardovia*

*wiggsiae* and *Bifidobacterium* spp. have been correlated with caries etiology (Henne et al. 2016).

To overcome this issue, further studies shall be done to analysis the microbiome profile of the microcosm biofilm and to correlate it with the microbiome profile of the donors (saliva and biofilm). Furthermore, it would be interesting to apply monospecies biofilm to better understanding the specific action of the mouthrinses on isolated microorganism.

In conclusion, the commercial mouthrinses containing chlorexidine (PerioGard® and Noplak® Max) or *Malva sylvestris* (with F, triclosan and xylitol, Malvatricin® Plus) had the best anticaries effect under this model. Considering the known side effects of CHX, it would be very interesting to further study the effect of Malvatricin® Plus using *in situ* and *in vivo* models, since it presents natural agents with low cytotoxic potential (Benso et al. 2015).

**Funding:** This work was supported by FAPESP (process number 2015/11635-2).

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Figure 1. Experimental protocol

Figure 2. Percentage of dead bacteria (%) in the biofilm formed on enamel after applying the tested treatments. A) Negative control, B) PerioGard®, C) Noplak® Max, D) Oral-B® Complete, E) Listerine® Zero, F) Malvatricin® Plus, G) Cepacol® Plus Advanced

Figure 3. Mean ± SD of the percentage (%) of live and dead microorganisms (viability assay using CLSM)
Different letters show significant differences among the groups (ANOVA/Tukey-Kramer, $p<0.0001$).

Figure 4. Boxplot of the lactic acid production (mmol/l BPW) using lactic dehydrogenase method
Different letters show significant differences among the groups (Kruskal-Wallis/Dunn, $p<0.0001$).

Figure 5. Representative TMR pictures (20x) of the artificial enamel lesions created using microcosm biofilm after applying the tested treatments. A) Negative control, B) PerioGard®, C) Noplak® Max, D) Oral-B® Complete, E) Listerine® Zero, F) Malvatricin® Plus, G) Cepacol® Plus Advanced

Table 1. Mouthrinses tested in the study

| Mouthrinse | Producer | Ingredients |
|---|---|---|
| PerioGard® | Colgate-Palmolive | 0.12% chlorhexidine digluconate, water, glycerin, propylene glycol, sorbitol, PEG-40 hydrogenated castor oil, cetylpyridinium chloride, aroma and citric acid. (pH 4.9) |
| Noplak® Max | Daudt | 0.12% chlorhexidine digluconate, cetylpyridinium chloride, EDTA disodium, *Echinacea angustifolia* extract, glycerin, *Hamamelis* extract (Hamamelis), PEG-40 castor oil, propolis extract, propylene glycol, sodium cyclamate, sodium fluoride (226 ppm F), sodium hydroxide, sodium saccharin, sorbitol, zinc acetate. (pH 5.5) |
| Oral-B® Complete | Procter & Gamble | 0.053% cetylpyridinium chloride monohydrate; 0.05% sodium fluoride (226 ppm F), water, glycerin, PEG-40 hydrogenated castor oil, methylparaben, aroma, sodium saccharin, sodium benzoate and propylparaben. (pH 5.4) |
| Listerine® Zero | Johnson & Johnson | 0.09% eucalyptol, 0.06% thymol, 0.05% methyl salicylate, 0.04% menthol, water, sorbitol, poloxamer 407, benzoic acid, sodium saccharin, aroma and sodium benzoate. (pH 4.1) |
| Malvatricin® Plus | Daudt | EDTA disodium, *Malva sylvestris* extract (Mallow), menthol, PEG-40 castor oil, propylene glycol, Copolymer PVM/MA, sodium benzoate, sodium fluoride (225 ppm F), sodium hydroxide, sodium dodecyl sulfate, sodium saccharin, sorbitol, triclosan, xylitol, zinc chloride and water. (pH 6.0) |
| Cepacol® Plus Advanced | Sanofi | 0.05% cetylpyridinium chloride; sodium fluoride (226.2 ppm F), water, sorbitol, glycerin, sodium benzoate, sodium saccharin, sodium cyclamate, poloxamer 407, hydrogenated ricinium oil, sodium monofluorophosphate, methylparaben, aroma (D-Limonene), citric acid and propylene glycol. (pH 6.0) |

Table 2. CFU counting ($log_{10}$ CFU/ml) for total microorganism, lactobacilli, total streptococci and mutans streptococci

| **Treatment** | **Total microorganism** | **lactobacilli** | **Total streptococci** | **mutans streptococci** |
|---|---|---|---|---|
| PBS (negative control) | 7.33±0.28 | 7.46(0.71) | 5.69(0.35) | 6.45±1.31 |
| PerioGard® | 7.03±0.18 | 7.51(0.87) | 5.58(0.28) | 6.01±0.82 |
| Noplak® Max | 7.15±0.29 | 7.30(0.41) | 5.60(0.80) | 6.40±0.99 |
| Oral-B® Complete | 7.27±0.18 | 7.15(0.26) | 5.20(0.29) | 5.98±1.21 |
| Listerine® Zero | 7.33±0.28 | 7.76(0.37) | 5.21(0.84) | 6.25±1.10 |
| Malvatricin® Plus | 7.23±0.27 | 7.02(0.24) | 5.60(0.67) | 6.25±1.07 |
| Cepacol® Plus Advanced | 7.16±0.09 | 7.14(0.47) | 5.64(0.53) | 6.07±1.27 |

Mean ± SD of total microorganism and mutans streptococci CFU (ANOVA/Tukey-Kramer, p=0.0906 and p=0.9651, respectively). Median (interquartile interval) of lactobacilli and total streptococci CFU (Kruskal-Wallis/Dunn, p=0.0756 and p=0.8473, respectively).

Table 3. Median (interquartile interval) of soluble and insoluble EPS (mg/g biofilm)

| Treatment | Soluble EPS (mg/g) | Insoluble EPS (mg/g) |
|---|---|---|
| PBS (negative control) | 0.18(0.11) | 0.25(0.08)$^{abc}$ |
| PerioGard® | 0.12(0.30) | 0.13(0.05)$^{a}$ |
| Noplak® Max | 0.30(0.22) | 0.41(0.14)$^{c}$ |
| Oral-B Complete® | 0.09(0.14) | 0.22(0.06)$^{abc}$ |
| Listerine® Zero | 0.18(0.19) | 0.29(0.35)$^{bc}$ |
| Malvatricin® Plus | 0.18(0.17) | 0.12(0.05)$^{a}$ |
| Cepacol® Plus Advanced | 0.66(0.60) | 0.10(0.13)$^{a}$ |

Different letters in the same column show significant differences among the groups. Soluble EPS (Kruskal-Wallis/Dunn, $p=0.0763$) and Insoluble EPS (Kruskal-Wallis/Dunn, $p<0.0001$).

Table 4. Median (interquartile interval) of the integrated mineral loss (ΔZ, %vol.µm), the average mineral loss (R, % vol) and lesion depth (LD, µm)

| Treatment | ΔZ (%vol. µm) | R (%vol) | LD (µm) |
|---|---|---|---|
| PBS (negative control) | 11555.0(1804.7)$^{c}$ | 49.5(7.85)$^{b}$ | 215.4(25.0)$^{b}$ |
| PerioGard® | 2948.0(940.0)$^{a}$ | 21.3(2.45)$^{a}$ | 134.2(40.6)$^{a}$ |
| Noplak® Max | 2900.0(1320.0)$^{a}$ | 19.9(10.1)$^{a}$ | 138.5(70.0)$^{a}$ |
| Oral-B Complete® | 6500.0(2567.3)$^{bc}$ | 26.1(10.65)$^{ab}$ | 208.5(45.5)$^{ab}$ |
| Listerine® Zero | 4530.0(1459.0)$^{abc}$ | 32.2(6.3)$^{ab}$ | 131.3(56.8)$^{ab}$ |
| Malvatricin® Plus | 3170.0(320.0)$^{a}$ | 22.7(3.7)$^{a}$ | 141.4(6.2)$^{a}$ |
| Cepacol® Plus Advanced | 4160.0(740.0)$^{abc}$ | 27.8(1.9)$^{b}$ | 155.3(15.9)$^{ab}$ |

Different letters in the same column show significant differences among the groups. All parameters were compared using Kruskal-Wallis/Dunn: ΔZ and R ($p<0.0001$) and LD ($p=0.0014$).

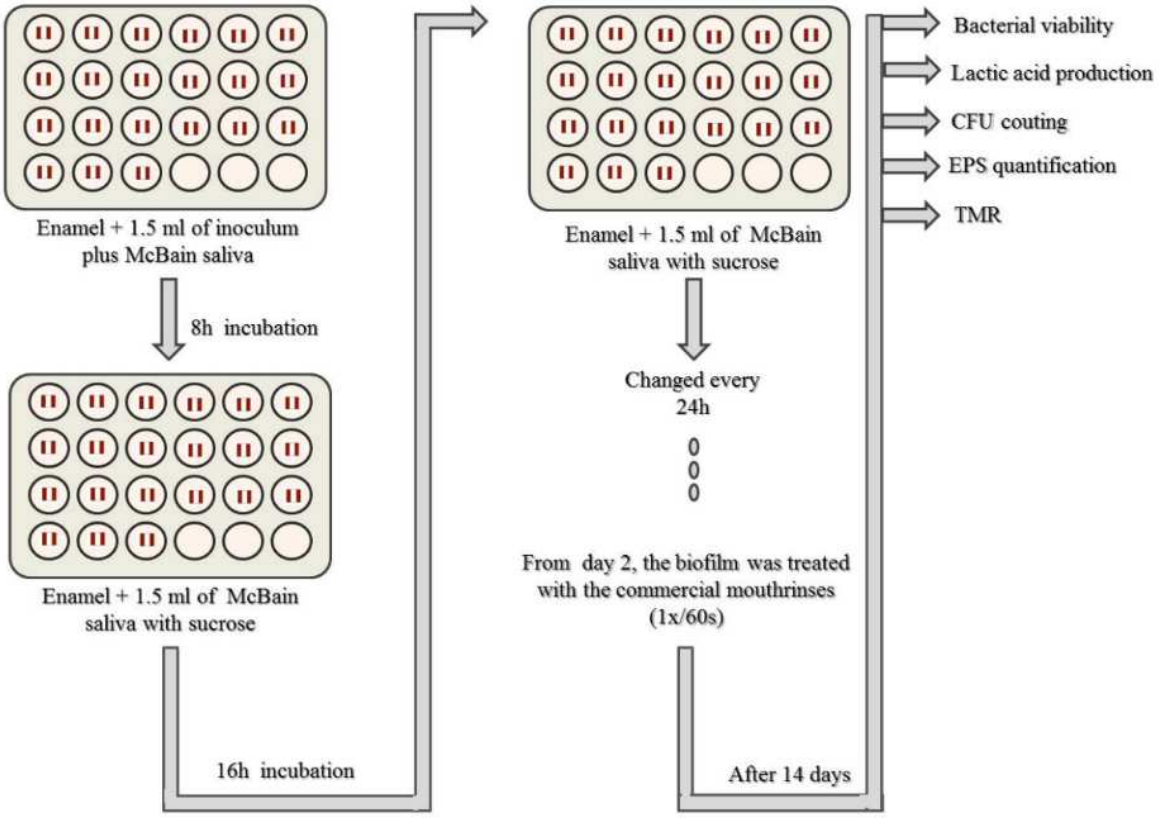

Enamel + 1.5 ml of inoculum plus McBain saliva
8h incubation
Enamel + 1.5 ml of McBain saliva with sucrose
16h incubation
Enamel + 1.5 ml of McBain saliva with sucrose
Changed every 24h
From day 2, the biofilm was treated with the commercial mouthrinses (1x/60s)
After 14 days
Bacterial viability
Lactic acid production
CFU couting
EPS quantification
TMR

Figure 1

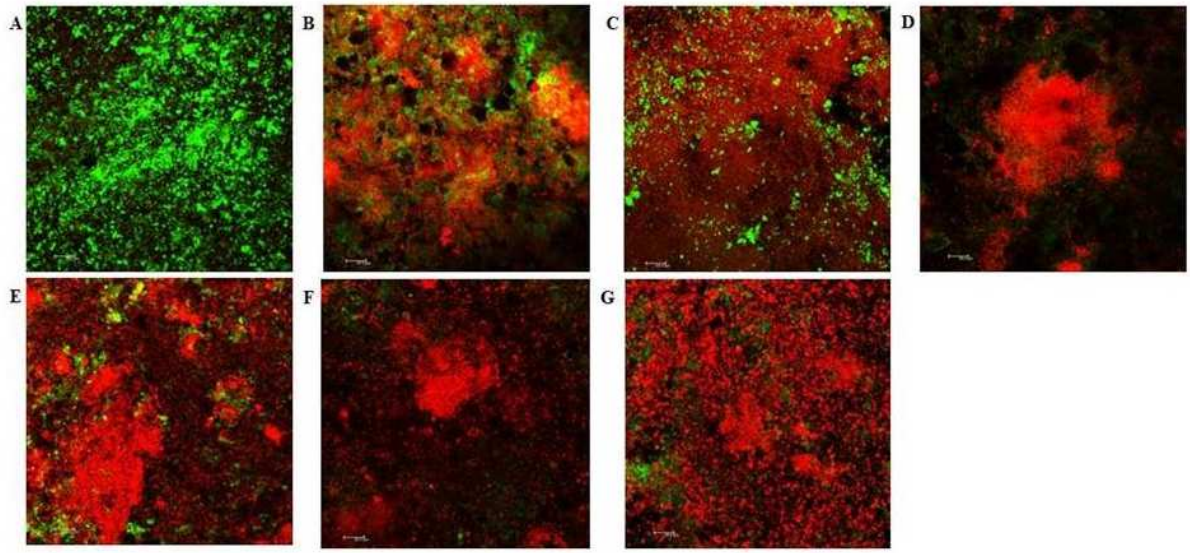
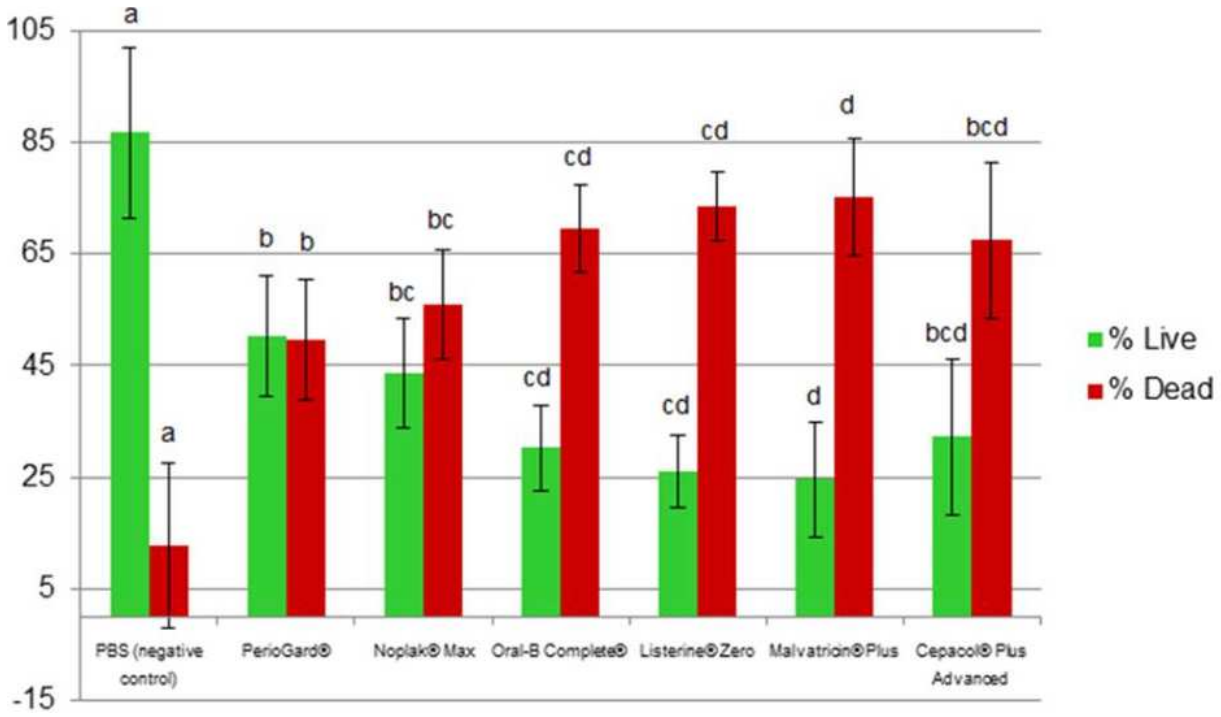



Figure 2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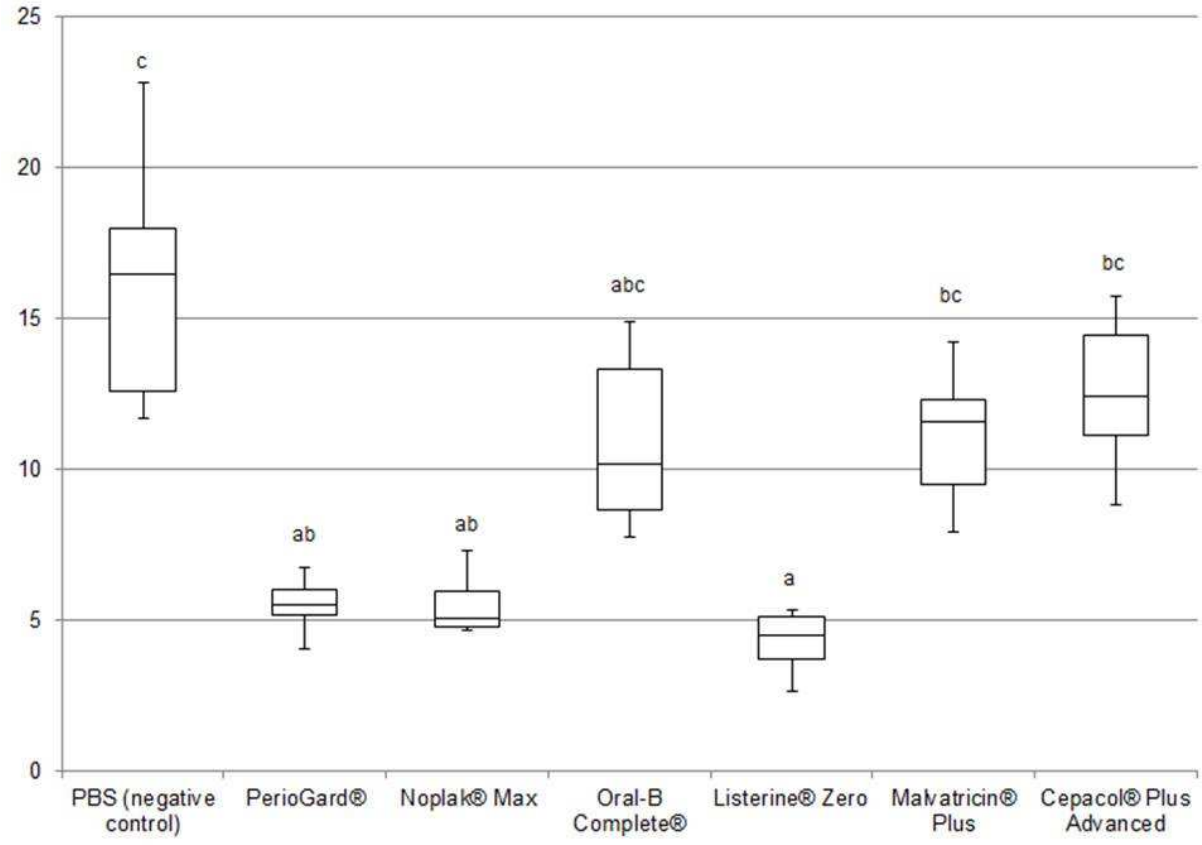
bcd
PBS (negative control)
PerioGard®
Noplak® Max
Oral-B Complete®
Listerine® Zero
Malvatricin® Plus
Cepacol® Plus Advanced
% Live
% Dead

Figure 3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PBS (negative control)
PerioGard®
Noplak® Max
Oral-B Complete®
Listerine® Zero
Malvatricin® Plus
Cepacol® Plus Advanced

Figure 4

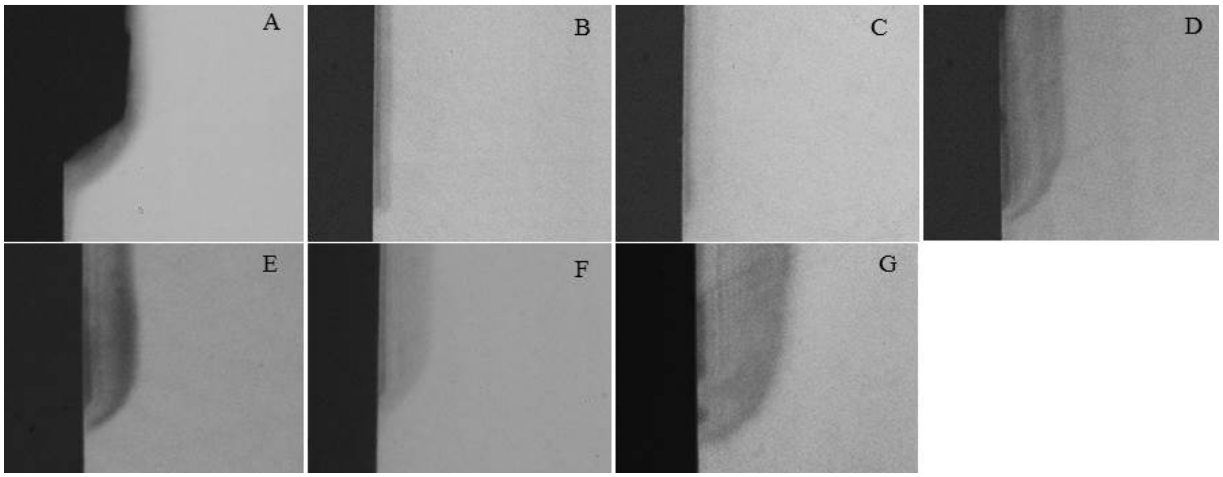


Figure 5

# 4- Discussion

## 4 DISCUSSION

The daily use of mouthrinses has been considered an alternative for the control of dental caries in patient who presents a poor oral hygiene or a systemic condition that compromise the oral health (VOZZA et al., 2015). According to Vozza et al., (2015), individual additional strategies for a preventive care should be applied for these patients at high risk for dental caries. Therefore, the present study compared the most known commercial mouthrinses containing different antimicrobial agents against enamel caries under a microcosm biofilm model. Microcosm biofilm is the most realistic *in vitro* model to induce biofilm formation using inoculum from pooled human saliva or *in vivo* biofilm mixture with nutrients. It is able to mimic the heterogeneity and variability of the supragingival biofilms (RUDNEY et al., 2012; AZEVEDO et al., 2014).

In our study, the saliva was collected from 2 donors, once we got the required volume for all experiments in one appointment. However, we have found a great variation in the number of donors (from 1 to 10) in the literature (LI et al., 2014; MASKE et al., 2016). We applied McBain saliva supplemented with 0.2% sucrose to induce the formation of a cariogenic biofilm as done previously (PRATTEN; WILSON; SPRATT, 2003). In the literature we have found differences in the sucrose concentration and the frequency of exposition (E.g: 1% for 6 h; 50 mM 8x15 min) (AZEVEDO et al., 2014; OWENS et al., 2017).

We had chosen six mouthrinses, considering their active agents and their availability in pharmacies and supermarkets. No previous study has compared this number of agents using a microcosm biofilm model. In general, the *in vitro* studies mostly apply monospecies or multispecies biofilm (mixed strains) and compare two or three agents in maximum (GUGGENHEIM; MEIER, 2011; RAMALINGAM et al., 2012; PANDIT et al., 2015).

All mouthrinses were alcohol-free. In general, the studies have shown no differences in the performance of mouthrinses with or without alcohol against cariogenic bacteria (GUGGENHEIM; MEIER, 2011; SREENIVASAN; HARASZTHY; ZAMBON, 2013; ULKUR; ARUN; OZDEMIR, 2013; SANTOS et al., 2017). There was only one study showing better anti-plaque effect of EO with alcohol compared to the free-alcohol version (MARCHETTI et al., 2011).

Commercial rinses containing 0.05% CPC, alcohol or free-alcohol, were compared with 0.05% fluoride mouthwash (F) and 0.12% chlorhexidine (positive control-CHX). MIC was firstly determined for each mouthrinse considering 25 microorganism species associated with oral diseases. The second part of the study evaluated the antimicrobial activity using supragingival biofilm collected from 15 subjects, which was exposed *ex vivo* to the mouthrinses for 5-7 days in anaerobic environment. MIC values were significantly lower for both CPC rinses compared to fluoride rinse especially against gram-negative bacteria (most involved in halitosis etiology), showing a broad-spectrum activity. CHX had the greatest antimicrobial effect. This *ex vivo* model showed no difference between CPC rinses formulated with alcohol or without alcohol. Both CPC (>90% killing) and CHX (98% killing) showed higher antimicrobial activity compared to F (SREENIVASAN; HARASZTHY; ZAMBON, 2013).

Some of the tested mouthrinses presented the antimicrobial agent combined with fluoride (Oral-B® Complete, Malvatricin Plus® and Cepacol® Plus Advanced). Latimer et al., (2015), showed that the presence of fluoride did not have influence on the antimicrobial effect of CPC, as well as Zhang et al., (2004), showed that fluoride did not have antimicrobial effect when combined with EO.

Zhang et al., (2004), evaluated the effect of EO with (100 ppm) or without fluoride (30 s, twice daily for 16 days) on the acid production and pH response after applying sucrose (10% sucrose solution for 1 min) *in vivo*. The results showed that after EO rinsing (with or without F) the production of lactate, acetate and propionate decreased in 36, 36 and 44%, respectively, compared to negative control. The dental plaque also exhibited a pH 0.42 unit higher after EO, rinsing with or without F) compared to negative control. EO, with or without fluoride, is effective in the reduction of plaque acidogenicity after a sucrose challenge.

Our results showed that all mouthrinses were able to reduce bacteria viability in accordance with Hannig et al., (2013), Babu; Garcia-Godoy, (2014), and Pandit et al., (2015). However, the lactic acid production was reduced by PerioGard®, Noplak® Max and Listerine® Zero only. Furthermore, the enamel demineralization was reduced by PerioGard®, Noplak® Max and Malvatricin® Plus only. Therefore, the antimicrobial and anticaries performance of the tested mouthrinses are different.

In the present study, the worst effect was found for Cepacol® Plus Advanced that contains CPC and fluoride. Also a limited effect was seen for Oral-B® Complete (CPC and fluoride), which reduced the bacteria viability, but not the development of dental caries. It is likely that CPC acts mostly against non-cariogenic bacteria. This result is in agreement with previous works that compared 0.05% CPC with other agents as 0.12% CHX and/or 0.042% menthol, 0.092% eucalyptol, 0.064% thymol, 0.06% methyl salicylate (EO) against cariogenic bacteria (planktonic phase and supragingival biofilm) (BABU; GARCIA-GODOY, 2014; VLACHOJANNIS et al., 2016). CPC has shown inhibitory effect on young *S. mutans* biofilm only, but not on the old-one (PANDIT et al., 2015).

On the other hand, Listerine® Zero (containing EO) has antimicrobial effect similar or higher than PerioGard® (containing CHX), in agreement with other studies (MARCHETTI et al., 2011; OYANAGI; TAGAMI; MATIN, 2012; WAKAMATSU et al., 2013). Listerine® Zero was able to reduce both viability and lactic acid production in the present study in accordance with Zhang et al., (2004). However, the previous studies did not evaluate the incidence of caries lesions along the time.

Different commercial mouthrinses containing CHX, in concentrations ranged from 0.05 to 0.2%, were compared to EO (formulae with alcohol) and water (negative control) using multispecies biofilms. The treatments were done after 16.5, 24.5, 40.5, and 48.5h of biofilm formation. After a total time of 64.5 h, CFUs were determined for total microorganisms (*A. naeslundii*, *V. dispar*, *F. nucleatum*, *S. mutans*, *S. oralis* and *C. albicans*), *S. mutans* and *S. oralis*. The total CFU numbers were not significant different among the mouthrinses. Biofilm formation was reduced in 7 $\log_{10}$ steps by 0.2% CHX (formulae with alcohol), and in 3 $\log_{10}$ steps by EO, 0.05% CHX (formulae with alcohol), and 0.12% and 0.2% CHX (without alcohol) solutions compared to water (GUGGENHEIM; MEIER, 2011).

An intriguing result was seen for Listerine® Zero in our study, its protection against demineralization was not as good as its effect on the lactic acid production. We suppose that other types of acid could have been produced by the Listerine-treated biofilm or that lactate consumer species (as *Veillonella* spp.) (DISTLER; KRONCKE, 1981; JANUS et al., 2016) might be absent in this biofilm. The hypothesis need to be further tested. There is only one study that observed anticaries

effect of EO (OYANAGI; TAGAMI, MATIN, 2012); however, the authors tested a version containing alcohol and compared it with a low concentrated CHX solution (0.05%).

Among the tested mouthrinses, Malvatricin® Plus was the only one containing a natural agent (*Malva sylvestris*) combined with fluoride and xylitol. *Malva sylvestris* is native from Europe, North Africa, and South-west Asia especially Iran (ELSAGH et al., 2015). It is worldwide applied as an alternative antiseptic, antifungal, anti-inflammatory agent and it also has antimicrobial effect against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Entrococcus faecalis* and *Candida albicans* (RAZAVI et al., 2011). *Malva sylvestris* contains malvone and different known monoterpenes, aromatic compounds, and tetrahydroxylated acyclic diterpense (VESHKOROVA et al., 2006). Malvone has been associated with the antimicrobial effect of *Malva sylvestris* (RAZAVI et al., 2011), which has been tested only on isolates cariogenic bacteria, *S. mutans*, *S. salivarius*, *S. oralis* and *L. casei* strains (DA SILVA et al., 2012).

Benso et al., (2015), investigated the anti-inflammatory activity of *Malva sylvestris* Extract (MSE) and its fractions. Co-culture of fibroblasts and keratinocytes was infected by *Aggregatibacter actinomycetemcomitans* (~1x$10^6$ CFU/ml) and treated with MSE or its fractions at concentrations of 0.1; 1; 10; 100 and 1000 µg/ml. A. *Malva sylvestris* and fractions lower than 100 µg/ml did not significantly reduce cell viability.

In the present study, Malvatricin® Plus reduced the bacteria viability, but not the acid production. As found in case of Oral-B® Complete, it is likely that Malvatricin® Plus acts mostly against non-cariogenic bacteria. Based on this statement, we would expect no anticaries effect by using this mouthrinse. Surprisingly, Malvatricin® Plus was able to reduce enamel caries lesion development. We justified this finding by the presence of fluoride, which has negligible antimicrobial effect, but it is known to be able to reduce demineralization (BUZALAF et al., 2011). Furthermore, the presence of xylitol could also have contributed to the protective effect. Xylitol is known to have antimicrobial effect on *S. mutans* biofilm (DECKER et al., 2008).

Decker et al., (2008), evaluated the antimicrobial effect of xylitol solution or xylitol plus CHX on *Streptococcus sanguis* and *Streptococcus mutans* during the

initial steps of biofilm formation on enamel samples. The bacterial vitality of the suspended and attached microrganisms to enamel was monitored using 2 fluorescent DNA stains and epifluorescence microscopy. Total bacterial cell counts on enamel slides and the suspended streptococci were quantified. *S. mutans* were sensible to pure CHX or combined with xylitol. The combination of xylitol/CHX showed a significant antimicrobial effect on *S. sanguis* compared to pure xylitol or chlorhexidine. The bacterial cell density on enamel and the bacterial reproduction on agar plates were similarly affected by the combination of xylitol/CHX or the isolated agents. Xylitol may have antimicrobial effect compatible to CHX depending on the target bacteria.

Previous study from our group has also shown positive effect of xylitol on enamel remineralization under abiotic models (CARDOSO et al., 2014). This is the first time that the antimicrobial effect of Malvatricin® Plus on biofilm and its anticaries effect on enamel have been demonstrated.

Finally, both Periogard® and Noplak® Max (containing CHX) reduced the bacteria viability and lactic acid production, as well as they reduced the enamel caries lesion development. CHX is known as the best antimicrobial agent and the present result is in agreement with the literature (SHAPIRO et al., 2002; HAERIAN-ARDAKANI et al., 2015; QUINTAS et al., 2015). Interesting that the effect of CHX compared to other agents is more pronounced *in vitro* than *in vivo* (HAERIAN-ARDAKANI et al., 2015).

Furthermore, no benefits of the application of chlorexidine varnish in the prevention of caries in children and adolescents have been discussed in systematic reviews (JAMES; PARNELL; WHELTON H, 2010; TWETMAN, 2004). CHX also is known to induce some side effects as tooth discoloration and astringent taste under uninterrupted use (BALAGOPAL; ARJUNKUMAR, 2013).

Our model was unable to show differences among the mouthrinses and the control in respect to CFU counting and EPS production. The microcosm biofilm presents the heterogeneity and variability of *in vivo* oral biofilms. Despite we applied sucrose to change the bacterial virulent properties and favor mutans (EPS producer) and lactobacillus species (DIAZ-GARRIDO et al., 2016), it might be expected other types of bacteria in the biofilm, which were not taken into account in the CFU

analysis and could have influenced the EPS production. Microbial species such as Scardovia wiggsiae and Bifidobacterium spp. have been correlated with caries etiology (HENNE et al., 2016). To overcome this issue, further studies shall be done to analysis the microbiome profile and metabolome of the microcosm biofilm and to correlate it with the microbiome profile of the donors (saliva and biofilm).

In conclusion, the mouthrinses containing chlorexidine or *Malva sylvestris* (with F, triclosan and xylitol) had the best anticaries effect under this model. Considering the anticaries properties rather than the antimicrobial effect, it would be very interesting to further study the effect of Malvatricin® Plus deeply using *in situ* and *in vivo* models, since it presents natural agents with low cytotoxic potential (BENSO et al., 2015), while CHX is known to induce local side-effects.

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# Annex

**Universidade de São Paulo**
**Faculdade de Odontologia de Bauru**

Comissão de Ética no Ensino e Pesquisa em Animais

Uso exclusivo da CEEPA/FOB/USP
Registro número: **005/2016**
Recebido em: 20/12/2016
Maristela Petenuci Ferrari
Secretária da CEEPA – SRTE 53052

**REGISTRO DE PESQUISA E/OU ENSINO, COM UTILIZAÇÃO DE CADÁVERES DE ANIMAIS, OU PARTE DELES**

| | |
|---|---|
| **Finalidade:** | Pesquisa |
| **Período:** | Abril/2015 à Fevereiro/2017 |
| **Título da pesquisa:** | Avaliação do efeito de diferentes exaguatórios bucais comerciais sobre a viabilidade e atividade de biofilme microcosmo e na desmineralização do esmalte |
| **Pesquisador Responsável:** | Profa. Dra. Ana Carolina Magalhães |
| **Pesquisador Executor:** | Aline Silva Braga |
| **Colaboradores:** | Giovana Bissoli Degand |
| **Dados Nota Fiscal/ Termo de Doação:** | Frigol S/A (Doação) |
| **Quantidade de Dentes Bovinos:** | 200 (utilizados na pesquisa = 54 dentes/12 grupos) |

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-101 – C.P. 73
e-mail: ceepa@fob.usp.br – Fone/FAX (0xx14) 3235-8356
http://www.fob.usp.br

**Universidade de São Paulo**
**Faculdade de Odontologia de Bauru**

Departamento de Ciências Biológicas

**Termo de Consentimento Livre e Esclarecido**

Caro aluno de pós-graduação da Faculdade de Odontologia de Bauru, através deste termo, lhe convidamos para participar da pesquisa "Avaliação do efeito de diferentes enxaguatórios bucais comerciais sobre a viabilidade e atividade de biofilme microcosmo e na desmineralização do esmalte".
A nossa pesquisa tem como objetivo testar o efeito de seis enxaguatórios comerciais: Oral-B Complete, Listerine, Cepacol, PerioGard, Noplak Max e Malvatricin Plus, sobre a viabilidade, contagem de UFC, produção de ácido por um biofilme microcosmo (placa rica em bactérias e fungos) submetido ao desafio cariogênico (exposição ao açúcar para simular a cárie) e o consequente efeito na desmineralização (perda mineral) do esmalte. Para isso, gostaríamos de pedir autorização ao senhor (a) para o uso da saliva. Esta pesquisa será feita pela Aline Silva Braga (eu) e pela Profa. Dra. Ana Carolina Magalhães (FOB – USP). É importante destacar que de acordo com o item IV.6.b da resolução 466/12 os sr(as) terão garantida a liberdade do consentimento para a participação ou não na pesquisa, sem qualquer represália. Portanto garantimos que não serão coagidos e nem sofrerão restrições de suas atividades usuais.

Assim, se o(a) Sr.(a) concordar, terá que mastigar uma parafina plástica- *Parafilm* (que é uma película plástica, sem cheiro, sem cor, resistente à água) com o objetivo de aumentar a quantidade de saliva para que então seja realizada a coleta que não é invasiva. Durante a coleta você cuspirá toda a saliva em um recipiente plástico. A saliva será coletada no período da manhã. Para a coleta você não poderá escovar os dentes, nem fazer uso de nenhum tipo de bochecho com fluoreto/agente antimicrobiano por um período de 24h, sendo que 2h antes da coleta, você não poderá fazer a ingestão de nenhum tipo de alimento. Após a utilização da sua saliva para este estudo, esta será descartada em local apropriado. A sua saliva será utilizada apenas para a formação do biofilme microcosmo no Laboratório.

O(a) sr(a) não precisarão passar por nenhum outro tipo de procedimento adicional. Isso quer dizer que iremos utilizar apenas a saliva. Queremos deixar claro que não existe a menor obrigação do(a) sr.(a) aceitar doar parte da saliva retirada para a pesquisa. Isso é totalmente voluntário.

Esta pesquisa gera o benefício do aumento do conhecimento sobre o funcionamento dos enxaguatórios bucais comerciais sobre as bactérias presente na boca e na formação da cárie, podendo, futuramente, auxiliar no tratamento desta doença. Não há benefícios imediatos para os participantes da pesquisa.

A sua participação neste trabalho acarretará em risco mínimo, que acontecerá no caso de você ter alergia ao plástico utilizado para mastigação ou se você apresentar enjoos na hora da coleta. Nestes casos, o(a) senhor(a) deverá comunicar o responsável pela pesquisa, que estará presente no momento da coleta, o qual o(a) liberará da participação na pesquisa, sem penalização alguma. Os gastos que forem gerados por este trabalho ficará a cargo da responsável pelo projeto. Importante ressaltar que não está sendo considerado nenhum pagamento ou recompensa material pela sua participação neste estudo. O Senhor (a) terá garantido o direito à indenização compensatória caso fique comprovado que a sua participação acarretou algum problema ao Senhor (a). Após a coleta, o sr(a) receberá uma profilaxia profissional. A saliva remanescente será descartada no lixo contaminado do laboratório de Bioquímica.

O sr(a) aluno de pós-graduação pode recusar-se em assinar este termo para a não participação na pesquisa e mesmo após assinar este termo, caso o senhor(a) mude de idéia e queira retirar seu consentimento em qualquer fase da pesquisa, poderá fazê-lo sem nenhuma represália. Todo o trabalho será feito sem a sua identificação, preservando completamente sua identidade. Ao concordar em participar desta pesquisa, o senhor(a) receberá uma via, igualmente válida deste termo. O direito à indenização lhe será permitido, caso ocorra algum dano decorrente da sua participação nesta pesquisa. Caso senhor (a) necessite de ajuda financeira de transporte para participar desta pesquisa ela poderá ser ressarcida pelo pesquisador.

Rubrica do Participante da Pesquisa

Rubrica do Pesquisador Responsável

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
e-mail: daniela@fob.usp.br – Fone (0xx14) 3235-8226- FAX (0xx14) 3223-4679
http://www.fob.usp.br

**Universidade de São Paulo**
**Faculdade de Odontologia de Bauru**

Departamento de Ciências Biológicas

Qualquer dúvida ou maiores esclarecimentos o sujeito da pesquisa poderá recorrer a qualquer um dos membros da equipe do projeto (Laboratório de Bioquímica 14-3235-8247) ou a pesquisadora responsável Aline Silva Braga (telefone 14 / 99766-9650, e-mail asbraga@usp.br). Caso possua preocupações quanto aos seus direitos como participante deste estudo, ou queira fazer denúncias quanto à condução do mesmo, sinta-se a vontade para procurar *o Comitê de Ética em Pesquisa, da Faculdade de Odontologia de Bauru/USP, Alameda Dr. Octávio Pinheiro Brisolla, 9-75, telefone (14)3235-8356 ou e-mail:* cep@fob.usp.br e a forma de contato com CONEP – Endereço: Esplanada dos Ministérios, Bloco G, Anexo B. Sala 104B, telefone: (61) 3315-5878, e-mail: cns@saude.gov.br.

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Rubrica do Pesquisador Responsável

**Universidade de São Paulo**
**Faculdade de Odontologia de Bauru**

Departamento de Ciências Biológicas

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____, portador da cédula de identidade 46767470-X, após leitura minuciosa das informações constantes neste TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicada pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, DECLARA e FIRMA seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em participar da pesquisa proposta. Fica claro que o participante da pesquisa, pode a qualquer momento retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar desta pesquisa e ciente de que todas as informações prestadas tornar-se-ão confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica).

Por fim, como pesquisador(a) responsável pela pesquisa, DECLARO o cumprimento do disposto na Resolução CNS nº 466 de 2012, contidos nos itens IV.3, item IV.5.a e na íntegra com a resolução CNS nº 466 de dezembro de 2012.

Por estarmos de acordo com o presente termo o firmamos em duas vias igualmente válidas (uma via para o participante da pesquisa e outra para o pesquisador) que serão rubricadas em todas as suas páginas e assinadas ao seu término, conforme o disposto pela Resolução CNS nº 466 de 2012, itens IV.3.f e IV.5.d.

Bauru, SP, 25 de Janeiro de 2016.

Assinatura do Participante da Pesquisa

Nome/Assinatura do Pesquisador(a)

O **Comitê de Ética em Pesquisa – CEP**, organizado e criado pela **FOB-USP**, em 29/06/98 (**Portaria GD/0698/FOB**), previsto no item VII da Resolução nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um Colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.
Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:

**Horário e local de funcionamento:**
Comitê de Ética em Pesquisa
Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira; no horário das **13h30 às 17 horas**, em dias úteis.
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**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

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Bauru, SP, 25 de Janeiro de 2016.

_____ _____

Assinatura do Participante da Pesquisa Nome/Assinatura do Pesquisador(a)

O **Comitê de Ética em Pesquisa – CEP**, organizado e criado pela **FOB-USP**, em 29/06/98 (**Portaria GD/0698/FOB**), previsto no item VII da Resolução nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um Colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.
Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:

**Horário e local de funcionamento:**
Comitê de Ética em Pesquisa
Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira, no horário das **13h30 às 17 horas**, em dias úteis.
Alameda Dr. Octávio Pinheiro Brisolla, 9-75
Vila Universitária – Bauru – SP – CEP 17012-901 Telefone/FAX(14)3235-8356 e-mail: cep@fob.usp.br

Projetos Cadastros Administrativo Reunião Relatórios USP - Faculdade de Odontolo

Você está em: CEP > Validar Protocolo de Pesquisa

## VALIDAR PROJETO DE PESQUISA

### DADOS DA VERSÃO DA EMENDA

**Título da Pesquisa:** AVALIAÇÃO DO EFEITO DE DIFERENTES ENXAGUATÓRIOS BUCAIS COMERCIAIS SOBRE A VIABILIDADE E ATIVIDADE DE BIOFILME MICROCOSMO E NA DESMINERALIZAÇÃO DO ESMALTE
**Pesquisador Responsável:** Aline Silva Braga
Área Temática:
**Versão:** 4
**CAAE:** 48100315.3.0000.5417
**Submetido em:** 23/06/2017
**Instituição Proponente:** Faculdade de Odontologia de Bauru
**Situação da Versão do Projeto:** Em Recepção e Validação Documental
**Localização atual da Versão do Projeto:** USP - Faculdade de Odontologia de Bauru da USP
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

EMENDA COORDENADOR

### DOCUMENTOS DO PROJETO DE PESQUISA

- Versão em Tramitação (E2) - Versão 4
  - Emenda (E2) - Versão 4
    - Documentos do Projeto
      - Comprovante de Recepção - Submissã
      - Folha de Rosto - Submissão 1
      - Informações Básicas do Projeto - Subm
      - Outros - Submissão 1
      - Projeto Detalhado / Brochura Investigad
      - TCLE / Termos de Assentimento / Justif
    - Apreciação 1 - USP - Faculdade de Odonto
- Versão Atual Aprovada (E1) - Versão 3
- Projeto Completo

| Tipo de Documento | Situação | Arquivo | Postagem | Ações |
|---|---|---|---|---|

Maristela Petenuci Ferrari
Secretária de Comissões
FOB/USP - DRT - 43052

### DOCUMENTOS POSTADOS

| Tipo Documento | Situação | Arquivo | Postagem | Autor | Perfil | Ação |
|---|---|---|---|---|---|---|
| Informações Básicas do Projeto | Aceito | PB_INFORMAÇÕES_BÁSICAS_948820_E2.pdf | 23/06/2017 16:43:04 | | | |
| Outros | Postado | JUSTIFICATIVA.pdf | 23/06/2017 16:41:02 | Aline Silva Braga | Pesquisador Principal | |
| Outros | Postado | Formulario_parcial.pdf | 23/06/2017 16:38:48 | Aline Silva Braga | Pesquisador Principal | |
| Outros | Aceito | oficio.pdf | 21/02/2017 11:26:21 | Aline Silva Braga | Pesquisador Principal | |
| Outros | Aceito | Registro.pdf | 21/02/2017 10:19:08 | Aline Silva Braga | Pesquisador Principal | |
| Projeto Detalhado / Brochura Investigador | Aceito | Projeto.pdf | 21/02/2017 08:46:30 | Aline Silva Braga | Pesquisador Principal | |
| Outros | Aceito | QUESTIONARIO.pdf | 04/09/2015 10:51:56 | Aline Silva Braga | Pesquisador Principal | |
| Outros | Aceito | TermodeAquiescenciadeCienciasBiologicas.pdf | 03/09/2015 10:10:28 | Aline Silva Braga | Pesquisador Principal | |
| TCLE / Termos de Assentimento / Justificativa de Ausência | Aceito | TCLEALINE.pdf | 03/09/2015 10:03:47 | Aline Silva Braga | Pesquisador Principal | |
| Outros | Aceito | TermodeAquiescencia.pdf | 03/09/2015 10:00:06 | Aline Silva Braga | Pesquisador Principal | |

« Ocorrência 1 a 10 de 13 registro(s) » »»

### HISTÓRICO DE TRÂMITES

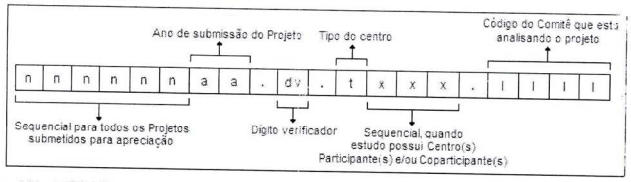
| Apreciação | Data/Hora | Tipo Trâmite | Versão | Autor | Perfil | Origem | Destino | Informações |
|---|---|---|---|---|---|---|---|---|
| E2 | 23/06/2017 16:43:03 | Submetido para avaliação do CEP | 4 | Aline Silva Braga | Pesquisador Principal | PESQUISADOR | USP - Faculdade de Odontologia de Bauru da USP | |

**LEGENDA:**

**(*) Apreciação**

| | | |
|---|---|---|
| PO = Projeto Original de Centro Coordenador | POp = Projeto Original de Centro Participante | POc = Projeto Original de Centro Coparticipante |
| E = Emenda de Centro Coordenador | Ep = Emenda de Centro Participante | Ec = Emenda de Centro Coparticipante |
| N = Notificação de Centro Coordenador | Np = Notificação de Centro Participante | |

**(*) Formação do CAAE**




Voltar | Pendência Documental | Aceitar | Salvar | Adicionar Documento

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