



UNESP - Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara



Patricia Milagros Maquera Huacho

**Descontaminação de superfícies de titânio com Terpinen-4-ol e Carvacrol:
estudo in vitro das propriedades físico-química, antimicrobiana e citotóxica**

Araraquara

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Orientadora:

Profa. Dra. Denise M. Palomari Spolidorio

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estudo in vitro das propriedades físico-química, antimicrobiana e citotóxica.**

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*“Dai-me a serenidade para
aceitar as coisas que eu não posso
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que eu possa, e sabedoria para que eu
saiba a diferença: vivendo um dia a cada
vez, aproveitando um momento de cada
vez; aceitando as dificuldades como um
caminho...”*

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Suporta a provação, não reclames. Aceita.
Não grites com ninguém, nem firas. Abençoa.
Lance de sofrimento, é o ensejo da fé. Silencia.
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RESUMO

A doença peri-implantar é um processo inflamatório de etiologia bacteriana. Diferentes tratamentos antimicrobianos disponíveis buscam reduzir a carga microbiana e eliminar a inflamação. Terapias alternativas com extratos naturais abrem novas perspectivas para controlar e reduzir a carga microbiana. Terpinen-4-ol e Carvacrol, principais constituintes naturais de óleos essenciais vêm se destacando por sua propriedade antimicrobiana. O objetivo deste estudo foi avaliar: I) as modificações físico-químicas em superfícies de titânio, adesão e proliferação de fibroblastos e osteoblastos; II) as propriedades antimicrobianas sobre biofilme de *P. gingivalis* e *F. nucleatum*; e citotóxicas em fibroblastos. III) efeito inibitório de biofilmes simples e multi-espécies de *S. sanguinis*, *S. gordonii*, *S. mitis*, *S. oralis*, *S. salivarius*, *S. mutans*, *S. sobrinus*, *A. viscosus*, *A. naeslundii*, *P. intermedia*, *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans* e *V. parvula* a partir de concentrações não tóxicas em queratinócitos. Para isto: I) Discos de titânio foram descontaminados com T4ol e Carvacrol e a influência na modificação do ângulo de contato, energia livre de superfície, estrutura química por MEV-EDS, viabilidade e adesão celular de fibroblastos e osteoblastos pelo ensaio Alamar Blue foram avaliados. II) Concentração inibitória mínima (CIM), concentração bactericida mínima (CBM), concentração mínima de inibição do biofilme (CMIB) por ensaio XTT, e biofilme em superfícies de titânio foi quantificada em UFC e por Microscopia de laser de varredura confocal (CLSM) e a citotoxicidade em fibroblastos foi avaliada pelo ensaio MTT. III) A citotoxicidade foi testada em queratinócitos orais por ensaio XTT, a inibição e erradicação do biofilmes simples foi analisada por cristal violeta e o efeito sobre biofilmes multi-espécie foi avaliado por qPCR. Resultados de MEV-EDS demonstraram presença de Na, O e Cl após o tratamento com T4ol e Carvacrol. Todas as superfícies apresentaram características hidrófilas e valores de Energia Livre de Superfície (ELS) entre 5,5 mN/m e 3,4 mN/m. Teste de viabilidade e microscopia de fluorescência não apresentaram diminuição da viabilidade celular e adesão. Os valores de CIM foram Carvacrol 0,007% e 0,002% respectivamente para *P. gingivalis* e *F. nucleatum* e T4ol 0,06% para ambos os microrganismos. Os valores de CBM foram semelhantes aos CIM. Os valores de CMIB foram Carvacrol 0,03%, 0,06% e T4ol 0,06%, 0,24%, respectivamente, para *P. gingivalis* e *F. nucleatum*. Observou-se por contagem de UFC e CLSM atividade antibiofilme usando Carvacrol (0,26%, 0,06%) e T4ol (0,95%, 0,24%) com atividade citotóxica semelhante à Clorexidina. T4ol 0.19% e Carvacrol 0.06% não afetaram a viabilidade das células epiteliais. Foi observada a inibição e erradicação de biofilmes simples e multi-espécie, especialmente em patógenos periodontais. Portanto, podemos concluir que T4ol e Carvacrol não alteram as propriedades da superfície do titânio e permitem interação adequada com células envolvidas no processo de re-osseointegração. Além disso, concentrações específicas destes compostos sem toxicidade sobre células humanas, reduziram o número de patógenos periodontais em biofilmes simples e multi-espécie.

Palavras-chave: Biofilmes. Doenças periodontais. Titânio. Medicamentos fitoterápicos. Técnicas in vitro.

Maquera-Huacho PM. Decontamination of titanium surfaces with Terpinen-4-ol and Carvacrol: in vitro study of physical-chemical, antimicrobial and cytotoxic properties. [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

ABSTRACT

Peri-implant disease is an inflammatory process of bacterial etiology. Different antimicrobial treatments available seek to reduce microbial load and eliminate inflammation. Alternative therapies with natural extracts open new perspectives to control and reduce microbial burden. Terpinen-4-ol and Carvacrol, the main natural constituents of essential oils; has been notable for its antimicrobial property. The objective of this study was to evaluate: I) physico-chemical modifications on titanium surfaces, adhesion and proliferation of fibroblasts and osteoblasts; II) the antimicrobial properties of *P. gingivalis* and *F. nucleatum* biofilm; and cytotoxic effects on fibroblasts. III) Inhibitory effect of single and multi-species biofilms of *S. sanguinis*, *S. gordonii*, *S. mitis*, *S. oralis*, *S. salivarius*, *S. mutans*, *S. sobrinus*, *A. viscosus*, *A. naeslundii*, *P. intermedia*, *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans* and *V. parvula* from non-toxic concentrations in keratinocytes. For this: I) Titanium discs were decontaminated with T4ol and Carvacrol and the influence on the modification of contact angle, surface free energy, chemical structure by MEV-EDS, viability and cellular adhesion of fibroblasts and osteoblasts by the Alamar Blue assay were evaluated. II) Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum concentration of biofilm inhibition (MBIC) by XTT assay, and biofilm on titanium surfaces was quantified in CFU and by Confocal Scanning Laser Microscopy (CLSM), and fibroblast cytotoxicity was evaluated by the MTT assay. III) Cytotoxicity was tested in oral keratinocytes by XTT assay, inhibition and eradication of single biofilms was analyzed by crystal violet and the effect on multi-species biofilms was evaluated by qPCR. SEM results showed the presence of Na, O and Cl after treatment with T4ol and Carvacrol. All surfaces showed hydrophilic characteristics and surface free energy (SFE) values between 5.5 mN/m and 3.4 mN/m. Viability test and fluorescence microscopy showed no decrease in cell viability and adhesion. MIC values were Carvacrol 0.007% and 0.002% respectively for *P. gingivalis* and *F. nucleatum* and T4ol 0.06% for both microorganisms. MBC values were similar to MICs. The MBIC values were Carvacrol 0.03%, 0.06% and T4ol 0.06%, 0.24%, respectively, for *P. gingivalis* and *F. nucleatum*. Anti-biofilm activity using Carvacrol (0.26%, 0.06%) and T4ol (0.95%, 0.24%) was observed through CFU and CLSM and showed cytotoxic activity like Chlorhexidine. T4ol 0.19% and Carvacrol 0.06% did not affect the viability of epithelial cells. Inhibition and eradication of simple and multi-species biofilms were observed, especially in periodontal pathogens. Therefore, we can conclude that T4ol and Carvacrol do not alter the surface properties of Ti surfaces and allow an adequate interaction with cells involved in the re-osseointegration process; and specific concentrations of these compounds without toxicity to human cells, reduced the number of periodontal pathogens in single and multi-species biofilms.

Key words: Biofilms. Periodontal diseases. Titanium. Phytotherapeutic medicines. In vitro techniques.

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1 INTRODUÇÃO

A cavidade oral é um sistema dinâmico continuamente colonizado por microrganismos, e a relação entre doença periodontal e biofilme dental já está bem estabelecida. A literatura relata a ocorrência de mais de 700 espécies microbianas, que podem colonizar diferentes tipos de superfícies naturais (dentes, mucosa) ou artificiais (prótese, implantes dentários)¹. Aproximadamente 415 espécies presentes no biofilme dental subgengival podem ser encontrados em diferentes casos de periodontite e doença peri-implantar, e a presença de microrganismos específicos e o acúmulo de biofilme bacteriano podem induzir à resposta inflamatória^{2,3}.

A doença periodontal é uma doença inflamatória multifatorial⁴ caracterizada clinicamente por inflamação, sangramento à sondagem e pronunciada perda de inserção e possui como fator etiológico o biofilme dental⁵. Assim como a periodontite, a doença peri-implantar se desenvolve quando ocorre um desequilíbrio entre a reposta do hospedeiro e a microbiota e, conseqüentemente, as alterações inflamatórias peri-implantares se iniciam como resultado do acúmulo de biofilme com predominância de microrganismos anaeróbios como *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis* e *Tanarella forsythus*^{6,7}. A colonização bacteriana nas superfícies de implantes e nos tecidos gengivais pode ocorrer minutos após a instalação dos implantes dentários^{8,1} e a composição da microbiota ao redor dos novos implantes instalados, após 10 dias, torna-se semelhante à microbiota de dentes comprometidos com doença periodontal⁹. No entanto, o padrão de colonização em implantes dentários parece ser inicialmente mais lento do que em dentes naturais¹.

No processo de formação do biofilme, superfícies naturais e de implantes dentários são imediatamente recobertos por mucoproteínas salivares formando a película adquirida, a qual é um fator imperativo para a adesão bacteriana¹⁰. Colonizadores primários no biofilme são predominantemente Gram-positivos do gênero *Streptococcus* (até 80%) e *Actinomyces*^{11,12} e as camadas subsequentes de bactérias se aderem pelo processo denominado coagregação¹³. Como a população bacteriana aumenta dentro do biofilme o conteúdo de oxigênio torna-se empobrecido, tornando o ambiente mais adequado para a colonização de bactérias anaeróbias. Esta depleção leva à proliferação dos colonizadores secundários predominantemente anaeróbios Gram-negativos e organismos filamentosos^{14,11}.

Fusobacterium nucleatum é considerado um microrganismo intermediário entre os colonizadores primários e secundários devido a sua capacidade de adesão e coagregação com todas as outras espécies dentro do biofilme¹⁵⁻¹⁷. Finalmente, as espiroquetas tais como *Treponema denticola*, devido a sua motilidade, ajudam na proliferação e invasão do biofilme aos tecidos adjacentes^{14,11}.

A presença de microrganismos é um fator importante para o desenvolvimento de peri-implantite e os tratamentos disponíveis buscam reduzir a carga microbiana, eliminar a inflamação da mucosa peri-implantar e descontaminar a superfície do implante com a finalidade de preservar o osso de suporte e, se possível realizar a regeneração óssea¹⁸. Superfícies de implantes de titânio podem ser descontaminadas utilizando-se meios mecânicos (curetas dentais, raspadores ultrassônicos, procedimentos abrasivos de ar-pó) e/ou químicos (ácido cítrico, H₂O₂, digluconato de clorexidina, e EDTA), geralmente associados com antibióticos locais ou sistêmicos^{19,20}. Desta forma, a antissepsia e o uso de antibióticos locais e sistêmicos como coadjuvantes são eficazes em curto prazo para a eliminação das bactérias⁶. Não existe consenso na literatura para o tratamento de infecções peri-implantares devido à heterogeneidade dos estudos e a variabilidade dos tipos de superfícies de implantes¹⁸. Entretanto, em alguns casos o tratamento aplicado parece não ser capaz de devolver ou manter a saúde peri-implantar, podendo ser explicado pela persistência ou recolonização de microrganismos²¹. Os diferentes tratamentos propostos para a peri-implantite combinam diferentes métodos, incluindo técnicas regenerativas e ressectivas, que envolvem ou não o uso de enxertos, membranas e implantoplastia²⁰. É comum em todos os tratamentos a necessidade da descontaminação da superfície afetada, o que é fundamental para alcançar a re-osseointegração²². Contudo, é importante destacar que alguns desses métodos poderiam danificar as propriedades de superfície dos implantes ou promover a resistência bacteriana²³.

A reação das células e dos tecidos com os biomateriais depende das propriedades do material, topografia superficial, composição e seu comportamento em contato com os fluidos corporais²⁴. Na adesão celular, a energia livre de superfície pode desempenhar um papel importante não só sobre a adsorção de proteínas, mas também em relação à adesão das células e ao posterior espalhamento destas^{25,26}. De acordo com Colvin et al.²⁷, as superfícies, quando hidrofóbicas, diminuem a quantidade de proteínas aderidas na superfície do

material. As mudanças físicas da superfície alteram a expressão dos sítios ligantes das mesmas que, conseqüentemente, irão interagir com os receptores celulares. Por conseguinte, é possível afirmar que as características físicas, tais como rugosidade e energia livre de superfície, assim com as propriedades químicas das mesmas, podem contribuir positivamente para a adesão de células epiteliais e conjuntivas^{28,29}.

A restauração da biocompatibilidade das superfícies de titânio contaminadas depende da preservação das propriedades de superfície do implante. As terapias utilizadas no tratamento das doenças peri-implantares assim como o biofilme residual induzem alterações nas propriedades químicas e físicas da superfície e, conseqüentemente, mudanças na camada de óxido relacionada ao pH, resultando assim em menor energia de superfície e, portanto, influenciando negativamente na adesão celular^{30,24,31,32}. Kotsakis et al.³³ mostraram que agentes químicos usualmente utilizados no tratamento da peri-implantite, tais como clorexidina 0,12%, ácido cítrico 20% e EDTA 24%-NaOCl 1.5% deixam resíduos que provocam alterações físico-químicas sobre as superfícies de titânio. Estes resíduos afetam negativamente a biocompatibilidade das superfícies assim como a resposta de células osteoblásticas, independente do seu efeito antimicrobiano. Os mesmos autores não recomendam a clorexidina para descontaminar superfícies de titânio pois produz efeitos citotóxicos nas superfícies descontaminadas e pode comprometer a biocompatibilidade das superfícies.

Grande atenção tem sido dada à identificação de potentes agentes antimicrobianos para a descontaminação da superfície do implante. A clorexidina tem sido utilizada amplamente no tratamento da redução do biofilme subgengival e controle da inflamação, devido a sua substantividade³⁴, propriedades biológicas e atividade antibacteriana de amplo espectro (bactérias Gram-positivas e Gram-negativas)³⁵. Apresenta eficácia na diminuição da carga bacteriana sobre superfícies de titânio quando comparado a outros descontaminantes como hipoclorito de sódio, peróxido de hidrogênio e ácido cítrico³⁶.

Entretanto, a clorexidina apresenta desvantagens frequentemente observadas, tais como, coloração dos dentes e alteração do paladar³⁷. Segundo Gianelli et al.³⁸ a clorexidina pode induzir apoptose e necrose celular devido a perturbação da função mitocondrial, aumento intracelular de Ca^{2+} e estresse oxidativo. A clorexidina também mostrou inibir a proliferação celular e síntese de colágeno³⁹. Portanto, a aplicação direta da clorexidina durante a terapia regenerativa

no tratamento de doenças peri-implantares poderia exercer efeito tóxico sobre fibroblastos gengivais, células endoteliais e especialmente sobre osteoblastos alveolares, interferindo assim negativamente no reparo de infecções orais³⁸. Esses efeitos negativos poderiam explicar a redução das contagens bacterianas, mas nenhuma melhora significativa na cicatrização dos tecidos. Desse modo, a comunidade odontológica ainda está a procura de novas drogas terapêuticas para a prevenção e tratamento de biofilmes orais assim como doenças relacionadas^{40,41}.

Os esforços atuais para o controle do biofilme abrem novas perspectivas no uso de terapias alternativas. Diversos produtos de origem natural apresentam resultados significativos sobre patógenos bucais e controle da resposta inflamatória mostrando que agentes fitoterápicos podem ser veículos de prevenção e controle de doenças bucais infecciosas assim como uma alternativa eficaz para superar a resistência microbiana⁴²⁻⁴⁶. Desta forma, diferentes produtos naturais têm sido investigados como promissores agentes para prevenção e tratamento das doenças periodontais e peri-implantares^{40,46}. O Carvacrol, um monoterpene fenol (2-metil- 5-(1-metiletil) fenol) presente nos óleos voláteis de *Thymus vulgaris*, *Carum copticum* e *Oreganum spp.*, é o principal constituinte natural (70%) destas plantas aromáticas⁴⁷. Apresentam diferentes atividades antimicrobianas, anti-inflamatória, antioxidante, antimutagênica, antigenotóxica e hepatoprotetora, destacando sua atividade antimicrobiana de largo espectro sobre bactérias patogênicas e leveduras incluindo microrganismos formadores de biofilmes resistentes a drogas⁴⁰. Além disso, o Carvacrol possui efeito inibitório sobre diferentes microrganismos, sendo capaz de desintegrar a membrana externa de bactérias Gram-negativas liberando lipopolissacarídeos da parede celular bacteriana^{48,49}, além de não possuir efeitos citotóxicos em baixas concentrações^{49,40}.

Outro fitoterápico que vem se destacando é o Terpinen-4-ol (porção solúvel da *Melaleuca alternifolia*) que atua na indução da perda da membrana, interferindo na integridade e fisiologia da célula do microrganismo⁵⁰. O mesmo apresenta amplo espectro de atividade antimicrobiana (antibacteriana, antiviral e antifúngica) e atividade anti-inflamatória⁵¹⁻⁵³. O Terpinen-4-ol é um monoterpene caracterizado por ser o principal composto da *Melaleuca alternifolia* e outras plantas tais como *Hajeb Layoun arborea* (Tunisia) e *Alpinia zerumbet* e, além das propriedades citadas acima, estudos indicam que possui efeito antitumoral^{50,54,53}.

Considerando que o Terpinen-4-ol e o Carvacrol possuem propriedades antimicrobianas, há poucos estudos na literatura avaliando esta propriedade sobre bactérias periodontopatogênicas. Além disso, estudos adicionais são necessários para entender o comportamento celular em resposta às superfícies de implantes descontaminadas com agentes de origem natural. Desse modo, o objetivo do presente estudo foi analisar as modificações físico-químicas da descontaminação de superfícies de titânio com Terpinen-4-ol e Carvacrol e a resposta celular de fibroblastos e osteoblastos, assim como avaliar *in vitro* suas propriedades antimicrobianas e citotóxicas sobre biofilmes periodontais.

2 PROPOSIÇÃO

Com base nas informações apresentadas temos as seguintes hipóteses:

- A. Os resíduos do Terpinen-4-ol e Carvacrol presentes após a descontaminação de superfícies de titânio não alteram as propriedades físico-químicas do titânio assim como a resposta celular de fibroblastos e osteoblastos.
- B. Estes agentes fitoterápicos são efetivos contra bactérias anaeróbias tais como *Porphyromonas gingivalis* e *Fusobacterium nucleatum*, envolvidas na doença peri-implantar, e não mostram efeito citotóxico sobre linhagem celular de fibroblastos.
- C. Concentrações de Terpinen-4-ol e Carvacrol não mostram efeito citotóxico sobre linhagem celular de queratinócitos e são efetivos contra biofilmes simples e multi-espécies de bactérias periodontopatogênicas.

Levando em consideração as hipóteses descritas, este trabalho tem como proposição:

- A. Verificar se ocorre alterações nas propriedades modificações físico-químicas de superfícies de titânio após a descontaminação com Terpinen-4-ol e Carvacrol, e adesão e viabilidade celular de fibroblastos e osteoblastos. (Publicação 1)
- B. Avaliar o efeito antimicrobiano do Terpinen-4-ol e Carvacrol sobre *Porphyromonas gingivalis* e *Fusobacterium nucleatum*, e sua citotoxicidade sobre linhagem celular de fibroblastos. (Publicação 2)
- C. Avaliar a inibição e o efeito antibiofilme de Terpinen-4-ol e Carvacrol sobre biofilmes simples e multi-espécies de bactérias periodontopatogênicas a partir de concentrações não tóxicas sobre linhagem celular de queratinócitos. (Publicação 3)

3 PUBLICAÇÕES

3.1 Publicação 1

Influence of titanium surfaces decontamination with natural compounds on physical-chemical modifications and cell behavior

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ABSTRACT

It is not defined if natural compounds may alter the structure of implant surfaces and affect the initial wound healing as well as the long-term behavior of the implants. The aim of this study was to evaluate in vitro the influence of physico-chemical modifications caused by the decontamination of titanium surfaces with Carvacrol and Terpinen-4-ol on cell viability and proliferation of fibroblasts and osteoblasts. Ti surfaces were treated for 5 minutes with Carvacrol 0.5%, T4ol 0.059%, Chlorhexidine 0.2%, DMSO 2% or ultrapure water (Control group). The physico-chemical modifications were evaluated by SEM-EDS system; surface wettability and the surface free energy (SFE) were calculated from the contact angle values using the OWRK (Owens-Wendt-Rabel-Kaelble) equation. Cells were seeded onto Ti surfaces treated and incubated for 24 h and 72 h. The specimens were evaluated by Alamar blue assay and fluorescence microscopy. The results were compared using the Kruskal Wallis and Mann Whitney test and ANOVA two-way, with a significance level of 5%. EDS peaks demonstrated presence of O and Cl after CHX treatment. On the other hand, Carvacrol and Terpinen-4-ol showed the presence of Na, O and Cl. All surfaces showed hydrophilic characteristics and SFE values between 5.5 mN/m and 3.4 mN/m. Viability test and fluorescence microscopy showed a decrease of cells viability and adhesion after 24 h and 72 h on surfaces treated with CHX. These results indicate that Carvacrol and Terpinen-4-ol on Ti surfaces did not alter the surface properties and permit an adequate interaction with cells involved in the re-ossseointegration process such as fibroblasts and osteoblasts in contrast to Chlorhexidine 0.2%.

Keywords:

Surface Properties, Peri-implantitis, Dental Implants, Plant Extracts, Fibroblasts, Osteoblasts

INTRODUCTION

The treatment of the peri-implant disease is not defined and the implant decontamination is the main objective for the eradication of the biofilm around the dental implant. Different studies have been proposed including the use of ultrasonic scalers, laser therapy, chemical methods and surgical therapies. However, adjunctive therapies may improve the efficacy of peri-implantitis treatment. (Schwarz et al., 2015) Chlorhexidine; an antimicrobial agent, is commonly used as adjuvants in surgical and non-surgical therapies due to its substantivity, (Suarez et al., 2013) biological properties and broad-spectrum antibacterial activity. (Jones, 1997)

However, researchers are interested in investigate the use of alternative therapies using herbal derivatives for the control of microorganisms. Terpinen-4-ol is the main component of the essential oil *Melaleuca alternifolia* (TTO) and has been highlighted mainly because of the antimicrobial and anti-inflammatory activity. (Hart et al., 2000, Nogueira et al., 2014) Carvacrol; the main natural constituent of *Thymus vulgaris*, *Carum copticum* and *Oreganum* spp. (volatile oils), (Baser, 2008) presents different properties: antimicrobial, antioxidant, antimutagenic, antigenotoxic and hepatoprotective, highlighting its wide spectrum antimicrobial activity on pathogenic bacteria and yeasts including drug-resistant biofilm forming microorganisms (Ciandrini et al., 2014).

In this way, various antimicrobial agents and methods have been suggested for implant surface decontamination in order to achieve the re-osseointegration. Some studies investigated the biocompatibility of implant surfaces with cells peri-implant tissues after different treatments. (Valderrama et al., 2014; Yuan et al., 2014; Chellini et al., 2017) Moreover, there is still lack information to get a general understanding the influence of natural compounds on titanium surface properties as chemical structure and mainly on cell adhesion and proliferation around of dental implant.

This study was conducted to evaluate the influence of the decontamination of titanium surfaces with naturals compounds as Carvacrol and Terpene-4-ol on physical-chemical proprieties. Additionally, evaluate the effect of these surface modifications on cell viability and cell proliferation of Fibroblasts L929 and Osteoblasts SaOs-2.

MATERIAL AND METHODS

Test Solutions and specimens treatment

Terpinen-4-ol and Carvacrol (Sigma-Aldrich, USA) stock solutions were solubilized in Dimethyl sulfoxide (DMSO) 0.4% and 2% (Sigma-Aldrich, USA) respectively. (Botelho et al. 2007; Nogueria et al. 2014) In the current study, Carvacrol and Terpinen-4-ol were prepared at 0.5% and 0.059% respectively due to their different antimicrobial and anti-inflammatory properties focused in the treatment of periodontal disease (Ciandrini et al., 2014; Botelho et al., 2009; Nogueira et al., 2014). Chlorhexidine 0.2% was used as a positive control antimicrobial agent. (Verkaik et al., 2011)

Sterile titanium discs with SLA surface produced using TiO₂ microparticles for blasting and subsequent acid conditioning (5 mm diameter; thickness 2 mm) kindly provided by Implacil De Bortoli (Brazil) were used. The specimens were treated with Carvacrol 0.5%, T4ol 0.059%, DMSO 2% and CHX 0.2%; the durations of treatment were 5 minutes. (Schou et al. 2003; Ungvári et al. 2010) Ultrapure water was used as a negative control group. Subsequently, the samples were dried at room temperature (Ungvári et al., 2010).

Surface Wettability and surface-free energy (SFE)

The surface wettability and SFE were calculated from the contact angle ($n = 9$) using the sessile drop technique (Young-Laplace) as described by Huacho et al. (2017). Liquids with different polarities: distilled water (52.2), ethylene glycol (19.0), polyethylene glycol (13.6) and diiodomethane (2.6) were dropped (0.08 μ L) onto the discs. After, the contact angle had been measured; the arithmetic mean and standard deviation were calculated for the surface-free energy of each group of samples, using the OWRK (Owens-Wendt-Rabel-Kaeble) equation (Janssen et al., 2006).

Scanning electron microscopy and energy dispersive spectrometry

Three samples of each group were analyzed using Scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS) (JEOL JSM-7500F, JEOL Ltda, USA) in order to identify the chemical elements present on the surfaces after decontamination with Carvacrol, T4ol, DMSO and CHX. The discs were examined at 10 000x magnifications and voltage 10 kV.

Cell growth and proliferation

Cell culture and viability assay

Fibroblasts L929 and Osteoblasts SaOs-2 were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), with 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mmol/l glutamine (Gibco, USA) in an humidified incubator with 5% CO₂ and 95% air at 37°C. The cells were sub-cultured until an adequate number of cells were obtained for the study.

Carefully the discs were placed in sterile 96-well plates and 5×10^4 cells/well (200 µl) were seeded on the surfaces and maintained in the incubator under the conditions described for 24 h and 72 h. Then, after each period the cells were evaluated using Alamar Blue® assay (n=9) and Fluorescence Microscope (n=3).

Alamar Blue® assay was performed to assess the metabolic activity by the fluorimetric indicator and cell proliferation after each period. This assay allows the evaluation of the viability of the cells in culture, without prejudice to them. For this, the reagent Alamar Blue® (Invitrogen, USA) was used, which is based on the incorporation of the oxidation indicator that shows color change in response to chemical reduction of the culture medium due to aerobic respiration. Thus, Alamar Blue dye (10%) in cell culture medium without SFB was added to the cells and incubated at 37 °C in 5% CO₂ for 4 h. After this period, an aliquot of each well (100 µL) was transferred to a new 96-well plate and the fluorescence of the samples was read in a spectrophotometer (Synergy - H1 - Biotek Winooski, USA) at the wavelength of 570 (reduction) and 600 nm (oxidation).

Analysis by fluorescence microscope

Proliferation of fibroblasts and osteoblasts on Ti discs after 24 h and 72 h were assessed by Actin Red 555 ReadyProbes® (Invitrogen, USA), which is a selective high affinity Reactant of Actin filaments where the actin cytoskeleton is stained with TRITC-phalloidin (red) and the nuclei with Hoechst 33242 (blue). Subsequently, samples were fixed with Paraformaldehyde for 15 minutes and washed with PBS. Then, Triton x-100 (0.1%) was added for 10 minutes and washed with PBS. Then the cells with actin marker were incubated for 30 minutes, the reagent was aspirated and Hoechst was added for 10 minutes. Finally, the cells were analyzed in the

Fluorescence Microscope EVOS FL Imaging System (Thermo Fisher Scientific, USA) using red and blue filters.

Statistical analysis

Statistical analysis was performed using SPSS software version 17.0 (USA). Contact angle values were submitted to Kruskal-Wallis and Mann-Whitney nonparametric tests for comparison of paired groups, since they did not present normal distribution. All the absorbance values of Alamar Blue analysis were compared using two-way ANOVA. All the statistical inferences were considered at the level of significance of 5 % ($p < 0.05$).

RESULTS

Surface Wettability and SFE

The contact angle and SFE were calculated using the contact of liquids (degree), which are given in Table 1. Surface wettability (using distilled water) showed that the different treatments with DMSO, CHX, T4ol and Carvacrol led to a decrease of the contact angles when compared to the control group: 67.8 ± 1.9 ($p < 0.05$).

After measuring the contact angles and determining the wettability on the tested surfaces, the SFE was calculated for each group in relation to the polarity of the liquids used by applying the OWRK (Owens-Wendt-Rabel-Kaelble) equation. According to the values obtained and corresponding to the polar capacity of the surfaces, control group presented higher SFE value: 5.5 mN/m, and the surfaces decontaminated with DMSO, CHX, T4ol and Carvacrol presented lower ELS values: 3.4 mN/m, 3.7 mN/m, 3.8 mN/m and 4.0 mN/m respectively.

Table 1. Values of Surface-free Energy (SFE), mean and standard deviation of the contact angles in the different groups evaluated

Groups	Control	DMSO 2%	CHX 0.2%	T4ol 0.059%	Carvacrol 0.5%
Contact angle (°)					
Distilled water	67.8±1.9	18.21±1.6*	31.1±0.8*	25.73±0.6*	33.9±1.3*
Ethylene glycol	28.3±0.4	20.7±0.5	21.4±0.7	18.7±0.8	23.7±0.7
Polyethylene glycol	24.9±2.8	30.0±0.8	25.5±0.8	24.6±0.7	22.6±0.9
Diiodomethane	41.2±1.6	26.2±0.9	42.0±1.3	28.0±0.9	25.6±1.3
SFE (mN/m)	5.5	3.4	3.7	3.8	4.0

*Significant difference ($p<0.05$) compared with control group.

Note. The surface-free energy (mN/m) is shown as the value of its polar component.

Scanning electron microscopy and energy dispersive spectrometry analysis

Specimens from all groups were analyzed descriptively based on SEM findings, chemical analysis (EDS) and showed in the Figure 1. The images of the control group and DMSO revealed the presence of chemical elements: Titanium (Ti) and Aluminum (Al). For CHX group, the following elements were identified: Oxygen (O), Titanium (Ti), Aluminum (Al) and Chlorine (Cl). Also, for T4ol and Carvacrol groups were observed mainly: Oxygen (O), Titanium (Ti), Sodium (Na) Aluminum (Al) and Chlorine (Cl).

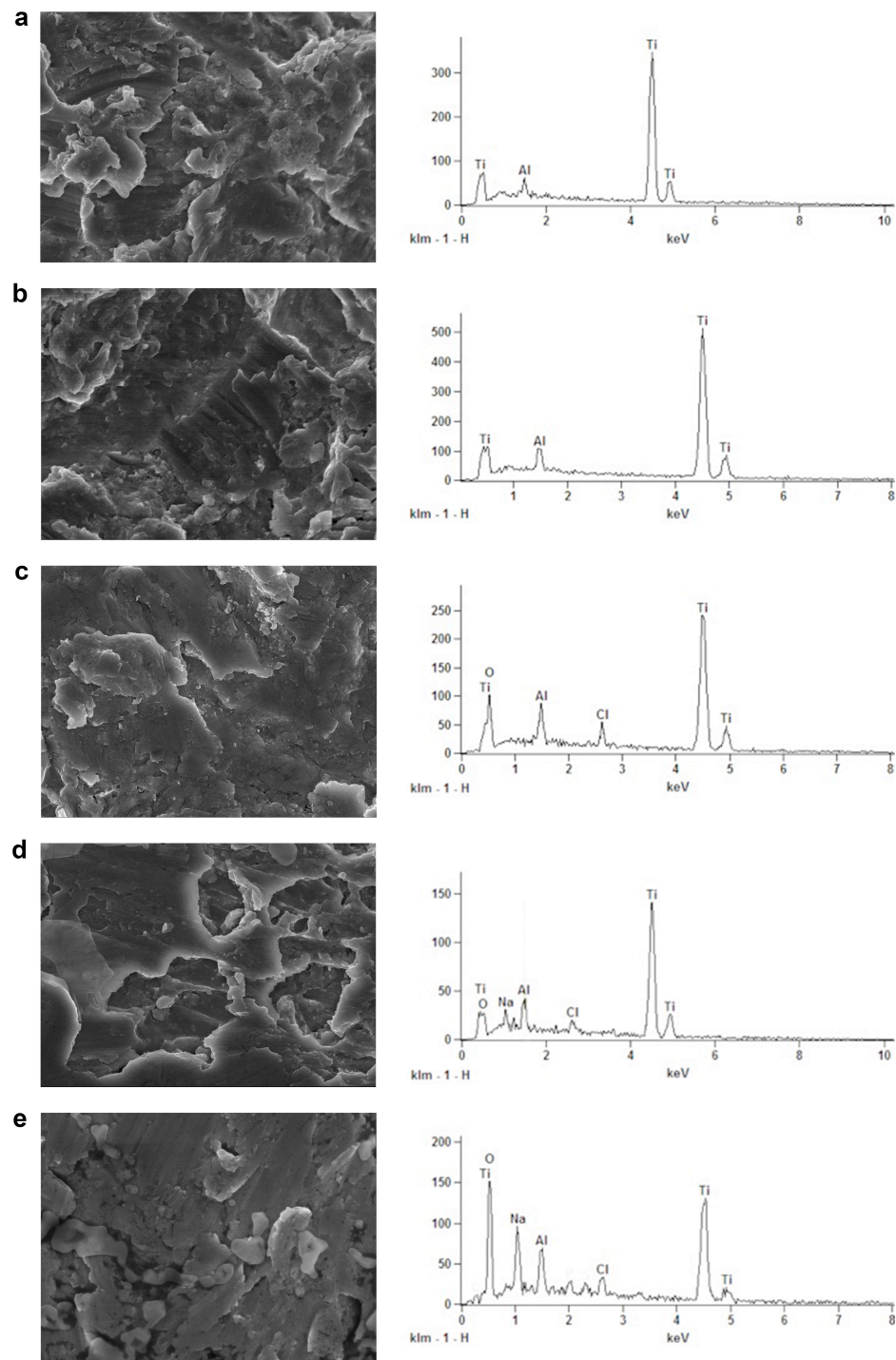


Figure 1. Photomicrographs and respective graphs representative of SEM/EDS analyses (original magnification 10 000x) of the chemical elements in each group: Control group (a), DMSO 2% (b), CHX 0.2% (c), T4ol 0.059% (d) and Carvacrol 0.5% (e).

Cell viability and cell proliferation

The results showed that the viability of fibroblast L929 (Figure 2) was affected when cultivated on surfaces treated with DMSO, CHX and T4ol at 72 h ($p < 0,0001$). A greater decrease of cell viability was observed on surfaces treated with CHX at 24 h and 72 h. However, the control group as well as those treated with Carvacrol did not affect cell viability during the evaluated periods ($p > 0.05$).

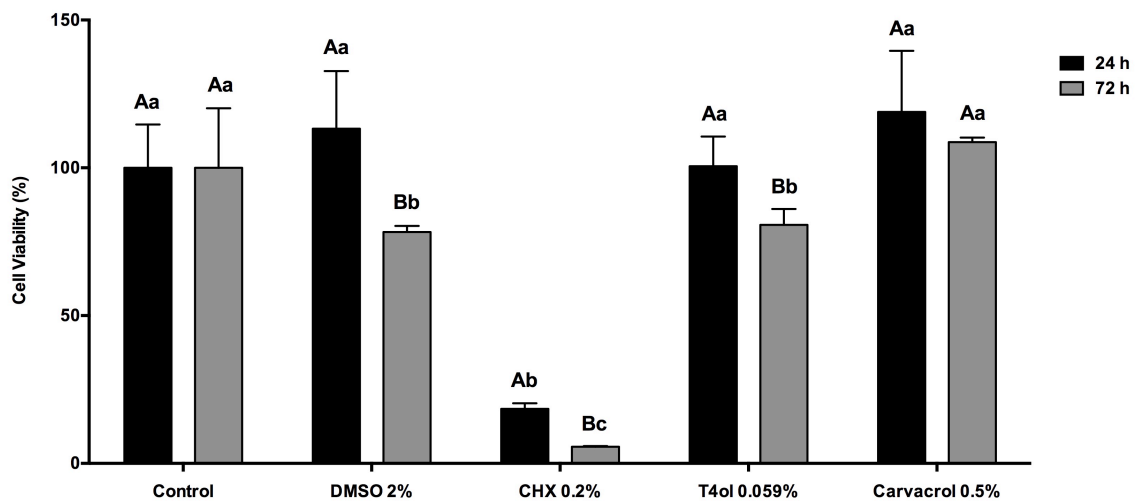


Figure 2. Cell viability of Fibroblast L929 (%) for each experimental group at the set evaluated periods. Uppercase letters allow comparison among the evaluated periods for each experimental group. Lowercase letters allow comparison among the groups within each evaluated period. Different letters show significant differences (ANOVA two-way, $p < 0,05$).

The results of Osteoblasts SaOs-2 viability are shown in Figure 3. Surfaces treated with DMSO and Carvacrol showed decreased cell viability after 72 h ($p < 0.0001$). However, surfaces treated with CHX revealed a significant decrease at 24 and 72 hours ($p < 0.0001$).

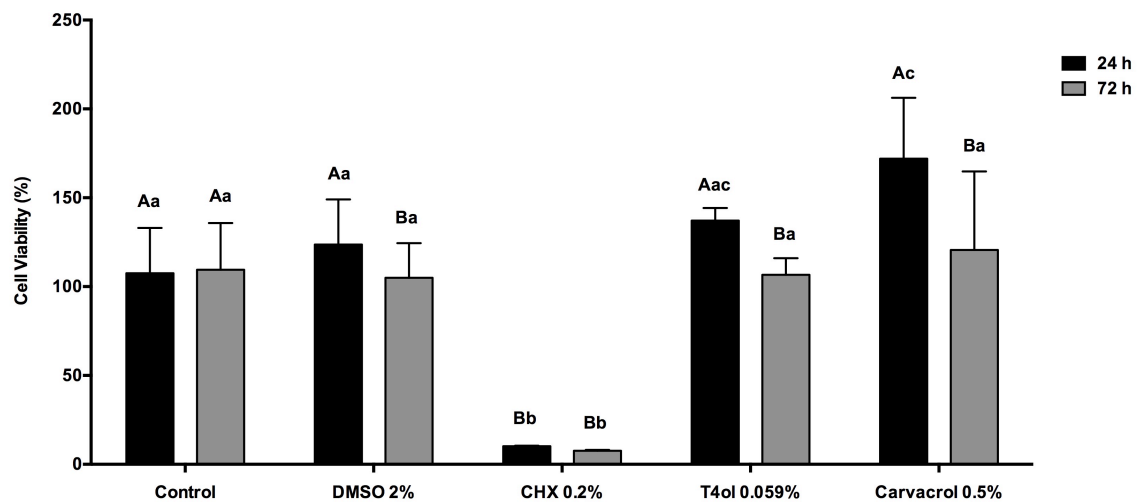


Figure 3. Cell viability of Osteoblasts SaOs-2 (%) for each experimental group at the set evaluated period. Uppercase letters allow comparison among the evaluated periods for each experimental group. Lowercase letters allow comparison among the groups within each evaluated period. Different letters show significant differences (ANOVA two-way, $p < 0,05$).

The proliferation of Fibroblasts L929 and Osteoblasts SaOs-2 (Figure 4) was also qualitatively assessed by the imuno-identification of actin filaments, which demonstrated similar results to cell viability data. For both cell lines, the photomicrographs showed extensive cell damage for surface treated with CHX.

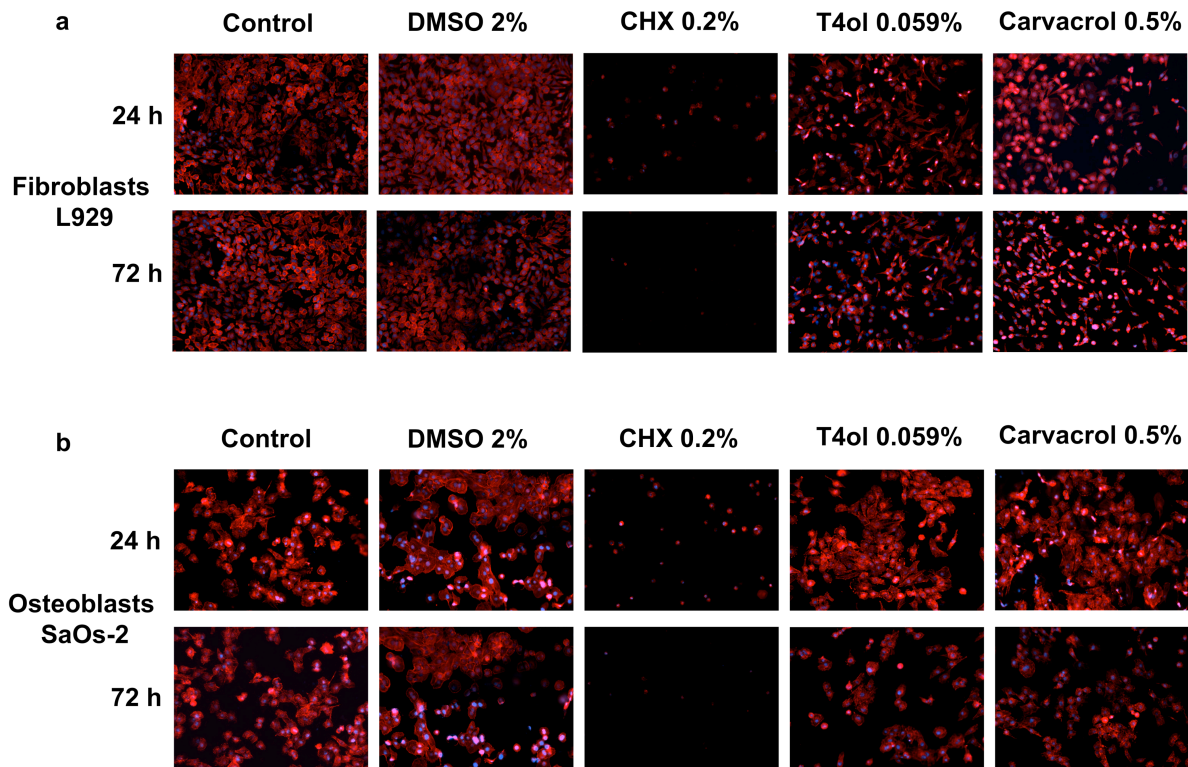


Figure 4. Photomicrographs of cytoskeletal identification of actin (red) of Fibroblasts L929 (a) and Osteoblasts SaOs-2 (b) adhered to treated surfaces for 24 and 72 h. Nuclei were stained with Hoescht (x10).

DISCUSSION

The main objective of the peri-implantitis treatment besides eliminating the biofilm is to achieve the re-osseointegration. Therefore, it is important that the surface decontaminated had characteristics suitable for cell adhesion and proliferation. In this context, the cell adhesion can be affected by different surface properties such as surface energy, roughness and chemical composition. (Anselme et al., 2010; Teng et al., 2012) Within these properties, the wettability is also an important parameter to understanding the cell behavior. Contact angle $<90^\circ$ has the tendency to wet the surface more and it become more interactive and hydrophilic. (Yua and Lee 2013) In this study, all surfaces showed a hydrophilic characteristic and groups treated showed a decrease value of contact angle compared with the control group (67.8 ± 1.9) thus, increasing their wettability propriety. (Tab 1) In the same way, Al-Radha et al. (2013) reported similar data after evaluate the surface wettability on titanium discs treated with *cinnamon oil*, *clove oil* and CHX.

The SFE represents the surface tension of each material and it is associated to the state of equilibrium of the atoms in the surface. So, if the SFE is high, there are more activated ions available on this surface and it can interact more easily with biological tissues improving the cell adhesion, provided that the cells have the same chemical nature as the surface (Yan et al., 2017). In this study, the results of SFE are presented in relation to the polar component and the surfaces treated with natural compounds showed SFE values of 3.8 and 4.0 mN/m when compared to the control group. This change did not influence in the cell adhesion at 24 h of fibroblasts and osteoblasts as shown in our results.

The surfaces treated with CHX, Carvacrol and T4ol (natural compounds) showed lipophilic characteristic and SFE values similar to the control group, only surfaces treated with natural compounds allow an adequate cell adhesion and proliferation of fibroblasts and osteoblasts (Figure 2, Figure 3, Figure 4) in contrast to CHX where this same cell behavior could not be observed. This can be attributed due to the presence of Chlorine found on its surface. When, treated discs with CHX come into contact with the cells, it will be in an aqueous solution, in this case, culture medium, which will lead to a dissociation of the CHX molecule, releasing to the medium hydrogen and chlorine atoms, allowing its ionic interaction, forming HCl molecules, making the pH acidified, damaging the cell viability. Our results differ from

those reported by Ungvári et al. (2010) that found the presence of Carbon and Oxygen bond on surface treated with CHX gel. On the other hand, surfaces treated with Carvacrol and T4ol showed the presence of Sodium and Chlorine atoms, which when bound, due to contact with aqueous medium would form a NaCl (salt), that would not alter pH and allows cells to adhere normally to the surface.

Additionally, although the CHX did not modify the surface properties, our results of viability for both cells showed that the surface treated with CHX induced a greater decrease in the cell viability and alterations of the actin cytoskeleton. So, this effect on the actin cytoskeleton reduces the adhesion ability of the cells affecting matrix components and receptors. (Chellini et al., 2017; Cline and Layman, 1992; Balloni et al., 2016) Our results showed high levels of cell death and apoptosis in both cell types at 24 h and 72 h. Similar results of osteoblast-like Saos2 behavior on surfaces pre-treated with CHX 0.2% for 5 minutes was related by Chellini et al. (2017). Also, due to cationic nature of the CHX molecule, rough titanium surface can adsorb and release it slowly over 24 h (CHX 0.2%); this effect is referred to as substantivity. (Kozlovsky et al., 2006) This property can improve its antibacterial effect and at the same time influence on the cell viability of fibroblasts and osteoblasts. Since, CHX has toxic effects on different cell types, causing total cell death by adenosine triphosphate (ATP) depletion and can induce the inhibition of mitochondrial activity, protein and DNA synthesis, and cell proliferation (Hidalgo and Dominguez, 2001).

Based on the findings of the present study, it could be demonstrated that titanium surfaces treated with natural compounds as Carvacrol and Terpene-4-ol permit the proliferation of fibroblasts and osteoblasts, cells involved in the re-osseointegration process. In addition, they can be considered as an option in the treatment of peri-implantitis because authors related their antimicrobial proprieties and the presence of them on the titanium surfaces did not affect the surface properties and cell viability as showed in this study. In contrast, the presence of Chlorhexidine showed to be toxic and influenced in the cell behavior and viability. However, further studies are necessary to understand this cell behavior considering that during the decontamination and re-osseointegration process there are other cell types and the presence of biofilm on the surface can be affect the surface properties and consequently may influence on the cell adhesion.

Disclosure statement

The authors declare that they have no conflict of interest.

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REFERENCES

1. Al-Radha AS, Younes C, Diab BS, Jenkinson HF. 2013. Essential oils and zirconia dental implant materials. *Int J Oral Maxillofac Implants*. 28(6):1497-1505.
2. Anselme K, Davidson P, Popa AM, Giazson M, Liley M, Ploux L. 2010. The interaction of cells and bacteria with surfaces structured at the nanometre scale. *Acta Biomater*. 6(10):3824-3846.
3. Balloni S, Locci P, Lumare A, Marinucci L. 2016. Cytotoxicity of three commercial mouthrinses on extracellular matrix metabolism and human gingival cell behaviour. *Toxicol in Vitro*. 34:88-96.
4. Baser KH. 2008. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. *Curr Pharm Des*. 14(29):3106-3119.
5. Botelho MA, Nogueira NA, Bastos GM, Fonseca SG, Lemos TL, Matos FJ, Montenegro D, Heukelbach J, Rao VS, Brito GA. 2007. Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. *Braz J Med Biol Res*. 40(3):349-356.
6. Botelho MA, Martins JG, Ruela RS, I R, Santos JA, Soares JB, França MC, Montenegro D, Ruela WS, Barros LP, Queiroz DB, Araujo RS, Sampo FC. 2009.

- Protective effect of locally applied carvacrol gel on ligature-induced periodontitis in rats: A tapping Mode AFM study. *Phytother Res.* 23(10):1439-1448.
7. Chellini F, Giannelli M, Tani A, Ballerini L, Vallone L, Nosi D, Zecchi-Orlandini S, Sassoli C. 2017. Mesenchymal stromal cell and osteoblast responses to oxidized titanium surfaces pre-treated with $\lambda = 808$ nm GaAlAs diode laser or chlorhexidine: in vitro study. *Lasers Med Sci.* 32(6):1309-1320.
 8. Ciandrini E, Campana R, Federici S, Manti A, Battistelli M, Falcieri E, Papa S, Baffone W. 2014. In vitro activity of Carvacrol against titanium-adherent oral biofilms and planktonic cultures. *Clin Oral Investig.* 18(8): 2001-2013.
 9. Cline NV, Layman DL. 1992. The effects of chlorhexidine on the attachment and growth of cultured human periodontal cells. *J Periodontol.* 63:598-602.
 10. Hart PH, Brand C, Carson CF, Riley TV, Prager RH, Finlay-Jones JJ. 2000. Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. *Inflamm res.* 49:619-626.
 11. Hidalgo E, Dominguez C. 2001. Mechanisms underlying chlorhexidine-induced cytotoxicity. *Toxicol In Vitro.* 15(4), 271-276.
 12. Huacho PMM, Nogueira MNM, Basso FG, Jafelicci Junior M, Francisconi RS, Spolidorio DMP. 2017. Analyses of Biofilm on Implant Abutment Surfaces Coating with Diamond-Like Carbon and Biocompatibility. *Braz Dent J.* 28(3):317-323.
 13. Janssen D, De Palma R, Verlaak S, Heremans P, Dehaen W. 2006. Static solvent contact angle measurements, surface energy and wettability determination of various self-assembled monolayers on silicon dioxide. *Thin solid films.* 515:1433-1438.
 14. Jones CG. 1997. Chlorhexidine: is it still the gold standard? *Periodontol* 2000. 15:55-62.
 15. Kozlovsky A, Artzi Z, Moses O, Kamin-Belsky N, Greenstein RB. 2006. Interaction of chlorhexidine with smooth and rough types of titanium surfaces. *J Periodontol.* 77:1194-1200.

16. Nogueira MN, Aquino SG, Rossa Junior C, Spolidorio DM. 2014. Terpinen-4-ol and alpha-terpineol (tea tree oil components) inhibit the production of IL-1 β , IL-6 and IL-10 on human macrophages. *Inflamm Res*. 63(9):769-778.
17. Schou S, Holmstrup P, Jørgensen T, Skovgaard LT, Stoltze K, Hjørting-Hansen E, Wenzel A. 2003. Implant surface preparation in the surgical treatment of experimental peri-implantitis with autogenous bone graft and ePTFE membrane in cynomolgus monkeys. *Clin Oral Implants Res*. 14(4):412-422.
18. Schwarz F, Becker K, Sager M. 2015. Efficacy of professionally administered plaque removal with or without adjunctive measures for the treatment of peri-implant mucositis. A systematic review and meta-analysis. *J Clin Periodontol*. 42:S202-213.
19. Suarez F, Monje A, Galindo-Moreno P, Wang HL. 2013. Implant surface detoxification: a comprehensive review. *Implant Dent*. 22(5): 465-473.
20. Teng FY, Ko CL, Kuo HN, Hu JJ, Lin JH, Lou CW, Hung CC, Wang YL, Cheng CY, Chen WC. 2012. A comparison of epithelial cells, fibroblasts, and osteoblasts in dental implant titanium topographies. *Bioinorg Chem Appl*. 2012: 687291. doi: 10.1155/2012/687291.
21. Ungvári K, Pelsöczy IK, Kormos B, Oszkó A, Rakonczay Z, Kemény L, Radnai M, Nagy K, Fazekas A, Turzó K. 2010. Effects on titanium implant surfaces of chemical agents used for the treatment of peri-implantitis. *J Biomed Mater Res B Appl Biomater*. 94(1): 222-229.
22. Valderrama P, Blansett J, Gonzalez MG, Cantu MG, Wilson TG. 2014. Detoxification of implant surfaces affected by peri-implant disease: an overview of non-surgical methods. *Open Dent J*. 8:77-84.
23. Verkaik M, Busscher H, Jager D, Slomp A, Abbas A, Van der Mei H. 2011. Efficacy of natural anti-microbials in toothpaste formulations against oral biofilms in vitro. *J Dent*. 39:218-224.
24. Yan Y, Chibowski E, Szcześ A. 2017. Surface properties of Ti-6Al-4V alloy part I: Surface roughness and apparent surface free energy. *Mater Sci Eng C Mater Biol Appl*. 70(1):207-215.

25. Yuan Y., Lee T.R. Surface Science Techniques. Berlin, Heidelberg: Springer Series. 2013. Chapter 1, Contact Angle and Wetting Properties; p. 3-34.
26. Yuan K, Chan YJ, Kung KC, Lee TM. 2014. Comparison of osseointegration on various implant surfaces after bacterial contamination and cleaning: a rabbit study. Int J Oral Maxillofac Implants. 29:32-40.

3.2 Publicação 2^{..}

In vitro antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces

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Abstract

The objective of this study was to evaluate the antibacterial properties of Carvacrol and Terpinen-4-ol against *Porphyromonas gingivalis* (*Pg*) and *Fusobacterium nucleatum* (*Fn*) and cytotoxic effects on fibroblasts cells. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were used. The minimum biofilm inhibition concentration (MBIC) was evaluated by XTT. Biofilm decontamination on titanium surfaces was quantified (CFU mL⁻¹), evaluated by Confocal Scanning Laser Microscopy (CLSM) and cytotoxic activity by MTT. MIC and MBC were Carvacrol 0.007% and 0.002% for *P. gingivalis* and *F. nucleatum* and Terpinen-4-ol 0.06% for both microorganisms. MBIC was Carvacrol 0.03%, 0.06% and Terpinen-4-ol 0.06%, 0.24%. It was observed through CFU mL⁻¹ and CLSM antibiofilm activity using Carvacrol (0.26%, 0.06%) and Terpinen-4-ol (0.95%, 0.24%), and its showed cytotoxic activity similar to CHX. However, Terpinen-4-ol 0.24% showed higher cell viability. Carvacrol and Terpinen-4-ol showed antibacterial activity reducing biofilms. Moreover, chlorhexidine-like cytotoxicity was observed.

Keywords: Carvacrol, Terpinen-4-ol, Peri-implantitis, antibacterial activity, biofilm, cytotoxic activity

Introduction

Formation of bacterial biofilms on dental implant material surfaces (titanium) may lead to the development of peri-implant diseases, influencing the long term success of dental implants (Mahato et al. 2016). The presence of periodontopathic bacteria is the main pathogens cause in the process of the development and progression of peri-implant diseases; and the microenvironment around implants may favor the colonization of anaerobic Gram-negative bacteria (Renvert and Quyrinen 2015). Thus, the microbiota associated with peri-implant disease may be described as a polymicrobial infection with predominance of anaerobic microorganisms (Belibasakis 2014; Renvert and Quyrinen 2015; Mombelli and Decaillet 2011).

Various treatment modalities have been put forward for the treatment of peri-implantitis, including regenerative techniques and decontamination methods (Figuro et al. 2014; Mahato et al. 2016). However, there is no consensus for the treatment of peri-implant infections due to the heterogeneity of the studies and the variability of the types of implant surfaces (Mellado-Valero et al. 2013). Thus, the application of antimicrobial agents for the surface biofilm removal seems to be a useful tool for the success of treatment outcome in the implanted patients (Mellado-Valero et al. 2013; Mahato et al. 2016; Lindhe and Meyle 2008).

Different natural products have been investigated as promising agents for surface biofilm removal, prevention and treatment of periodontal and peri-implant diseases (Ciandrini et al. 2014; Botelho et al. 2007). Carvacrol, a monoterpene phenol (2-methyl-5- (1-methylethyl) phenol) is found in the volatile oils of *Thymus vulgaris*, *Carum copticum* and *Oreganum spp* and it is the main natural constituent (70%) of these aromatic plants (Baser, 2008). It presents different activities, and its antimicrobial activity of broad spectrum of pathogenic microorganisms including bacteria and yeast biofilms drug-resistant can be highlighted (Ciandrini et al. 2014; Dalleau et al. 2008).

Another phytotherapeutic agent that has been highlighted is the Tea Tree essential oil (*Melaleuca alternifolia* - TTO), which has Australian origin. TTO has antibacterial, antifungal, antiviral, anticancer and anti-inflammatory proprieties (Catalán et al. 2008; Kwieciński et al. 2009; Greay et al. 2010; Hart et al. 2000). TTO comprises about 100 parts, and the Terpinen-4-ol, the major component (30-40%)

that has been highlighted by assigning mainly antimicrobial and anti-inflammatory activities (Hart et al. 2000; Nogueira et al. 2014).

The first purpose of this research was to evaluate in vitro the effectiveness of antibacterial properties of Carvacrol and Terpinen-4-ol against peri-implant pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in planktonic state, single-species and two-species biofilms on titanium discs. Additionally, the cytotoxic effects of Carvacrol and Terpinen-4-ol were evaluated on fibroblasts cells.

Material and methods

Bacterial conditions

Porphyromonas gingivalis (ATCC 33277) and *Fusobacterium nucleatum* (NCTC 10326), used in this study, were grown on Brucella agar (Becton Dickinson, Heidelberg, Germany) supplemented with 5% sheep blood, hemin (10 mg/mL) and menadione (5 mg/mL) for 72 h at 37 °C in anaerobic chamber (atmosphere of 85% N₂, 10% H₂ and 5% CO₂) (Don Whitley – England). Broth cultures were prepared in Brain-heart infusion (BHI) broth supplemented with 1% yeast extract, hemin (5 mg/mL) and menadion (10 mg/mL) and incubated for 48 h in anaerobic conditions.

Antimicrobials preparation

Serial dilutions of Carvacrol (0.26 – 0.0005 % v/v) (Sigma, Chemical Co., St. Louis, MO.) with 2% dimethylsulfoxide (DMSO) (Sigma, Chemical Co.)(Botelho et al. 2007) and Terpinen-4-ol (3.8 – 0.001 % v/v) with 0.4% DMSO were prepared in BHI medium (Nogueira et al. 2014). Chlorhexidine stock solution (CHX 2%) was used as a positive control and was prepared at 0.2%.

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined according to the CLSI M11-A8 (2012). Serial doubling dilutions of Carvacrol and Terpinen-4-ol were prepared and distributed in 96-well plate. The final inoculum concentration (200 µL) of each bacteria was 1.0×10^5 CFU mL⁻¹ per well. Bacteria suspension without Carvacrol or Terpinen-4-ol served as a positive control and BHI medium as a blank and negative control.

After incubation under anaerobic conditions at 37°C for 48 h (Ciandrini et al. 2014), bacterial growth was monitored by absorbance at 600 nm using a micro plate reader (Synergy H1 Multi-Mode Reader – BioTek). The MIC was defined as the lowest Carvacrol and Terpinen-4-ol concentration without bacteria growth. MBC was obtained after plating 10 µL aliquots from each well on Brucella agar and defined as the lowest concentration of Carvacrol and Terpinen-4-ol at which 99.9% of the bacteria were killed (Kwieciński et al. 2009). The plates were incubated at 37°C for 72 h under anaerobic conditions.

Biofilm assays

Biofilm metabolic activity assessment using the XTT

Single-specie biofilms formed by each bacteria (*P. gingivalis* and *F. nucleatum*) were developed in 96-well plates as described by Ciandrini et al. 2014 with some modifications. Unstimulated human saliva (CAAE 49473115.0.0000.5416) was collected in the morning from healthy individual without active caries lesions and periodontal diseases (Moura et al. 2006) For the formation of a salivary pellicle, 50 µL of sterile human saliva was added into each well and incubated with for 4 h with gently shaken at 37 °C (Ciandrini et al. 2014). After, the saliva was removed and the wells were washed with PBS (Phosphate Buffered Saline) and 200 µL of each bacterial suspension (1.0×10^7 CFU mL⁻¹) was inoculated. The plates were incubated at 37° C for 48 h. In brief, after 48 h each well was washed with PBS, and dilutions of Carvacrol and Terpinen-4-ol were added (200 µL) and incubated for 24 h in anaerobic conditions. BHI medium was used as non-treated group and blank control. The bacterial metabolic activity following the exposure to Carvacrol and Terpinen-4-ol was measured by XTT reduction assay (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide – Sigma, Chemical Co.) that metabolically reduces sodium to a soluble formazan product by the cells (Banzi et al. 2014). The plates were incubated at 37°C for 3 h and the absorbance of the supernatant was measured at 492 nm using an ELISA reader (Synergy H1 Multi-Mode Reader – BioTek). The minimum biofilm inhibition concentration (MBIC) was defined as the lowest concentration tested, which was inhibited by 50% of the biofilm formation (Miladi et al. 2016).

Biofilm formation on specimens

Single and two-species biofilms were developed on sterile titanium discs with SLA surface produced using TiO₂ microparticles for blasting and subsequent acid conditioning (5 mm; thickness 2 mm) kindly provided by Implacil De Bortoli (São Paulo, Brazil) as described by Ciandrini et al. 2014 with modifications. Subsequently, the titanium discs were placed in sterile 24-well plates and the salivary pellicle was preformed as described for the single biofilm. Titanium discs were placed in sterile 24-well plate and 1 mL of each bacteria for single biofilms or 500 µL of each oral bacterial suspension (1.0×10^7 CFU mL⁻¹) for two-species biofilm was inoculated. Biofilms were incubated at 37° C for 5 days in BHI supplemented as described, and the medium was changed every 24 h. After the period the discs were transferred to new wells containing 1 mL of Carvacrol 0.26% and 0.06% or Terpinen-4-ol 0.95% and 0.24%, these were, among others, the lowest and the best tested concentrations from MBIC, that revealed antimicrobial effectiveness and prevention of biofilm formation on *P. gingivalis* and *F. nucleatum*. The discs were incubated in anaerobic conditions at 37° C for 5 minutes, according to the usual clinical protocols (Schou et al. 2003; Ungvári et al. 2010). After this, all discs were washed with PBS and transferred to sterile eppendorfs with 1 mL of physiological saline. Then, titanium discs were maintained in ultrasonic for 20 min, scraped carefully for 1 minute and harvested by vigorous vortexing for 1 min. The resulting material (bacteria adherent) was diluted and plated on Brucella agar for 48-72 h at 37° C under anaerobic conditions for further quantitative analysis of CFU mL⁻¹.

Laser-scanning confocal microscopy (CLSM)

After the exposure with the different solutions, titanium discs were washed gently with sterile PBS to remove unattached bacteria. The bacteria were then labeled with 0.01 mM of Syto-9 and 0.06 mM of propidium iodide (PI), Live/Dead stain BacLight Bacterial Viability Kit (Invitrogen Corporation) for 15 minutes, according to the manufacturer's instructions. The excitation/emission wavelengths were 488/500 nm for SYTO 9 and 488/635 nm for propidium iodide. Fluorescence from the stained cells was viewed by CLSM (LSM 780 inverted, Zeiss, Germany). CLSM images were acquired using software (ZEN 2012, Zeiss) at a resolution of 1,024 by 1,024 pixels. Images from a single focal plane of the biofilm were captured

by the system using a 20x magnification lens. The area selected for analysis of the biofilm was defined randomly and not so close to the edge of the specimens.

L929 fibroblasts culture and cytotoxicity analysis

L929 mouse fibroblast cells were seeded at a density of 1.0×10^4 cells/well into a 96-well plates (100 μ L) in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Chemical Co.) culture medium supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/mL penicillin and 100 μ g/mL streptomycin (Gibco, Grand Island, NY); and incubated at 37°C in an humidified incubator with 5% CO₂ for 24 h. Thereafter, the culture medium of the confluent cell monolayers was replaced by Terpinen-4-ol 0.95%, 0.24%, Carvacrol 0.26%, 0.06%, DMSO 0.4%, 2%, CHX 0.2% and DMEM alone (negative control group). Then the plates were incubated for 5 minutes, 1 h and 24 h. After each period, the cytotoxic effects were evaluated using the MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide – Sigma, Chemical Co.). (Basso et al. 2013) Afterwards, the culture medium was aspirated and replaced by 100 μ L of MTT solution (5 mg/mL) and incubated at 37° C for 4 h. Then, the culture medium with the MTT solution was replaced by 100 μ L of acidified isopropanol solution (0.04 N HCl) to dissolve the violet formazan crystals producing a homogenous bluish solution resulting from the cleavage of the MTT salt ring by the SDH enzyme present in the mitochondria of viable cells. Cell viability was evaluated by spectrophotometry (570 nm) with an ELISA plate reader (Synergy H1 Multi-Mode Reader – BioTek).

Statistical analysis

The SPSS 17.0 software (IBM) was used to perform the statistical analysis. The antimicrobial activity and the comparison of the numbers of bacteria ($\log_{10}$) after antimicrobial treatments for single biofilms formed on titanium discs were statistically analyzed by Kruskal–Wallis non-parametric test complemented by Mann-Whitney tests, while the biofilm inhibition and numbers of bacteria for two-species biofilm on titanium discs were evaluated using ANOVA and Tukey tests. All the absorbance values of the cytotoxicity analysis were compared using two-way ANOVA. All the statistical inferences were considered at the level of significance of 5 %. All experiments were repeated three times at different days.

Results

Antibacterial activity and inhibition of biofilm development

The bacteriostatic, bactericidal and the inhibition of biofilm effectiveness were tested in planktonic cultures and biofilm developed in well plates, using different concentration ranges of the Carvacrol (from 0.0005% to 0.26%) and Terpinen-4-ol (0.001% to 3.8%). The MIC, MBC and MBIC values of the antimicrobials agents tested against *P. gingivalis* and *F. nucleatum* are shown in Table 1. *P. gingivalis* and *F. nucleatum* were sensitive to Carvacrol with MICs values of 0.007% and 0.002%, respectively and Terpinen-4-ol with MICs values of 0.06% to both bacteria. The results of the MIC and MBC experiments were consistent. MBICs values of Carvacrol were 0.03% and 0.06% and Terpinen-4-ol 0.06% and 0.24% to *P. gingivalis* and *F. nucleatum*, respectively.

[Table 1 near here]

Effect of carvacrol and terpinen-4-ol on biofilm viability

The inhibitory activity of Carvacrol and Terpinen-4-ol against single and two-species biofilm of *P. gingivalis* and *F. nucleatum* developed on titanium surfaces were determined. (Fig. 1, 2 and 3) The efficacy of Terpinen-4-ol (0.95% and 0.24%) and Carvacrol (0.26% and 0.06%) induced a statistically significant reduction of viable cell as compared with untreated biofilm of *P. gingivalis* ($p < 0.0001$) (Fig. 1a). The Figure 2a and 3a showed antimicrobial activity of Terpinen-4-ol 0.95%, Carvacrol 0.26 and 0.06% similar to CHX 0.2% and significantly different to control group biofilm of *F. nucleatum* and two-species biofilm ($p < 0.0001$). *F. nucleatum* and two-species biofilms treated with Terpinen-4-ol 0.24% was not significantly lower compared to the control groups ($p > 0.05$). This Terpinen-4-ol concentration showed similar results that CHX 0.2% on two-species biofilm of *P. gingivalis* and *F. nucleatum*.

CLSM images (Fig. 1b, 2b and 3b) revealed the presence of microorganisms throughout the extension of the substrate. The biofilm thickness after 5 days of cultivation ranged from 25–30 μm in this experimental setting. In the images, the live biofilms were stained and emitted a green fluorescence signal, while the dead one was stained and produce red fluorescence. The no treated control biofilm emitted

green fluorescence in all biofilms. Biofilm of *P. gingivalis* treated with CHX 0.2% emitted obvious red fluorescence and inconspicuous green fluorescence and almost completely red fluorescence with Terpinen-4-ol (0.95% and 0.24%) and Carvacrol (0.26% and 0.06%)(Fig. 1b). Compared to the control group, CHX 0.2% and Carvacrol 0.06% had a significant impact on the viability of *F. nucleatum* biofilm. After the treatment with Terpinen-4-ol 0.95% and 0.24%, *F. nucleatum* biofilms exhibited extensive green areas, and a great deal of cells still survived. In contrast, treatment with Carvacrol 0.26% almost completely killed the bacterial cells within single species biofilms formed on titanium discs by *F. nucleatum* (Fig. 2b). Furthermore, the treatment with CHX 0.2% and Carvacrol 0.06% had little effect on two-species biofilm; and the treatment with Terpinen-4-ol 0.95% and 0.24% led to a significant increase in the red areas of the two-species biofilms. However, Carvacrol 0.26% was able to effectively kill the majority of cells in the biofilm (Fig. 3b)

[Figure 1 near here]

[Figure 2 near here]

[Figure 3 near here]

Cell viability analysis (MTT assay)

The viability of the L929 mouse fibroblast cells are summarized in Figure 4. The number of cells in the control group was considered as 100%. The number of cells progressively increased after 5 min ($93.75 \pm 16.42\%$), 1 h ($95.67 \pm 19.12\%$) and 24 h ($121.87 \pm 10.95\%$). DMSO 2% and DMSO 0.4% did not significantly change the relative growth of fibroblast cells in comparison with the control group after 5 min and 1h of exposure ($p > 0.05$). However, DMSO 2% and 0.4 caused a statistically significant reduction of fibroblast cells after 24 h of exposure in comparison with the control group ($p < 0.0001$). Chlorhexidine 0.2% caused a decrease in the number of cells as compared with the control group after 5 min, 1 h and 24 h ($p < 0.0001$). Terpinen-4-ol 0.95% and 0.24% caused a significant decrease in the number of cells after 5 min ($7.89 \pm 5.07\%$ and $52.8 \pm 16.19\%$) 1 h ($11.23 \pm 3.9\%$ and $28.66 \pm 10.40\%$) and 24 h ($3.09 \pm 0.92\%$ and $2.97 \pm 0.75\%$). The differences were significant in comparison with the control group ($p < 0.0001$). However, the fibroblast cells after 5 min of exposure with Terpinen-4-ol 0.24% showed high viability as compared with CHX 0.2%, the difference was significant: $52.80 \pm 16.19\%$ vs. $7.99 \pm 3.44\%$

($p < 0.0001$). Furthermore, after 5 min, 1 h and 24 h of exposure with Carvacrol 0.26% ($6.83 \pm 1.90\%$, $6.36 \pm 5.50\%$ and $2.32 \pm 1.04\%$) and Carvacrol 0.06% ($3.85 \pm 0.90\%$, $6.10 \pm 2.37\%$ and $5.62 \pm 3.03\%$) cell viability showed a significant decrease as compared with the control group ($p < 0.0001$).

[Figure 4 near here]

Discussion

The present study showed antibacterial effect of Carvacrol and Terpinen-4-ol against peri-implant pathogens *P. gingivalis* and *F. nucleatum*. There are few data in the literature about the antimicrobial effect of Carvacrol on periodontopathic bacteria. MIC and MBC results (Table 1) showed values lower than those reported by Ciandrini et al. 2014 who, like us, tested Carvacrol on *P. gingivalis* and *F. nucleatum* and obtained MIC values of 0.25% and MBC values of 0.50%. On the other hand, the novelty of this study lies in the fact that there are not studies in the literature to comparing the antibacterial activity of Terpinen-4-ol on *P. gingivalis* and *F. nucleatum*. Terpinen-4-ol has been shown to be the main active component of *Melaleuca alternifolia* (TTO) (Hart et al. 2000; Nogueira et al. 2014) and several studies have reported TTO antimicrobial activity, promoting bactericidal and bacteriostatic effects at low concentrations against *P. gingivalis* W83 (Graziano et al. 2016) and ATCC 33277 (Takarada et al. 2004), and against *F. nucleatum* and *P. gingivalis* isolates from oral cavity (Hammer et al. 2003).

Like in natural teeth, on the implant surface the salivary mucoproteins form a pellicle and it is a crucial event for the bacterial adhesion (Busscher et al. 2010; Hahnel et al. 2008). Thus, in the current study unstimulated human saliva was used for the biofilm formation to simulate clinical conditions. A preliminary assessment of MBIC by XTT assay was carried out to evaluate the lowest concentration tested, which was inhibited by 50% of the biofilm formation (Miladi et al. 2016) for each bacteria. Although XTT is a semi quantitative colorimetric assay, it has been demonstrated that this method correlates well with other quantitative techniques such as ATP and CFU assays (Ramage et al. 2002; Jin et al. 2004). In the presence of Carvacrol and Terpinen-4-ol, the metabolic activity of cells in biofilms was distinctly reduced after 24 h of incubation on preformed biofilm of 48 h (Table 1). The results

also showed that the two reference strains were more susceptible to the Carvacrol at lower concentrations than Terpinen-4-ol. MBIC of *P. gingivalis* and *F. nucleatum* varies between from 0.03 to 0.06% (Carvacrol) and 0.06 to 0.24% (Terpinen-4-ol) suggesting the ability of these compounds to prevention of biofilm formation. These results indicate that both Carvacrol and Terpinen-4-ol used in this study have an effect on the metabolic activity of the biofilm. In contrast to our results, Ciandrini et al. 2014 showed biofilm inhibition concentration (BIC) values of Carvacrol 1% tested on single biofilm of *P. gingivalis* and *F. nucleatum*.

Biofilms are organized and structured microbial communities, embedded in polymeric matrices and release planktonic cells to colonize surfaces (Belibasakis 2014). Implant surfaces have been modified in various ways to increase bone formation around the implant and to increase the healing capacity of the bone tissue. Surfaces sandblasting with Titanium Oxide produced high osteoconductivity and good bone formation (Gehrke et al. 2016). In this way, this type of surfaces benefit in the osseointegration process. However, the physicochemical properties (higher free energy) and texture characteristics (higher roughness) of the implant surfaces may favor bacterial colonization. John et al. 2015 showed that SLA has higher plaque accumulation in comparison with machined-modified acid-etched surface. Thus, this surface type can be a disadvantage for the removal of the mineralized and mature plaque in patients. In the present in vitro study by the characteristics of the surface and the maturity of biofilms the main difficulty in the evaluation of the biofilms development on titanium surfaces in spite of the procedures performed was during the detachment of biofilms.

As observed from CLSM and CFU, the results showed a statistical difference significant of Carvacrol (0.26% and 0.06%), Terpinen-4-ol (0.95%) and CHX 0.2% on the biofilm viability of single and two-species mature biofilm of *P. gingivalis* and *F. nucleatum* compared to the control group. (Fig. 1, 2 and 3) In contrast to the results shown by Ciandrini et al. 2014, where Carvacrol was most effective on immature biofilms, the results in this study showed that Carvacrol and Terpinen-4-ol were effective on mature biofilms of 5 days. In addition, single biofilms of *P. gingivalis* seem to be more susceptible to Carvacrol and Terpinen-4-ol when compared to CHX. Although the results in this study do not show statistically significant differences between the test groups and CHX, it is possible to observe that on mature biofilm model of *F. nucleatum* and two pathogenic bacterial species, Carvacrol 0.26% shows

a greater effect in the inhibition of biofilm and this significant effect can be justified due to its mechanism of action. Monoterpenes, such as Carvacrol and Terpinen-4-ol, due to its lipophilic character have a mechanism of bacterial action based on inhibition peptidoglycan synthesis, damage microbial membrane structures (Cox et al. 2000) and modification bacterial membrane surface hydrophobicity, increasing its fluidity and permeability (Ultee et al. 1999). Due to, the expansion of the membrane phospholipids by increasing spaces, ion leakage might occur and consequently attenuate the permeable barrier role of the cell membrane (Turi et al. 1997; Ultee et al. 1999). Also, interrupts genetic material synthesis, induces coagulation of cytoplasmic constituents, forms toxic compounds, interrupts normal cell communication (quorum sensing) and reduces pH inside the cell (Radulovic et al., 2013). In contrast to monoterpenes, Chlorhexidine cause membrane disruption and inhibition of proteolytic and glycosidic enzymes, inhibiting and reducing the viability of microorganisms (Ready et al. 2015). Furthermore, CHX seems to have a limited distribution in the extracellular polymer matrix and therefore not have total efficacy in eradicating biofilms (Corbin et al. 2011; Brindle et al. 2011), which corroborate with the results of this study.

Focusing on the clinical application of new substances such as Carvacrol and Terpinen-4-ol, is necessary to determine their possible toxic effect on the cells. In this study, the cells were exposed for different contact times to the substances tested and regardless of the concentration, the results revealed a concentration-and-time-dependent decrease in all concentrations assayed for Carvacrol, Terpinen-4-ol and CHX (Fig. 4). The monoterpenes Carvacrol and Terpinen-4-ol are isomeric. Despite having the same molecular formula, they exhibit different levels of cytotoxic activity against cell lines. Thus, unlike the other concentrations evaluated, after 5 minutes of contact, Terpinen-4-ol 0.24% showed mild grade of cytotoxicity when compared with the control group (93.75%) according to the ISO 10993-5 2009 Standard: Biological Evaluation of Medical Devices: In vitro Method. In this concern, the aim of this study was evaluated the toxic effects of Carvacrol and Terpinen-4-ol on the fibroblasts line, since these cells are involved in the re-osseointegration and osseointegration process.

The results show the cytotoxic effect of Carvacrol on fibroblasts based on the best concentrations with antibiofilm effect. Nevertheless, few studies have been reported on the cytotoxicity of Carvacrol in fibroblasts. Melo et al. 2014 reported IC₅₀

value (Concentration that inhibited 50% of cell growth) 62.5 µg/mL of Carvacrol in the MRC5 (normal human fibroblast). In contrast, Houdkova et al. 2017, showed lowest cytotoxicity for Carvacrol with IC₅₀ value 5.09 µg/mL using the same cells line used by Melo et al. 2014. On the other hand, several studies reported cytotoxic effects of Carvacrol on different cells such as HepG2 (Yin et al. 2012), SiHa, Hela (Mehdi et al. 2011), Caco 2 cells (Dusan et al. 2006; Llana-Ruiz-Cabello et al. 2014), human oral cancer (Liang and Lu 2012), on human metastatic breast cancer cell line (Arunasree 2010) and HL-60 promyelocytic and Jurkat T lymphoma cells (Bhakkiyalakshmi et al. 2016). There are no studies that evidence the cytotoxic effect of Terpinen-4-ol on fibroblasts line. However, this compound exhibited cytotoxic effects on murine AE17 mesothelioma and B16 melanoma tumor cell lines (Greay et al. 2010) and Jurkat cells (Döll-Boscardin et al. 2012).

It is difficult to compare the results of this study with other investigations about these monoterpenes because some factors as pH, bacteria strains, incubation time, temperature, concentration tests and others factors can influence the results. A limitation of this study was the use of only two pathogen oral species to develop single and two-species biofilm. However, this is a preliminary study and further studies with multi-species oral biofilm will be necessary in order to mimic the complex peri-implant microbiology. Although some clinical and animal studies reported the efficacy of Carvacrol 0.02% and 0.5% in the treatment of periodontitis, these studies are limited to the total reduction of the biofilm and do not differentiate the effect of Carvacrol on each of the bacteria present in this complex biofilm (Shahab et al. 2011; Botelho et al. 2009). Furthermore, considering the few number of investigations about cytotoxic effects of Carvacrol and Terpinen-4-ol on fibroblasts, this paper can also open the way for future development of therapeutic opportunities against peri-implantitis.

Conclusion

Carvacrol and Terpinen-4-ol showed antimicrobial activity, promoting bactericidal and bacteriostatic effects at low concentrations and due to its antimicrobial and antibiofilm properties, both monoterpenes might represent a promising approach for prevention and treatment of peri-implant diseases. Also, considering the better concentration of antimicrobial and antibiofilm activity of Carvacrol and Terpinen-4-ol, chlorhexidine-like cytotoxicity was observed. Nevertheless results suggest that Terpinen-4-ol 0.24% has a lower cytotoxicity profile than CHX.

Disclosure statement

The authors declare that they have no conflict of interest.

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References

1. Arunasree, KM. 2010. Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB231. *Phytomedicine*. 17:581-588.
2. Banzi ÉC, Costa AR, Puppim-Rontani RM, Babu J, García-Godoy F. 2014. Inhibitory effects of a cured antibacterial bonding system on viability and metabolic activity of oral bacteria. *Dent Mater*. 30(9):238-244.
3. Baser KH. 2008. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. *Curr Pharm Des*. 14(29):3106-3119.

4. Basso FG, Oliveira CF, Kurachi C, Hebling J, de Souza Costa CA. 2013. Biostimulatory effect of low-level laser therapy on keratinocytes in vitro. *Lasers Med Sci.* 28:367-374.
5. Belibasakis GN. 2014. Microbiological and immuno-pathological aspects of peri-implant diseases. *Arch Oral Biol.* 59(1):66-72.
6. Bhakkiyalakshmi E, Suganya N, Sireesh D, Krishnamurthi K, Saravana Devi S, Rajaguru P, Ramkumar KM. 2016. Carvacrol induces mitochondria-mediated apoptosis in HL-60 promyelocytic and Jurkat T lymphoma cells. *Eur J Pharmacol.* 772:92-98.
7. Botelho MA, Nogueira NA, Bastos GM, Fonseca SG, Lemos TL, Matos FJ, Montenegro D, Heukelbach J, Rao VS, Brito GA. 2007. Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. *Braz J Med Biol Res.* 40(3):349-356.
8. Botelho MA, Martins JG, Ruela RS, I R, Santos JA, Soares JB, França MC, Montenegro D, Ruela WS, Barros LP et al. 2009. Protective effect of locally applied carvacrol gel on ligature-induced periodontitis in rats: a tapping mode AFM study. *Phytother Res.* 23(10):1439-1448.
9. Brindle ER, Miller DA, Stewart PS. 2011. Hydrodynamic deformation and removal of *Staphylococcus epidermidis* biofilms treated with urea, chlorhexidine, iron chloride, or DispersinB. *Biotechnol Bioeng.* 108:2968-2977.
10. Busscher HJ, Rinastiti M, Siswomihardjo W, van der Mei HC. 2010. Biofilm formation on dental restorative and implant materials. *J Dent Res.* 89(7):657-665.
11. Catalán A, Pacheco JG, Martínez A, Mondaca MA. 2008. In vitro and in vivo activity of *Melaleuca alternifolia* mixed with tissue conditioner on *Candida albicans*. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 105(3):327-332.
12. Ciandrini E, Campana R, Federici S, Manti A, Battistelli M, Falcieri E, Papa S, Baffone W. 2014. In vitro activity of Carvacrol against titanium-adherent oral biofilms and planktonic cultures. *Clin Oral Investig.* 18(8):2001-2013.
13. CLSI – Clinical and Laboratory Standard Institute. 2012. Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard. 8thed. Wayne, PA, CLSI document, M11-A8.

14. Corbin A, Pitts B, Parker A, Stewart PS. 2011. Antimicrobial penetration and efficacy in an in vitro oral biofilm model. *Antimicrob Agents Chemother.* 55:3338-3344.
15. Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmington JR, Wyllie SG. 2000. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J Appl Microbiol.* 88:170-175.
16. Dalleau S, Cateau E, Bergès T, Berjeaud JM, Imbert C. 2008. In vitro activity of terpenes against *Candida* biofilms. *Int J Antimicrob Agents.* 31:572-576.
17. Döll-Boscardin PM, Sartoratto A, Sales Maia BH, Padilha de Paula J, Nakashima T, Farago PV, Kanunfre CC. 2012. In Vitro Cytotoxic Potential of Essential Oils of *Eucalyptus benthamii* and Its Related Terpenes on Tumor Cell Lines. *Evid Based Complement Alternat Med.* 2012:342652.
18. Dusan F, Marián S, Katarína D, Dobroslava B. 2006. Essential oils-their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability *Toxicol in Vitro.* 20,1435-1445.
19. Figuero E, Graziani F, Sanz I, Herrera D, Sanz M. 2014. Management of peri-implant mucositis and peri-implantitis. *Periodontol 2000.* 66(1):255-273.
20. Gehrke SA, Ramírez-Fernandez MP, Granero Marín JM, Barbosa Salles M, Del Fabbro M, Calvo Guirado JL. 2016. A comparative evaluation between aluminium and titanium dioxide microparticles for blasting the surface titanium dental implants: an experimental study in rabbits. *Clin Oral Implants Res.* 0:1-6.
21. Graziano TS, Calil CM, Sartoratto A, Franco GC, Groppo FC, Cogo-Müller K. 2016. In vitro effects of *Melaleuca alternifolia* essential oil on growth and production of volatile sulphur compounds by oral bacteria. *J Appl Oral Sci.* 24(6):582-589.
22. Greay SJ, Ireland DJ, Kissick HTA, Levy MW, Beilharz TV, Riley CF, Carson CF. 2010. Induction of necrosis and cell cycle arrest in murine cancer cell lines by *Melaleuca alternifolia* (tea tree) oil and terpinen-4-ol. *Cancer Chemother Pharmacol.* 65:877-888.
23. Hahnel S, Rosentritt M, Handel G, Burgers R. 2008. Influence of saliva substitute films on initial *Streptococcus mutans* adhesion to enamel and dental substrata. *J Dent.* 36(12):977-983.

24. Hart PH, Brand C, Carson CF, Riley TV, Prager RH, Finlay-Jones JJ. 2000. Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. *Inflamm res.* 49:619-626.
25. Hammer KA, Dry L, Johnson M, Michalak EM, Carson CF, Riley TV. 2003. Susceptibility of oral bacteria to *Melaleuca alternifolia* (tea tree) oil in vitro. *Oral Microbiol Immunol.* 18:389-392.
26. Houdkova M, Rondevaldova J, Dorskocil I, Kokoska L. 2017. Evaluation of antibacterial potential and toxicity of plant volatile compounds using new broth microdilution volatilization method and modified MTT assay. *Fitoterapia.* 118:56-62.
27. ISO - International Organization for Standardization. 1992. Biological evaluation of medical devices-part 5. Tests for cy-toxicity: in vitro methods. Geneve, ISO document, 10993-5.
28. Jin Y, Samaranayake LP, Samaranayake Y, Yip HK. 2004. Biofilm formation of *Candida albicans* is variably affected by saliva and dietary sugars. *Arch Oral Biol.* 49,789-798.
29. John G, Becker J, Schwarz F. 2015. Modified implant surface with slower and less initial biofilm formation. *Clin Implant Dent Relat Res.* 17(3):461-468.
30. Kwiecinski J, Eick S, Wójcik K. 2009. Effects of tea tree (*Melaleuca alternifolia*) oil on *Staphylococcus aureus* in biofilms and stationary growth phase. *Int J Antimicrob Agents.* 33:343-347.
31. Liang WZ, Lu CH. 2012. Carvacrol-induced $[Ca^{2+}]$ rise and apoptosis in human glioblastoma cells. *Life Sci.* 90:703-711.
32. Lindhe J, Meyle J. 2008. Peri-implant diseases:Consensus report of the sixth european workshop on periodontology. *J Clin Periodontol.* 35(8):282-285.
33. Llana-Ruiz-Cabello M, Gutiérrez-Praena D, Pichardo S, Moreno FJ, Bermúdez JM, Aucejo S, Cameán AM. 2014. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. *Food Chem Toxicol.* 64:281-290.
34. Mahato N, Wu X, Wang L. 2016. Management of peri-implantitis: a systematic review, 2010-2015. *Springerplus.* 5:105.
35. Mehdi SJ, Ahmad A, Irshad M, Manzoor N, Rizvi MMA. 2011. Cytotoxic effect of Carvacrol on human cervical cancer cells. *Biol Med.* 3(2):307-312.

36. Mellado-Valero A, Buitrago-Vera P, Solá-Ruiz MF, Ferrer-García JC. 2013. Decontamination of dental implant surface in peri-implantitis treatment: a literature review. *Med Oral Patol Oral Cir Bucal*. 18(6):869-876.
37. Melo JO, Fachin AL, Rizo WF, Jesus HC, Arrigoni-Blank MF, Alves PB, Marins MA, França SC, Blank AF. 2014. Cytotoxic effects of essential oils from three *Lippia gracilis* Schauer genotypes on HeLa, B16, and MCF-7 cells and normal human fibroblasts. *Genet Mol Res*. 13(2):2691-2697.
38. Miladi H, Mili D, Ben Slama R, Zouari S, Ammar E, Bakhrouf A. 2016. Antibiofilm formation and anti-adhesive property of three mediterranean essential oils against a foodborne pathogen *Salmonella* strain. *Microb Pathog*. 93:22-31.
39. Mombelli A, Decaillet F. 2011. The characteristics of biofilms in peri-implant disease. *J Clin Periodontol*. 38(11):203-213.
40. Moura JS, da Silva WP, Pereira T, Del Bel Cury AA, Garcia RCR. 2006. Influence of acrylic resin polymerization methods and saliva on the adherence of four *Candida* species. *J Prosthet Dent*. 96(3):205-211.
41. Nogueira MN, Aquino SG, Rossa Junior C, Spolidorio DM. 2014. Terpinen-4-ol and alpha-terpineol (tea tree oil components) inhibit the production of IL-1 β , IL-6 and IL-10 on human macrophages. *Inflamm Res*. 63(9):769-778.
42. Ramage G, Vande Walle K, Bachmann SP, Wickes BL, López-Ribot JL. 2002. In vitro pharmacodynamic properties of three antifungal agents against preformed *Candida albicans* biofilms determined by time-kill studies. *Antimicrob Agents Chemother*. 46:3634-3636.
43. Ready D, Theodoridis G, Green I, Ciric L, Pratten J, Tay W, McDonald A. 2015. In vitro evaluation of the antibiofilm properties of chlorhexidine and delmopinol on dental implant surfaces. *Int J Antimicrob Agents*. 45:662-666.
44. Renvert S, Quirynen M. 2015. Risk indicators for peri-implantitis. A narrative review. *Clin Oral Implants Res*. 26(11):15-44.
45. Schou S, Holmstrup P, Jørgensen T, Skovgaard LT, Stoltze K, Hjørting-Hansen E, Wenzel A. 2003. Implant surface preparation in the surgical treatment of experimental peri-implantitis with autogenous bone graft and ePTFE membrane in cynomolgus monkeys. *Clin Oral Implants Res*. 14(4):412-422.

46. Shahab A, Haghighati F, Baeeri M, Jamalifar H, Abdollahi M. 2011. A clinical, microbiological and immunological comparison between subgingival irrigation with Dentol and chlorhexidine in advanced periodontitis. *Arch Med Sci.* 7(1):154-160.
47. Takarada K, Kimizuka R, Takahashi N, Honma K, Okuda K, Kato T. 2004. A comparison of the antibacterial efficacies of essential oils against oral pathogens. *Oral Microbiol Immunol.* 19(1):61-64.
48. Turi M, Turi E, Koljalg S, Mikelsaar M. 1997. Influence of aqueous extracts of medicinal plants on surface hydrophobicity of *Escherichia coli* strains of different origin. *APMIS.* 105:956-962.
49. Ultee A, Bennink MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol.* 68:1561-1568.
50. Ungvári K, Pelsöczy IK, Kormos B, Oszkó A, Rakonczay Z, Kemény L, Radnai M, Nagy K, Fazekas A, Turzó K. 2010. Effects on titanium implant surfaces of chemical agents used for the treatment of peri-implantitis. *J Biomed Mater Res B Appl Biomater.* 94(1):222-229.
51. Yin Q, Yan F, Zu XY, Wu Y, Wu X, Liao M, Deng S, Yin L, Zhuang Y. 2012. Anti-proliferative and pro-apoptotic effect of carvacrol on human hepatocellular carcinoma cell line HepG-2. *Cytotechnology.* 64(1):43-51.

Table

Table 1 Antibacterial activity and inhibition of biofilm development of Carvacrol and Terpinen-4-ol against *Porphyromonas gingivalis* and *Fusobacterium nucleatum*

Bacterial strains	Carvacrol			Terpinen-4-ol		
	^a MIC(%)	^b MBC(%)	^c MBIC(%)	^a MIC(%)	^b MBC(%)	^c MBIC(%)
<i>Porphyromonas gingivalis</i>	0.007	0.007	0.03	0.06	0.06	0.06
<i>Fusobacterium nucleatum</i>	0.002	0.002	0.06	0.06	0.06	0.24

^a Minimum inhibitory concentration.

^b Minimum bactericidal concentration

^c Minimum biofilm inhibition concentration

Figures

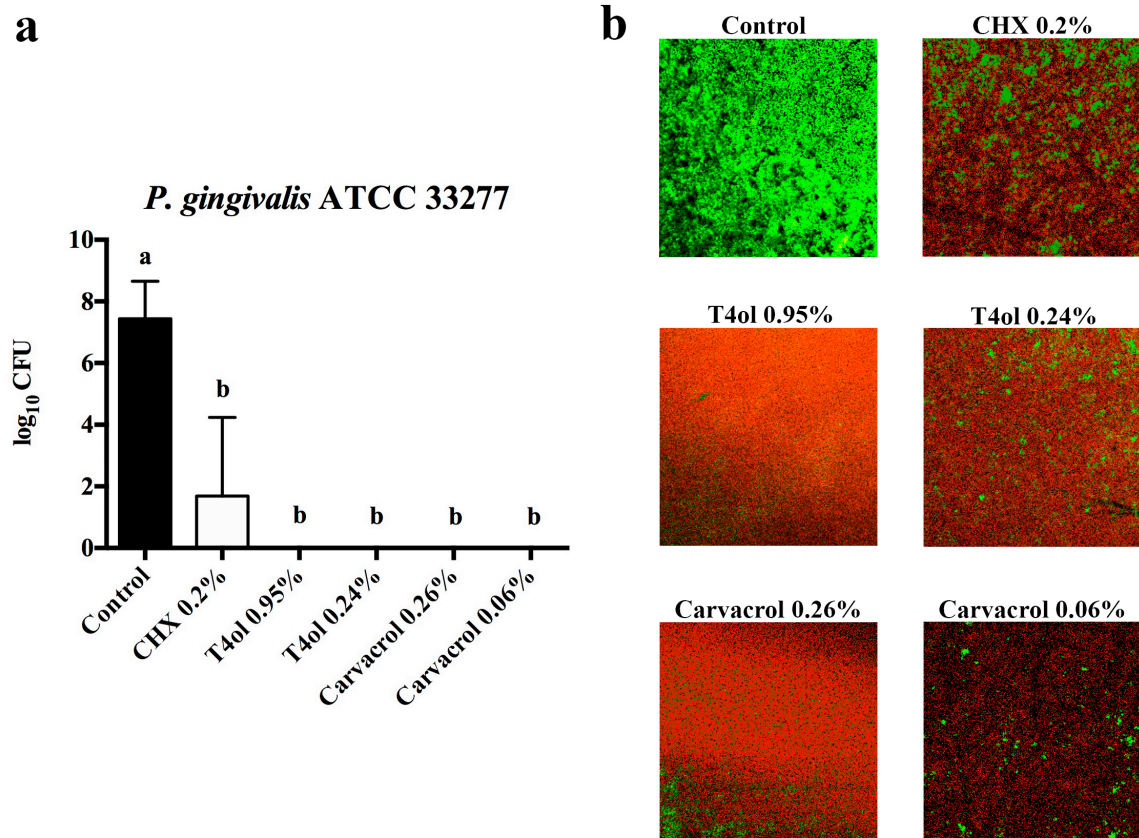


Fig. 1 (a) Effect of Carvacrol (0.26% and 0.06%) and Terpinen-4-ol (0.95% and 0.24%) in comparison with CHX 0.2%, against single biofilm of *P. gingivalis* (on titanium surfaces after 5 days of incubation). Statistical analysis was performed using Kruskal–Wallis non-parametric test complemented by Mann-Whitney tests ($p < 0.05$). (b) Representative CLSM images of untreated *P. gingivalis* biofilm (control), *P. gingivalis* biofilm treated with 0.2% chlorhexidine (CHX), Terpinen-4-ol 0.95%, Terpinen-4-ol 0.24%, Carvacrol 0.26% and Carvacrol 0.06%. Dead cells stained red, live cells stained green.

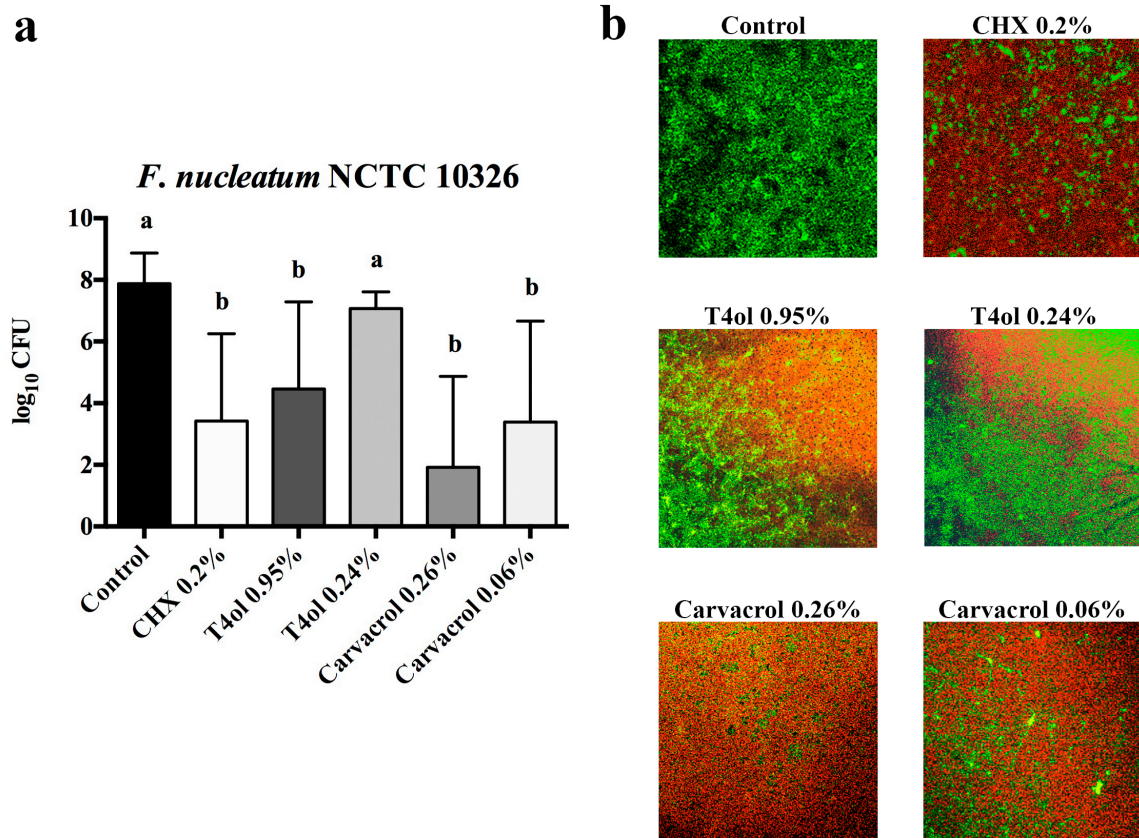


Fig. 2 (a) Effect of Carvacrol (0.26% and 0.06%) and Terpinen-4-ol (0.95% and 0.24%), in comparison with CHX 0.2%, against single biofilm of *F. nucleatum* (on titanium surfaces after 5 days of incubation). Statistical analysis was performed using Kruskal–Wallis non-parametric test complemented by Mann-Whitney tests ($p < 0.05$). (b) Representative CLSM images of untreated *F. nucleatum* biofilm (control), *F. nucleatum* biofilm treated with 0.2% chlorhexidine (CHX), Terpinen-4-ol 0.95%, Terpinen-4-ol 0.24%, Carvacrol 0.26% and Carvacrol 0.06%. Dead cells stained red, live cells stained green.

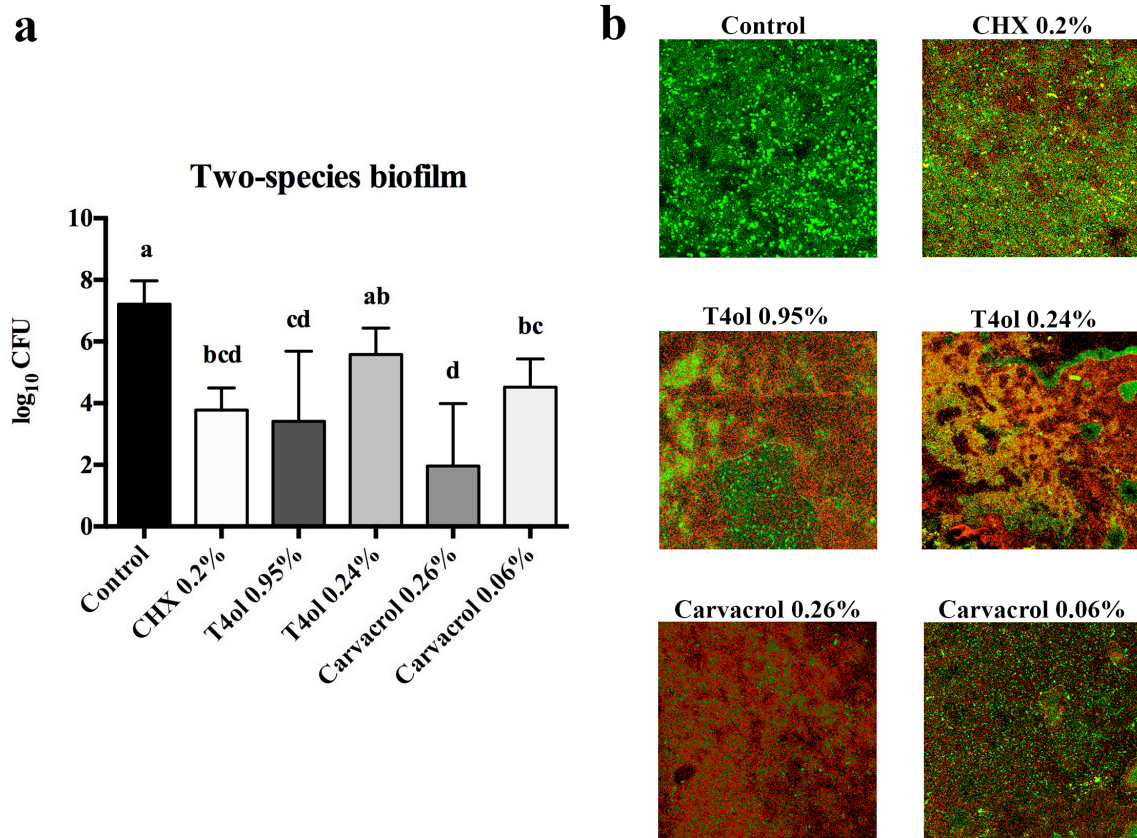


Fig. 3 (a) Effect of Carvacrol (0.26% and 0.06%) and Terpinen-4-ol (0.95% and 0.24%), in comparison with CHX 0.2%, against two-species biofilm of *P. gingivalis* and *F. nucleatum* (on titanium surfaces after 5 days of incubation). Statistical analysis was performed using ANOVA and Tukey tests ($p < 0.05$). (b) Representative CLSM images of two-species biofilm of *P. gingivalis* and *F. nucleatum* (control), two-species biofilm treated with 0.2% chlorhexidine (CHX), Terpinen-4-ol 0.95%, Terpinen-4-ol 0.24%, Carvacrol 0.26% and Carvacrol 0.06%. Dead cells stained red, live cells stained green.

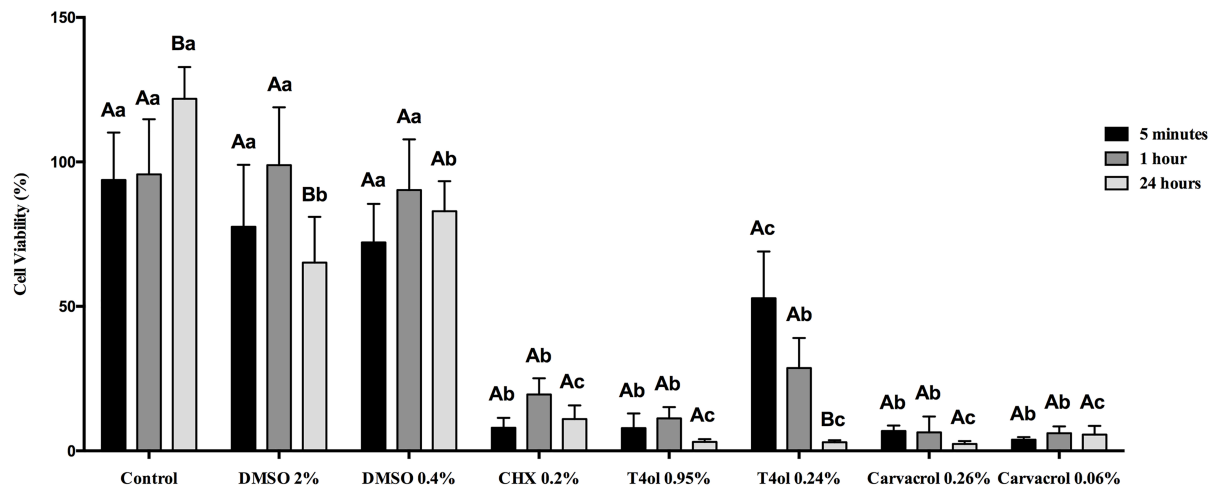


Fig. 4 Cell viability (% of control) of fibroblasts L929 according to the treatment of Carvacrol and Terpine-4-ol for different exposure time lengths. Capital letters allow comparisons among the times for the same group and lowercase letters allow comparisons among the groups for the same time. Groups represented by different letters are statistically different (two-way ANOVA, $p < 0.05$)

3.1 Publicação 3

Terpinen-4-ol and Carvacrol affect multi-species biofilms

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ABSTRACT

Terpinen-4-ol (T4ol) and carvacrol are well-known natural antimicrobial compounds. However, only a limited number of studies provide robust evidence of their effects on the growth of periodontal pathogens. The aim of this study was to investigate the cytotoxic activity and inhibitory effect of T4ol and Carvacrol against single- and multi-species biofilms. The toxicity of each compound was tested on oral keratinocytes and evaluated by XTT assay. Inhibition and eradication of single-species biofilm was analyzed by crystal violet assay and the effect on multi-species biofilms was evaluated by qPCR. T4ol and carvacrol did not affect the epithelial cell viability, in contrast to CHX, which showed a high cytotoxic effect. Inhibition and eradication of single-species biofilms treated with T4ol and carvacrol were observed. The same inhibitory effect was observed for multi-species biofilms, especially on periodontal pathogens. In conclusion, specific concentrations of T4ol and carvacrol without toxicity towards the human cells reduced the numbers of periodontal pathogens in single- and multi-species biofilms.

Keywords: Terpinen-4-ol, carvacrol, peri-implantitis, periodontal disease, multi-species biofilm

INTRODUCTION

Periodontal and peri-implant diseases are oral biofilm induced infections that result in a local and systematic inflammatory response, leading to periodontal or peri-implant tissue destruction (Ivanovski and Lee, 2018; Paster et al., 2006). Therefore, their treatment is focused on the removal of the oral biofilm, which is often supplemented with adjuvants like antibiotics (systemic or local) or antiseptic agents (Smeets et al., 2014; Fellman et al., 2010). On the other hand, regular use of these adjuvants can cause various side effects in patients, as well as possible bacterial drug resistance and bacterial necrotrophy (Robert and Moellering, 1998, Rodriguez Herrero et al., 2017). Chlorhexidine (CHX), a commonly used antimicrobial agent, reduces the biofilm formation of pathogenic microorganisms and exhibits substantivity for a long period (Seymour et al., 1992). However, toxic effects of CHX on different cell types at clinical concentrations have already been reported (Hidalgo and Dominguez, 2001).

Currently, medicinal plants and their constituents are extensively studied because of their biological anti-inflammatory, antioxidant, antifungal and antibacterial properties (Ciandrini et al., 2014; Catalán et al., 2008; Botelho et al., 2007). For instance, *Thymus vulgaris*, *Carum copticum*, *Oreganum spp.* and *Melaleuca alternifolia* were found to have a broad-spectrum *in vitro* activity against Gram-positive and Gram-negative bacteria including antimicrobial-resistant and multi-resistant organisms (Baser, 2008; Carson et al., 1995; Hammer et al., 1996). Terpinen-4-ol and carvacrol are the major components that contribute to the antimicrobial activity of essential oils (Cox et al., 2000; Baser, 2008).

Several *in vitro* biofilm models have been developed in an attempt to reproduce the complexity and heterogeneity of oral biofilms in order to study the effect of antimicrobial treatments (Busscher et al., 2010; Belibasakis et al., 2015). Oral biofilms are defined as complex and heterogeneous bacterial communities that are attached to different oral surfaces such as teeth, dental implants and prosthetic devices (Belibasakis et al., 2015). Since the biofilm structure partially abolishes the effectiveness of antimicrobials, mainly by reducing the diffusion of the antimicrobial compounds (Hoiby et al., 2010), it is necessary to find new antibacterial compounds to effectively combat oral biofilms.

Therefore, the aim of this study was to investigate the antimicrobial effect of non-toxic concentrations of terpinen-4-ol and carvacrol against periodontal single- and multi-species biofilms.

MATERIALS AND METHODS

Stock solutions

Terpinen-4-ol (Sigma, Chemical Co., USA) and carvacrol (Sigma, Chemical Co., USA) were prepared in 2% and 0.4% dimethylsulfoxide (DMSO) (Sigma, Chemical Co., USA) respectively. (Botelho et al. 2007; Nogueria et al. 2014). Later dilutions were made with cell culture medium or bacteria medium.

Cell Culture

Human oral keratinocytes (HOK-18A) were cultured in Keratinocyte-SFM (Serum-free keratinocyte medium, Gibco, USA) containing bovine pituitary extract (25 µg/mL) and recombinant epidermal growth factor (5 ng/mL) in a humidified incubator with 5% CO₂ and 95% air at 37 °C.

Cytotoxicity assay

Keratinocytes were seeded in 96-well tissue-culture plates (Greiner Bio-One, Belgium) at a concentration of 20,000 cells/well in 100 µL culture medium and incubated for 24 h. After a state of confluence was reached, the cell culture medium was removed from each well and substituted by DMSO in KSFM (2% and 0.4%), CHX in KSFM (0.2% to 0.04% v/v), T4ol in KSFM (0.31% to 0.03% v/v) and carvacrol in KSFM (0.26% to 0.02% v/v), Triton X-100 in KSFM (1%, positive control) or KSFM alone (control group). After 20 h of incubation, an XXT assay was performed to determine cell viability as described by Li et al. (2017).

Bacterial strains and growth conditions

The bacterial strains *Streptococcus sanguinis* LM14657, *Streptococcus gordonii* ATCC 49818, *Streptococcus mitis* DSM 12643, *Streptococcus oralis* DSM 20627, *Streptococcus salivarius* TOVE-R, *Streptococcus mutans* ATCC 20523, *Streptococcus sobrinus* ATCC 20742, *Actinomyces viscosus* DSM 43327, *Actinomyces naeslundii* ATCC 51655, *Prevotella intermedia* ATCC 25611,

Porphyromonas gingivalis ATCC 33277, *Fusobacterium nucleatum* ATCC 20482, *Aggregatibacter actinomycetemcomitans* ATCC 43718 and *Veillonella parvula* DSM 2008 were maintained on blood agar (Oxoid, UK) supplemented with 5 mg/mL hemin (Sigma, USA), 1 mg/mL menadione (Calbiochem-Novabiochem, USA) and 5% sterile horse blood (E&O Laboratories, Scotland). Overnight cultures were prepared in Brain Heart Infusion (BHI) broth (Difco, USA) under aerobic (5% CO₂) or anaerobic (80% N₂, 10% H₂ and 10% CO₂) conditions.

Susceptibility tests on single-species biofilms

T4ol (0.19% to 0.03% v/v) and carvacrol (0.06% and 0.02%) in BHI were used as antimicrobial compounds. Overnight cultures of *P. gingivalis* and *P. intermedia* were adjusted to 1x10⁸ CFU/mL or 1x10⁹ CFU/mL. In the first set-up, 200 µL of each bacterial culture was incubated together with T4ol and carvacrol for 24 h or 48 h under anaerobic conditions. In the second set-up, 200 µL of each bacterial culture was incubated for 24h or 48 h under anaerobic conditions, after which the planktonic bacteria were removed and the biofilms were exposed to 200 µL of T4ol or carvacrol for another 24h under anaerobic conditions. The amount of biofilm was measured by crystal violet (CV) staining. Briefly, the supernatant was removed and the biofilm washed with phosphate-buffered saline (PBS). After that, 100 µL of 96% EtOH was added for 20 minutes to allow cell fixation and dried at room temperature. Bacterial biofilms were stained with 100 µL CV solution (1%) for 15 minutes and washed with PBS to remove remaining CV. After drying at room temperature, the attached CV was solubilized by adding 75 µL of acetic acid (5%) for 45 minutes after which the optical density of the solutions was measured at 630 nm (OD₆₃₀, GeneQuant Spectrophotometer, Buckinghamshire, UK).

Multi-species biofilm formation and treatments

Titanium discs (diameter 5 mm, thickness 2 mm) with SLA surface produced using TiO₂ microparticles for blasting and subsequent acid conditioning (Implacil De Bortoli, Brazil) and Calcium Deficient Hydroxyapatite (CAD-HA) discs (diameter 12.7 mm, thickness 1.02-1.52 mm) were used. A stable 14-species community (multi-species biofilm) was grown in a BIOSTAT B TWIN (Sartorius, Germany) bioreactor as described by Herrero et al. (2016). After 72 h, 1800 µL fresh BHI-2 (Alvarez et al., 2013) and 200 µL bioreactor culture were added to each well of a 24-well plate and

discs were placed in a vertical position using the Amsterdam Active Adhesion biofilm model (Exterkate et al., 2010), incubated under anaerobic conditions for 24 h with shaking at 170 rpm. After biofilm formation, the discs containing the biofilms were washed with PBS to remove non-attached cells, and transferred to a new 24-well plate containing T4ol 0.19% and carvacrol 0.06%. In the first set-up, the pre-formed biofilms were exposed to T4ol or carvacrol for 24 hours under anaerobic conditions with shaking at 170 rpm. The second set-up consisted of a 5 minutes exposure to T4ol and carvacrol at room temperature with shaking at 250 rpm. Afterwards, the exposed biofilms were washed with PBS and transferred to a new plate with fresh medium and incubated for another 24 h under anaerobic conditions with shaking at 170 rpm. Subsequently, the discs were transferred to 13 mL-tubes and the biofilms were detached using 1.5 mL of 0.05% trypsin-EDTA (Gibco, USA) for 45 min at 37°C with shaking at 230 rpm. After 15 seconds of vortexing, the detached cells were transferred to Eppendorf tubes followed by sonication for 2 minutes to deposit the remaining hydroxyapatite. The resulting supernatant was transferred to a new Eppendorf tube, which was centrifuged ($6010 \times g$, 5 min). Afterwards, the obtained pellets were resuspended in 500 μ L PBS. Vitality extraction of bacterial DNA using propidium monoazide (PMA) and subsequent qPCR assay were performed as described by Loozen et al. (2011). qPCR assays were performed according to Herrero et al. (2018).

Statistical analysis

Data obtained in this study were analyzed using the D'Agostino-Pearson normality test. Afterwards, one-way ANOVA and Dunnett's test for comparisons with the control group were set up for every model separately and performed utilizing GraphPad Prism software (GraphPad Software, Inc.). Differences were considered statistically significant at $p < 0.05$. For all outcomes, three independent experiments were performed in triplicate.

RESULTS

Cytotoxicity on human oral keratinocyte cells

Antimicrobial agents should be safe to use within the oral cavity at the concentrations for which they are effective. Therefore, the cytotoxic effect of T4ol and carvacrol was tested on oral keratinocytes. The cytotoxic effects of CHX, T4ol and carvacrol on keratinocytes are shown in Figure 1. All CHX concentrations tested showed a significant decrease ($p < 0.05$) in cell viability after 24 h of exposure. However, T4ol (0.19% to 0.03% v/v) and carvacrol (0.06% and 0.02%) did not show cytotoxic effects ($p > 0.05$).

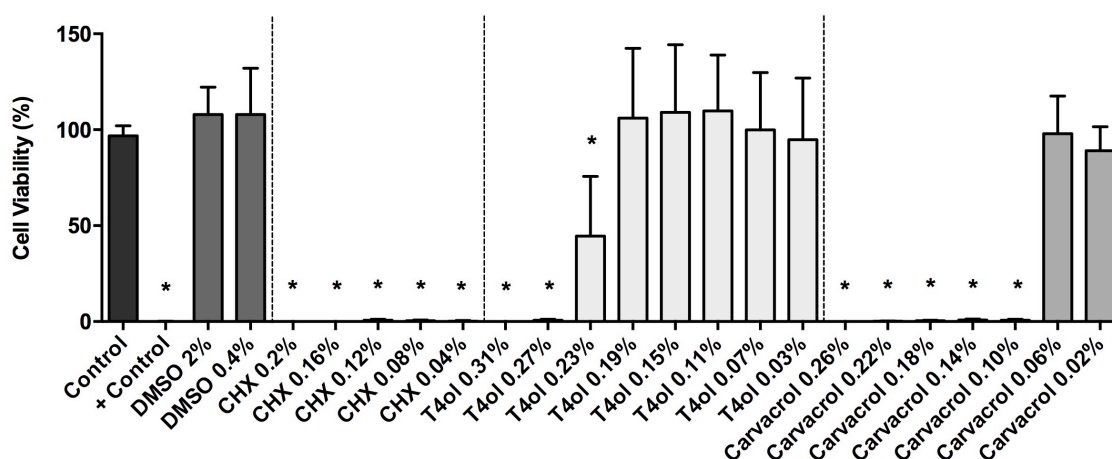


Figure 1. Viability percentage of keratinocyte cells after treatment with various concentrations of CHX, T4ol and carvacrol. *Significant difference in comparison with the control group (ANOVA, $p < 0.05$).

Inhibition of single-species biofilm formation

The not-cytotoxic concentrations of T4ol and carvacrol were further tested on their ability to inhibit single-species biofilm formation after 24 h and 48 h (Table 1 and Table 2). In general, it was observed that both T4ol and carvacrol show a concentration dependent inhibitory effect on single-species biofilm formation with T4ol 0.19% and carvacrol 0.06% showing the strongest inhibitory effects for both periods. The inhibitory effect of T4ol was however lower after 48 hours than after 24

hours. Carvacrol exhibited a broader spectrum and a more pronounced biofilm inhibition than T4ol.

T4ol inhibited biofilm formation of all bacteria except for *S. mutans*, *S. sobrinus* and *S. gordonii* after 24 h and *F. nucleatum*, *V. parvula*, *A. viscosus* and *A. naeslundii* after 48 h ($p > 0.05$). Interestingly, *S. mutans* and *S. sobrinus* biofilm formation was significantly higher when compared to the control group after 24 h growth in presence of T4ol 0.03%. At the same concentration, *S. sobrinus* and *A. viscosus* biofilms showed a significant increase in biomass after 48 h of growth.

Despite the strong broad-spectrum effect of carvacrol, it did not inhibit *F. nucleatum* after 24 and 48 hours ($p > 0.05$). Carvacrol 0.02% also induced an increase in *S. sobrinus* biofilm after 48 h ($p < 0.05$).

Table 1. Inhibition of single-species biofilm formation by T4ol and carvacrol after 24 h

	Control	T4ol					Carvacrol	
	-	0.19%	0.15%	0.11%	0.07%	0.03%	0.06%	0.02%
<i>A. actinomycetemcomitans</i>	0.21±0.07	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*
<i>F. nucleatum</i>	0.17±0.04	0.09±0.04*	0.09±0.04*	0.12±0.03*	0.14±0.05	0.18±0.03	0.12±0.01	0.21±0.02
<i>P. gingivalis</i>	0.07±0.01	0.01±0.00*	0.00±0.00*	0.00±0.00*	0.05±0.01	0.05±0.00	0.00±0.00*	0.01±0.00*
<i>S. mutans</i>	0.27±0.08	0.28±0.04	0.31±0.05	0.31±0.08	0.31±0.05	0.40±0.08#	0.00±0.00*	0.00±0.00*
<i>S. sobrinus</i>	0.17±0.05	0.20±0.04	0.20±0.04	0.22±0.04	0.23±0.03#	0.31±0.07#	0.00±0.00*	0.01±0.00*
<i>P. intermedia</i>	2.10±0.58	0.00±0.00*	0.00±0.01*	0.01±0.01*	0.01±0.01*	0.02±0.01*	0.00±0.00*	0.00±0.00*
<i>V. parvula</i>	0.10±0.01	0.01±0.00*	0.01±0.00*	0.02±0.01*	0.06±0.01*	0.07±0.00*	0.02±0.00*	0.02±0.00*
<i>S. sanguinis</i>	0.26±0.03	0.05±0.01*	0.08±0.02*	0.08±0.02*	0.11±0.03*	0.14±0.03*	0.00±0.00*	0.11±0.02*
<i>S. salivarius</i>	0.11±0.02	0.05±0.01*	0.06±0.02*	0.06±0.02*	0.06±0.01*	0.06±0.02*	0.00±0.00*	0.00±0.00*
<i>S. oralis</i>	0.22±0.03	0.07±0.01*	0.07±0.02*	0.08±0.03*	0.08±0.02*	0.14±0.03*	0.00±0.00*	0.00±0.00*
<i>S. gordonii</i>	0.57±0.10	0.67±0.13	0.65±0.14	0.67±0.13	0.64±0.07	0.62±0.10	0.00±0.00*	0.18±0.03*
<i>S. mitis</i>	0.10±0.02	0.04±0.01*	0.04±0.01*	0.04±0.02*	0.08±0.02	0.11±0.02	0.00±0.00*	0.00±0.00*
<i>A. viscosus</i>	0.63±0.24	0.31±0.05*	0.35±0.12*	0.41±0.11*	0.43±0.10*	0.41±0.08*	0.00±0.00*	0.01±0.01*
<i>A. naeslundii</i>	1.10±0.26	0.02±0.02*	0.02±0.03*	0.06±0.02*	0.13±0.07*	0.31±0.14*	0.03±0.04*	0.00±0.00*

Biofilm formation was determined by crystal violet staining and is expressed as the optical density at 630 nm (OD₆₃₀) (Mean ± Standard Deviation).

#Statistically significant increase in biomass in comparison with the control group (ANOVA, $p < 0.05$)

*Statistically significant decrease in biomass in comparison with the control group (ANOVA, $p < 0.05$)

Table 2. Inhibition of single-species biofilm formation by T4ol and carvacrol after 48 h

	Control	T4ol					Carvacrol	
	-	0.19%	0.15%	0.11%	0.07%	0.03%	0.06%	0.02%
<i>A. actinomycetemcomitans</i>	0.50±0.12	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*
<i>F. nucleatum</i>	0.22±0.07	0.15±0.04	0.13±0.06	0.15±0.06	0.19±0.08	0.23±0.10	0.15±0.06	0.28±0.04
<i>P. gingivalis</i>	0.22±0.04	0.01±0.01	0.01±0.01*	0.11±0.03*	0.19±0.04	0.17±0.01*	0.00±0.00*	0.01±0.00*
<i>S. mutans</i>	0.35±0.11	0.35±0.07	0.33±0.07	0.37±0.05	0.38±0.09	0.29±0.05	0.00±0.00*	0.67±0.06*
<i>S. sobrinus</i>	0.25±0.03	0.31±0.03	0.30±0.05	0.32±0.04#	0.31±0.03	0.33±0.04#	0.00±0.00*	0.40±0.02#
<i>P. intermedia</i>	1.56±0.20	0.00±0.00*	0.00±0.00*	0.02±0.01*	0.03±0.01*	0.01±0.00*	0.01±0.00*	0.01±0.00*
<i>V. parvula</i>	0.07±0.01	0.10±0.00#	0.11±0.02#	0.10±0.02	0.09±0.02	0.09±0.02	0.01±0.00*	0.01±0.00*
<i>S. sanguinis</i>	0.31±0.03	0.09±0.03*	0.10±0.04*	0.12±0.03*	0.15±0.05*	0.20±0.03*	0.00±0.00*	0.16±0.03*
<i>S. salivarius</i>	0.11±0.02	0.07±0.02*	0.07±0.01*	0.07±0.01*	0.07±0.02*	0.08±0.01*	0.00±0.00*	0.01±0.00*
<i>S. oralis</i>	0.20±0.03	0.08±0.01*	0.07±0.01*	0.09±0.02*	0.08±0.02*	0.13±0.03*	0.00±0.00*	0.00±0.00*
<i>S. gordonii</i>	0.62±0.15	0.43±0.07*	0.51±0.06*	0.50±0.07*	0.52±0.08	0.55±0.08	0.00±0.00*	0.11±0.05*
<i>S. mitis</i>	0.23±0.05	0.16±0.01*	0.14±0.02*	0.13±0.04*	0.22±0.05	0.22±0.04	0.00±0.00*	0.00±0.00*
<i>A. viscosus</i>	1.23±0.25	1.43±0.34	1.39±0.26	1.37±0.13	1.49±0.17#	1.62±0.07#	0.01±0.00*	0.00±0.00*
<i>A. naeslundii</i>	0.29±0.04	0.27±0.03	0.27±0.03	0.31±0.04	0.33±0.03	0.31±0.03	0.00±0.00*	0.01±0.01*

Biofilm formation was determined by crystal violet staining and is expressed as the optical density at 630 nm (OD₆₃₀) (Mean ± Standard Deviation).

#Statistically significant increase in biomass in comparison with the control group (ANOVA, $p < 0.05$)

*Statistically significant decrease in biomass in comparison with the control group (ANOVA, $p < 0.05$)

Eradication of pre-formed single-species biofilms

Although inhibition of biofilm formation is a well-known property of antimicrobials, eradication of biofilms is much more difficult and less well studied. Therefore, the effects of T4ol and carvacrol on pre-formed single-species biofilms after 24 h and 48 h of growth were evaluated (Table 3 and Table 4). In general, T4ol 0.19% and carvacrol 0.06% show strongest eradication effects single-species biofilm for both periods. The eradication of biofilms of T4ol was however lower after 48 hours than after 24 hours. Carvacrol exhibited a broader spectrum and a more pronounced biofilm inhibition than T4ol.

None of the tested concentrations of T4ol was able to eradicate the 24 h pre-formed biofilm of *S. sobrinus*, *S. salivarius* and *A. viscosus* and all tested carvacrol concentrations were unable to eradicate *S. salivarius* biofilm ($p > 0.05$). Interestingly, after 24 h evaluation of pre-formed biofilm of *P. intermedia* and *A. viscosus* growth in presence of T4ol 0.11 and 0.07% significantly higher biomass were observed when

compared to the control group. At the same concentration, *S. sobrinus* and *A. viscosus* biofilms showed a significant increase in biomass on 48 h pre-formed biofilm. Similar with inhibition the strong broad-spectrum effect of carvacrol was observed, it did not inhibit *S. salivarius* after 24 h and *S. sobrinus* and *S. oralis* after 48 h ($p > 0.05$). Carvacrol also induced an increase in *S. salivarius* pre-formed biofilm of 48 h ($p < 0.05$).

Table 3. Effect of T4ol and carvacrol concentrations on different pre-formed single-species biofilms after 24 h of growth

	Control		T4ol					Carvacrol	
	-		0.19%	0.15%	0.11%	0.07%	0.03%	0.06%	0.02%
<i>A. actinomycetemcomitans</i>	1.41±0.09		0.30±0.06*	0.31±0.06*	0.44±0.08*	0.72±0.06*	0.77±0.11*	0.33±0.13*	0.34±0.09*
<i>F. nucleatum</i>	2.49±0.57		1.45±0.30*	1.56±0.48*	1.82±0.22*	1.80±0.30*	1.72±0.23*	0.66±0.17*	0.73±0.15*
<i>P. gingivalis</i>	0.68±0.31		0.28±0.03*	0.26±0.08*	0.27±0.09*	0.26±0.09*	0.23±0.08*	0.40±0.08*	0.29±0.12*
<i>S. mutans</i>	3.19±0.55		0.97±0.25*	0.89±0.42*	1.05±0.31*	1.23±0.30*	2.21±0.52*	1.05±0.11*	1.00±0.09*
<i>S. sobrinus</i>	1.24±0.19		1.16±0.06	1.08±0.23	1.23±0.22	1.27±0.17	1.32±0.07	0.71±0.16*	0.79±0.09*
<i>P. intermedia</i>	1.23±0.40		0.15±0.03*	0.37±0.19*	1.89±0.18#	1.79±0.32#	1.94±0.49#	0.07±0.02*	0.06±0.02*
<i>V. parvula</i>	0.54±0.08		0.15±0.04*	0.18±0.04*	0.22±0.08*	0.35±0.09*	0.41±0.01*	0.24±0.05*	0.21±0.08*
<i>S. sanguinis</i>	0.49±0.07		0.34±0.10*	0.37±0.07	0.36±0.09*	0.37±0.06*	0.38±0.07	0.17±0.08*	0.16±0.10*
<i>S. salivarius</i>	0.43±0.21		0.30±0.14	0.51±0.16	0.55±0.14	0.52±0.16	0.47±0.13	0.52±0.14	0.47±0.15
<i>S. oralis</i>	0.41±0.06		0.28±0.08*	0.31±0.05*	0.30±0.01*	0.33±0.03	0.33±0.07	0.32±0.06	0.32±0.04*
<i>S. gordonii</i>	1.81±0.29		1.20±0.20*	1.23±0.18*	1.38±0.16*	1.39±0.24*	1.43±0.20*	0.54±0.06*	0.70±0.16*
<i>S. mitis</i>	0.89±0.18		0.24±0.06*	0.27±0.10*	0.41±0.09*	0.46±0.12*	0.67±0.10*	0.24±0.08*	0.42±0.07*
<i>A. viscosus</i>	1.68±0.34		2.40±0.17#	2.34±0.21#	2.20±0.29#	2.14±0.13#	2.16±0.16#	0.36±0.05*	0.37±0.04*
<i>A. naeslundii</i>	2.90±0.28		2.58±0.11*	2.54±0.22*	2.62±0.15*	2.58±0.35*	2.59±0.34*	0.74±0.13*	0.75±0.12*

Biofilm formation was determined by crystal violet staining and is expressed as the optical density at 630 nm (OD₆₃₀) (Mean ± Standard Deviation).

#Statistically significant increase in biomass in comparison with the control group (ANOVA, $p < 0.05$)

*Statistically significant decrease in biomass in comparison with the control group (ANOVA, $p < 0.05$)

Table 4. Effect of T4ol and carvacrol concentrations on different pre-formed single-species biofilms after 48 h of growth

	Control		T4ol				Carvacrol	
	-	0.19%	0.15%	0.11%	0.07%	0.03%	0.06%	0.02%
<i>A. actinomycetemcomitans</i>	1.67±0.23	0.56±0.07*	0.60±0.05*	0.59±0.06*	0.57±0.06*	0.81±0.11*	0.65±0.11*	0.62±0.16*
<i>F. nucleatum</i>	3.81±0.30	1.69±0.24*	1.59±0.25*	2.78±0.47*	3.18±0.27*	3.19±0.32*	1.21±0.16*	1.41±0.18*
<i>P. gingivalis</i>	0.79±0.26	0.20±0.08*	0.16±0.05*	0.17±0.08*	0.25±0.09*	0.23±0.06*	0.46±0.11*	0.38±0.02*
<i>S. mutans</i>	1.92±0.41	0.52±0.15*	0.63±0.31*	1.09±0.35*	1.35±0.39*	1.64±0.35	0.45±0.11*	0.41±0.15*
<i>S. sobrinus</i>	1.67±0.55	2.09±0.48	2.74±0.61#	2.88±0.66#	2.83±0.67#	2.88±0.39#	1.11±0.08	1.04±0.16
<i>P. intermedia</i>	3.76±0.16	0.55±0.05*	0.86±0.20*	1.70±0.29*	2.41±0.40*	3.48±0.41	0.33±0.07*	0.34±0.05*
<i>V. parvula</i>	0.70±0.15	0.19±0.05*	0.15±0.02*	0.34±0.12*	0.43±0.05*	0.43±0.13*	0.26±0.06*	0.26±0.09*
<i>S. sanguinis</i>	0.55±0.10	0.30±0.07*	0.27±0.09*	0.48±0.07	0.53±0.08	0.55±0.07	0.19±0.03*	0.23±0.05*
<i>S. salivarius</i>	0.31±0.03	0.21±0.04	0.23±0.09	0.27±0.07	0.30±0.08	0.30±0.03	0.53±0.07#	0.45±0.10#
<i>S. oralis</i>	0.26±0.06	0.17±0.04*	0.16±0.04*	0.16±0.06*	0.17±0.06*	0.17±0.06*	0.21±0.03	0.19±0.05
<i>S. gordonii</i>	1.61±0.29	1.45±0.16	1.33±0.23	1.45±0.22	1.60±0.28	1.58±0.32	0.63±0.07*	0.75±0.07*
<i>S. mitis</i>	0.75±0.12	0.28±0.08*	0.29±0.09*	0.38±0.06*	0.48±0.10*	0.53±0.15*	0.31±0.03*	0.35±0.02*
<i>A. viscosus</i>	1.76±0.16	1.54±0.26	2.03±0.25#	2.15±0.12#	2.07±0.09#	1.93±0.13	0.40±0.03*	0.38±0.07*
<i>A. naeslundii</i>	1.39±0.28	1.12±0.14	1.08±0.12*	1.09±0.20*	1.09±0.19*	1.16±0.14	0.83±0.08*	0.94±0.06*

Biofilm formation was determined by crystal violet staining and is expressed as the optical density at 630 nm (OD₆₃₀) (Mean ± Standard Deviation).

#Statistically significant increase in biomass in comparison with the control group (ANOVA, $p < 0.05$)

*Statistically significant decrease in biomass in comparison with the control group (ANOVA, $p < 0.05$)

Effect on multi-species biofilms

Multi-species biofilms were pre-formed on HA and titanium discs, and exposed to T4ol 0.19% or carvacrol 0.06% for 24 h (Figure 2 and Figure 3). Both compounds were effective against pre-formed multi-species biofilms on both tested surfaces. Treatment of pre-formed multi-species biofilms on HA surfaces with T4ol or carvacrol resulted in a significant decrease of *F. nucleatum*, *P. gingivalis*, *S. sobrinus*, *P. intermedia*, *V. parvula*, *S. oralis* and *A. viscosus* ($p < 0.05$). Additionally, T4ol also decreased *A. naeslundii* ($p < 0.05$).

On biofilms pre-formed on titanium discs, T4ol and carvacrol showed a significant reduction of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *S. oralis*, *F. nucleatum*, *V. parvula* and *A. viscosus* ($p < 0.05$). Additionally, T4ol decreased in *S. sanguinis* whereas carvacrol decreased *A. naeslundii* ($p < 0.05$). In

general, the effect of both compounds was more pronounced on titanium disc biofilms than on HA disc biofilms

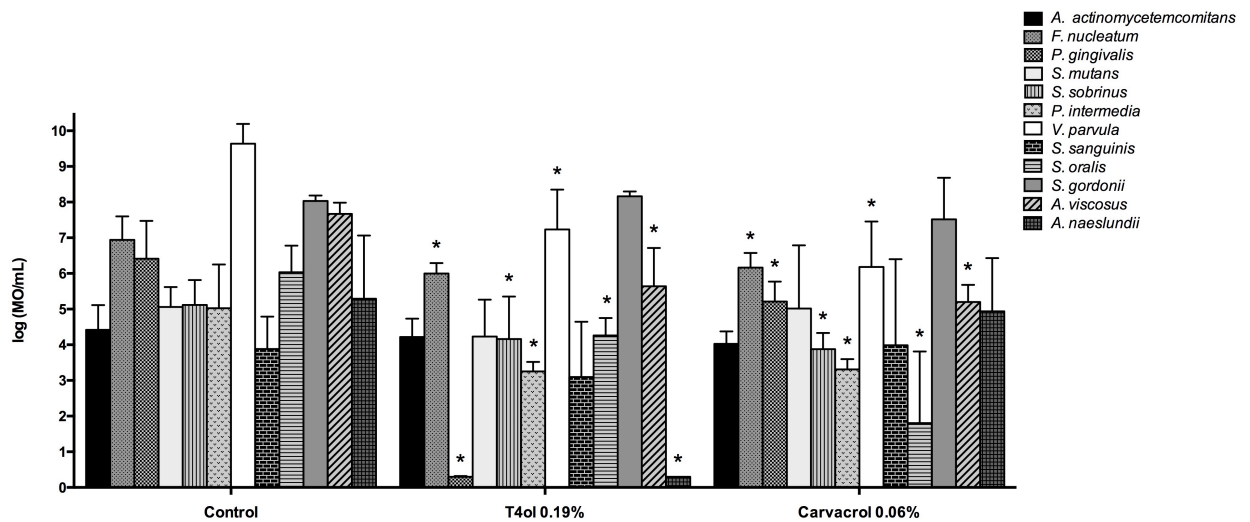


Figure 2. Effect of T4ol and carvacrol on pre-formed multi-species biofilms grown on HA discs after 24 hours of treatment. *Significant difference in comparison with the control group (ANOVA, $p < 0.05$).

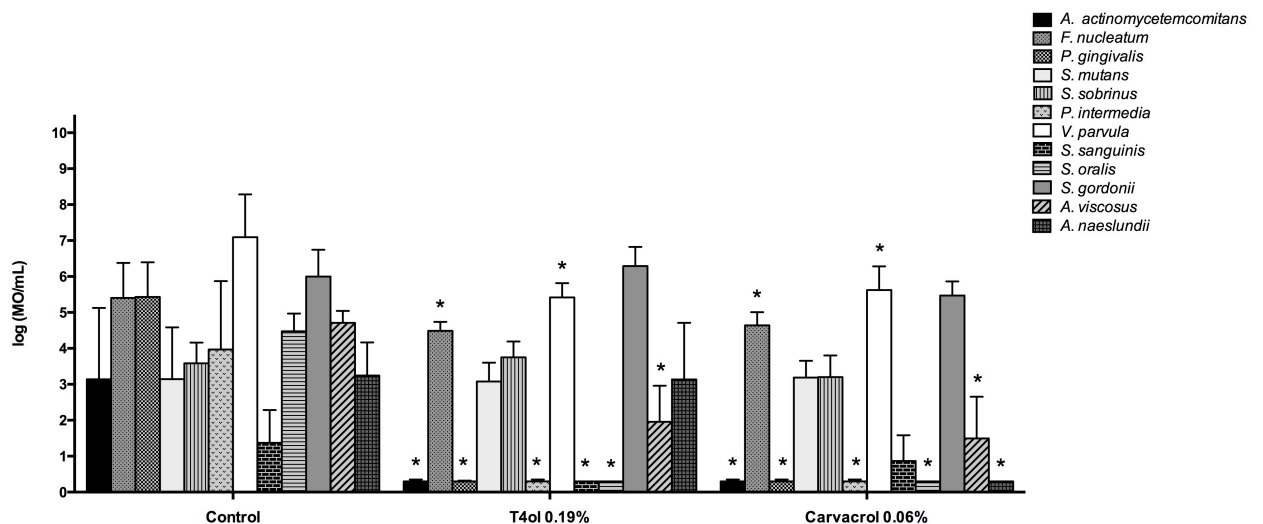


Figure 3. Effect of T4ol and carvacrol on pre-formed multi-species biofilms grown on titanium discs after 24 hours of treatment. *Significant difference in comparison with the control group (ANOVA, $p < 0.05$).

The effects of these compounds on pre-formed multi-species biofilms on HA and titanium surfaces and treated for 5 minutes are shown in Figure 4 and Figure 5. The effects were clearly less strong after 5 minutes exposure than after 24 hour exposure. The composition of multi-species biofilms on HA after treatment with T4ol 0.19% showed a significant decrease in *P. intermedia*, whereas carvacrol 0.06% treatment significantly decreased the presence of *S. sanguinis* and *P. intermedia* ($p < 0.05$). On titanium surfaces, T4ol showed a strong significant decrease in *P. gingivalis*, *F. nucleatum* and *S. sobrinus*. However, *V. parvula* and *S. gordonii* only showed a significant decrease after treatment with carvacrol ($p < 0.05$).

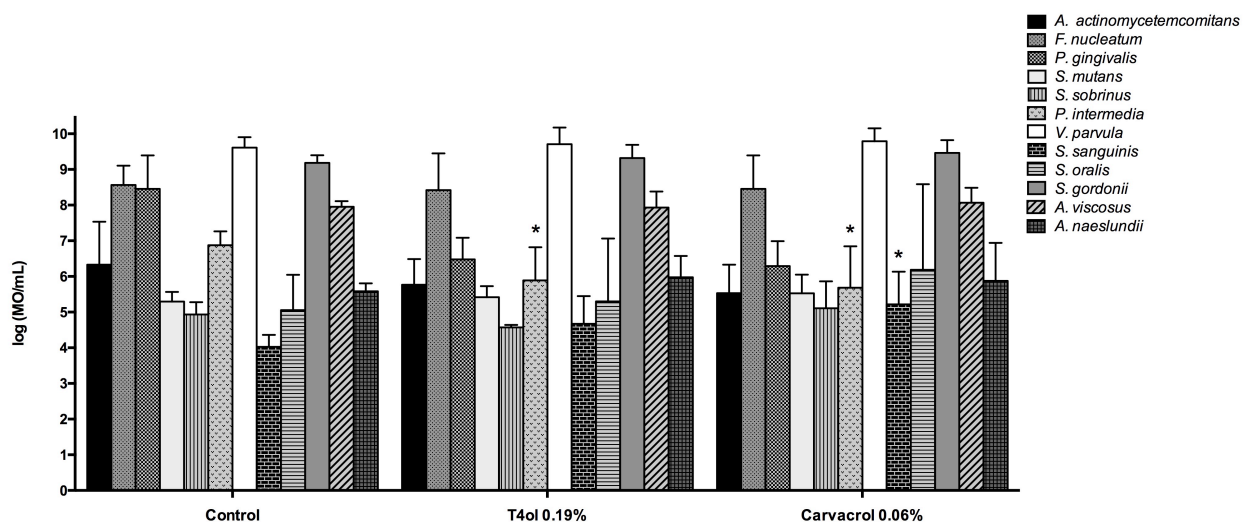


Figure 4. Effect of T4ol and carvacrol on multi-species biofilms pre-formed on HA discs after 5 minutes of treatment. *Significant difference in comparison with the control group (ANOVA, $p < 0.05$).

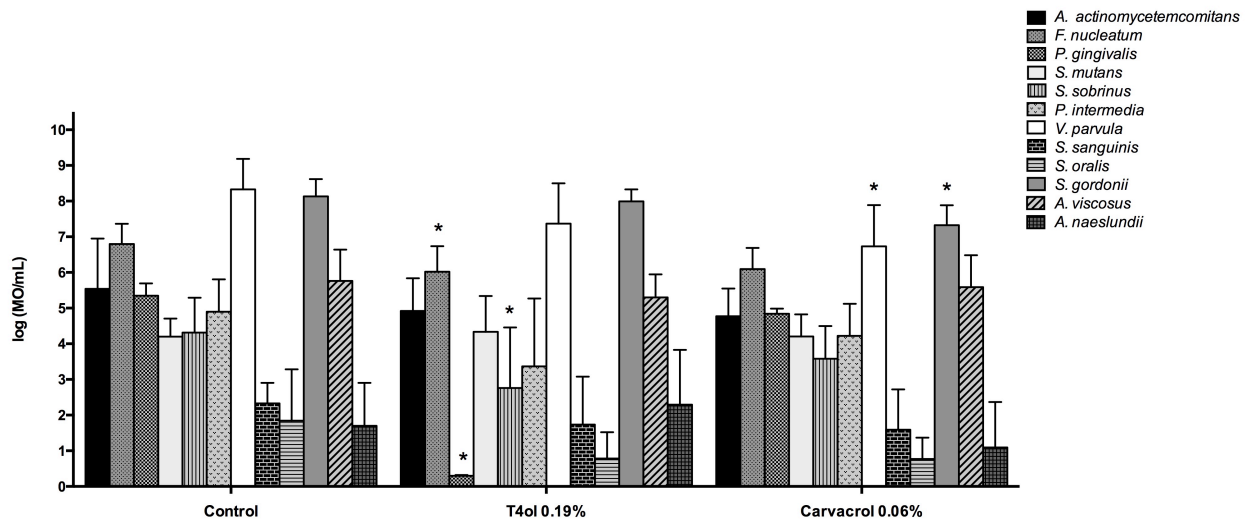


Figure 5. Effect of T4ol and carvacrol on multi-species biofilms pre-formed on titanium discs after 5 minutes of treatment. *Significant difference in comparison with the control group (ANOVA, $p < 0.05$).

DISCUSSION

In the present study, the cytotoxic effect on epithelial cells and the antimicrobial activity against single- and multi-species biofilms of T4ol and carvacrol were evaluated. In terms of potential future clinical applications, cytotoxicity tests are essential in the selection of potentially new antimicrobial agents.

In the current study, the toxicity of different concentrations of CHX (0.2% - 0.04% v/v), T4ol (0.31% - 0.03%) and carvacrol (0.26% - 0.02% v/v) on an immortalized human oral keratinocytes cell line were tested. Human oral keratinocytes were selected because they are predominant in the oral cavity and form the first surface that comes into contact with the antimicrobial agents during the treatment of peri-implantitis or periodontal diseases. Our results showed that T4ol (0.19% - 0.03% v/v) and carvacrol (0.06% and 0.02%) were concentrations that did not cause cytotoxic effects on the keratinocytes. The novelty of this study lies in the fact that the cytotoxic activity of T4ol and carvacrol has never been studied on human oral keratinocytes before. A previous study conducted by Loughlin et al. (2008) showed that T4ol 0.125%, 0.25%, 0.5% and 1% did not have cytotoxic effects on murine fibroblast cells after 24 h of exposure. However, other studies revealed cytotoxic effects of carvacrol on HL-60 promyelocytic, jurkat T lymphoma and Caco-2

cell lines with concentration- and time-dependent experiments (Bhakkiyalakshmi et al., 2016; Llana-Ruiz-Cabello et al., 2014). Although CHX is widely used in the treatment of peri-implant and periodontal diseases, our data show that all tested CHX concentrations inflicted damage to oral keratinocyte cells. This can be explained by the main mechanisms of action of this antiseptic, which are the inhibition of protein synthesis (Pucher and Daniel, 1992), induction of apoptosis at low concentrations, necrosis at high concentrations and inhibition of DNA synthesis (Hidalgo and Dominguez, 2001).

In a next step, the anti-biofilm effect of T4ol and carvacrol was tested on different biofilm models. Most published studies only evaluated the antimicrobial activity of T4ol and carvacrol against planktonic bacteria, thereby underestimating the influence of the biofilm structure on the outcome of the treatment. For instance, the polymer matrix present around microcolonies in a complex biofilm structure can contribute to the neutralization of the effect of antimicrobials agents, when compared with planktonic bacteria (Hoiby et al., 2010). Our results of the crystal violet assay indicate that T4ol and carvacrol were able to inhibit the formation of all single-species biofilms at all tested concentrations, except for *S. mutans* and *S. sobrinus* after 24 h and 48 h for T4ol, and for *F. nucleatum* in the case of carvacrol. This is in contrast to a previous study that evaluated *S. mutans* biofilm formation in presence of 200 µg/mL (0.2%) carvacrol by crystal violet staining after 24 h and found to that carvacrol inhibited biofilm formation (Khan et al., 2017). On the other hand, T4ol and carvacrol were able to eradicate all pre-formed biofilms. This was except for *S. sobrinus* and *S. salivarius*, which were not susceptible to any of the tested T4ol and carvacrol concentrations and for *S. oralis*, which was only inhibited by carvacrol after 48 h pre-formed biofilm. In the same way, Ciandrini et al. (2014) reported an inhibitory effect of carvacrol concentrations of respectively 0.25% on *S. mutans* and 1% on *F. nucleatum* and *P. gingivalis* biofilms after 48 h and quantified by plate counting (CFU mL⁻¹).

Since the roughness, free energy and other properties of a biomaterial surface can influence biofilm formation on this surface (Teughels et al., 2006), biofilms were grown as well on HA as on titanium discs. Surprisingly, our data show differences in the inhibitory effect of T4ol and carvacrol on the biofilms depending on the type of surfaces on which the biofilm was formed. A stronger inhibition using T4ol and carvacrol on biofilms pre-formed on titanium surfaces than on HA was shown.

Bacterial persistence and re-colonization after antimicrobial treatment are factors that can determine the outcome of periodontal treatments (Drisko, 1998). Our multi-species biofilms results showed a reduction of *P. intermedia*, *S. sanguinis*, *F. nucleatum*, *P. gingivalis*, *S. sobrinus*, *V. parvula* and *S. gordonii* after 5 minutes of treatment with T4ol and carvacrol, and this for biofilms grown on different surfaces. However, treatment for 24 h using both compounds was generally stronger and found to be more effective. The antimicrobial activity of essential oils has been studied extensively. Our study showed the antimicrobial/anti-biofilm activity of two different compounds present in essential oils. There were differences in the anti-biofilm activity of T4ol and carvacrol, which can be explained by their mechanism of action or their effect on the bacterial membrane (Hąc-Wydro and Szydło K, 2016). T4ol is the major active component of *Melaleuca alternifolia*, known as tea tree oil (TTO) (Nogueria et al. 2014). Due to its lipophilic properties, it can penetrate through the cell wall and cytoplasmic membrane of bacteria, causing structural damage and loss of cytoplasmic material (Li et al., 2016). Similarly, carvacrol interacts with the lipid bilayer of the cytoplasmic membrane due to its hydrophobic characteristics, thereby causing membrane destabilization and increasing its fluidity and permeability (Nostro and Papalia, 2012; Ultee et al., 1999).

Although the inhibitory effect of carvacrol on periodontal pathogens was already demonstrated and concentrations of 0.5% and 0.02% were found to be effective in reducing the total count of bacteria in subgingival biofilms (Botelho et al., 2009; Shahab et al., 2011), these studies only reported on the total reduction of the biofilm, without looking at the specific values of each individual bacterial strain. Additionally, the number of studies focused on investigating the inhibition and/or eradication of single- and multi-species biofilms by T4ol or carvacrol is rather limited. The data of our present study shows for the first time the inhibitory effects of these compounds on the composition of multi-species biofilms. Further research should focus on the *in vivo* effects of T4ol and carvacrol and on the host inflammatory response during peri-implantitis and periodontal treatments.

In conclusion, low and non-cytotoxic concentrations of terpinen-4-ol and carvacrol can inhibit and eradicate periodontal pathogens both in single- and multi-species biofilms grown on HA and titanium surfaces.

Disclosure statement

The authors declare that they have no conflict of interest.

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REFERENCES

1. Alvarez G, González M, Isabel S, Blanc V, León R. Method to quantify live and dead cells in multi-species oral biofilm by real-time PCR with propidium monoazide. *AMB Express* 2013; 3(1): 1.
2. Baser KH. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. *Curr Pharm Des* 2008; 14(29): 3106-19.
1. Belibasakis GN, Charalampakis G, Bostanci N, Stadlinger B. Peri-implant infections of oral biofilm etiology. *Adv Exp Med Biol* 2015; 830: 69-84.
2. Bhakkiyalakshmi E, Suganya N, Sireesh D, Krishnamurthi K, Saravana Devi S, Rajaguru P, Ramkumar KM. Carvacrol induces mitochondria-mediated apoptosis in HL-60 promyelocytic and Jurkat T lymphoma cells. *Eur J Pharmacol* 2016; 772: 92-8.
3. Botelho MA, Martins JG, Ruela RS, I R, Santos JA, Soares JB, França MC, Montenegro D, Ruela WS, Barros LP, Queiroz DB, Araujo RS, Sampaio FC. Protective effect of locally applied carvacrol gel on ligature-induced periodontitis in rats: a tapping mode AFM study. *Phytother Res* 2009; 23(10): 1439-48.
4. Botelho MA, Nogueira NA, Bastos GM, Fonseca SG, Lemos TL, Matos FJ, Montenegro D, Heukelbach J, Rao VS, Brito GA. Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. *Braz J Med Biol Res* 2007; 40(3): 349-56.

5. Busscher HJ, Rinastiti M, Siswomihardjo W, van der Mei HC. Biofilm formation on dental restorative and implant materials. *J Dent Res* 2010; 89: 657-65.
6. Carson CF, Cookson BD, Farrelly HD, Riley TV. Susceptibility of methicillin-resistant *Staphylococcus aureus* to the essential oil of *Melaleuca alternifolia*. *J Antimicrob Chemother* 1995; 35: 421-4.
7. Catalán A, Pacheco JG, Martínez A, Mondaca MA. In vitro and in vivo activity of *Melaleuca alternifolia* mixed with tissue conditioner on *Candida albicans*. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008; 105(3): 327-32.
8. Ciandrini E, Campana R, Federici S, Manti A, Battistelli M, Falcieri E, Papa S, Baffone W. In vitro activity of Carvacrol against titanium-adherent oral biofilms and planktonic cultures. *Clin Oral Investig* 2014; 18(8): 2001-13.
9. Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmington JR, Wyllie SG. The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J Appl Microbiol* 2000; 88(1): 170-5.
10. Drisko CH. The use of locally-delivered doxycycline in the treatment of periodontitis. Clinical results. *J Clin Periodontol* 1998; 25: 947-52.
11. Exterkate RA, Crielaard W, Ten Cate JM. Different response to amine fluoride by *Streptococcus mutans* and polymicrobial biofilms in a novel high-throughput active attachment model. *Caries Res* 2010; 44(4): 372-9.
12. Fellman M. Pharmacology and periodontal disease: implications and future options. *CDHA Journal* 2010; 25(2): 9-11.
13. Hąc-Wydro K, Szydło K. The influence of environmentally friendly pesticide - Eucalyptol - alone and in combination with terpinen-4-ol - on model bacterial membranes. *Colloids Surf B Biointerfaces* 2016; 146: 918-23.
14. Hammer KA, Carson CF, Riley TV. Susceptibility of transient and commensal skin flora to the essential oil of *Melaleuca alternifolia* (tea tree oil). *Am J Infect Control* 1996; 24: 186-9.
15. Herrero ER, Slomka V, Boon N, Bernaerts K, Hernandez-Sanabria E, Quirynen M, Teughels W. Dysbiosis by neutralizing commensal mediated inhibition of pathobionts. *Sci Rep* 2016; 6: 38179.
16. Herrero ER, Fernandes S, Verspecht T, Ugarte-Berzal E, Boon N, Proost P, Bernaerts K, Quirynen M, Teughels W. Dysbiotic Biofilms Deregulate the Periodontal Inflammatory Response. *J Dent Res* 2018; doi: 10.1177/0022034517752675.

17. Hidalgo E, Dominguez C. Mechanisms underlying chlorhexidine-induced cytotoxicity. *Toxicol In Vitro* 2001; 15(4): 271-6.
18. Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 2010; 35(4): 322-32.
19. Ivanovski S, Lee R. Comparison of peri-implant and periodontal marginal soft tissues in health and disease. *Periodontol 2000* 2018; 76(1): 116-30.
20. Khan ST, Khan M, Ahmad J, Wahab R, Abd-Elkader OH, Musarrat J, Alkhathlan HZ7, Al-Kedhairy AA. Thymol and carvacrol induce autolysis, stress, growth inhibition and reduce the biofilm formation by *Streptococcus mutans*. *AMB Express* 2017; 7(1): 49.
21. Li WR, Li HL, Shi QS, Sun TL, Xie XB, Song B, Huang XM. The dynamics and mechanism of the antimicrobial activity of tea tree oil against bacteria and fungi. *Appl Microbiol Biotechnol* 2016; 100(20): 8865-75.
22. Li X, Yoshihara K, De Munck J, Cokic S, Pongprueksa P, Putzeys E, Pedano M, Chen Z, Van Landuyt K, Van Meerbeek B. Modified tricalcium silicate cement formulations with added zirconium oxide. *Clin Oral Investig* 2017; 21(3): 895-905.
23. Llana-Ruiz-Cabello M1, Gutiérrez-Praena D1, Pichardo S2, Moreno FJ3, Bermúdez JM4, Aucejo S4, Cameán AM1. Cytotoxicity and morphological effects induced by carvacrol and thymol on the human cell line Caco-2. *Food Chem Toxicol* 2014; 64: 281-90.
24. Loozen G, Boon N, Pauwels M, Quirynen M, Teughels W. Live/dead real-time polymerase chain reaction to assess new therapies against dental plaque-related pathologies. *Mol Oral Microbiol* 2011; 26(4): 253-61.
25. Loughlin R, Gilmore BF, McCarron PA, Tunney MM. Comparison of the cidal activity of tea tree oil and terpinen-4-ol against clinical bacterial skin isolates and human fibroblast cells. *Lett Appl Microbiol* 2008; 46(4): 428-33.
26. Nostro A, Papalia T. Antimicrobial activity of carvacrol: current progress and future perspectives. *Recent Pat Antiinfect Drug Discov* 2012; 7(1): 28-35.
27. Ninomiya K, Maruyama N, Inoue S, Ishibashi H, Takizawa T, Oshima H, Abe S. The essential oil of *Melaleuca alternifolia* (tea tree oil) and its main component, terpinen-4-ol protect mice from experimental oral candidiasis. *Biol Pharm Bull* 2012; 35(6): 861-5.

28. Paster BJ, Olsen I, Aas JA, Dewhirst FE. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol 2000* 2006; 42: 80-7.
29. Pucher JJ, Daniel JC. The effects of chlorhexidine dielugonate on human fibroblasts *in vitro*. *J Periodontol* 1992; 63(6): 526-32.
30. Robert C, Moellering Jr. Antibiotic resistance: Lessons for future. *Clin Infect Dis* 1998; 27: 135-40.
31. Rodriguez Herrero E, Boon N, Pauwels M, Bernaerts K, Slomka V, Quirynen M, Teughels W. Necrotrophic growth of periodontopathogens is a novel virulence factor in oral biofilms. *Sci Rep* 2017; 7(1): 1107 doi: 10.1038/s41598-017-01239-9.
32. Seymour RA, Heasman PA, MacGregor IDM. Anti-plaque and anti-calculus agents. In: Seymour RA, Heasman PA (eds) *Drugs, diseases and the periodontium*. Oxford Medical Publications, Hong Kong 1992; 153–179.
33. Shahab A, Haghighati F, Baeeri M, Jamalifar H, Abdollahi M. A clinical, microbiological and immunological comparison between subgingival irrigation with Dentol and chlorhexidine in advanced periodontitis. *Arch Med Sci* 2011; 7(1): 154-60.
34. Smeets R, Henningsen A, Jung O, Heiland M, Hammächer C, Stein JM. Definition, etiology, prevention and treatment of peri-implantitis-a review. *Head Face Med* 2014; 10: 34.
35. Teughels W, Van Assche N, Sliepen I, Quirynen M. Effect of material characteristics and/or surface topography on biofilm development. *Clin Oral Implants Res* 2006; 2: 68-81.
36. Ultee A, Kets EP, Smid EJ. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* 1999; 65(10): 4606-10.

4 CONCLUSÃO

Com base nos achados do presente estudo, pode-se demonstrar que Terpinen-4-ol e Carvacrol usados durante a descontaminação de superfícies de titânio não afeta nas propriedades de superfície e permite uma adequada proliferação e adesão celular de células envolvidas no processo de re-osseointegração. Estes compostos mostraram atividade antimicrobiana, promovendo efeitos bactericidas, bacteriostáticos, antibiofilme e não citotóxica em baixas concentrações. Além disso, concentrações baixas e não citotóxicas de Terpinen-4-ol e Carvacrol podem inibir e erradicar patógenos periodontais tanto em biofilmes simples e multi-espécie. Devido a estas propriedades, ambos os monoterpenos podem representar uma abordagem promissora para prevenção e tratamento de doenças peri-implantes.

REFERÊNCIAS*

1. Belibasakis GN. Microbiological and immuno-pathological aspects of peri-implant diseases. *Arch Oral Biol.* 2014; 59(1): 66-72.
2. Heitz-Mayfield LJ, Lang NP. Comparative biology of chronic and aggressive periodontitis vs. peri-implantitis. *Periodontol 2000.* 2010; 53: 167-81.
3. Malcolm J, Millington O, Millhouse E, Campbell L, Adrados Planell A, Butcher JP et al. Mast cells contribute to *Porphyromonas gingivalis*-induced bone loss. *J Dent Res.* 2016; 95(6): 704-10.
4. Andersen R, Loebel N, Hammond D, Wilson M. Treatment of periodontal disease by photodisinfection compared to scaling and root planning. *J Clin Dent.* 2007; 18(2): 1-5.
5. Qin YL, Luan XL, Bi LJ, Sheng YK, Zhou CN, Zhang ZG. Comparison of toluidine blue-mediated photodynamic therapy and conventional scaling treatment for periodontitis in rats. *J Periodontal Res.* 2008; 43(2): 162-7.
6. Smeets R, Henningsen A, Jung O, Heiland M, Hammächer C, Stein JM. Definition, etiology, prevention and treatment of peri-implantitis: a review. *Head Face Med.* 2014 Sep 3; 10: 34.
7. Maruyama N, Maruyama F, Takeuchi Y, Aikawa C, Izumi Y, Nakagawa I. Intraindividual variation in core microbiota in peri-implantitis and periodontitis. *Sci Rep.* 2014; 13(4): 6602.
8. Fürst MM, Salvi GE, Lang NP, Persson GR. Bacterial colonization immediately after installation on oral titanium implants. *Clin Oral Implants Res.* 2007; 18(4): 501-8.
9. Quirynen M, Vogels R, Peeters W, Van Steenberghe D, Naert I, Haffajee A. Dynamics of initial subgingival colonization of “pristine” peri-implant pockets. *Clin Oral Implants Res.* 2006; 17(1): 25-37.
10. Kolenbrander PE1, Palmer RJ Jr, Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol.* 2010; 8(7): 471-80.
11. Teughels W, Quirynen M, Jakubovics N. Periodontal microbiology. In: Newman M, Takei H, Klokkevold P, Carranza F. *Clinical periodontology.* 11th ed. St Louis: Elsevier; 2012. p. 232-70.
12. Jenkinson HF, Lamont RJ. Oral microbial ecology. In: Lamont RJ, Hajishengallis GN, Jenkinson HF. *Oral microbiology and immunology.* 2nd ed. Washington, DC: ASM Press; 2014. p. 97-102.

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: <http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacaoatualizado.pdf>

13. Egland PG, Marquis RE. Oral microbial physiology. In: Lamont RJ, Hajishengallis GN, Jenkinson HF. Oral microbiology and immunology. 2nd ed. Washington, DC: ASM Press; 2014. p. 113-38.
14. Scannapieco FA. The oral environment. In: Lamont RJ, Hajishengallis GN, Jenkinson HF. Oral microbiology and immunology. 2nd ed. Washington, DC: ASM Press; 2014. p. 57-72.
15. Sundqvist GK, Eckerbom MI, Larsson AP, Sjogren UT. Capacity of anaerobic bacteria from necrotic dental pulps to induce purulent infections. *Infect Immun*. 1979; 25(2): 685-93.
16. Feuille F, Ebersole JL, Kesavalu L, Stepfen MJ, Holt SC. Mixed infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in a murine lesion model: potential synergistic effects on virulence. *Infect Immun*. 1996; 64(6): 2094-100.
17. Ebersole JL, Feuille F, Kesavalu L, Holt SC. Host modulation of tissue destruction caused by periodontopathogens: effects on a mixed microbial infection composed of *P. gingivalis* and *F. nucleatum*. *Microb Pathog*. 1997; 23(1): 23-32.
18. Mellado-Valero A, Buitrago-Vera P, Solá-Ruiz MF, Ferrer-García JC. Decontamination of dental implant surface in peri-implantitis treatment: a literature review. *Med Oral Patol Oral Cir Bucal*. 2013; 18(6): 869-76.
19. Norowski PA Jr, Bumgardner JD. Biomaterial and antibiotic strategies for peri-implantitis: a review. *J Biomed Mater Res B Appl Biomater*. 2009; 88(2): 530-43.
20. Figuero E, Graziani F, Sanz I, Herrera D, Sanz M. Management of peri-implant mucositis and peri-implantitis. *Periodontol 2000*. 2014; 66(1): 255-73.
21. Drisko CH. The use of locally-delivered doxycycline in the treatment of periodontitis. Clinical results. *J Clin Periodontol*. 1998; 25(11): 947-52.
22. Tastepe CS, Liu Y, Visscher CM, Wismeijer D. Cleaning and modification of intraorally contaminated titanium discs with calcium phosphate powder abrasive treatment. *Clin Oral Implants Res*. 2013; 24(11): 1238-46.
23. Louropoulou A, Slot DE, Van der Weijden FA. Titanium surface alterations following the use of different mechanical instruments: a systematic review. *Clin Oral Implants Res*. 2012; 23(6): 643-58.
24. Louropoulou A, Slot DE, Van der Weijden F. Influence of mechanical instruments on the biocompatibility of titanium dental implants surfaces: a systematic review. *Clin Oral Implants Res*. 2015; 26(7): 841-50.
25. Jansen JA, Den Braber ET, Walboomers XF, De Ruijter JE. Soft tissue and epithelial models. *Adv Dent Res*. 1999; 13: 57-66.

26. Rompen E, Domken O, Degidi M, Pontes AEF, Piattelli A. The effect of material characteristics, of surface topography and of implant components and connections on soft tissue integration: a literature review. *Clin Oral Impl Res.* 2006; 17 (Suppl. 2): 55-67.
27. Colvin RB. Fibrinogen–fibrin interactions with fibroblasts and macrophages. *Ann N Y Acad Sci.* 1983; 408: 621-33.
28. Teng F, Ko C, Kuo H, Hu J, Lin J, Lou C et al. A comparison of epithelial cells, fibroblasts, and osteoblasts in dental implant titanium topographies. *Bioinorg Chem Appl.* 2012; 2012: 687291. Epub 2012 Jan 12.
29. Sardin S, Morrier J, Benay G, Barsotti O. In vitro streptococcal adherence on prosthetic and implant materials. Interactions with physicochemical surface properties. *J Oral Rehabil.* 2004; 31(2): 140-8.
30. Valderrama P, Blansett JA, Gonzalez MG, Cantu MG, Wilson TG. Detoxification of implant surfaces affected by peri-implant disease: an overview of non-surgical methods. *Open Dent J.* 2014; 8: 77-84.
31. Wheelis SE, Gindri IM, Valderrama P, Wilson TG Jr., Huang J, Rodrigues DC. Effects of decontamination solutions on the surface of titanium: investigation of surface morphology, composition, and roughness. *Clin Oral Implants Res.* 2016; 27(3): 329-40.
32. Kasemo B, Lausmaa J. Biomaterial and implant surfaces: a surface science approach. *Int J Oral Maxillofac Implants.* 1988; 3(4): 247-59.
33. Kotsakis GA, Lan C, Barbosa J, Lill K, Chen R, Rudney J, et al. Antimicrobial agents used in the treatment of peri-implantitis alter the physicochemistry and cytocompatibility of titanium surfaces. *J Periodontol.* 2016; 87(7): 809-19.
34. Suarez F, Monje A, Galindo-Moreno P, Wang HL. Implant surface detoxification: a comprehensive review. *Implant Dent.* 2013; 22(5): 465-73.
35. Jones CG. Chlorhexidine: is it still the gold standard? *Periodontol 2000.* 1997; 15: 55-62.
36. Gosau M, Hahnel S, Schwarz F, Gerlach T, Reichert TE, Bürgers R. Effect of six different peri-implantitis disinfection methods on in vivo human oral biofilm. *Clin Oral Implants Res.* 2010; 21(8): 866-72.
37. Guggenheim B, Meier A. In vitro effect of chlorhexidine mouth rinses on polyspecies biofilms. *Schweiz Monatsschr Zahnmed.* 2011; 121(5): 432-41.
38. Giannelli M, Chellini F, Margheri M, Tonelli P, Tani A. Effect of chlorhexidine digluconate on different cell types: a molecular and ultrastructural investigation. *Toxicol In Vitro.* 2008; 22(2): 308-17.
39. Lee TH, Hu CC, Lee SS, Chou MY, Chang YC. Cytotoxicity of chlorhexidine on human osteoblastic cells is related to intracellular glutathione levels. *Int Endod J.* 2010; 43(5): 430-5.

40. Ciandrini E, Campana R, Federici S, Manti A, Battistelli M, Falcieri E et al. In vitro activity of Carvacrol against titanium-adherent oral biofilms and planktonic cultures. *Clin Oral Investig*. 2014; 18(8): 2001-13.
41. Rasooli I, Shayegh S, Taghizadeh M, Astaneh SD. Phytotherapeutic prevention of dental biofilm formation. *Phytother Res*. 2008; 22(9): 1162-7.
42. Abe S, Sato Y, Inoue S, Ishibashi H, Maruyama N, Takizawa T et al. Anti-*Candida albicans* activity of essential oils including Lemongrass (*Cymbopogon citratus*) oil and its component, citral. *Nihon Ishinkin Gakkai Zasshi*. 2003; 44(4): 285-91.
43. Almeida FC, Lemonica IP. The toxic effects of *Coleus barbatus* B. on the different periods of pregnancy in rats. *J Ethnopharmacol*. 2000; 73(1-2): 53-60.
44. Catalán A, Pacheco JG, Martínez A, Mondaca MA. In vitro and in vivo activity of *Melaleuca alternifolia* mixed with tissue conditioner on *Candida albicans*. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2008; 105(3): 327-32.
45. Maggi F, Bramucci M, Cecchini C, Coman MM, Cresci A, Cristalli G et al. Composition and biological activity of essential oil of *Achillea ligustica* All. (Asteraceae) naturalized in central Italy: ideal candidate for anti-cariogenic formulations. *Fitoterapia*. 2009; 80(6): 313-9.
46. Botelho MA, Nogueira NA, Bastos GM, Fonseca SG, Lemos TL, Matos FJ et al. Antimicrobial activity of the essential oil from *Lippia sidoides*, carvacrol and thymol against oral pathogens. *Braz J Med Biol Res*. 2007; 40(3): 349-56.
47. Baser KH. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. *Curr Pharm Des*. 2008; 14(29): 3106-19.
48. La Stora A, Ercolini D, Marinello F, Di Pasqua R, Villani F, Mauriello G. Atomic force microscopy analysis shows surface structure changes in carvacrol-treated bacterial cells. *Res Microbiol*. 2011; 162(2): 164-72.
49. Gursoy UK, Gursoy M, Gursoy OV, Cakmakci L, Könönen E, Uitto VJ. Antibiofilm properties of *Satureja hortensis* L. essential oil against periodontal pathogens. *Anaerobe*. 2009; 15(4): 164-7.
50. Oliveira ACM, Fontana A, Negrini TC, Nogueira MNM, Bedran TBL, Andrade CR et al. Use of *Melaleuca alternifolia* Cheele (Myrtaceae) oil in dentistry: perspectives on its use as alternative antimicrobial to infectious diseases of oral origin. *Rev Bras Pl Med*. 2011; 13(4): 492-9.
51. Catalán A, Pacheco JG, Martínez A, Mondaca MA. In vitro and in vivo activity of *Melaleuca alternifolia* mixed with tissue conditioner on *Candida albicans*. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2008; 105(3): 327-32.
52. Kwiecinski J, Eick S, Wójcik K. Effects of tea tree (*Melaleuca alternifolia*) oil on *Staphylococcus aureus* in biofilms and stationary growth phase. *Int J Antimicrob Agents*. 2009; 33(4): 343-7.

53. Nogueira MN, Aquino SG, Rossa Junior C, Spolidorio DM. Terpinen-4-ol and alpha-terpineol (tea tree oil components) inhibit the production of IL-1 β , IL-6 and IL-10 on human macrophages. *Inflamm Res*. 2014; 63(9): 769-78.
54. Hart PH, Brand C, Carson CF, Riley TV, Prager RH, Finlay-Jones JJ. Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* (tea tree oil), suppresses inflammatory mediator production by activated human monocytes. *Inflamm Res*. 2000; 49(11): 619–26.

APÊNDICES

Apêndice A: Material e Métodos – Publicação 1

Corpos de prova

Discos de titânio esterilizados com superfície tratada (jateamento e ataque ácido) foram cedidos pela empresa Implacil De Bortoli, SP, Brasil. Os discos foram confeccionados nas dimensões de 5 mm de diâmetro por 2 mm de espessura aproximadamente.

Preparo das soluções

Foi utilizado Carvacrol (Sigma, USA) e Terpinen-4-ol (Sigma, USA) diluídos em tampão fosfato (PBS). DMSO 0,4% (Dimethyl sulfoxide) (Sigma, USA) e DMSO 2% foram acrescentados respectivamente para Terpinen-4-ol e Carvacrol como agente solubilizante. Foram utilizados como controles água ultrapura e clorexidina 0.2%. A clorexidina foi a solução de escolha por ser mais comumente utilizada em protocolos clínicos habituais no tratamento de peri-implantite.

Tratamento das superfícies

Discos de titânio foram divididos em cinco grupos de acordo com o tipo de tratamento recebido: Terpinen-4-ol 0.059%, Carvacrol 0.5%, Clorexidina 0.2%, DMSO e água ultrapura. Em câmara de fluxo laminar, todos os discos foram colocados individualmente em poços de placas de microtitulação (24 poços) contendo 1 mL de cada solução previamente preparada e mantidos por 5 minutos a 37°C. Após o tratamento, as amostras foram deixadas à temperatura ambiente para secagem e armazenados até o momento das análises estabelecidas.

Ângulo de contato e Energia Livre de Superfície

As medidas dos ângulos de contato ($n=9$) foram calculados com os líquidos: Água (polaridade 52,20), Etilenoglicol (polaridade 19), Polietilenoglicol (polaridade 13,60) e Diiodometano (polaridade 2,60). Foi utilizada a técnica da gota séssil (Young-Laplace) a 37°C em goniômetro “Contact Angle System OCA” de DataPhysics Instruments GmbH, Germany acoplado a computador com software SCA 20 (Laboratório Materiais Magnéticos e Coloides do Departamento de Físico Química, Instituto de Química – UNESP Araraquara, SP). Este equipamento possui

uma câmera que grava a imagem da gota, que por sua vez é tratada num algoritmo para a determinação do ângulo de contato e uma fonte de luz difusa. Com ajuda de uma pinça as amostras foram colocadas sobre a base plana e em seguida com uma micro seringa “Hamilton DS 500/GT” de 500 μL acoplada no equipamento foi depositada uma gota de aproximadamente 0,08 μL do líquido sobre os discos testados e 10 segundos da gota entrar em contato com a superfície, se obteve os valores dos ângulos dos lados direito e esquerdo da imagem da gota formada com as diferentes soluções e calculado automaticamente θ médio. Depois de mensurado o ângulo de contato, a média aritmética e desvio padrão foram calculados para obter a energia livre de superfície de cada grupo de amostras, no software SCA 20 com a equação do OWRK (Owens-Wendt-Rabel-Kaelble).

Espectrometria de raios-X por Dispersão de Energia (EDS) em Microscópio Eletrônico de Varredura (MEV)

Tres amostras de cada grupo foram utilizadas para caracterizar a superfície por meio do Espectrômetro de raios-X por Dispersão de Energia ou EDS acoplado ao microscópio eletrônico de varredura (Philips - XL 30 FEG), detectores que captam raios-X característicos utilizados por dispersão de energia, com o objetivo de demonstrar os elementos químicos das amostras após a descontaminação com Carvacrol 0.5%, Terpinen-4-ol 0.059%, Clorexidina 0.2%, DMSO e água ultrapura, e antes dos testes de viabilidade e adesão celular. Previamente a esta análise os discos foram armazenados em dissecador.

Análise da adesão celular

Cultura celular e inicio do experimento

Foram utilizadas linhagem celulares de fibroblastos L929 e linhagens celulares de osteoblastos linhagem SaOs-2, as quais foram cultivadas e mantidas em meio de cultura Dulbecco's Medium (DMEM) (SIGMA Chemical Co., St. Louis, MO, USA) suplementado com 10% de soro fetal bovino (SFB – Cultulab, Campinas, SP, Brasil) e 1% de penicilina, estreptomicina e glutamina (100 UT/mL de penicilina, 100 $\mu\text{g/mL}$ de estreptomicina e 2 mmol/L de glutamina) (Gibco, Grand Island, NY, EUA) em atmosfera umidificada a 5% de CO_2 a 37°C. As substituições do meio de cultura foram realizadas a cada 2 dias e o crescimento celular foi avaliado a cada 24

horas. As células foram sub-cultivadas a partir da obtenção de sub-confluência, até que fosse obtido o número de células adequado para a realização do estudo.

A adesão celular foi realizada em 11 corpos de prova (9 para Alamar Blue e 2 para as análises no microscópio de Fluorescência) para cada grupo. Os experimentos foram realizados em três momentos diferentes. Utilizando uma pinça estéril, os espécimes foram colocados individualmente em placas de 96 poços. Em seguida, 5×10^4 células/poço (100 μ L), mantidas em incubadora umidificada por 24 h e 72 h e após cada período de a adesão celular foram avaliados utilizando o ensaio de Alamar Blue.

Análise da viabilidade celular pelo ensaio de Alamar Blue

O ensaio Alamar Blue® foi realizado para detectar a atividade metabólica celular através de indicador fluorimétrico. Este ensaio permite a avaliação da viabilidade das células em cultura, sem prejuízo para as mesmas, permitindo análises pareadas, de acordo com os períodos estabelecidos. Para isso, foi utilizado o reagente Alamar Blue® (Invitrogen), o qual se baseia na incorporação de um indicador de oxirredução que apresenta alteração de cor em resposta a redução química do meio de cultura em decorrência da respiração aeróbica. Assim, foi adicionado às células o equivalente a 10% do volume de cultura da solução de Alamar Blue® conjuntamente ao meio de cultura sem SFB (20 μ L de solução de Alamar Blue® em 180 μ L de meio de cultura). As células permaneceram em contato com a solução em incubadora em condições adequadas (37°C e 5% de CO₂) por 4 h. Após este período, 1 alíquota de cada compartimento (100 μ L) foram transferidas para uma placa de 96 poços e a fluorescência das amostras foi avaliada em espectrofotômetro (Synergy – H1 – Biotek Winooski, VT, USA) no comprimento de onda de 570 e 600 nm, nos períodos de 24 h e 72 h.

Análise da adesão e proliferação celular por Microscópio de Fluorescência

A adesão celular de linhagem celular de fibroblastos L929 osteoblastos SaOs-2 sobre os discos descontaminados com CHX 0.2%, DMSO 2%, Terpinen-4-ol 0.059% e Carvacrol 0.5% nos respectivos tempos de incubação (24 e 72 horas) foram estudadas mediante a aplicação de Actin Red 555 ReadyProbes® que é um Reagente de alta afinidade selectiva de filamentos de Actina onde o citoesqueleto de actina é corado com TRITC-phalloidin (vermelho) e os núcleos com Hoechst 33242

(azul). Na sequência, as amostras foram fixadas com Paraformaldeído por 15 minutos, lavadas com PBS. Adicionou-se Triton x-100 (0.1%) por 10 minutos e lavadas com PBS. Seguidamente as células receberam o marcador de actina e foram incubadas por 30 minutos, o reagente foi aspirado e adicionou-se o Hoechst por 10 minutos. As células foram analisadas em Microscópio de Fluorescência utilizando filtros vermelho e azul, com excitações nos comprimentos de onda (λ) de 540/565 nm de acordo com o fluorocromo utilizado.

Análise Estatística

A análise estatística foi realizada utilizando-se software SPSS versão 17.0 (Chicago, IL, USA) e os resultados expressos em média $\pm$ desvio padrão. Os dados foram analisados em relação à presença de outliers e quanto aos pressupostos de normalidade (teste de Shapiro-Wilks) e homogeneidade das variáveis (teste de Levene). Valores de ângulo de contato foram submetidos aos testes não paramétricos de Kruskal-Wallis e Mann-Whitney, para comparação dos grupos aos pares, uma vez que não apresentaram distribuição normal. Os dados da viabilidade celular foram transformados em porcentagem considerando ao grupo controle como 100% e em seguida os dados foram comparado utilizando ANOVA two-way. Todos os testes estatísticos foram considerados em nível de significância de 5% ($p < 0.05$).

Apêndice B: Material e Métodos – Publicação 2

Microrganismos

Foram utilizadas duas cepas de referência, *Fusobacterium nucleatum* (NCTC 10326) e *Porphyromonas gingivalis* (ATCC 32277).

Reativação das espécies

As cepas de referência foram cultivadas individualmente em Ágar Brucella (Becton Dickinson, Alemanha) suplementado com 5% de sangue de carneiro desfibrinado, hemina (10 mg / mL) e menadiona (5 mg / mL) durante períodos de 5 - 7 dias a 37 °C em câmara de anaerobiose (85% N₂, H₂ 10%, 5% de CO₂) (Don Whitley - Inglaterra). Quatro colônias bacterianas foram transferidas a um tubo contendo caldo BHI (Brain Heart Infusion, Acumedia, Lansing, USA) suplementado com 1% de extrato de levedura, hemina (5 mg/mL) e menadiona (10 mg/mL) e incubadas durante 48 horas em câmara de anaerobiose. Após crescimento, alíquotas foram transferidas para outro tubo contendo caldo BHI para completar o tempo adequado de crescimento segundo a curva de crescimento de cada microrganismo.

Coleta da saliva para utilização na adesão de microrganismos

Um único voluntário foi convidado a participar da pesquisa para a doação de saliva (CAAE 49473115.0.0000.5416– Comitê de Ética). Um voluntário sadio, que não apresentava presença de cárie ativa, doença periodontal, doenças sistêmicas graves ou doenças bucais e não fazia uso de medicamentos que pudesse alterar as propriedades da saliva. A coleta da saliva foi fracionada em 10 minutos pela manhã até que o volume total necessário fosse conseguido. No momento da coleta, o voluntário estava em jejum e não tinha realizado higienização bucal durante o período da manhã. O participante recebeu um frasco estéril onde foi depositada a saliva. Após a coleta, a saliva foi centrifugada a 10000 rpm por 5 minutos a 4°C e posteriormente o sobrenadante foi aspirado e filtrado. O resultante da saliva foi imediatamente armazenado a -70 °C até à utilização.

Preparo das soluções

As soluções foram preparadas em meio BHI caldo fazendo diluições seriadas de Carvacrol (0,26-0,0005% v/v) (Sigma, USA) e Terpinen-4-ol (3,8 a 0,001% v/v) (Sigma, USA) solubilizados em Dimetilsulfóxido (DMSO) (Sigma, USA) a 2% e 0.4% para Carvacrol e Terpinen-4-ol respectivamente. Clorexidina 2% (solução stock) foi utilizada como controle positivos e preparada a uma concentração de 0.2%.

Determinação da Concentração Inibitória Mínima (CIM) e Determinação da Concentração Bactericida Mínima (CBM)

O método de microdiluição em caldo foi realizado para a determinação da concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM) em placas de 96 poços de microtitulação estéreis (TPP, Suíça) de acordo com a Comissão Nacional de Orientações Clinical Laboratory Standards (CLSI). Foi preparado uma série de diluições de Carvacrol e Terpinen-4-ol. As diferentes concentrações (190 µL) de Carvacrol e Terpinen-4-ol foram distribuídas em placas de 96 poços (n=12). Cada cultura bacteriana, obtida por incubação em caldo BHI tal como descrito acima para as estirpes anaeróbicas, foi diluída para 1×10^7 UFC/mL e, em seguida, inoculados (10 µL) para cada poço. A concentração final do inoculo bacteriano (200 µL) foi 5×10^5 CFU/mL. BHI e uma suspensão bacteriana de cada estirpe serviu como controle negativo. Ensaios preliminares com DMSO foram realizados para excluir sua possível atividade bacteriostática e/ou bactericida; o solvente não mostrou qualquer atividade antibacteriana.

Após incubação em condições de anaerobiose a 37°C durante 48 horas, o nível de desenvolvimento microbiano foi medido a 600 nm em espectrofotômetro (Synergy H1 Multi-Mode Reader - BioTek, USA). A concentração inibitória mínima (CIM) foi definida como a menor diluição de Carvacrol e Terpinen-4-ol que restringiu o crescimento para uma absorvância de $\leq 0,1$. Para determinar a concentração mínima bactericida (CBM), alíquotas de 10 µL foram removidas assepticamente a partir de cada poço, semeadas em Ágar Brucella (duplicata) e incubadas sob condições anaeróbicas a 37°C durante 48-72 h. CBM foi determinada como a menor concentração de Carvacrol e Terpinen-4-ol em que 99,9% das bactérias foram mortas.

Concentração Mínima para Erradicação de Biofilmes (CMEB)

Biofilme simples de *P. gingivalis* ATCC 33277 e *F. nucleatum* NCTC 10326 foram desenvolvidas em placas de microtitulação de 96 poços estéreis (n=12). Para a formação da película adquirida, 50 µL de saliva humana não estimuladas foi adicionado a cada poço e incubou-se durante 4 horas sob agitação (75 rpm) a 37°C. Seguidamente a saliva foi removida por aspiração e os poços foram lavados com PBS estéril 10 mM (pH 7). Cada poço recebeu 200 µL de cada suspensão bacteriana correspondendo a 1×10^7 UFC/mL, obtida após a incubação em caldo de BHI. As placas de cultura celular foram incubadas em anaerobiose a 37°C durante 48 h. O meio foi mudado após 24 h por aspiração do meio gasto e a adição de meio fresco (100 µL). Finalmente, após 48 h cada poço foi lavado duas vezes com PBS estéril, e adicionou-se 200 µL de diluições de Carvacrol (0,13-0,0005%, v/v) e Terpinen-4-ol (3,8-0,001% v/v). Um controle negativo (200 µL de BHI) para cada espécie foram utilizados. As placas foram cobertas com folha de papel alumínio para protege-las da luz e incubadas em condições anaeróbias à 37°C durante 24 h. Depois da incubação, o biofilme foi analisado por ensaio de XTT.

Análise da viabilidade celular do biofilme (teste do XTT) para CMEB

O ensaio de XTT (2,3-bis (2-metoxi-4-nitro-5-sulfofenil) -5 - [(fenilamino) carbonil] -2H- Hidróxido de tetrazólio – Sigma, USA) é um teste colorimétrico e permite uma estimativa do metabolismo ativo microbiano por redução das enzimas desidrogenase presentes no sistema de transporte de elétrons em um cristal solúvel pelas células, o formazano. XTT (Sigma, USA) foi preparado em água ultrapura purificada na concentração final de 1 mg/mL. A solução foi esterilizada por filtração e armazenada a -80°C até à sua utilização. Solução de menadiona (Sigma, USA) foi preparada em acetona a 0,4 mM, imediatamente antes de cada ensaio. Após as 24 horas de incubação, as soluções testadas foram removidas e os poços foram lavados 2 vezes com solução salina estéril. Cada poço recebeu 200 µL de solução de XTT (contendo 158 µL de PBS com 200 mM glicose, 40 µL de XTT e 2 µL de menadiona diluída). As placas foram incubadas por 3 h no escuro a 37°C e a absorbância determinada em 492 nm utilizando um espectrofotômetro (Synergy H1 Multi-Mode Reader - BioTek, USA).

Efeito do Terpinen-4-ol e Carvacrol em biofilme formado sobre superfícies de titânio

Corpos de prova

Discos de titânio esterilizados com superfície tratada com jateamento e ataque ácido foram cedidos pela empresa Implacil De Bortoli, SP, Brasil. Os discos foram confeccionados nas dimensões de 5 mm de diâmetro por 2 mm de espessura aproximadamente.

Formação do biofilme

Com uma pinça estéril os discos (n=12) foram colocados em placas de microtitulação estéreis de 24 poços, adicionado 1 mL de saliva humana e mantidos durante 4 h a 37°C sob agitação (75 RPM), para a formação da película adquirida sobre as superfícies. Em seguida, as amostras foram removidas e lavadas duas vezes com PBS estéril e transferidas para poços de uma nova placa de microtitulação.

Para a formação dos biofilmes sobre as superfícies de titânio, suspensões de 1 mL de *F. nucleatum* ou *P. gingivalis* individualmente assim como suspensões de 500 µL de *F. nucleatum* e *P. gingivalis* associadas foram colocadas sobre os discos de titânio e mantidas à 37°C por 24 horas em anaerobiose que corresponde a fase de adesão. Após este intervalo, a suspensão foi removida e os discos lavados duas vezes em PBS estéril, para remoção das bactérias não aderidas. Em seguida, as amostras foram colocadas em novos poços contendo 1 mL de BHI caldo com a finalidade de promover o crescimento do biofilme, e a placa foi levada à câmara de anaerobiose pelo período de 5 dias, onde o meio gasto foi trocado a cada 24 horas.

Efeito do Carvacrol e Terpinen-4-ol e análise quantitativa do biofilme

Após a formação do biofilme, os discos foram lavados duas vezes com PBS estéril e transferidos para novos poços contendo 1 mL das soluções testes Carvacrol 0,26 e 0,06%, Terpinen-4-ol 0,94, e 0,24%, estas foram, entre as outras, as menores e melhores concentrações testadas que mostraram eficácia antimicrobiana e prevenção na formação de biofilme de *F. nucleatum* e *P. gingivalis*. Clorexidina 0,2% e PBS foram utilizados como controle positivo e negativo respectivamente. Todas as soluções testadas foram aplicadas e as placas mantidas em condições anaeróbias

por 5 minutos. Posteriormente os discos de titânio foram lavados com PBS estéril para remoção das soluções testes e análise quantitativa do biofilme.

Os biofilmes foram analisados por meio da quantificação de UFC/mL (Unidade formadora de colônias por mililitro). Após o término de incubação e a lavagem para a remoção dos biofilmes, os discos de titânio foram imersos em tubos tipo eppendorfs contendo 1 mL de PBS e submetidos à ultrassom (Cristófoli, potencia 160 Watts/Frequência ultrassônica 42 KHz) por 20 min. Seguidamente a massa do biofilme aderido também foi raspada cuidadosamente durante 1 min. Todo o procedimento foi padronizado e realizado por um único operador. Finalmente a suspensão resultante do biofilme contendo os microrganismos foi agitada por um minuto e essa suspensão final foi diluída e inoculada em placas de Ágar Brucella para seu crescimento. As placas foram mantidas a 37°C em anaerobiose por 48-72 horas para posterior contagem de colônias e transformados em UFC/mL.

Análise por microscopia confocal de varredura a laser (MCVL)

A fim de estabelecer se as soluções testes mantem os microrganismos viáveis ou não e aderidos ou não após o tratamento do biofilme formado sobre os discos de titânio, foi realizada análise por microscopia confocal de varredura laser (MCVL) (n=2).

Após o período de exposição com as diferentes soluções, os discos foram removidos e lavados delicadamente duas vezes com PBS para a remoção de microrganismos não aderidos. Para a análise no MCVL os discos foram corados com o kit Live/Dead BacLight - L7012 (Invitrogen) seguindo as recomendações do fabricante e analisados em microscópio confocal de fluorescência Zeiss (modelo LSM 780 invertido - FAPESP 2009/54035-4) (Zeiss, Alemanha) localizado no Instituto de Física de São Carlos – Universidade de São Paulo.

Para preparar uma solução estoque, 15 µL da solução A (SYTO 9) e 15µL de solução B (Iodeto de Propídio) foram adicionados a 15.000 µL de PBS estéril e, após a homogeneização completa, 800 µL foi adicionado a cada poço contendo os discos corpos com os biofilmes a serem analisados. Após aplicação dos corantes em cada disco individualmente, a placa foi fechada e envolta por um papel alumínio, com objetivo de realizar a difusão dos corantes no biofilme com ausência de luz, temperatura de 37°C e condições anaeróbias por 15 minutos. A seguir, as amostras foram posicionadas sobre uma lamínula, de modo a deixar a superfície a ser

analisada em contato com a mesma, o que possibilitou a análise do biofilme formado em microscópio confocal.

Os comprimentos de onda de excitação/emissão foram 488/500 nm para SYTO 9 e 488/635 nm para iodeto de propídio. As imagens obtidas foram adquiridas usando o software ZEN 2012 (Zeiss, Alemanha) com uma resolução de 1.024 por 1.024 pixels. As imagens de um único plano focal do biofilme foram capturadas pelo sistema usando uma lente de ampliação de 20x. A área selecionada para análise do biofilme foi definida aleatoriamente e não tão próxima à borda dos espécimes.

Avaliação da citotoxicidade do Carvacrol e Terpinen-4-ol

Cultura Celular e início do experimento

Foi utilizada linhagem celular de fibroblastos L929 as quais foram cultivadas e mantidas em meio de cultura Dulbecco's Medium (DMEM) (SIGMA Chemical Co., USA) suplementado com 10% de soro fetal bovino (SFB – Cultulab, Brasil) e 1% de penicilina, estreptomicina e glutamina (100 UT/mL de penicilina, 100 µg/mL de estreptomicina e 2 mmol/L de glutamina) (Gibco, USA) em atmosfera umidificada a 5% de CO₂ a 37°C. As substituições do meio de cultura foram realizadas a cada 2 dias e o crescimento celular foi avaliado a cada 24 horas. As células foram subcultivadas a partir da obtenção de sub-confluência, até que fosse obtido o número de células adequado para a realização do estudo. Em seguida, 1x10⁴ células/poço foram semeadas (n=12) em placas de cultura celular de 96 poços (100 µL) e mantidas em incubadora umidificada por 24 h para a formação de uma monocamada de células confluentes. Seguidamente, o meio de cultura foi aspirado e substituído por Carvacrol 0,26 e 0,06%, Terpinen-4-ol 0,94, e 0,24%, DMSO 0,4% e 2%, CHX 0,2% e DMEM sem SFB em contato com as células.

As placas foram incubadas por períodos de 5 minutos, 1 hora e 24 horas e após cada período de exposição determinado os efeitos citotóxicos foram avaliados utilizando o ensaio de MTT (metiltetrazolium).

Análise do metabolismo celular

A análise colorimétrica do metiltetrazolium (ensaio de MTT) avalia o metabolismo celular por meio da atividade citoquímica da enzima desidrogenase

succínica (SDH), a qual representa a taxa de respiração mitocondrial das células viáveis.

Cada poço dos grupos experimentais e controle receberam uma solução composta por 90 μ L de meio de cultura DMEM sem soro fetal bovino e 10 μ L de solução de MTT (Sigma, USA), a qual foi preparada por meio da dissolução de 5 mg do sal de MTT em 1 mL de PBS esterilizado. Após a incubação das células pelo período de 4 horas, a 37°C, o meio de cultura com a solução de MTT foi aspirado e, posteriormente, aplicado 100 μ L de solução de isopropanol acidificado em HCL a 0.04 N, para solubilizar os cristais formados na presença de mitocôndrias ativas. A coloração produzida foi quantificada, considerando-se que as células com atividade mitocondrial normal serão coradas em violeta intenso. A viabilidade celular foi avaliada por espectrofotometria (570 nm) utilizando um espectrofotômetro (Synergy H1 Multi-Mode Reader - BioTek, USA).

Todos os experimentos foram realizados em triplicata em três momentos diferentes.

Análise Estatística

A análise estatística foi realizada por meio do software SPSS versão 17.0 (IBM, USA) e os resultados expressos como média $\pm$ desvio padrão. Os dados foram analisados em relação à presença de outliers e quanto aos pressupostos de normalidade (teste de Shapiro-Wilk) e homogeneidade das variáveis (teste de Levene). Os dados obtidos referentes à CIM e biofilmes simples formados sobre superfícies de titânio ($\log_{10}$) apresentaram distribuição não normal e, portanto, foram utilizados os testes não-paramétricos de Kruskal-Wallis e U de Mann-Whitney para a comparação dos tratamentos; enquanto os dados de CMEB e biofilme dupla-espécie em discos de titânio ($\log_{10}$) foram avaliados utilizando ANOVA one-way seguido pelo teste de Tukey. Todos os valores de absorbância da análise da citotoxicidade foram transformados em porcentagem considerando o grupo controle não tratado como 100%, em seguida os dados foram comparados utilizando ANOVA two-way. Todos os testes estatísticos foram considerados em nível de significância de 5% ($p < 0,05$).

Apêndice C: Material e Métodos – Publicação 3

Corpos de prova

Discos de titânio esterilizados com superfície tratada (jateamento e ataque ácido) foram cedidos pela empresa Implacil De Bortoli, SP, Brasil. Os discos foram confeccionados nas dimensões de 5 mm de diâmetro por 2 mm de espessura aproximadamente. Adicionalmente discos de hidroxipatita de cálcio (CAD-HA) com 12,7 mm de diâmetro e 1,02-1,52 mm de espessura foram utilizados.

Preparo das soluções

Foi utilizado Carvacrol (Sigma, USA) e Terpinen-4-ol (Sigma, USA) diluídos em tampão fosfato (PBS). DMSO 0,4% (Dimethyl sulfoxide) (Sigma, USA) e DMSO 2% foram acrescentados respectivamente para Terpinen-4-ol e Carvacrol como agente solubilizante.

Biofilme multi-espécies derivado de Biorreator

Biofilme multi-espécies foi crescido em biorreator (BIOSTATVR B, Germany) simulando as condições bucais. Foram utilizadas 14 cepas bacterianas: *Streptococcus sanguinis* LM14657, *Streptococcus gordonii* ATCC 49818, *Streptococcus mitis* DSM 12643, *Streptococcus oralis* DSM 20627, *Streptococcus salivarius* TOVE-R, *Streptococcus mutans* ATCC 20523, *Streptococcus sobrinus* ATCC 20742, *Actinomyces viscosus* DSM 43327, *Actinomyces naeslundii* ATCC 51655, *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* ATCC 20482, *Aggregatibacter actinomycetemcomitans* ATCC 43718 e *Veillonella parvula* DSM 2008.

Inicialmente, foram adicionados ao recipiente do biorreator 750 mL de BHI II (pH 7,4) contendo 37 g/L brain heart infusion broth, 2,5 g/L de mucina tipo III (Sigma, USA), extrato de levedura de 1 g/L, 0,1 g/L de L-cisteína, 2 g/L de bicarbonato de sódio, 5,0 mg/mL hemina, 1,0 mg/mL menadiona e 200 µl/L Anti-espuma Y-30 (Sigma, USA).

Separadamente 2L de BHI II fresco foi preparado e esterilizado para refrescar o meio gasto do biorreator. Após o processo de esterilização, o biorreator foi configurado com 300 rpm de agitação na presença de 100% N₂ e 5% CO₂ a 37°C e

deixado durante 24h. O pH foi ajustado em 6,7 +/- 0,1. Simultaneamente as cepas bacterianas foram crescidas em BHI overnight à 37°C em condições adequadas para cada estirpe. Após este período, 10 mL de BHI contendo *S. Mitis* foi adicionado ao biorreator e após 7 horas, as seguintes cepas bacterianas foram adicionadas (750 µL). A absorbância de cada bactéria foi ajustada a OD de 1,4. Biofilme estável de várias espécies foram obtidos após 72 h. Durante as primeiras 48 horas, o meio não foi substituído. Depois disso, o meio foi substituído com um volume de 200 mL a cada 24 horas.

Formação de biofilme em posição vertical

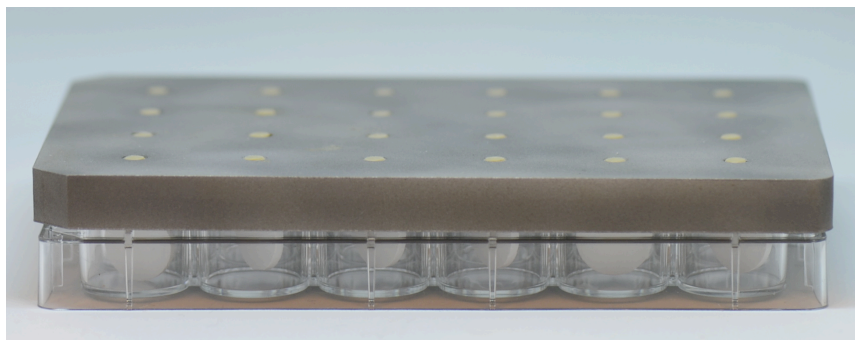
Em placas de 24 poços, foram adicionados 1800 µL de BHI-II fresco e 200 µL de cultura de biorreator, seguidamente discos (HA e titânio) foram posicionados verticalmente seguindo o modelo de adesão de biofilme “Amsterdam Active” (Figura 1 e 2) e incubados durante 24 h sob agitação a 170 rpm em condições anaeróbias. Após a formação do biofilme, os discos foram lavados com PBS para remover as células não aderidas, então os discos foram posicionados em uma nova placa de 24 poços contendo T4ol 0.19%, Carvacrol 0.06% e BHI (grupo controle). Foram seguidas duas condições: 1) Os biofilmes foram expostos à soluções testes durante 24 h sob agitação em condições anaeróbias. 2) Os biofilmes foram expostos por 5 minutos à temperatura ambiente com agitação de 250 rpm e posteriormente lavados com PBS, e transferido à uma nova placa contendo BHI-II fresco e incubados por mais 24 h sob agitação (170 rpm) em condições anaeróbias.

Figura 1 - Dispositivo utilizado para modelo de adesão de biofilme “Amsterdam Active”



Fonte: Elaboração própria.

Figura 2 - Posição dos discos no modelo de adesão de biofilme “Amsterdam Active”



Fonte: Elaboração própria.

Para a remoção dos biofilmes, os discos foram transferidos à tubos contendo 1,5 mL de tripsina-EDTA 0,05% (Gibco, USA) e mantidos sob agitação (230 rpm) a 37°C durante 45 minutos. Após este período, cada tubo foi vortexado durante 15 segundos e o líquido transferido para eppendorfs, sonicados durante 2 minutos e centrifugados (6010 x g, 5 min). Posteriormente o pellet foi ressuspendido em 500 µL de PBS.

Extração de DNA

A extração de células viáveis foi realizada utilizando PMA (propidium monoazide) (Biotium, USA), um corante de ligação ao DNA fotorreativo usado para detectar microorganismos viáveis por qPCR. PMA foi dissolvido em DMSO numa concentração final de stock de 1mg/mL. Então, 10 µL de PMA foi adicionado para cada 90 µL de cada amostra e mantidos durante 5 minutos no escuro. Seguidamente as amostras foram expostas durante 10 minutos à uma fonte de luz de 650 W a uma distancia de 20 cm e mantidas em gelo para evitar o excesso de aquecimento. DNA de cada bactéria foi extraída usando QIAamp DNA Mini Kit seguindo as instruções do fabricante.

Real time qPCR

As reações de quantificação por qPCR foram realizadas em equipamento CFX96 Real-Time System (BioRad, Hercules, CA. Para quantificação de DNA de cada estirpe bacteriana foi utilizado o sistema Taqman 5' e primers e sondas específicos. (Tabela 1) As reações de Taqman continham 12,5 µL de Mastermix (Eurogentec, Seraing, Bélgica), 4,5 µL de H₂O estéril, 1 µL de cada primer e sonda e 5 µL DNA de cada amostra. Os primers e sondas foram utilizados em diferentes

concentrações, dependendo da bactéria utilizada. As condições de amplificação foram: 2 min a 50°C, 10 min a 95°C, seguido de 45 ciclos de 95°C durante 15 s e 60 s a 60°C. A quantificação foi baseada em uma curva padrão de plasmídeo.

Quadro 1 - Primers e Sondas usados para detecção e quantificação de PMA-qPCR (Vitalidade bacteriana)

Cepa bacteriana		Primer/Sondas(5'-3')	Comprimento do fragmento
<i>A. actinomycetemcomitans</i>	Forward	GAA CCT TAC CTA CTC TTG ACA TCC GAA	80 bp
	Reverse	TGC AGC ACC TGT CTC AAA GC	
	Probe	AGA ACT CAG AGA TGG GTT TGT GCC TTA GGG	
<i>F. nucleatum</i>	Forward	GGA TTT ATT GGG CGT AAA GC	162 bp
	Reverse	GGC ATT CCT ACA AAT ATC TAC GAA	
	Probe	CTC TAC ACT TGT AGT TCC G	
<i>P. gingivalis</i>	Forward	GCG CTC AAC GTT CAG CC	68 bp
	Reverse	CAC GAA TTC CGC CTG C	
	Probe	CAC TGA ACT CAA GCC CGG CAG TTT CAA	
<i>P. intermedia</i>	Forward	CGG TCT GTT AAG CGT GTT GTG	99 bp
	Reverse	CAC CAT GAA TTC CGC ATA CG	
	Probe	TGG CGG ACT TGA GTG CAC GC	
<i>S. mutans</i>	Forward	GCC TAC AGC TCA GAG ATG CTA TTC T	114 bp
	Reverse	GCC ATA CAC CAC TCA TGA ATT GA	
	Probe	TGG AAA TGA CGG TCG CCG TTA TGA A	
<i>S. sobrinus</i>	Forward	TTC AAA GCC AAG ACC AAG CTA GT	88 bp
	Reverse	CCA GCC TGA GAT TCA GCT TGT	
	Probe	CCT GCT CCA GCG ACA AAG GCA GC	
<i>A. naeslundii</i>	Forward	TCG AAA CTC AGC AAG TAG CCG	96 bp
	Reverse	AGA GGA GGG CCA CAA AAG AAA	
	Probe	GGG TAC TCT AGT CCA AAC TGG CGG ATA GCG	
<i>S. gordonii</i>	Forward	CGG ATG ATG CTA ATC AAG TGA CC	177 bp
	Reverse	GTT AGC TGT TGG ATT GGT TGC C	
	Probe	AGA ACA GTC CGC TGT TCA GAG CAA	
<i>A. viscosus</i>	Forward	GTG AAG GAG CCA GCT TGC TGG TTC TG	155 bp
	Reverse	CGG AAC AAA CCT TTC CCA GGC	
	Probe	ATG AGT GGC GAA CGG GTG AGT AAC	
<i>S. salivarius</i>	Forward	AAC GTT GAC CTT ACG CTA GC	192 bp
	Reverse	ACC GTA ACG TGG GAA AAC TG	
	Probe	GTA GCG TCA GAG TGG TTG AC	
<i>S. oralis</i>	Forward	ACC AGC AGA TAC GAA AGA AGC AT	229 bp
	Reverse	AGG TTC GGG CAA GCG ATC TTT CT	
	Probe	AAG GCT GCT GTT GCT GAA GAA GT	
<i>S. mitis</i>	Forward	GGC TCG TAG TCT GGA GAT GG	133 bp
	Reverse	TAG GTC GTC GTC CCA AGG AA	
	Probe	CGA AGA GCA CCA ATA GCA CCT CCC	
<i>S. sanguinis</i>	Forward	CAA AAT TGT TGC AAA TCC AAA GG	75 bp
	Reverse	GCT ATC GCT CCC TGT CTT TGA	
	Probe	AAA GAA AGA TCG CTT GCC AGA ACC GG	
<i>V. parvula</i>	Forward	GAC GAA AGT CTG ACG GAG CA	171 bp
	Reverse	TGC CAC CTA CGT ATT ACC GC	
	Probe	AGC TCT GTT AAT CGG GAC GAA AGG C	

Fonte: Elaboração própria.

Análise estatística

Os dados obtidos neste estudo foram analisados utilizando o teste de normalidade D'Agostino-Pearson. Posteriormente, ANOVA-one way e o teste de Dunnett foram utilizados para comparações com o grupo controle utilizando o software GraphPad Prism (GraphPad Software, Inc.). As diferenças foram consideradas estatisticamente significativas em $p < 0,05$.

ANEXOS

Anexo A - Carta de submissão – publicação 2

05-Feb-2018

Dear Dr Maquera Huacho:

Your manuscript entitled "In vitro antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces" has been successfully submitted online and is presently being given full consideration for publication in Biofouling.

Your manuscript ID is GBIF-2018-0038.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to Manuscript Central at <https://mc.manuscriptcentral.com/gbif> and edit your user information as appropriate.

You can also view the status of your manuscript at any time by checking your Author Centre after logging in to <https://mc.manuscriptcentral.com/gbif>.

Thank you for submitting your manuscript to Biofouling.

Sincerely,
Biofouling Editorial Office

Author Dashboard

- 1 Submitted Manuscripts >
- 1 Manuscripts with Decisions >
- Start New Submission >
- Legacy Instructions >
- 5 Most Recent E-mails >
- English Language Editing Service >

Manuscripts with Decisions

ACTION	STATUS	ID	TITLE	SUBMITTED	DECISIONED
a revision has been submitted (GBIF-2018-0038.R1)	ADM: Evans, Len - Major Revision (05-Mar-2018) - a revision has been submitted view decision letter	GBIF-2018-0038	In vitro antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces View Submission	05-Feb-2018	05-Mar-2018

Submission Confirmation

 Print

Thank you for your revision

Submitted to

Biofouling

Manuscript ID

GBIF-2018-0038.R1

Title

In vitro antibacterial and cytotoxic activities of carvacrol and terpinen-4-ol against biofilm formation on titanium implant surfaces

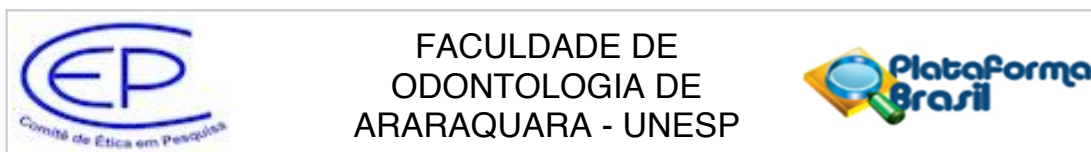
Authors

Maquera Huacho, Patricia Milagros
Tonon, Caroline
Correia, Marilia
Francisconi, Renata
Bordini, Ester
Marcantonio Junior, Elcio
Spolidorio, Denise

Date Submitted

20-Mar-2018

Anexo B - Comitê de ética – publicação 2



PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito da descontaminação de superfícies de titânio. Avaliação físico-química e estudo in vitro sobre modelo de biofilme periodontal e cultura celular.

Pesquisador: Denise Madalena Palomari Spolidorio

Área Temática:

Versão: 2

CAAE: 49473115.0.0000.5416

Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP

Patrocinador Principal: Faculdade de Odontologia de Araraquara - UNESP

DADOS DO PARECER

Número do Parecer: 1.330.377

Apresentação do Projeto:

Projeto apresentado de forma clara e objetiva, com todos os documentos exigidos.

Objetivo da Pesquisa:

O objetivo do presente estudo será analisar a descontaminação de superfícies de titânio com Carvacrol e Terpinen-4-ol, assim como avaliar in vitro suas propriedades antimicrobianas, citotóxicas e anti-inflamatórias sobre biofilmes de *Porphyromonas gingivalis* e *Fusobacterium nucleatum* desenvolvidas em superfícies de titânio

Avaliação dos Riscos e Benefícios:

Riscos: não há riscos sociais ou físicos para o voluntário. No entanto, para evitar qualquer desconforto durante a coleta da saliva não estimulada, ele será esclarecido quanto as condições da coleta que será realizada em sala isolada por apenas 10 minutos pela manhã, antes do período do jejum e da higienização oral. A coleta será fracionada em um período de 6 meses obtenção do volume total de 150mL. **Benefícios:** facilitar aplicação clínica de componentes antimicrobianos naturais, que não causem resistência bacteriana, para obtenção de melhores resultados no tratamento das doenças peri-implantares

Endereço: HUMAITA 1680

Bairro: CENTRO

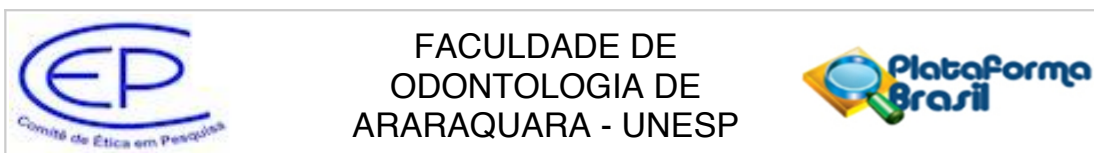
UF: SP

Município: ARARAQUARA

Telefone: (16)3301-6459

CEP: 14.801-903

E-mail: cep@foar.unesp.br



Continuação do Parecer: 1.330.377

Comentários e Considerações sobre a Pesquisa:

Pesquisa considera os fatores éticos e usará somente a saliva doada de pacientes esclarecidos sobre a pesquisa, por um TCLE

Considerações sobre os Termos de apresentação obrigatória:

Os termos estão sendo apresentados em conformidade com as exigências normativas.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Não existem pendências.

Considerações Finais a critério do CEP:

Protocolo APROVADO em reunião de 19 de Novembro de 2015.

O pesquisador deverá encaminhar relatórios parciais a cada 01 (um) ano até o prazo final da pesquisa, quando deverá encaminhar o relatório final.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_591325.pdf	15/10/2015 16:08:54		Aceito
Projeto Detalhado / Brochura Investigador	PROJETO2.pdf	15/10/2015 16:08:22	Denise Madalena Palomari Spolidorio	Aceito
Cronograma	CRONOGRAMA2.pdf	15/10/2015 16:03:34	Denise Madalena Palomari Spolidorio	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE2.pdf	15/10/2015 15:59:55	Denise Madalena Palomari Spolidorio	Aceito
Folha de Rosto	folha_de_rostro.pdf	21/09/2015 11:27:37	Denise Madalena Palomari Spolidorio	Aceito

Situação do Parecer:

Aprovado

Necessita apreciação da CONEP:

Não

Endereço: HUMAITA 1680

Bairro: CENTRO

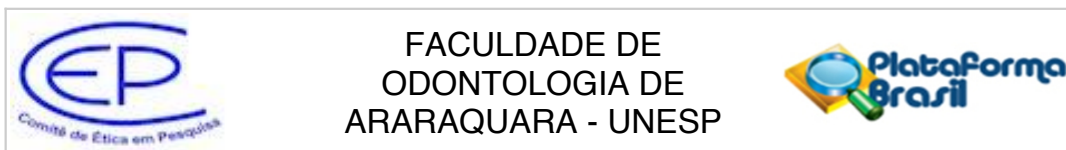
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Município: ARARAQUARA

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E-mail: cep@foar.unesp.br



Continuação do Parecer: 1.330.377

ARARAQUARA, 19 de Novembro de 2015

Assinado por:
Lígia Antunes Pereira Pinelli
(Coordenador)

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**Não autorizo a publicação deste trabalho pelo prazo de 2 anos após a data de
defesa**

(Direitos de publicação reservado ao autor)

Araraquara, 04 de Abril de 2018

Patricia Milagros Maquera Huacho