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

Carmélia Isabel Vitorino Lobo

Eficácia de compostos com alvos distintos no controle de biofilmes de *Streptococcus mutans* e *Candida albicans*

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Orientadora: Marlise Inêz Klein Furlan

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Carmélia Isabel Vitorino Lobo

Eficácia de compostos com alvos distintos no controle de biofilmes de *Streptococcus mutans* e *Candida albicans*

Comissão Julgadora

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DADOS CURRICULARES

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RESUMO

A doença cárie é um problema de saúde pública mundial, causada por biofilme patogênico modulado por uma dieta rica em açúcares (principalmente, sacarose). A associação de *Candida albicans* e *Streptococcus mutans* tem sido encontrada em biofilmes de lesões clínicas de cárie da infância. O biofilme misto dessas duas espécies é mais tolerante à tratamentos tópicos com agentes que controlam e diminuiem a virulência de biofilme simples de *S. mutans*. Portanto, avaliou-se as atividades antimicrobiana, antibiofilme e citotóxica de seis compostos com possíveis alvos em *S. mutans* e *C. albicans*: terpenóide (*tt*-farnesol ou Far), flavonoide (mirecetina ou Mir), três hidroxichalconas (2' hidroxichalconas: AGN e R815; 4' hidroxichalcona: C135) e um agente modulador da síntese de ácido lipoteicóico (composto 1771). Assim, determinou-se o potencial antimicrobiano (antibacteriano e antifúngico) e antibiofilme (modelos em placas de poliestireno: biofilmes iniciais de 24h e pré-formados de 48h) dos compostos isolados contra os microrganismos isolados. Após, combinou-se os compostos e concentrações promissoras entre si e com e sem flúoreto de sódio (F) para avaliação antibiofilme contra biofilmes mistos (iniciais e pré-formados). Posteriormente, avaliou-se a eficácia da aplicação tópica (5 min) dos compostos promissores contra biofilmes mistos formados em discos de hidroxiapatita com película salivar em 28h (expressão gênica) e 43h [população microbiana, biomassa, proteinas na porção insolúvel, componentes da matriz extracelular (exopolissacarídeos solúveis em água e em álcali, eDNA e proteinas), estrutura 3D e microscopia eletrônica de varredura (MEV) do biofilme]. Ainda, determinou-se o efeito citotóxico dos tratamentos usados para a aplicação tópica sobre monocamanda de queratinócitos orais. Os testes estatísticos consideraram α=0,05. Dentre os agentes avaliados, C135, Far, Mir e 1771 foram promissores para as atividades antimicrobiana e antibiofilme sobre biofilme simples de cada espécie (24 e 48h). Esses compostos combinados com e sem flúor foram eficazes contra as duas espécies microbianas (biofilme misto inicial); entretanto C135+Far+Mir, C135+Far+F e todos compostos combinados foram eficazes contra biofilmes mistos pré-formados. Os biofilmes mistos tratados topicamente com C135+Far+F apresentaram menor expressão gênica para genes *de C. albicans* associados à síntese (BGL2, FKS1) e remodelagem de β-glucanos (XOG1, PHR1, PHR2) (componentes da matriz extracelular), estresse oxidativo (SOD1), efluxo de fármacos (CDR1, ERG11) e maior expressão do gene de *S. mutans nox1* (tolerância à estresses oxidativo e ácido); já C135+Far resultou em menor quantidade de exopolissacarídeos insolúveis (quantificação bioquímica), biomassa e proteínas na porção insolúvel dos biofilmes, e redução de expressão de BGL2, ERG11, SOD1 e PHR2. A quantificação dos microrganismos e expopolissacarídeos a partir da microscopia confocal mostrou menor biovolume e porcentagem de cobertura da estrutura 3D para C135+F, C135+Far+F e C135; e nas imagens de MEV, C135+Far e C135+Far+F mostraram *C. albicans* apenas na forma de levedura. A exposição aos tratamentos por 30 min, mostrou citotoxicidade moderada para C135+F e C135 enquanto todos tratamentos contendo Far e a clorexidina (0,12%) foram altamente citotóxicos. Portanto, as formulações avaliadas afetaram alvos distintos dos biofilmes simples e misto de *S. mutans* e *C. albicans*; com efeito mais pronunciado contra o fungo em biofilmes mistos.

Palavras – chave: Biofilmes. *Streptococcus mutans*. *Candida albicans.*

Lobo CIV. Efficacy of compounds with distinct targets in the control of *Streptococcus mutans* and *Candida albicans* biofilms.[Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2022.

ABSTRACT

Dental caries is a worldwide public health problem caused by a pathogenic biofilm modulated by a diet rich in sugars (mainly sucrose). *Candida albicans* has been found in association with *Streptococcus mutans* in biofilms of clinical childhood dental caries lesions. Dual-species biofilms of these two species are more tolerant to topical treatments with agents that can control and decrease the virulence of single-species *S. mutans* biofilm. Therefore, the antimicrobial, antibiofilm, and cytotoxic activity of six compounds with possible targets in *S. mutans* and *C. albicans* were evaluated: a terpenoid (*tt*-farnesol or Far), a flavonoid (myricetin or Mir), three hydroxychalcones (2'-hydroxychalcones: AGN and R815; 4'-hydroxychalcone: C135) and a lipoteichoic acid synthesis modulating agent (compound 1771). Thus, the antimicrobial (antibacterial and antifungal) and antibiofilm (polystyrene model plates: initial 24h and 48h pre-formed biofilms) potential of the isolated compounds against isolated microorganisms were determined. Afterward, the promising compounds and concentrations were combined with each other and with or without sodium fluoride (F) for antibiofilm evaluation against dual-species biofilms (initial and pre-formed). After, the effectiveness of topical application (5 min) of the promising compounds with and without F against dual-species biofilms formed on hydroxyapatite discs with salivary pellicle was evaluated via different analyzes at 28h (gene expression) and 43h [microbial population, biomass, proteins of the insoluble portion, extracellular matrix components (water- and alkali-soluble exopolysaccharides, eDNA, and proteins), 3D structure and scanning electron microscopy (SEM) of the biofilm]. Also, the cytotoxicity of treatments used for topical application on oral keratinocytes (monolayer) was determined. Statistical tests followed α=0.05. Among the agents evaluated, C135, Far, Mir, and 1771 were promising for antimicrobial and antibiofilm activities on single-species biofilms of both species (24 and 48h). Combined compounds with and without fluoride were effective against both microbial species (initial dual-species biofilm); C135+Far+Mir, C135+Far+F and all compounds combined were effective against pre-formed dual-species biofilms. The topical treatments on dual-species biofilms with C135+Far+F showed lower gene expression for *C. albicans* genes associated with synthesis (BGL2, FKS1) and remodeling of β-glucans (XOG1, PHR1, PHR2) (extracellular matrix components), oxidative stress (SOD1), drug efflux (CDR1, ERG11) and increased expression of the *S. mutans* gene nox1 (tolerance to oxidative and acidic stress). C135+Far resulted in a lower amount of insoluble exopolysaccharides (biochemical quantification), biomass, and proteins in the insoluble portion of the biofilms and reduced the expression of BGL2, ERG11, SOD1, and PHR2. However, the quantification of microorganisms and exopolysaccharides from confocal microscopy showed lower biovolume and percentage of coverage of the 3D structure for C135+F, C135+Far+F, and C135. For SEM, biofilms treated with C135+Far e C135+Far+F presented *C. albicans* only as yeast form. The exposure to treatments (30 min) demonstrated moderate cytotoxicity for C135+F and C135, while treatments containing Far or chlorhexidine (0.12%) were highly cytotoxic. Therefore, the evaluated formulations affected different targets in single- and dual-species biofilm of *S. mutans* and *C. albicans*, with a more pronounced effect against *C. albicans* cells in dual-species biofilms.

Keywords: Biofilms. *Streptococcus mutans*. *Candida albicans.*

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1 INTRODUÇÃO

Muitas doenças infecciosas humanas são causadas por biofilmes virulentos, incluindo os biofilmes orais. Os biofilmes são comunidades de células microbianas altamente dinâmicas, estruturadas e organizadas, em que as células estão cobertas e imersas em uma matriz extracelular (MEC) tridimensional (3D) de substâncias poliméricas tais como exopolissacarídeos (EPS)[1]. A MEC serve como um esqueleto 3D para o desenvolvimento do biofilme, ajudando a moldar a heterogeneidade espacial e metabólica, e contribuindo para a criação de microambientes (nichos) que favorecem o crescimento e a proliferação dos microrganismos[2-4].

Dentre as doenças causadas por biofilmes, a cárie dentária é um problema de saúde pública mundial, afetando crianças e adultos[5,6]. A cárie dentária resulta de interações complexas entre organismos orais específicos, fatores do hospedeiro e dieta (rica em carboidratos, principalmente sacarose), que promovem o estabelecimento de um biofilme cariogênico na superfície dos dentes[7]. Ainda, a cárie da infância ou *ECC* (do inglês *early childhood caries*) é uma forma agressiva da doença, que se não tratada, pode resultar na rápida e extensiva cavitação dos dentes e outras complicações[8]; além disso, é dolorosa e onerosa para tratar, podendo afetar o bem-estar e o desenvolvimento integral das crianças[9].

A construção do biofilme cariogênico é orquestrado pela espécie *Streptococcus mutans,* através da formação da MEC, composta principalmente por EPS[10], mas também DNA extracelular (eDNA), ácidos lipoteicóicos e proteínas também estão presentes[11]. *S. mutans* é o principal produtor de MEC e modula a formação de biofilme cariogênico quando a sacarose e o amido da dieta do hospedeiro estão disponíveis[12,13], embora não seja a espécie mais numerosa e existam outros microrganismos igualmente acidogênicos e acidúricos na cavidade bucal[14,15]. As exoenzimas de *S. mutans* denominadas glicosiltransferases (Gtfs) e frutosiltransferases (Ftfs) utilizam a sacarose como substrato e os hidrolisados de amido como aceptores para a síntese de EPS (glicanos e frutanos)[10]; Gtfs também se adsorvem na superfície de outros microrganismos orais convertendo os mesmos em produtores de glicano[10]. Adicionalmente, *S. mutans* apresenta tolerância ao ambiente ácido[16,17], principalmente através atividade da bomba de prótons F0F1-ATPAse (codificada pelos genes do operon *atpCDGAHF*, dos quais *atpD* apresenta a maior

expressão)[18]. A resposta de *S. mutans* à acidificação do ambiente também torna esse microrganismo mais tolerante ao estresse oxidativo, especialmente via uma NADH oxidase (codificada por *nox1*)[19].

C. albicans é um dos microrganismos aos quais as Gtfs se ligam e onde as Gtfs formam quantidades elevadas de EPS[20]. Este fungo é acidogênico e acidúrico[21]; também produz MEC de composição variada, contendo eDNA, β glicanos, mananas, proteínas[22-25]; e essa matriz tem sido associada à resistência contra antifúngicos[26,27.] Ainda, a biogênese da matriz produzida por *C. albicans* é coordenada extracelularmente, refletindo ações de cooperação na comunidade do biofilme[27].

C. albicans produz polissacarídeos do tipo β-glicanos que estão presentes na parede celular e na matriz extracelular com ligações β-1,3 e β-1,6. A presença desses polissacarídeos é fator importante para aos antifúngicos[23,26], bem como eDNA também pode estar relacionado a proteção contra difusão de antifúngicos[25]. Em biofilmes maduros, os genes BGL2 (codifica beta-1,3- glicosiltransferase) PHR1 (codifica uma glicosidase), FKS1 (β-1,3 sintase de glicano) e XOG1 (codificador de β-1,3 exoglicanase) de *C. albicans* fazem a modificação e distribuição de carboidratos na matriz do biofilme maduro conferindo e reforçando a barreira contra os antifúngicos[26]. Ainda, o gene ERG11 esta relacionado com a síntese de ergosterol (principal esterol das membranas fungicas e análogo do colesterol nos mamíferos), e a sua expressão aumentada deste gene é associada a resistência antifúngica[28]. Ainda, o gene ERG11 esta relacionado com o crescimento filamentar, invasivo e virulência de *C. albicans*; assim como reduz a capacidade fúngica de adaptação ao estresse oxidativo; e dessa forma as vias da biosíntese de ergosterol também podem estar envolvidas na transição morfológica de *C. albicans*[29]. A expressão de BGL2 está relacionada a formação de EPS na matriz via biosíntese da parede celular e as funções do biofilme; PHR1 desempenha papel importante na estrutura da parede celular (*crosslinking* de β-1,3 e β-1,6 glicanos)[30] e na virulência de infeções quando o pH $\geq$ 5.5 (interferindo na morfologia do fungo). A expressão aumentada de PHR2 quando o pH < 5 (codifica uma glicosidade que também atua na ligação cruzada dos beta-glicanos fúngicos)[30] pode ajudar a reforçar a proteção da parede celular[31].

As modalidades terapêuticas contemporâneas para o controle e modulação da formação de biofilme dental são limitadas. Por exemplo, a clorexidina é um agente antimicrobiano de amplo espectro que suprime os níves de estreptococos do grupo

mutans na saliva, mas não é muito efetiva contra biofilmes maduros e seu uso preventivo e ou terapêutico (diário e contínuo) não é adequado[32]; e o flúor apesar de ser o padrão ouro para a prevenção da cárie (relacionado ao processo de remineralização), oferece proteção incompleta contra a doença[33].

Assim, uma estratégia atraente e superior seria o uso e/ou a inclusão de outros agentes bioativos, incluindo substâncias naturais[34-38] ou compostos sintéticos derivados de tais substâncias, que afetem a virulência e/ou a habilidade dos microrganismos desenvolverem biofilmes patogênicos. Nesse sentido, devem ser considerados os agentes inibitórios não biocidas, por preservarem a microbiota natural na boca e serem menos propensos ao desenvolvimento de resistência à terapia, como tem revelado os estudos com flavonoides e terpenóides[34-37].

Dentre os agentes promissores, *tt*-farnesol é um agente que tem como alvo a membrana celular, afetando a tolerância de *S. mutans* ao ácido; e mirecetina inibe a síntese de EPS *in situ*. Ambos agentes não têm efeito biocida pronunciado[35,36], mas sua combinação com flúor resulta em menos lesões de cárie, com menor severidade[36]. Ainda, farnesol é um derivado da via de biosíntese de esterol em células eucarióticas, e uma molécula de *quorum sensing* de *C. albicans*[38], mas aparenta não ter efeito contra *S. mutans* nas concentrações produzidas quando ambos microrganismos são co-cultivados em biofilmes[39,40]. Além disso, a combinação de *tt*-farnesol, mirecetina e flúor mostrou-se promissora em afetar o desenvolvimento do biofilme misto[41], entretanto, esta estratégia precisa ser aperfeiçoada (*e.g.,* aumentando a concentração dos agentes ou combinando-os com outros para potencializar seu efeito combinado) para o melhor controle desses biofilmes.

Farnesol em concentrações acima do *threshold* (*i.e.,* concentração fisiológica da espécie) é capaz de induzir apoptose em células de *C. albicans* em culturas planctônicas[42]. Farnesol é um composto lipofílico que se conjuga com glutadiona, um antioxidante importante para a detoxificação celular contra compostos que podem causar danos à célula. Conjugados de glutadiona agem como substratos para transportadores do tipo ABC (do inglês *ATP-binding cassete*, onde ATP é a abreviação do inglês *adenosine triphosphate*, que em português é o trifosfato de adenosina), que são transportadores de membrana dependentes de ATP; esses conjugados de glutadiona são transportados para fora das células por esses transportadores. Um estudo usando concentrações distintas de farnesol em contato com células por

tempos diferentes, demonstrou o envolvimento de efluxo de glutadiona mediado por CDR1 como o mecanismo precedente do processo apoptótico induzido por farnesol em *C. albicans*[43]. Adicionalmente, os genes SOD (do inglês *superoxide dismutase*), que codificam as enzimas superóxido dismutases primárias responsáveis pela defesa contra o estresse oxidativo, são induzidos [especialmente *superoxide dismutase* 1 (SOD1), sem gerar mudança na expressão de CDR1[43].

Além dos supracitados, as hidroxichalconas são intermediários metabólicos precursores de flavonoides e isoflavonoides e podem inibir a atividade de Gtfs[44]. Ainda, interferir na biologia dos ácidos lipoteicóicos (ALT) afetaria o desenvolvimento dos biofilmes, visto que ALT presentes na parede celular de espécies Gram positivas também estão presentes na MEC de biofilmes cariogênicos de *S. mutans*, e interagem com EPS durante o desenvolvimento e a maturação dos biofilmes[11]. Portanto, como o alvo do composto 1771 é a proteina LtaS (do inglês *lipoteichoic acid synthase*) que é a enzima sintetase de ALT em *S. aureus*[45], o seu efeito sobre *S. mutans* e sobre o desenvolvimento dos biofilmes pode ser promissor[46-48].

Assim, foram investigados compostos selecionados (com alvos distintos nas células e vias metabólicas de *S. mutans* e *C. albicans*) para identificar agentes seletivos antibiofilme que podem ter aplicações potenciais na prevenção da cárie dentária onde as duas espécies estão presentes.

2 PROPOSIÇÃO

Avaliar a eficácia antimicrobiana e antibiofilme de seis compostos selecionados com possíveis alvos tanto na bactéria *S. mutans* quanto no fungo *C. albicans*: um terpenóide (*tt*-farnesol), um flavonoide (mirecetina), três hidroxichalconas [duas 2' (AGN e R815) e 4' (C135)] e um agente que modula a síntese de ácidos lipoteicóicos em bactérias Gram positivas (composto 1771).

2.1 Objetivos Específicos

1. Verificar o potencial antimicrobiano (antibacteriano e antifúngico) de cada composto para as duas espécies cultivadas isoladamente via concentração inibitória mínima (CIM) e concentração bactericida ou fungicida mínima (CBM/CFM).

2. Analisar o potencial antibiofilme dos compostos isolados e combinados para as duas espécies cultivadas isoladamente e em conjunto (modelo em placas de 96-poços).

3. Selecionar os compostos e as concentrações do objetivo específico 2 para avaliar a eficácia dos tratamentos tópicos na inibição da formação e desenvolvimento do biofilme misto de *S. mutans* e *C. albicans* em modelo sobre discos de hidroxiapatita com película salivar.

4. Determinar o efeito citotóxico dos tratamentos tópicos com eficácia contra biofilmes.

3 PUBLICAÇÕES

A execução dos objetivos 1 e 2 resultou no artigo 1 publicado no periódico científico *Journal of Fungi* (ISSN2309-608X). Os desfechos dos objetivos seguintes foram utilizados para a confecção do artigo 2. Assim, à seguir estão apresentados os dois artigos.

3.1 ARTIGO 1[1]

Lobo, C.I.V.; Lopes, A.C.U.d.A.; Klein, M.I. **Compounds with Distinct Targets Present Diverse Antimicrobial and Antibiofilm Efficacy against *Candida albicans* and *Streptococcus mutans,* and Combinations of Compounds Potentiate Their Effect**. *J. Fungi* 2021, *7*, 340. doi.org/10.3390/Jof7050340.

Compounds with Distinct Targets Present Diverse Antimicrobial and Antibiofilm Efficacy against *Candida albicans* and *Streptococcus mutans,* and Combinations of Compounds Potentiate Their Effect

Abstract: *Candida albicans* and *Streptococcus mutans* interact synergistically in biofilms associated with a severe form of dental caries. Their synergism is driven by dietary sucrose. Thus, it is necessary to devise strategies to hinder the development of those biofilms and prevent cavities. Six compounds [*tt*-farnesol (sesquiterpene alcohol that decreases the bacterium acidogenicity and aciduricity and a quorum sensing fungal molecule), myricetin (flavonoid that interferes with *S. mutans* exopolysaccharides production), two 2'-hydroxychalcones and 4'-hydroxychalcone (intermediate metabolites for flavonoids), compound 1771 (inhibitor of lipoteichoic synthase in Gram-positive bacteria)] with targets in both fungus and bacterium and their products were investigated for their antimicrobial and antibiofilm activities against single-species cultures. The compounds and concentrations effective on single-species biofilms were tested alone and combined with or without fluoride to control initial and pre-formed dual-species biofilms. All the selected treatments eliminated both species on initial biofilms. In contrast, some combinations eliminated the bacterium and others the fungus in pre-formed biofilms. The combinations 4'-hydroxychalcone+*tt*-farnesol+myricetin, 4'-hydroxychalcone+*tt*-farnesol+fluoride, and all compounds together with fluoride were effective against both species in pre-formed biofilms. Therefore, combinations of compounds with distinct targets can prevent *C. albicans* and *S. mutans* dual-species biofilm build-up in vitro.

Keywords: biofilm; *Candida albicans*; *Streptococcus mutans*; extracellular matrix; antimicrobial agents; antibiofilm agents.

[1] Artigo escrito seguindo as normas do periódico: https://www.mdpi.com/journal/jof/instructions (ANEXO A)

INTRODUCTION

Several human diseases are caused by dysbiotic biofilms, including tooth decay, periodontal diseases, and oral candidiasis [1]. *Candida albicans* is an opportunistic species that, when associated with *Streptococcus mutans,* contributes to forming a complex and organized biofilm that is more tolerant to environmental stresses, including antimicrobial [2]. The interaction between these two species is synergistic in the presence of dietary sucrose and leads to severe dental caries lesions [3]. Therefore, it is necessary to devise strategies to hinder the development of those biofilms.

Within the complex oral microbiota, *S. mutans* is a producer of the extracellular matrix and modulates cariogenic biofilm formation when sucrose from the host's diet is available [4]. This bacterium is aciduric and acidogenic but not the most numerous species in the mouth, and there are other acidogenic and aciduric microorganisms [5,6]. However, its exoenzymes glucosyltransferases (Gtfs) and fructosyltransferase (Ftf) use sucrose as a substrate for the synthesis of exopolysaccharides (α-glucans and fructans), important components of biofilm construction [4]. Gtfs also adsorb on the surface of other oral microorganisms, converting them into glucan producers [4]. *C. albicans* is one of the microorganisms to which Gtfs binds and form high amounts of exopolysaccharides [7]. This fungus is also acidogenic and aciduric [8], and oral biofilms could serve as reservoirs for it.

The extracellular matrix of *C. albicans* biofilms contains extracellular DNA, β-glucans, mannans, proteins, and lipids [9-12]. This matrix has been associated with resistance against antifungals [13,14]. Moreover, the biogenesis of this matrix is coordinated extracellularly, reflecting cooperative actions in the biofilm community [14]. Therefore, the production of exopolysaccharides synthesized on surfaces *in situ* allows adhesion and microbial accumulation [4] and contributes to the construction of the 3D matrix that surrounds and supports cells, forming an environment with acidic niches and limited diffusion [6,15]. Thus, strategies that control the matrix formation could prevent pathogenic biofilms development [13] and perhaps control the amount of *C. albicans* in oral biofilms that could flourish when conditions are favorable.

The therapeutic modalities for controlling dental biofilm formation are still limited. Chlorhexidine is a broad-spectrum antimicrobial agent that suppresses microorganisms levels in saliva but is not effective against mature biofilms, and its daily

and continuous use is not recommended [16]. Fluoride is the mainstay of caries prevention, but its protection against the disease is incomplete [17]. Therefore, an attractive and superior strategy would be to use or include bioactive agents targeting virulence factors and the mechanisms for biofilm development.

Several studies have prioritized finding new antibiofilm agents, including natural substances [18-20]. Among the promising agents, *tt*-farnesol (a membrane-targeting sesquiterpenoid) and myricetin (a flavonoid) hinder the development of cariogenic biofilm formed by *S. mutans*. Myricetin inhibits the *gtfs* gene expression and Gtfs activity, thereby hindering the exopolysaccharides synthesis *in situ* [18,19]. *tt*-farnesol targets the cellular membrane, affecting the tolerance of *S. mutans* to acid stress. Both agents have a moderate biocidal effect [18,19], and their combination with fluoride results in fewer and less severe carious lesions [19]. In addition, *tt*-farnesol is a derivative of the sterol biosynthesis pathway in eukaryotic cells and a *quorum-sensing* molecule of *C. albicans* [21], which keeps the fungus in yeast form. However, it appears not to affect *S. mutans* in concentrations produced when both microorganisms are co-cultivated in biofilms, possible due to the thickness and amount of biomass of these biofilms [2,3]. Additionally, at concentrations above the threshold (*i.e.*, the physiological concentration of the species), *tt*-farnesol can induce apoptosis in planktonic cultures of *C. albicans* cells [22]. Therefore, the antibiofilm effect of *tt*-farnesol and myricetin against *C. albicans* and *S. mutans* biofilms still needs to be investigated [23].

Hydroxychalcones are precursor metabolic intermediates for flavonoids and isoflavonoids. These agents inhibit the streptococcal Gtfs activity [24], impair *S. mutans* survival in planktonic culture [25]; thus, possibly impairing the structure of biofilms. In addition, flavonoids interfere with *C. albicans* cell wall formation, cause disruption of the plasma membrane and mitochondrial dysfunction, affect cell division, protein synthesis, and the efflux-mediated pumping system [26]. Also, synthetic hydroxychalcones were shown to have anti-*Candida* activity [25,27]. Nevertheless, the efficacy of hydroxychalcones on dual-species *S. mutans* and *C. albicans* biofilms is unexplored.

Also, the interference in the metabolism of lipoteichoic acids (LTA) would affect the development of biofilms by Gram-positive bacteria. The compound 1771 targets LtaS, an LTA synthase enzyme in *S. aureus* [28] and *Enterococcus faecium* [29]. This

compound also hinders *S. mutans* biofilm formation [30]. However, the effect of compound 1771 on *C. albicans* is unknown.

Thus, considering the virulence and difficulty of controlling mature biofilms, this study evaluated the antimicrobial and antibiofilm activities of six compounds (*tt*-farnesol, myricetin, two 2'-hydroxychalcones, 4'-hydroxychalcone, and compound 1771) against *C. albicans* and *S. mutans* single- and dual-species settings.

MATERIALS AND METHODS (APÊNDICE A)

Experimental Design

Antimicrobial activity was evaluated using planktonic cultures of *C. albicans and S. mutans* in microdilution assay to determine the minimum inhibitory concentration (MIC) and minimum fungicidal and bactericidal concentrations (MFC and MBC). Next, single- species biofilms were used to investigate the antibiofilm activities of compounds during initial biofilm formation (24 h) and against pre-formed biofilms (48 h). Finally, promising compounds (and their corresponding concentrations) on both species were combined to analyze the antibiofilm activity on dual-species biofilms formed by *C. albicans* and *S. mutans* on initial biofilm formation (24 h) and pre-formed biofilms (48 h). At that time, fluoride was also added, and groups with and without fluoride were evaluated. All tests were performed using a 96-polystyrene microplate to determine viable microbial population (colony forming units or CFU). At least three independent experiments were performed in triplicate for the antimicrobial and antibiofilm tests (n = 9). The data were statistically analyzed according to the factorial design of this study, considering each microplate well as a statistical block. The hypothesis was that elimination or reduction of at least 50% of microbial cells (of both species) from biofilm using the proposed agents and their combinations substantially affect the development of dual-species biofilms.

Test Agents

An *in vitro* study with *C. albicans* and *S. mutans* was conducted to investigate the antimicrobial and antibiofilm activity of six compounds: *tt*-farnesol or (E,E)-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol, trans,trans-3,7,11-Trimethyl-2,6,10-dodecatrien-1-

ol (Sigma-Aldrich Co., St Louis, MO; Cat.#46193; 96% purity), myricetin or 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-chromen-4-one (AK Scientific, Inc.; Cat.#J10595; 95% purity), three hydroxychalcones [2'-hydroxichalcone or 1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one (Angene; Cat.#AGN-PC-015IM; 95% purity), 2'-hydroxichalcone or (2E)-1-(2-hydroxyphenyl)-3-phenylprop-2-en-1-one (AK Scientific, Inc.; Cat.#R815; 98% purity), 4'-hydroxichalcone or (2E)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one (AK Scientific, Inc.; Cat.#C135; 98% purity)], and compound 1771 [(5-phenyl-1,3,4-oxadiazol-2-yl)carbamoyl]methyl 2-{naphtho[2,1-b]furan-1-yl}acetate)] (UkrOrgSynthesis Ltd.; Cat.#PB25353228; purity not available). The compounds were diluted with 84.15% ethanol (EtOH; Sigma-Aldrich; Cat.#E7023) and 15% dimethyl sulfoxide (DMSO; Sigma-Aldrich; Cat.#D8418) to have stock solutions at 15 mg/mL, except for compound 1771 that was used at 2 mg/mL. The concentration for compound 1771 was lower than the other agents because of solubility issues. Also, sodium fluoride was prepared at 5000 ppm (Sigma-Aldrich; Cat.#71519). These stock solutions were diluted to distinct concentrations for assays. For antimicrobial assays, the agents with stock concentration at 15 mg/mL were tested using concentrations of 1250, 1000, 500, 250, 125, 62.5, 31.25, 15.625 µg/mL. For compound 1771 (stock concentration at 2 mg/mL), the concentrations used were 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, 0.977, 0.488, 0.244 µg/mL.

Microbial Strain and Growth Conditions

C. albicans SC5314 and *S. mutans* UA159 (serotype c; ATCC 700610) strains preserved in a freezer -80°C were thaw, platted on a blood agar plate (5% sheep's blood; Laborclin), and incubated (48h, 37ºC, 5% CO_2; Steri-Cult™Thermo Scientific, Waltham, USA). Next, five colonies of each microorganism were inoculated into a liquid culture medium (2.5% tryptone with 1.5% yeast extract or TY, pH 7.0; Difco) containing 1% of glucose (w/v) (TY+1% glucose) and incubated (16h, 37ºC, 5% CO_2). After that, the starter cultures were diluted 1:20 in the same culture medium and grown until mid-log growth phase: *S. mutans* OD_{562nm} 0.500 (±0.100) and *C. albicans* OD_{562nm} 0.482 (±0.058) (ELISA plate reader, Biochrom Ez, Cambourne, UK). These cultures were diluted in TY+1% glucose for each microorganism inoculum with $2x10^6$ colony-forming units per milliliter (CFU/mL) for antimicrobial evaluation. However, the mid-log growth

phase cultures were diluted in TY+1% sucrose (w/v) to yield $2x10^6$ CFU/mL for single-species antibiofilm assays.

Antimicrobial Activity

The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) and minimum fungicidal and bactericidal concentration (MFC and MBC, respectively) using broth microdilution following the Clinical and Laboratory Standards Institute [31-33], with some modifications [34]. The compounds were evaluated according their stock concentration ranging from 0 to 1250 µg/mL (when stock concentration 15 mg/mL) and 0 to 250 µg/mL (for compound 1771 with stock concentration at 2 mg/mL). Of note, 0 µg/mL was the vehicle-control. For most of the newest compounds, MIC has been described with some caveats, as when the visual inspection and the optical density are compromised by precipitation, for example [35]. Here, most agents complexed when in contact with the culture medium, making visual inspection subjective and interfering with optical density readings, and most of them did not present a clear dose-dependent effect. Therefore, the abbreviation 'IC_{50}' was defined as the inhibitory concentration of the agent that inhibited the growth of the microorganism by 50% [34], considering microbial growth as the CFU/mL counts. Thus, MIC abbreviation was not employed to state the outcomes**.**

All selected compounds [*tt*-farnesol, myricetin, 2'-hydroxychalcone (AGN), 2'-hydroxychalcone (R815), 4'-hydroxychalcones (C135), and compound 1771 (1771)] were tested against *S. mutans* planktonic culture. However, only the effective compounds against the bacterium (*tt*-farnesol, myricetin, C135, and compound 1771) were used for *C. albicans* because when both species are together, they form robust biofilms mediated primarily by the exopolysaccharides produced by the bacterium in a rich-sucrose environment [3].

The inoculum culture (100 µL of bacterium or fungus) was transferred to 96-well plates containing TY+1% glucose and distinct concentrations of agents were arranged from the highest to the lowest for a final volume of 200 µL (yielding $1x10^6$ CFU/mL for each species). As controls for each experiment, wells containing only culture medium, only inoculum (growth control without treatment), and inoculum plus vehicle (final concentration of 7% EtOH and 1.25% DMSO) were added to rule out any effect of the vehicle on microbial cells. The assembled plates were incubated (24h, 37ºC, 5% CO_2),

followed visual inspection (turbidity: microbial growth, clear: no growth), and OD_{562nm} readings (ELISA plate reader). Next, the plates were incubated to homogenize the cultures (5 min, 75 rpm, 37ºC) (Quimis, São Paulo, BR). After that, an aliquot from each well was removed for serial dilution in saline solution (0.89% NaCl; Synth), plating (undiluted and 10^{-1} to 10^{-5}), and incubation (48h, 37ºC, 5% CO_2) to determine CFU/mL quantification and inhibitory concentration (IC_{50}). The MBC and MFC were measured by CFU/mL count and defined as the lowest compound concentration that inhibited microbial growth (or absence of colony growth on agar). However, for some compounds that may target the extracellular matrix production in biofilms, the concentrations that inhibited at least 50% of microbial growth (*i.e.,* 50% of CFU/mL reduction *vs.* vehicle) were considered promising antimicrobial activity [34,35].

Antibiofilm Activity

This analysis was conducted after determining antimicrobial activity and was performed using single- and dual-species settings and different exposure to compounds: activity against initial biofilm formation (24h) and pre-formed biofilms (48h). For initial biofilm formation, the agents were introduced at the time 0 h, and biofilms were evaluated at 24h of development. For pre-formed biofilms, biofilms were grown for 24h and then treated, being evaluated at 48h of growth. Thus, it was evaluated the inhibition of biofilm formation for biofilms at 24h and the eradication of biofilm growth for 48h-old biofilms. The measurement was considered effective when the CFU/mL count was reduced by 50% (*vs.* vehicle) for 24 and 48h-old biofilms [34,36].

The strains were grown and prepared using the methodology described above. However, the culture medium and inoculum of the experiments were prepared using TY+1% sucrose. The selected compounds and their concentrations were based on antimicrobial data: C135 (from 1250 to 62.5 µg/mL), myricetin (from 1250 to 125 µg/mL), *tt*-farnesol (from 1250 to 31.25 µg/mL), and compound 1771 (from 250 to 1.953 µg/mL). Previous studies evaluated the antibiofilm activity of myricetin, compound 1771, and *tt*-farnesol for *S. mutans* [18,19,30], but here distinct concentrations were tested. Tests with compound 1771 for *C. albicans* and C135 for both species will be presented for the first time.

Initially, single-species biofilm to prevent initial biofilm formation (24h) were analyzed. On a 96-well plate, 100 µL of final inoculum with $2x10^6$ CFU/mL (for both species) were added to each well, containing test concentrations and culture medium (TY+1% sucrose), totalizing 200 µL ($1x10^6$ CFU/mL). Controls of experiments were also added (wells containing only culture medium, wells containing only the inoculum of the experiment, and wells containing the inoculum plus vehicle or 0 µg/mL). The plate was incubated (24h, 37ºC, 5% CO_2). After incubation, visual inspection was performed, followed by orbital incubation (5 min, 75 rpm, 37ºC). The remaining biofilms on the wells were rinsed three times with 200 µL of 0.89% NaCl solution to remove any loose material. Next, these biofilms were scraped with pipet tips five times (5X) with 200 µL of 0.89% NaCl, totalizing 1 mL of biofilm suspension (from each well). This biofilm suspension was placed in a microtube, subjected to serial dilutions (10^{-1} to 10^{-5}), which were plated, as were the undiluted suspensions. The plates were incubated (48h, 37ºC, 5% CO_2), and the CFU/mL was calculated. Next, the microbial growth inhibition of each concentration was compared to vehicle control.

Subsequently, prevention of build-up pre-formed biofilm (48h) was evaluated [36]. Here, 96-well plates were assembled using 100 µL final inoculum of *C. albicans* or *S. mutans* ($2x10^6$ CFU/mL) and 100 µL of TY+1% sucrose (to reach $1x10^6$ CFU/mL). The microplate was incubated (24h, 37°C, 5% CO_2) without any treatment or vehicle control. After incubation and biofilm formation, visual inspection was performed, followed by culture medium removal and washing of remaining biofilms (three times with 200 µL 0.89% NaCl solution). Next, fresh culture medium TY+1% sucrose and test concentrations of agents or the vehicle were added. The microplate was incubated again (24h, 37°C, 5% CO_2). After incubation (when biofilms were 48 h-old), the same processing protocol applied for 24h-old biofilms was conducted until obtaining 1 mL of biofilm suspension. The biofilm suspensions were sonicated (30 s at 7 w, Sonicator QSonica, Q125), subjected to serial dilutions (10^{-1} to 10^{-5}), and plated. The undiluted suspensions were also plated. The CFU/mL counts were evaluated and compared to vehicle control.

Finally, the antibiofilm activity for dual-species biofilms of *C. albicans* and *S. mutans* was also performed at 24 and 48h. These analyzes were performed with the same methodology used for single-species biofilms (24 and 48h). However, the inoculum of the dual-species culture was prepared with $2x10^6$ CFU/mL of *S. mutans*

and $2x10^4$ CFU/mL of *C. albicans* [3] to reach $1x10^6$ CFU/mL of *S. mutans* and $1x10^4$ CFU/mL of *C. albicans* after adding culture medium or treatments.

The concentrations of agents with better results against each species in the single-species biofilm setting were selected: 125 µg/mL (C135 and *tt*-farnesol), 500 µg/mL (myricetin), and 3.906 µg/mL (1771). Then, compounds with selected concentrations were combined with each other and with or without sodium fluoride (250 ppm) or F totalizing 16 groups. The elected combinations included groups without fluoride (C135, C135+*tt*-farnesol, C135+1771, C135+myricetin, C135+*tt*-farnesol+1771, C135+*tt*-farnesol+myricetin, C135+*tt*-farnesol+myricetin+1771, C135+1771+myricetin, *tt*-farnesol+myricetin, 1771+myricetin, *tt*-farnesol+1771+myricetin) and groups with fluoride (250 ppm) (C135+fluoride, C135+*tt*-farnesol+fluoride, C135+1771+fluoride, C135+myricetin+fluoride, C135+*tt*-farnesol+1771+myricetin+fluoride). The concentration of fluoride was selected based on the commercially available fluoride-based mouth rinse [19,37].

Statistical Analyses

The statistical analyses for CFU/mL values were performed using descriptive and inferential statistics. Normality was evaluated with the Shapiro-Wilk test employing a significance level of 5%. The data were non-parametric; thus, the data were evaluated with Kruskal–Wallis test, followed by Dunn's post-test, considering $\alpha = 0.05$ (Prism 9 software, GraphPad Software, Inc. 2021). The microbial growth inhibition of each agent and concentration was compared to vehicle control. In addition, the CFU/mL data were transformed to $\log_{10}$ or log to verify the log reduction.

RESULTS

Antimicrobial Activity

S. mutans

Three compounds (AGN, C135, R815) complexed with the culture medium, making the visual inspection analysis inaccurate; turbidity was also present for myricetin (but in minor proportion than for the three aforementioned compounds), and compound 1771 (at concentrations equal of more than 31.25 µg/mL). The observation of culture medium turbidity occurred immediately after adding the compound into the culture media, without microbial inoculation and incubation (controls per each concentration tested). The compound that did not complex with culture medium was *tt*-

farnesol, and the absence of visual growth was observed at 31.25 µg/mL, which was also its IC_{50}.

Regarding MBC, as per CLSI definition, the absence of colony growth on an agar plate was found for *tt*-farnesol and compound 1771, as 62.5 µg/mL and 250 µg/mL, respectively. Thus, a potential antimicrobial activity was achieved for the compounds when the compound at a specific concentration yielded a 50% reduction of CFU/mL counts compared to the vehicle control (IC_{50}), as follows.

For C135, the lowest concentration that yielded 50% reduction was 62.5 µg/mL, but the same reduction was observed for 125, 250, and 500 µg/mL (Figure 1). Thus, the antibacterial activity of C135 was not dose-dependent.

There was an absence of expressive effect on growth inhibition for all concentrations of AGN and R815 (*vs*. vehicle). However, some concentrations of R815 showed statistical differences and some inhibition of growth at 500 and 250 µg/mL (2 and 1 log reduction, respectively), 125 and 62.5 µg/mL (0.5 log reduction). These reductions were not dose-dependent and are not within the cutoff established for an effective compound (Figure 1). Thus, AGN and R815 were not used in the downstream assays.

Three concentrations of myricetin presented statistical differences compared to the vehicle (250, 500, and 1000 µg/mL) but were not dose-dependent. However, a better effect was obtained for 500 µg/mL, which was considered the IC_{50}. For compound 1771, the IC_{50} was 7.813 µg/mL; higher concentrations also demonstrated significative statistical differences (at least 4 log reduction *vs*. vehicle) and, as mentioned above, 250 µg/mL rendered absence of CFU/mL quantification on agar (MBC).

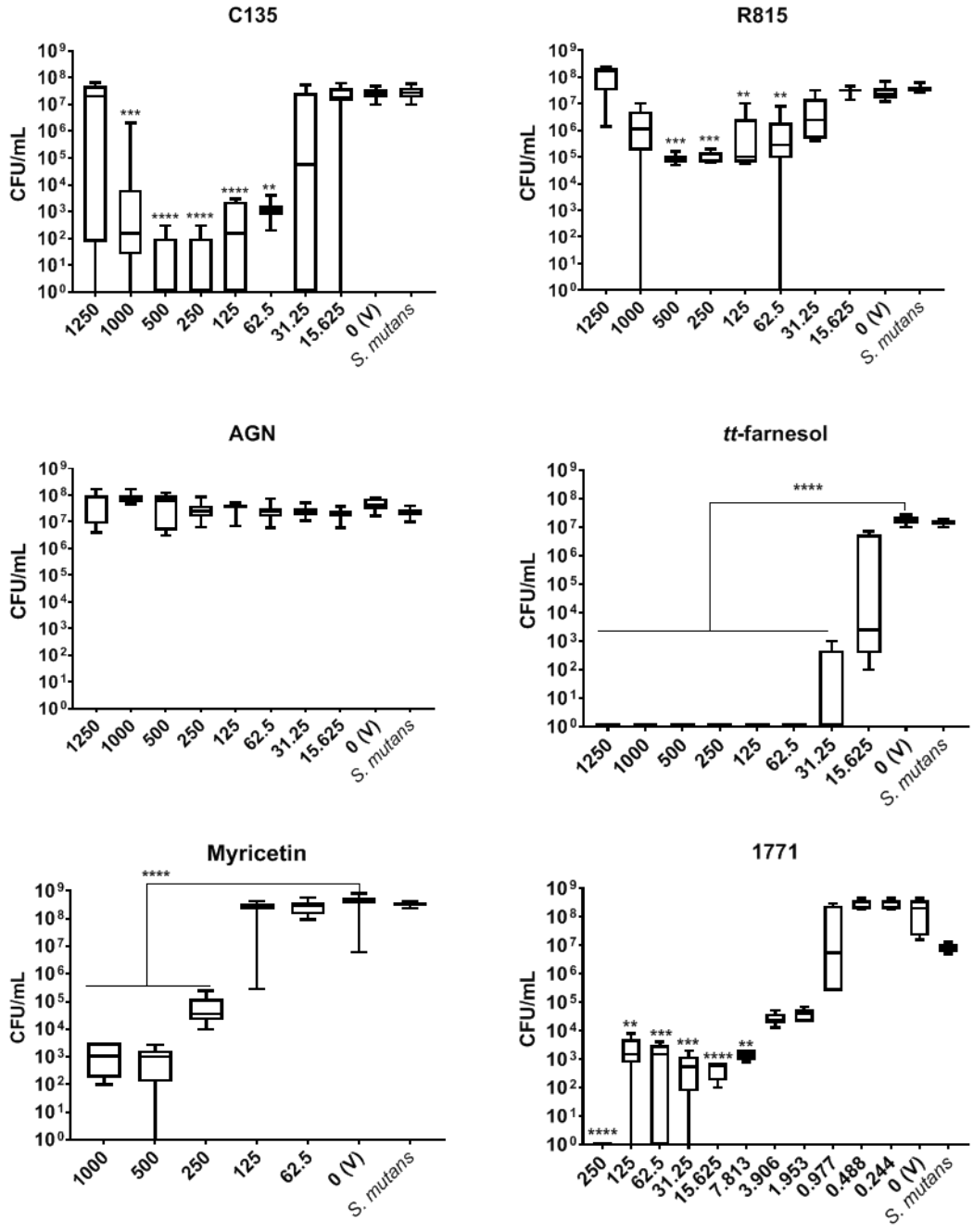


Figure 1. Antimicrobial activity of *S. mutans* using six compounds: C135, R815, AGN, *tt*-farnesol, myricetin and 1771. Data represents median and interquartile ranges (n = 9). Statistical differences are represented with ** ($p < 0.05$), *** ($p < 0.001$), and **** ($p < 0.0001$) (Kruskal Wallis, followed by Dunn's test). The tabulated results are in Table S1.

C. albicans

The antimicrobial activity of *C. albicans* was analyzed using four agents (C135, *tt*-farnesol, myricetin, and compound 1771) that were selected based on the effective antimicrobial activity founded for *S. mutans*. Among them, only *tt*-farnesol did not

complex with the culture medium; the absence of visual growth was observed at 125 µg/mL, which was also its IC_{50}.

C135 and *tt*-farnesol presented a dose-dependent effect on viable fungal growth. C135 was the most effective in inhibiting fungal viability (Figure 2). All its concentrations above 31.25 µg/mL hindered colony growth on agar plates; thus, its IC_{50} and MFC were determined as 62.5 µg/mL (absence of colony growth on agar plates). The MFC for *tt*-farnesol was 1000 µg/mL (Figure 2). Both myricetin and compound 1771 did not demonstrate significant antimicrobial activities as per the cutoff of 50% colony growth reduction (IC_{50}), although statistical differences were observed, as depicted in Figure 2.

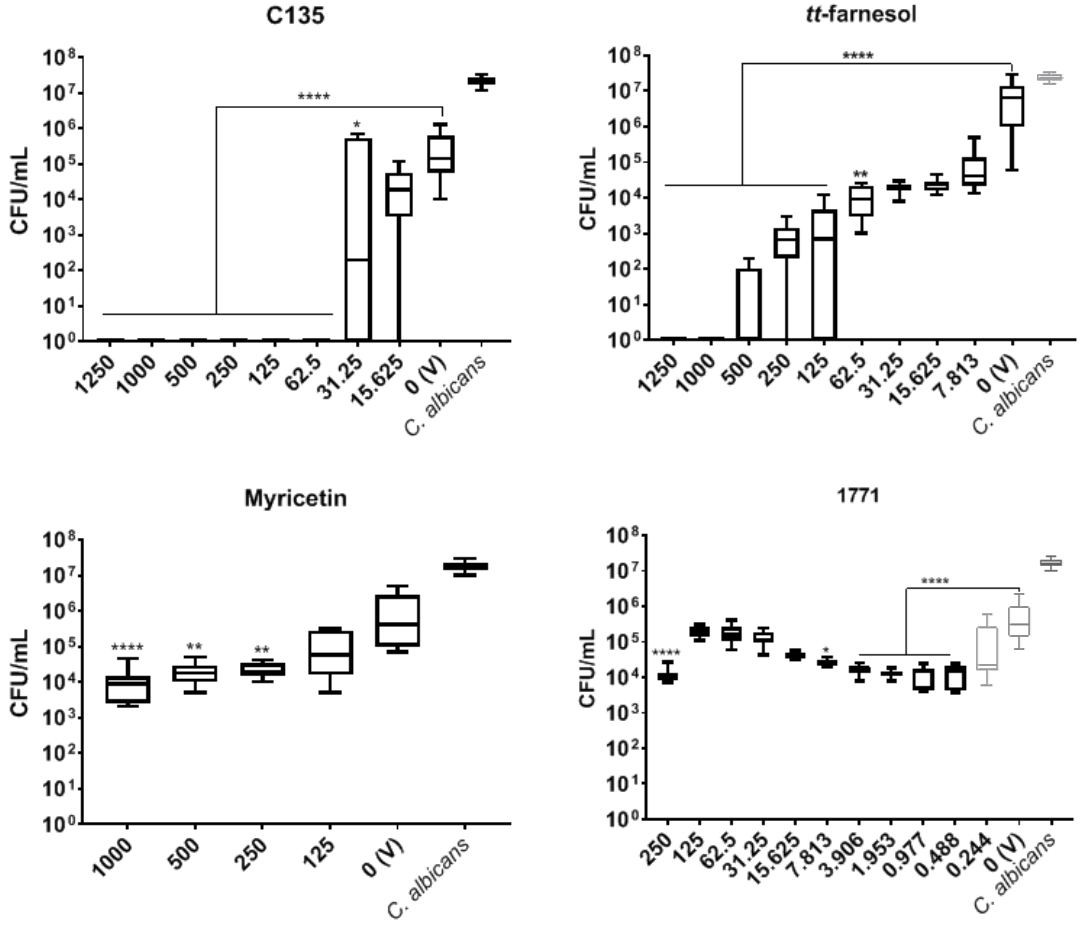


Figure 2. Antimicrobial activity of *C. albicans* with compounds: C135, *tt*-farnesol, myricetin and 1771. Data represents median and interquartile ranges (n = 9). Statistical differences are represented with * (C135 $p = 0.048$; 1771 $p = 0.013$), ** ($p < 0.05$) and **** ($p < 0.0001$) (Kruskal Wallis, followed by Dunn's test). The tabulated results are in Table S2.

Antibiofilm activity

Single-species *S. mutans* biofilm

On 24h biofilm (initial biofilm formation), all concentrations of tested compounds (C135, myricetin, *tt*-farnesol, and compound 1771) demonstrated antibiofilm activity, specially myricetin and *tt*-farnesol concentrations that eliminated bacterial growth (Figure 3). C135 eliminated the bacterium at 62.5, 125, and 250 µg/mL, but not at higher concentrations. Also, all concentrations of compound 1771 reduced about 5 logs of bacterium growth (*vs.* vehicle).

However, on *S. mutans* pre-formed biofilms (48h) a greater inhibitory effect was achieved with *tt*-farnesol; where concentrations of 62.5 µg/mL and higher eliminated the bacterium. For C135 the best concentration was 125 µg/mL with 5 logs reduction (*vs.* vehicle). In contrast, a lower inhibitory activity was observed for myricetin and compound 1771 (although they presented statistical differences, the reduction was about 1 log *v*s. vehicle) (Figure 3).

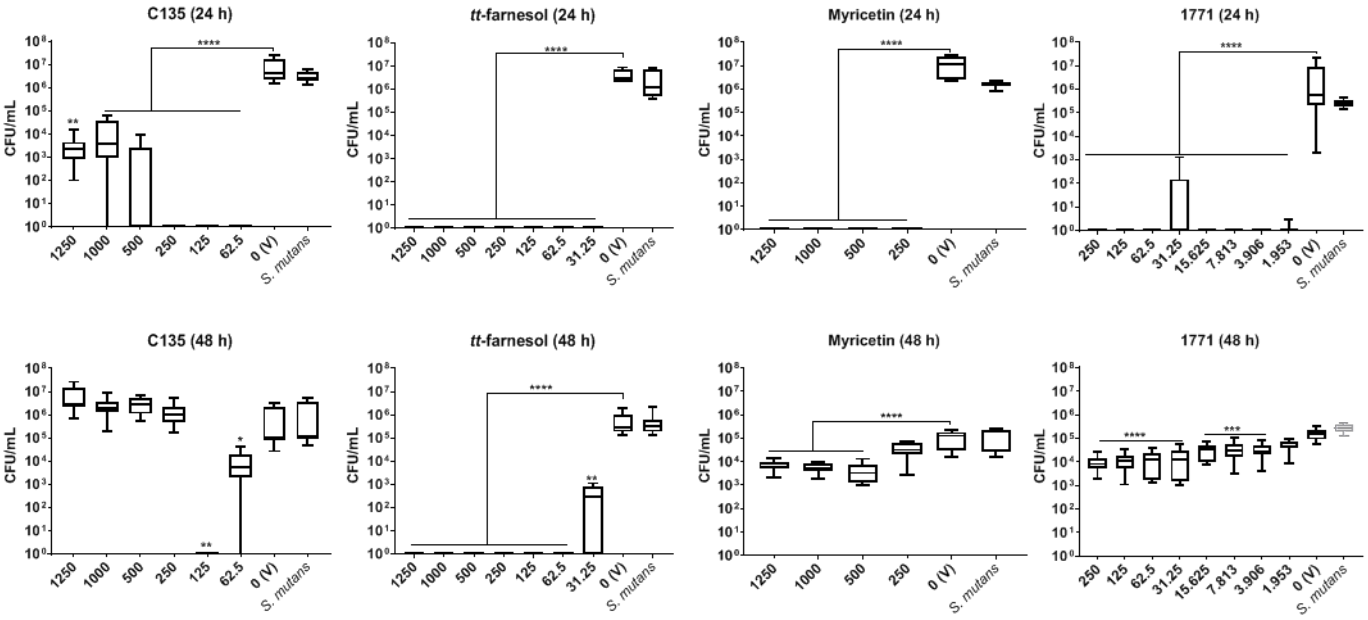


Figure 3. Antibiofilm activity of *S. mutans* with compounds: C135, *tt*-farnesol, myricetin, and 1771. On the first line are presented data of 24 h biofilm (initial biofilm formation); and right below are the data of pre-formed biofilms (48 h). The data represents median and interquartile ranges (n = 9). Statistical differences are represented with * (p = 0.026), ** (p < 0.05), ***(p < 0.001), and **** (p < 0.0001) (Kruskal Wallis, followed by Dunn's test). The tabulated results are in Table S3.

Single-species *C. albicans* biofilm

The best antibiofilm activity for *C. albicans* 24h biofilm was observed for C135 and *tt*-farnesol; both eliminated the fungus, except at 31.25 µg/mL of *tt*-farnesol that reduced 4 logs (*vs.* vehicle). Compound 1771 reduced 3 logs from 3.906 µg/mL to higher concentrations. However, the lowest antibiofilm effect was observed for myricetin with about 1 log reduction (*vs*. vehicle) in all tested concentrations, although statistical differences were observed (Figure 4).

The antibiofilm activity against *C. albicans* pre-formed biofilm (48h) was achieved effectively only by C135. C135 eliminated the fungus at 125, 500, and 1250 µg/mL; it also decreases CFU/mL counts by 4 logs (62.5 µg/mL) and 5 logs (250 and 1000 µg/mL) (*vs*. vehicle). A weak activity was observed using *tt*-farnesol, where concentrations above 125 µg/mL presented about 2 log reduction (*vs*. vehicle). However, no effect was observed using myricetin and compound 1771 (except 1771 at 250 µg/mL with 2 logs reduction *vs.* vehicle) (Figure 4).

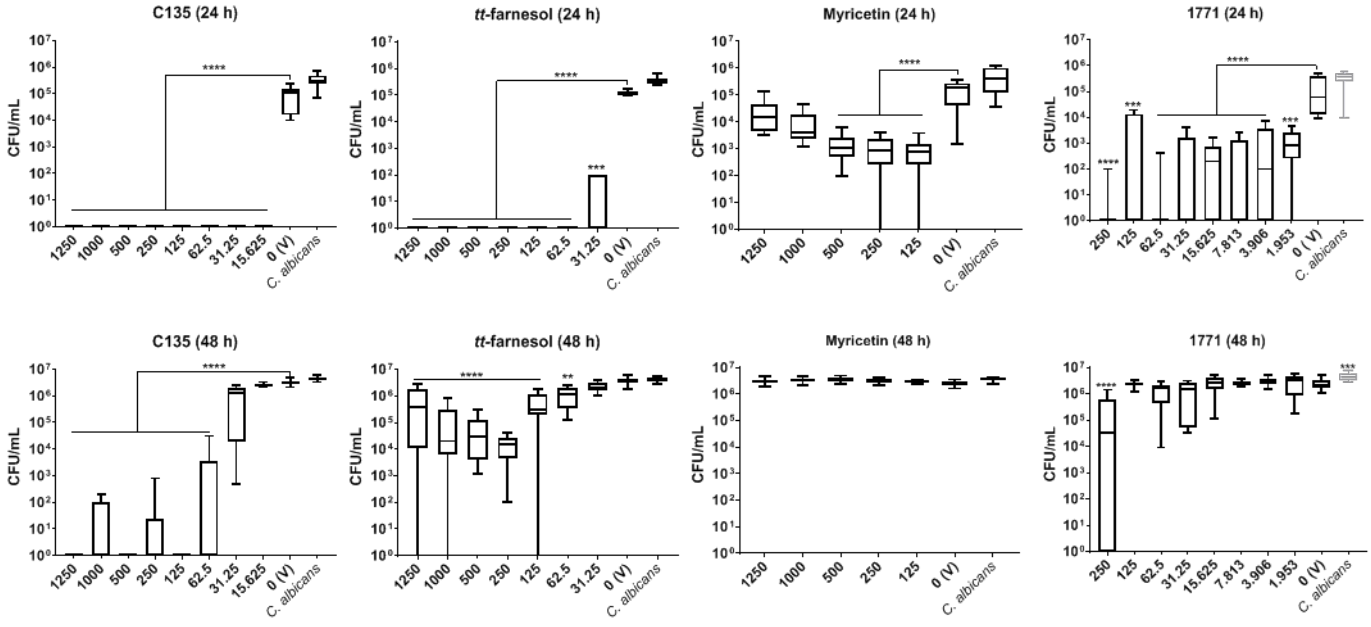


Figure 4. Antibiofilm activity of *C. albicans* with compounds: C135, *tt*-farnesol, myricetin, and 1771. On the first line are presented data of 24 h biofilm (initial biofilm formation); and right below are the data of pre-formed biofilms (48 h). The data represents median and interquartile ranges (n = 9). Statistical differences are represented with ** (p = 0.002), *** ($p \leq 0.0003$), and **** ($p \leq 0.0001$) (Kruskal Wallis, followed by Dunn's test). The tabulated results are in Table S4.

Dual-species *C. albicans* and *S. mutans biofilms.*

Based on the previously presented data, compounds and their most effective concentrations were selected for combinations of compounds tested on dual-species *C. albicans* and *S. mutans* biofilms (see data summarized in Table 1). The selected concentrations were: 125 µg/mL for C135 and *tt*-farnesol, 500 µg/mL for myricetin, and 3.906 µg/mL for compound 1771. In addition, C135 was used alone or combined with the other agents with and without sodium fluoride because it was the most effective agent against the fungus growth.

Table 1. Summary of antimicrobial and antibiofilm activity on single-species cultures for selection of compounds concentrations (µg/mL) to test against dual-species biofilms

Compound	Antimicrobial activity				Antibiofilm activity (single-species)			
	S. mutans		*C. albicans*		*S. mutans*		*C. albicans*	
	IC_{50}	MBC	IC_{50}	MFC	24h	48h	24h	48h
C135	62.5	-	62.5	62.5	62.5	125	15.625	125
tt-farnesol	31.25	62.5	125	1000	31.25	62.5	62.5	125*
Myricetin	500	-	-	-	250	500*	500*	-
1771	7.813	250	-	-	3.906	3.906*	3.906*	250*

* represent selected concentrations that did not reduce 50% of CFU/mL but had a significative statistical reduction vs. vehicle between all tested concentrations. IC_{50}: the inhibitory concentration of the agent that inhibited the growth of the microorganism by 50%, considering microbial growth as the CFU/mL counts. MBC: minimum bactericidal concentration. MFC: minimum fungicidal concentration. 24 h: 24 h-old biofilms. 48 h: 48 h-old biofilms.

For 24 h-old biofilms (initial biofilm formation), all 16 formulations were effective, impeding the growth of both species (bacterium and fungus) (Figure 5). However, the microbial growth inhibition of pre-formed (48 h-old biofilms) was different between treatments and species (Figure 5). Among the 16 formulations tested, three inhibited the growth of both species completely: C135+*tt*-farnesol+myricetin, C135+*tt*-farnesol+fluoride, and C135+*tt*-farnesol+1771+myricetin+fluoride (all compounds combined with fluoride). Furthermore, considering the total inhibition of bacterial growth in the dual-species setting, four formulations were effective (C135+*tt*-farnesol+1771+myricetin, C135+fluoride, C135+1771+fluoride, and C135+myricetin+fluoride). Considering the total inhibition of fungal growth in the dual-species setting, four treatments were effective (C135, C135+*tt*-farnesol, C135+1771, and C135+myricetin). Also, some formulations reduced at least 50% CFU/mL (*vs.* vehicle) of *S. mutans* (C135+*tt*-farnesol, C135+*tt*-farnesol+1771), or *C. albicans* (C135+*tt*-farnesol+1771+myricetin and C135+myricetin+fluoride).

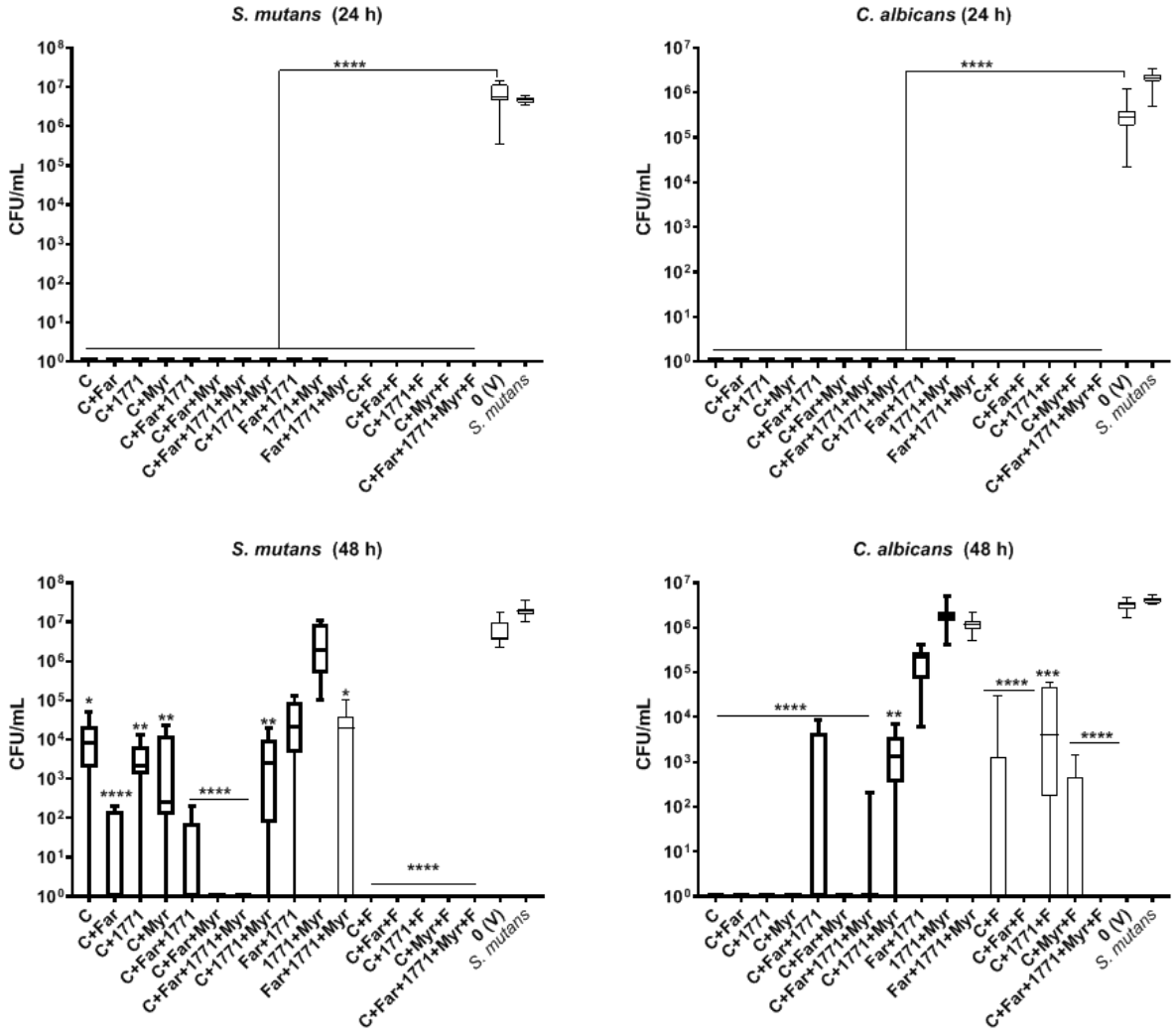


Figure 5. Antibiofilm activity of dual-species *S. mutans* and *C. albicans* biofilms with combined compounds (with and without sodium fluoride): C135 (C), *tt*-farnesol (Far), myricetin (Myr), 1771, and sodium fluoride (F). The top graphs presented data of 24 h biofilm (initial biofilm formation). The bottom graphs depict data of pre-formed biofilms (48 h). The data represents median and interquartile ranges ($n = 9$). Statistical differences are represented with * ($p = 0.04$), ** ($p \leq 0.002$), *** ($p = 0.0007$), and **** ($p \leq 0.0001$) (Kruskal Wallis, followed by Dunn's test). The tabulated results are in Table S5.

Discussion

Several strategies can be used to control biofilms to prevent oral diseases. The classical strategies for oral biofilm control include brushing/flossing (mechanical removal of biofilms) and restricting dietary sugar intake (mainly frequency) to prevent biofilm build-up. Both diet restriction and brushing/flossing require behavioral compliance, which can be challenging. In addition, fluoride is used to avoid teeth demineralization and promote remineralization as part of oral hygiene products

(toothpaste and mouthwashes) and/or supplied in tap drinking water. However, they may not be appropriate to all individuals, such as those without adequate dexterity (e.g., young children, elderly, people with disabilities, people in ICUs), which may require supervision for brushing/flossing and aids to weaken the overall biofilm structure to facilitate its mechanical removal, or even substances to enhance biofilm control.

Single targets for biofilm prevention and antimicrobial control can be difficult and limit the treatment options. Thus, combining agents with distinct targets can be an effective approach to access different sites in biofilms, which present complex biological traits and protected niches [1,19]. Therefore, this study evaluated the antimicrobial and antibiofilm activities of six compounds [*tt*-farnesol, myricetin, two 2'-hydroxychalcones (AGN and R815), 4'-hydroxychalcone (C135), and compound 1771] with different targets against *C. albicans* and *S. mutans* single- and dual-species settings.

Of note, an antimicrobial substance/molecule may not be an antibiofilm agent, and a compound with antibiofilm activity may not be an antimicrobial *per se* (*e.g.*, molecules that affect extracellular enzymes responsible for the extracellular matrix construction). This scenario is depicted by the findings in both antimicrobial and antibiofilm assays performed as some agents were effective against the microorganisms in planktonic cultures and were not in the biofilms' settings. Also, the effect of compounds was mostly non-dose-dependent for both *C. albicans* and *S. mutans*.

The antimicrobial outcome for *S. mutans* showed the lowest IC_{50} value for compound 1771 (7.813 µg/mL), followed by *tt*-farnesol (31.25 µg/mL), C135 (62.5 µg/mL), while the highest value was observed for myricetin (500 µg/mL). However, IC_{50} values for *C. albicans* were C135 (62.5 µg/mL) and *tt*-farnesol (125 µg/mL). Furthermore, *tt*-farnesol eliminated CFU/mL count of both species reaching a MBC (62.5 µg/mL) and a MFC (1000 µg/mL). The compound 1771 presented MBC (250 µg/mL) and C135 reached MFC (62.5 µg/mL). The findings for C135 for both species and 1771 for *C. albicans* are presented for the first time here. Thus, C135 presented a promising antimicrobial effect for both species, and compound 1771 did not inhibit *C. albicans* growth.

A previous study with *S. mutans* using different concentrations of compound 1771 did not eliminate the bacterium [30], but the total elimination of CFU/mL count

was observed here using the greatest concentration. In the same study [30], the antimicrobial activity of myricetin for *S. mutans* was at a lower concentration than the one found here. The antimicrobial effect of *tt*-farnesol on both species corroborates previous findings [18,22]. Among the three chalcones tested, the antimicrobial activity was better for C135, a 4'-hydroxychalcone. The other two 2'-hydroxychalcones (AGN and R815) did not present antimicrobial effect for *S. mutans*. These results can be explained by the differences between the chemical structure of the selected hydroxychalcones, suggesting that the presence of hydroxyl groups on the ring of the 4'-hydroxychalcone scaffold is crucial for the growth inhibition [24,38,39].

The presence of an extracellular matrix is essential for the existence of biofilm and the complete expression of virulence by microbial pathogens and pathobionts, hindering the action of antimicrobial agents and preventing their access to microbial cells [40]. Sucrose can modulate microbial synergism and ecology of the oral microbiota because its hexoses (glucose and fructose) are used for exopolysaccharides and organic acid production that influences the structure and composition of dental biofilms [1,6]. Cariogenic biofilms promote interactions and mechanisms that control dysbiosis [1,6] as observed on dual-species biofilms of *S. mutans* and *C. albicans in vitro* [2,3]. Therefore, it is important to understand the mechanisms of possible antibiofilm compounds.

Myricetin and some hydroxychalcones inhibit *S. mutans* F-ATPase activity (acid tolerance mechanism) [19], glycolysis (organic acid production or acidogenicity) [19], and synthesis of extracellular matrix glucans (by interfering with *gtfs* gene expression and Gtfs activity) [19,24]. The deficit in glucan production can compromise the integrity and 3D structure biofilms [4], facilitating their disruption. These findings can explain the antibiofilm effect of myricetin on *S. mutans* initial biofilms (24h) and the greatest potential of C135 (4'-hydroxychalcone) on initial (24h) and mature *S. mutans* biofilm (48h). The weaker inhibition of myricetin on pre-formed biofilms (48h—biofilm eradication) can be promoted by the presence of pre-formed microcolonies and their 3D extracellular matrix.

tt-farnesol eliminated the bacterium in 24- and 48h-old single-species biofilms (at 62.5 µg/mL and higher concentrations). The antimicrobial and antibiofilm effect of this compound can be related to the targets on *S. mutans* cytoplasmatic membrane, altering its proton permeability, decreasing its tolerance to acid stress [18]. Compound 1771 also had a promising antibiofilm effect for the bacterium, especially on initial

biofilm (24 h-old single-species biofilms). This compound inhibits the LTA synthesis [28], interfering with the cell wall composition, making the cytoplasmatic membrane an easy target for environmental stresses. Also, LTA from the cell wall are released in the matrix and interact with exopolysaccharides during the development and maturation of biofilms [41]; hence, interfering with LTA metabolism can impair cell wall and extracellular matrix composition. Thus, *tt*-farnesol and compound 1771 hindered *S. mutans* biofilms by promoting antimicrobial and antibiofilm activities.

Hydroxychalcones inhibit the cell wall formation of *C. albicans* cells [42,43], while *tt*-farnesol keeps the fungus in its yeast form [21]. However, it is unknown whether myricetin inhibits *C. albicans* biofilm formation or its extracellular matrix development or whether compound 1771 could target this fungus or its metabolism. The effect on the extracellular matrix components and construction could make the fungal cells more susceptible to antimicrobial agents. Here, single-species *C. albicans* biofilms were not greatly affected by myricetin. However, the 4'-hydroxychalcone C135 presented a promising effect in the initial (24h) and mature (48) fungal biofilms. Also, *tt*-farnesol and 1771 were effective on initial fungal biofilm, but only *tt*-farnesol eliminated the fungus (at 62.5 μg/mL and higher concentrations). In addition, *tt*-farnesol had a weak effect, while 1771 had practically no effect on fungal growth on pre-formed biofilms. Thus, eradication of *C. albicans* biofilm (48h) was achieved only with C135.

Previous studies with *C. albicans* biofilms demonstrate that chalcones inhibited enzymes involved in resistance pathways [42]. Thus, the effect of C135 on *C. albicans* biofilms can be related to targets on resistance pathways; nevertheless, this hypothesis must be better explained. In addition, as observed on the data above from antimicrobial activity of *tt*-farnesol, this compound can inhibit fungal growth, induce apoptosis in *C. albicans* cells, and inhibit the fungal hyphae [21,22]. As described for initial biofilms (24h), the treatment was applied at 0h, so it may be that the effect of *tt*-farnesol (preventing filamentous morphology) supports the inhibition of fungal growth in 24h while hampered its effect on pre-formed biofilm (48h). Therefore, combine compounds could improve the effect in mature biofilms. This hypothesis is confirmed by the potentiated effect on *C. albicans* cells in dual-species biofilm (48h) when C135 and *tt*-farnesol were combined, suggesting inhibition of the resistance mechanisms when both compounds are present.

Altogether, the data from antimicrobial and single-species biofilms assays enabled the selection of specific concentrations of the four compounds (C135,

myricetin, *tt*-farnesol, and compound 1771) that were combined, with or without sodium fluoride, to assess the antibiofilm activity of formulations against dual-species *S. mutans* and *C. albicans* biofilm. All formulations without fluoride [(C135, C135+*tt*-farnesol, C135+1771, C135+myricetin, C135+*tt*-farnesol+1771, C135+*tt*-farnesol+myricetin, C135+*tt*-farnesol+1771+myricetin, C135+1771+myricetin, *tt*-farnesol+1771, 1771+myricetin, *tt*-farnesol+1771+myricetin)] and with fluoride [(C135+fluoride, C135+*tt*-farnesol+fluoride, C135+1771+fluoride, C135+J10595+fluoride , C135+*tt*-farnesol+1771+myricetin+fluoride completely inhibited the initial biofilm formation (24h). These findings showed that there might be a potential synergism between the compounds and a greater effect when they are combined and applied since the beginning of biofilm formation and during the 24h of dual-species biofilm development. Part of the effect can be because of the antimicrobial effect *per ser*, as microbial cells were exposed to formulations in their free form before adhesion to the surface. Also, the effect on extracellular matrix formation can not be ruled out.

In contrast, dual-species biofilm eradication (48h) in which both species did not grow occurred for three formulations: C135+*tt*-farnesol+myricetin, C135+*tt*-farnesol+fluoride, and C135+*tt*-farnesol+1771+myricetin+fluoride. Four formulations only eradicated the bacterial growth (C135+*tt*-farnesol+1771+myricetin, C135+fluoride, C135+1771+fluoride, and C135+myricetin+fluoride), while other four formulations eradicated fungal growth (C135, C135+*tt*-farnesol, C135+1771, and C135+myricetin). Of note, the formulations with fluoride exhibited a greater antibiofilm activity (mainly for the bacterium), reinforcing the importance of its inclusion in strategies to prevent and control cariogenic biofilms [18]; fewer and less severe carious lesions were observed using combined treatments and fluoride on a rodent model of dental caries [19]. Fluoride can interfere with microbial metabolism, especially on glycolytic enzymes and proton gradient dissipation on the cell cytoplasmatic membrane (when the extracellular pH is higher than the intracellular pH) [44]. This effect can hamper cell growth. Nevertheless, C135 alone or combined with other agents (even those without pronounced effect on singles-species 48h-old biofilms) prevented fungal and bacterial growth in dual-species biofilms, making it a promising agent.

The present findings provided insights about: (i) compounds as inhibitors of biofilm formation of single-species biofilm (24h); (ii) compounds that can eradicate pre-formed biofilm (48h); and (iii) formulations with combined compounds for biofilm inhibition (24h) and eradication (48h) of both species in dual-species biofilms. C135 is a novel compound with possible distinct targets alone or in combination with other agents. The formulations that combined agents with distinct targets prevented *C. albicans* and *S. mutans* dual-species biofilm build-up *in vitro.* The formulation C135+*tt*-farnesol with or without fluoride may represent a potential alternative approach that deserves further investigation, including cytotoxicity to host [30, 45]. Therefore, the outcomes of this study could be applied to future studies using the compound alone or combined as an adjuvant strategy to control oral biofilms using shorter exposure times, as mouthwashes.

Supplementary Materials (APÊNDICE B)

Author Contributions: Conceived and designed the experiments: C.I.V.L. and M.I.K. Carried out the experiments: C.I.V.L. Analyzed the data C.I.V.L. and M.I.K. Reagents/materials/analysis tools: M.I.K. Wrote the paper: C.I.V.L., A.C.U.d.A.L., and M.I.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available as supplementary material. Additional data are available on request from the corresponding author.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3.2 Artigo 2[2]

Lobo, C.I.V.; Barbugli, P.A.; Rocha, G.R.; Klein, M.I. **Topical application of a 4'-hydroxychalcone combined with *tt*-farnesol is effective against *Candida albicans* and *Streptococcus mutans* biofilms.** Molecules 2022.

Abstract

Candida albicans and *Streptococcus mutans* interaction yield a complex biofilm with an organized and structured extracellular matrix that increases the tolerance to environmental stresses, including antimicrobials. Thus, the compounds 4'-hydroxychalcone (C135) (flavonoids intermediate metabolites), *tt*-farnesol (Far) (terpenoid), and sodium fluoride (F) were tested isolated or combined against *C. albicans* and *S. mutans* dual-species biofilms grown on saliva-coated hydroxyapatite discs (twice-daily topical treatments). The biofilms were evaluated for gene expression, microbial population, biochemical components, 3D structural organization via confocal microscopy and scanning electron microscopy (SEM). The cytotoxicity of formulations was tested on keratinocytes monolayer. C135+Far+F promoted lower gene expression for fungal genes associated with β-glucan synthesis (BGL2, FKS1) and remodeling (XOG1, PHR1, PHR2), oxidative stress (SOD1), and drug tolerance (CDR1, ERG11); and higher expression for bacterial *nox1* (oxidative and acidic stresses tolerance). C135+Far yielded less insoluble exopolysaccharides, biomass, and proteins (insoluble portion) and lower expression of BGL2, ERG11, SOD1, and PHR2. C135+F, C135+Far+F, and C135 rendered lower biomass, thickness, and coverage percentage (confocal microscopy). C135+Far and C135+Far+F maintained *C. albicans* as yeast morphology (SEM). Formulations containing *tt*-farnesol and chlorhexidine were highly cytotoxic. Therefore, the formulations with C135 affected fungal and bacterial targets but exerted a more pronounced effect against fungal cells.

Keywords: Biofilm; *Candida albicans*; *Streptococcus mutans*; topical treatments.

Introduction

Over time, organized biofilms in the oral cavity can lead to severe diseases with complex management such as dental caries. Dental caries is a multifactorial disease

[2] Artigo escrito seguindo as normas do periódico: https://www.mdpi.com/journal/molecules/instructions (ANEXO C)

affecting people of all social classes and ages [1]. Early childhood caries or ECC is a serious public health problem, considered one of the most common preventable diseases affecting children. Its prevalence is related to the interaction of the type of microorganisms, diet, and caregivers' instruction [2]. Essentially, dental caries is a biofilm-diet-derived disease in which the organic acids from microbial metabolism are trapped inside the biofilm and at the interface biofilm/teeth surface by its extracellular matrix, leading to teeth demineralization [3] that can result in teeth loss that affects oral and systemic health [4].

The interaction of microorganisms, especially *Streptococcus mutans* and *Candida albicans,* makes a complex biofilm with an organized and structured extracellular matrix that contributes to increasing the environmental stress tolerance from both species [5]. This association appears to be primarily mediated by a physical interaction that relies on glucans produced by bacterial exoenzymes glucosyltransferases (Gtfs) on yeast and hyphal cell surfaces [6]. Gtfs convert a moderately cariogenic organism into a major contributor to the formation of virulent plaque biofilms and dental caries in a susceptible and vulnerable population *in vivo* [7].

The antimicrobial effect against microorganisms is usually compromised by their cell wall barrier and the extracellular matrix. The *S. mutans* Gtfs synthesize exopolysaccharides (α-glucans) using dietary sucrose as the substrate for the extracellular matrix construction [8]. During *S. mutans* biofilm development in the presence of a cariogenic diet, genes related to biofilm's matrix construction (e.g., *gtfB, gtfC, gtfD*), acid production, and tolerance to acids (e.g., *atpD*) and oxidative stresses (e.g., *nox1*) are expressed [9,10]. Also, the *C. albicans* cell wall has important components (β-glucans) that contribute to fungal virulence [11]. Genes BGL2, FKS1, PHR1, and XOG1 are related with β-1,3 glucan (and antifungal resistance) and β-1,6 glucan (PHR1) synthesis and are essential for the extracellular matrix construction [12-14]. In addition, the genes PHR1 and PHR2 are expressed in imbalanced pH and contributes to the acid production by *C. albicans* and its survival in acidic environments [12]; SOD and ERG11 genes are related with oxidative stresses, and CDR1 and ERG11 are associated with antimicrobial resistance [15-17]. In addition, the ERG11 gene is part of the pathways for the biosynthesis of ergosterol and can be related with the morphogenesis of hyphal form of *C. albicans* as well as with fungal virulence [18].

Disrupting pathogenic local niches to eliminate the embedded microorganisms and facilitate access to the action of the agents by targeting biofilm structure and its microenvironment can be an effective therapeutic strategy, especially if the agents generate minimal cytotoxicity and minimize the drug resistance issue [19].

Fluoride and chlorhexidine digluconate are the gold standards for topical treatments of dental biofilms. Fluoride helps balance the remineralization process after demineralization [20], enhancing remineralization in the presence of dental plaque. However, it has limited effect against biofilms *per se*; it has a small action on glycolysis or acid production [21]. On the other hand, chlorhexidine is a broad-spectrum antimicrobial, important for Gram-positive bacteria control like *S. mutans*; still, its daily use is limited (up to 15 days) [22]. Thus, treating cariogenic biofilms is still a public health challenge; hence, finding an alternative that could be used daily for cariogenic biofilm control for dental caries prevention becomes interesting.

Several compounds have been studied to serve as antimicrobial and antibiofilm strategies. Hydroxychalcones inhibited the Gtfs activity of *S. mutans* [23]. Chalcones affect the cell wall formation of *C. albicans,* contributing to mitochondrial dysfunction and for the efflux-mediated pumping system [24]. A 4' hydroxychalcone (C135) presented promising effects against *C. albicans* and *S. mutans* growth in planktonic cultures and single- and dual-species biofilms after log exposures to it [25]. *tt*-farnesol is a *C. albicans* quorum sensing molecule that hinders fungal filamentation (hyphal formation) and targets *S. mutans* cytoplasmatic membrane, affecting the bacterium stress tolerance [26]. Myricetin is a flavonoid that interferes with *S. mutans* exopolysaccharides production [27].

The topical treatment using the association of *tt*-farnesol with myricetin and fluoride decreases the number of dental caries lesions, and the lesions are less severe than the controls in rats colonized by *S. mutans* [27]. However, that formulation (*tt*-farnesol plus myricetin plus fluoride) applied topically (1.5 minutes) had little effect on *C. albicans* and *S. mutans* dual-species *in vitro* [28]. In addition, 24h-exposure to the combination of 4' hydroxychalcone (C135) with *tt*-farnesol and fluoride hindered that dual-species biofilm development and killed both bacterium and fungi in the pre-formed biofilms (96-well polystyrene plates model) [25]. Nonetheless, further research is needed to pinpoint formulations for effective topical treatments to control fungal-bacterium biofilms

Therefore, finding a formulation with an effective combination of compounds for topical application to prevent *S. mutans* and *C. albicans* biofilm development may result in a strategy for preventing dental caries lesions. Here, the promising agents (4'-hydroxychalcone and *tt*-farnesol) [25] and their combinations with and without fluoride were tested as topical treatments against dual-species biofilms formed on saliva-coated hydroxyapatite (HA) discs.

Methods (APÊNDICE C)

Compounds and their combinations (formulations tested)

Previously, our research group selected and combined compounds against single- and dual-species biofilms of *C. albicans* and S. *mutans* on 96-well plates (24h and 48h) [25]. Also, based on pilot tests (not reported) the promising compounds were: 4'-hydroxychalcone (C135): (2E)-1-(4-hydroxyphenyl)-3-phenylprop-2-en-1-one (AK Scientific, Inc.; Cat.#C135; 98% purity) and *tt*-farnesol (Far): (E,E)-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol, trans,trans-3,7,11-Trimethyl-2,6,10-dodecatrien-1-ol (Sigma-Aldrich Co., St Louis, MO; Cat.#46193; 96% purity) diluted with 84.15% ethanol (EtOH; Sigma-Aldrich; Cat.#E7023) and 15% dimethyl sulfoxide (DMSO; Sigma-Aldrich; Cat.#D8418) for stock solutions at 15 mg/mL. These compounds were used alone and combined, with and without sodium fluoride (F) (stock solution at 5000 ppm; Sigma-Aldrich; Cat.# 71519). The final concentrations for both compounds (C135 and *tt*-farnesol) were 125 µg/mL based on previous data [25] and 250 ppm for fluoride [25,29]. The test groups were the following formulations: 1. C135, 2. C135+Far, 3. C135+F, 4. C135+Far+F, 5. Far, 6. F, and 7. vehicle—V (or control group: the solution used to dilute the compounds at their working concentrations: 7% EtOH + 1.25% DMSO). Chlorhexidine digluconate solution 0.12% (Sigma-Aldrich; Cat.# C9394) was used for cytotoxicity assay.

Strains cultivation and Biofilm formation

The strains *C. albicans* SC5314 and *S. mutans* UA159 (serotype c; ATCC 700610) were plated separately on blood agar (5% sheep's blood; Laborclin, São Paulo, Brazil) and incubated for 48h at 37ºC and 5% CO_2 (Steri-Cult™Thermo Scientific, Waltham, USA). Then, for starter cultures, 5 colonies of each microorganism were transferred to individuals Falcon tubes containing 10 mL of culture medium [9,5

mL (2.5% tryptone and 1.5% yeast extract or TY, pH 7.0; Difco) + 0,5 mL (20% glucose): TY+ 1% glucose; all these % are w/v]. These tubes were incubated for 16h (37ºC, 5% CO_2); then diluted (1:20) using the same culture medium for the inoculum of each strain. The inoculum was incubated (37ºC, 5% CO_2), and each culture was used at their corresponding mid-log growth phase: *S. mutans* Optical Density (OD_{562nm}) 0.500 (±0.100) and *C. albicans* OD_{562nm} 0.482 (±0.058) (ELISA plate reader, Biochrom Ez, Cambourne, UK). The final culture for biofilm formation containing both microorganisms was prepared using TY and 1% sucrose (w/v) to yield $2x10^6$ CFU/mL *S. mutans* and $2x10^4$ CFU/mL *C. albicans* [7].

Biofilms were formed on hydroxyapatite (HA) discs [2.7 ± 0.2 cm^2 (Clarkson Chromatography Products, Inc. South Williamsport, PA, US)]. Wire apparatuses were used to place the HA discs vertically in 24-well plates (KASVI, China). Before the biofilm formation, the discs were coated with saliva collected by three health donors (approved by Institutional Ethical Committee CAAE: 08189119.7.0000.5416) (ANEXO B). This saliva was stimulated (chewing a piece of parafilm), sterilized by filtration (500 mL Rapid – Flow Filter Unit, 0.2 µm aPES membrane, 75mm dia, Mexico), and prepared following a previous protocol (Lemos et al., 2010). For salivary pellicle formation, the HA discs were hydrated (sterilized MilliQ water, 20 min), incubated in saliva (1h, 37ºC, 75 rpm; Quimis, São Paulo, BR), then dipped into adsorption buffer [(50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, 1 mM$MgCl_2$, 0.1 mM PMSF (phenylmethylsulfonyl fluoride – Sigma), in dd-H_2O, pH 6.5] for washing to remove excess saliva. Next, the discs with the pellicle were treated (as described in ***Treatment Regimen***) and transferred to the inoculated culture medium for biofilm formation. The biofilms were treated at 6, 21, and 29 h.

At 19 and 27h of the experiment, biofilms were transferred for a new plate with fresh culture medium (TY + 1% sucrose) and incubated again (37ºC, 5% CO_2). After two hours of incubation (21 and 29 h), the biofilms were treated, as described below. The spent culture medium (from 19, 27, 28, and 43 h) was used for pH analysis. The biofilms were grown up to 28 h for gene expression analysis and 43 h for microbial population, biomass, biochemical (matrix components—exopolysaccharides, proteins, and extracellular DNA or eDNA), structural organization and scanning electron microscopy. The experimental design is depicted in Figure 1.

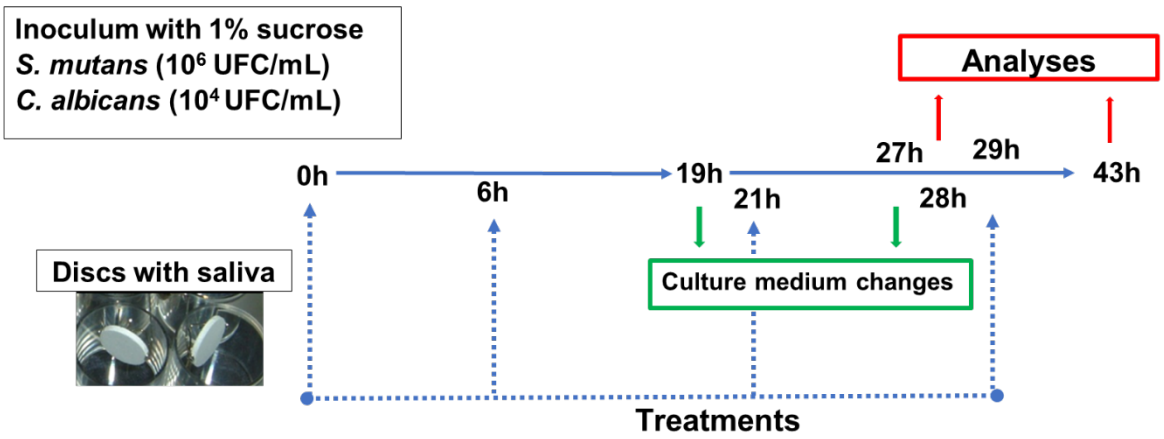


Figure 1. Experimental design. Blue dotted arrows represent the age of biofilm treatments; Green arrows indicate the period (biofilm age) when the culture medium was changed, and red arrows are the ages when biofilms were analyzed.

Treatment Regimen (Topical application)

Formulations were applied at 0, 6, 21, and 29 hours. For topical application, 2 mL of each formulation (or control) was prepared on the 24-well plate. First, the discs (with salivary pellicle at 0 h, and with biofilms at 6, 21, and 29 h) were rinsed to remove the excess of saliva (after pellicle formation at 0 h) or of the culture medium (at 6, 21, and 29 h) by dipping them into adsorption buffer after pellicle or 0.89% NaCl solution during biofilm treatment. Next, these discs were submerged for 5 min into the formulations (or control), then washed using a new adsorption buffer or 0.89% NaCl solution and transferred to the culture media, followed by incubation (37ºC, 5% CO_2).

Gene expression analysis

The Reverse Transcription-quantitative Polymerase Chain Reaction or RT-qPCR analysis included RNA isolation, cDNA synthesis, and qPCR using specific primers for selected genes of *S. mutans* (*atpD*, *gtfB*, *nox1*, and the normalizer *16S rRNA*) and *C. albicans* (BGL2, CDR1, ERG11, PHR1, PHR2, FKS1, SOD1, XOG1, and RPP2B) (Table 1).

Biofilms were processed and evaluated at 28 h. Here, four discs were used for each experimental group on three distinct experimental occasions. So, after 28 h of incubation, the biofilms were dip washed on a 24-well plate containing 0.89% NaCl, and each disc was transferred to an individual glass tube containing 1 mL of RNAlater (Ambion – Molecular Probes). All tubes were subjected to an ultrasonic bath (Bransonic 3800, Emerson, Mexico) for 10 min; the discs were scraped using a metallic spatula (to remove any remaining biofilm attached on HA discs). Then, the

suspensions were transferred from glass tubes to a Falcon tube. Here, biofilms from the same group were pooled (biofilms from 4 discs from the same treatment). Additionally, the glass tubes were washed with 1 mL of RNAlater and transferred to the correspondent Falcon tube. Thus, each falcon tube had 4 mL of biofilm suspension. These suspensions were stored at -80ºC.

RNA Isolation

The protocol used for RNA isolation was established before [30], with some modifications [5]. First, the phenol-chloroform separation method was used for RNA extraction, followed by DNAse treatments in column (RNeasy Micro Qiagen, Germany) and solution (TURBO DNAse; Ambion) for purification. In addition, the Rneasy MinElute cleanup kit (Qiagen) was employed, followed by agarose gel electrophoresis (1%) (Ultra-Pure ™ Invitrogen) to verify the integrity of purified total RNA. Then, the RNA quantity (260 nm) and purity (260/280) were analyzed by spectrophotometry (Nano-spectrophotometer DS-11+, Denovix).

cDNA synthesis

The cDNA synthesis was performed using the iScript kit (Cat.#1708891; BioRad). Thus, to prepare +RT samples 1 µg of total RNA (corresponding to 5 µL of RNA at 200 ηg/µL) was mixed with 4 µL of the 5X iScript buffer, 1 µL of the reverse transcriptase enzyme, and molecular grade water to complete 20 µL (10 µL). The same reaction without reverse transcriptase enzyme was used for -RT samples (control group) [5]. The reactions were incubated [25ºC/5 min, 42ºC/30 min, 85ºC/5 min, hold at 4ºC; CFX96 thermocycler (BioRad)], and stored at -20ºC.

Quantification of gene expression via qPCR

The cDNA samples were diluted (+RT 1:5 for all genes except *16SrRNA*, which used 1:1000; and -RT was applied undiluted to ensure no DNA contamination) and amplified with specific primers, following standard protocols [32]. For qPCR reactions, 5 µL of cDNA were amplified with the adequate volume of 10 µM corresponding primers (Table 1), 12.5 µL of 2X SYBR Green Supermix (BioRad), and molecular grade water up to 25 µL. The reactions were carried out according to the optimal concentration and the ideal temperature of each primer using the CFX96 (BioRad) and

StepOnePlus Real-time PCR System (Applied Biosystems). Standard curves based on the PCR product were used [31], respecting the parameters of $R^2 \cong 1$, reaction efficiency of 90–100%, and slope of ≅ −3.3. The 16S rRNA gene act as normalizer gene for *S. mutans* samples [32], and RPP2B (structural ribosome constituent) gene was selected for *C. albicans* samples [33]; however, under this experimental design, it did not work as normalizer as observed before [5].

The biofilm samples were obtained from a pool of four replicates per treatment in three independent experiments (after RNA isolation, the pooled biofilm results in two samples of cDNA used, each one used in duplicate) (n = 3).

Table 2: *Primers* for RT-qPCR.

Microorganism	Gene	Sequence of *Primers* (*forward* e *reverse*)	Tm (ºC)	Primer concentration (nM)	Product size (bp)	References
S. mutans	16S rRNA	ACCAGAAAGGGACGGCTAAC TAGCCTTTTACTCCAGACTTTCCTG	58	200	122	[34]
	gtfB	AAACAACCGAAGCTGATAC CAATTTCTTTTACATTGGGAAG	58	250	90	
	nox1	GGACAAGAATCTGGTGTTGA CAATATCAGTCTCTACCTTAGGC	58		91	[32]
	atpD	GGCGACAAGTCTCAAAGAATTG AACCATCAGTTGACTCCATAGC	58		115	
C. albicans	ERG11	GGTGGTGGTAGACATAGA TGCTGGTTCAGTAGGTAA	58	300	159	[17]
	RPP2B	TGCTTACTTATTGTTAGTTCAAGGTGGTA CAACACCAACGGATTCCAATAAA	60	350	83	[33]
	XOG1	TAATGAACCATTGGGTCCAG GTGACTGATCCAGTTTGTCT	60		93	This study
	BGL2	ATGGGTGATTTGGCTTTCAA CAGCTGGACCAAGGTTTTGT	62		163	[35]
	PHR1	GGTTTGGTTCTGGTTGATGG AGCAGCAGTTCCTGGACATT	62		156	
	PHR2	CTCCTCCATTTCCAGAACCA CGTCTGAATCAACCTTGTCG	62		146	
	FKS1	CATGCCATTGGGTGGTTTAT GATTTCCATTTCCGTGGTAGC	60	400	481	[36]
	CDR1	CCATTAACCATCAGCACTTACC CCGTTCTACCACCTTTTTGCA	60		71	[37]
	SOD1	TTGAACAAGAATCCGAATCC AGCCAATGACACCACAAGCAG	60		396	[15]

Biofilm processing and analyses to determine viable microbial population, biomass, protein content, and extracellular matrix composition

At 43 h of development, biofilms were processed to quantify viable bacterial and fungal counts, biomass (insoluble dry-weight), protein content (in the insoluble and soluble portions of the biofilms), exopolysaccharides in the matrix [water-soluble polysaccharides (WSP) and alkali-soluble polysaccharides (ASP)] [38-40] and eDNA [41].

Each disc was dip washed on 0.89% NaCl (3X) and transferred to a glass tube containing 2 mL of 0.89% NaCl. The tubes were subjected to an ultrasonic bath (Bransonic 3800, Emerson - Mexico) for 10 min, and the discs were scrapped with a metal spatula to remove all remaining biofilm. Each biofilm suspension was transferred to a 15 mL Falcon tube. The glass tubes were washed with 3 mL of 0.89% NaCl, and this volume was added to the correspondent Falcon tube. Thus, the final suspension of biofilm was 5 mL. This volume was sonicated using a probe (30 s / 7 w; Sonicator QSonica, Q125, Newtown CT, USA). Next, 0.1 mL were subjected to serial dilutions (10^{-1} to 10^{-5}) and plated (on blood or BHI agar plate); these plates were incubated (37ºC, 5% CO_2, 48 h) for quantification of the culturable viable microbial population (colony forming units or CFU counting). The remaining biofilm suspension volume (4.9 mL) was centrifuged (4000 rpm / 20 min / 4°C; Eppendorf, Centrifuge 5810R). The supernatant was transferred to a new Falcon tube, and the pellet was washed with 2.6 mL of MilliQ water. The suspension was homogenized, centrifuged (4000 rpm / 20 min / 4°C), and the supernatant was transferred to the corresponding Falcon tube containing the initial supernatant. Again, the pellet was washed with 2.5 mL of MilliQ water, and the process was repeated (homogenization and centrifugation) to obtain 10 mL of the biofilm suspension supernatant.

From this 10 mL of biofilm supernatant suspension: 0.5 mL were used for eDNA, 0.5 mL for proteins in the soluble portion (Bradford method), and 6 mL were precipitated with three volumes of 99% ethanol (18 h, −20°C) for WSP analysis [38].

The pellet was resuspended with 1 mL MilliQ water, where 0.05 mL was used to quantify the total proteins (from the insoluble portion) (Bradford method), and the remaining suspension (0.995 mL) was transferred to a pre-weighed microtube. The microtube was centrifuged (12000 rpm / 10 min / 4ºC; Eppendorf, Centrifuge 5430R), the supernatant was discarded, and the pellets were dried for 270 min (Speed Vac

Concentrator RVC 2–18 CD Plus, Christ). Next, all tubes were weighed again, and the difference between the final and initial weight was the biomass (dry-weight) of the biofilm. The same pellet was used to extract ASP with 1 M NaOH (0.3 mL of 1 M NaOH per 1 mg of dry-weight). The extracted solution was precipitated with ethanol, followed by carbohydrate quantification [38], as detailed before [39].

All tests were performed in three independent experiments, where each group had discs per treatment (n = 6).

Structural organization of biofilms (confocal microscopy)

Biofilms were analyzed at 43h via confocal microscopy. All procedures for biofilm formation and topical treatments were as described above, except that 13.4 µL of 1 mM Alexa Fluor 647 fluorophore-labeled dextrans (647/668 nm; Molecular Probes, Carlsbad, CA, EUA) were added to the culture medium to label the extracellular matrix. This reagent was added in the inoculum (at 0 h) and after every culture medium exchange (19 and 27h). At the end of the experiment (43h), the biofilms were dip washed on 24-well plate containing 0.89% NaCl and incubated in another 24-well plate containing 0.89% NaCl with 1.5 µL of SYTO9 (485/498 nm; Molecular Probes) (30 min) for microorganisms labeling. After, they were dip washed on a new 24-well plate with 0.89% NaCl; then, the samples were ready to be analyzed.

Confocal fluorescence microscope (CARLS ZEISS LSM 800 with Airyscan with GaAsp detector, Germany) was used with EC Plan-Neofluar 20x/0.50 Oil DIC M27 objective, with laser wavelengths (488 nm: 2.10% and 561 nm: 1.81%), increments of 1.5 µm, at the configuration of 1024x1024. The images were analyzed using the ZEN Blue software (3D reconstruction) and were used to quantify biomass, maximum thickness, and percentage of coverage area using COMSTAT2.

Three experimental occasions were performed. Two discs represented each treatment group (formulation), and three images were acquired per disc avoiding the disc's edges (n = 6). All data files were used for the quantification analyses, and a representative image from each group was selected to illustrate the findings.

Scanning electron microscopy (SEM)

Biofilms were grown and treated as described above up to 43 h, then prepared for SEM analysis. The discs with the biofilms were washed with 0,89% NaCl, and the biofilms were fixed using glutaraldehyde solution at 2.5% (1h at room temperature).

Next, the samples were washed three times with 0,89% NaCl, dehydrated [by incubation on 70% ethanol (1x / 1h), 90% ethanol (1x / 1h) and 99% ethanol (5x / 30 min)] and dried on silica vacuum desiccator (7 days). After, the biofilms were metalized with gold (Denton Vaccum, Desc V) and the images were acquired on amplification of 1,000 and 5,000X in a scanning electron microscope (JEOL JSM-6610LV).

Each treatment group (formulation) was represented by two discs and at least three images of each amplification were acquired per disc.

Cytotoxicity activity

Cells and culture conditions

Culture cells of NOK-si keratinocytes [42] were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM, GIBCO, Grand Island, NY, EUA) with 2.0 mM/L of glutamine with 10% bovine serum albumin (BSA, GIBCO, Grand Island, NY, EUA), penicillin G (10.000 µg/mL), streptomycin (10.000 µg/mL), amphotericin (25 µg/mL) (Invitrogen) and incubated (CO_2 / 5% / 37ºC) (Midi 40™Thermo Scientific, USA). At 90% confluence, keratinocytes were washed with phosphate-buffered saline-PBS 1X pH 7.2 (140 mM/L NaCl, 3.0 mM/L KCl, 4.30 mM/L Na_2HPO_4, 1.40 mM/L KH_2PO_4), detached with 0.05% trypsin / 0.53 mM/L EDTA (Invitrogen) and centrifuged (400 xg / 5 min). The cells were resuspended in DMEM fresh culture medium and replanting. The culture medium was replaced every 2 days, and experiments were performed between third- and fourth-passage. For that, 20.000 cells/well were counted using a Neubauer chamber and platting in 96-well plates. Therefore, 8 wells were used per treatment in three independent occasions.

Cell viability by the MTT assays

All formulations used against biofilms were tested for cytotoxic assays via measurements by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] colorimetric assay. The principle of MTT assay reduce metabolically the tetrazolium salt MTT into formazan crystals by living cells, which determines mitochondrial activity. OD measurements detected formazan crystals concentrations. Thus, viable cells have competent mitochondria, which promote cellular respiration and release reducing species from the cellular respiratory chain into the medium, promoting the reduction of yellow MTT salt in purple color.

Thus, a monolayer cell culture was incubated (37ºC / 5% CO_2) for 30 or 60 min with 200 µL of the treatments (C135; C135+Far; C135+F; C135+Far+F; Far; F; and chlorhexidine digluconate 0.12%) or controls [the vehicle of the treatments (EtOH + DMSO); the culture medium (DMEM) and triton X-100 (dead control)]. After, all treatments or controls were discarded and 100 µL of the MTT solution (3.0 mg/mL) diluted in RPMI without phenol was incubated for 4 h (37ºC / 5% CO_2). Next, the MTT solution was replaced by 100 µL of 2-propanol to solubilize the crystals formed by MTT reaction, and the plate was sonicated (15 min; Limp Sonic, model LS-0,8D-3/X). Then, spectrophotometric measurements were carried out using OD_{562nm} (Reader 400 EZ – Biochrom).

Statistical analyses

The quantitative data were analyzed using descriptive and inferential statistics. Shapiro-Wilk test was applied for normality considering 5% of significance, and Brown-Forsythe test was applied for homogeneity of variances. Analyses were performed using one-way or mixed ANOVA tests, followed by Tukey post-test (Prism 9 software, GraphPad Software, Inc. 2021). In addition, qualitative and descriptive analyses of confocal and scanning electron microscopy images were performed.

Results

pH of the spent culture medium

The pH was measured when the culture medium was changed at 19h, 27h, and at the end of the experiments at 28h (gene expression) and 43h (all other analyses) (Figure 2). The data were analyzed by time, treatment, and time versus treatment using a mixed ANOVA test: $p<0.0001$; followed by Tukey post-test). The comparison of time for all groups demonstrated that the lowest pH was measured at 19h, followed by 43h, and 27h; all groups showed pH≥4.5 except 28h when all groups had pH above 6.0 (mixed ANOVA: $p\leq0.0001$). However, analyzing each group by time, at 19h and 43h, the treatments containing fluoride (C135+F; C135+Far+F; F) present pH values slightly higher than groups without fluoride (Tukey post-test: $p<0.0001$); while no significant statistic differences were observed when fluoride groups were compared to each other. Moreover, at 27h, the higher pH value was observed for C135+Far (Tukey post-test: $p\leq0.0069$); and treatments containing C135 and *tt*-farnesol (C135+Far or

C135+Far+F) had a higher pH than other treatments (Tukey post-test: p<0.0001); also, significative differences were observed between C135 versus C135+F (Tukey post-test: p=0.012), Far (Tukey post-test: p=0.0188) and F (Tukey test: p=0.0329); C135+F versus V (Tukey post-test: p=0.0364) and Far versus V (Tukey post-test: p=0.0449). The higher pH (>6.0) was observed at 28h, with statistic differences only for C135+Far versus C135, C135+F, Far, F, and V (Tukey post-test: p≤0.0331) and C135+Far+F versus C135 (Tukey post-test: p=0.0006).

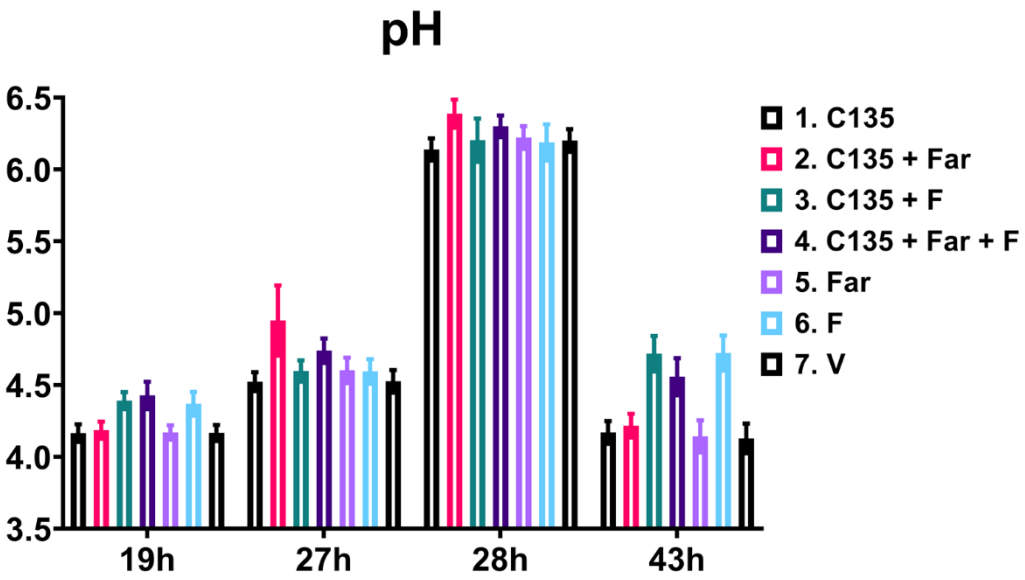


Figure 2: pH of spent culture medium. The bars represent the mean containing the standard deviations.

Gene expression

The data of *S. mutans* genes (*atpD*, *gtfB*, and *nox1*) were normalized by 16S rRNA (Figure 3). The expression of *nox1* was higher (than other groups) when biofilms were treated with C135+Far and C135+Far+F, but statistic differences *versus* vehicle were only observed for C135+Far+F (one-way ANOVA: p=0.003; Tukey post-test p=0.0238). For this gene, significant statistical differences were also observed for C135+Far+F *versus* C135 (one-way ANOVA: p=0.003; Tukey post-test p=0.012), Far (one-way ANOVA: p=0.003; Tukey post-test p=0.0384) and F (one-way ANOVA: p=0.003; Tukey post-test p=0.0188). However, no differences were observed for *atpD* and *gtfB* gene expression (one-way ANOVA: p=0.4419 and p=0.5688).

Figure 3. *S. mutans* Gene expression of *S. mutans* and *C. albicans* biofilms with 28 h. Biofilms were treated with combined compounds of C135, *tt*-farnesol (Far) and sodium fluoride (F) [1.C135; 2.C135 + Far; 3.C135 + F; 4.C135 + Far + F; 5.Far; 6.F and 7.Vehicle or V (EtOH + DMSO)]. Data represent means and standard deviations (n = 3), where differences are presented by * (*one-way ANOVA: p=0.003; Tukey post-test: p≤0.0384).

For *C. albicans*, eight genes were analyzed: BGL2, ERG11, CDR1, FKS1, PHR1, PHR2, RPP2B, SOD1, and XOG1 (Figure 4). The gene RPP2B was selected as a normalizer gene for *C. albicans*, but because of statistical differences for C135 versus C135 + Far + F (one-way ANOVA: p=0.0187; Tukey post-test: p=0.0322), this gene did not work as a normalizer; thereby, the expression of fungal genes was not normalized by a gene, it has been normalized by the same amount of RNA used for cDNA synthesis.

A lower fungal gene expression was observed for all genes when all compounds were combined (C135+Far) with or without sodium fluoride. Specifically, for BGL2 (a cell wall 1,3 β-glucosyltransferase associated at cell wall biosynthesis) [14], the lowest

expression values were was observed for C135+Far+F [*vs*. C135*, C135+F, and F***, and V**** (one-way ANOVA: p<0.0001; Tukey post-test:*p=0.0341, ***p=0.0002, ****p≤0.0001)], and for C135+Far [*vs*. C135 + F*, F* and V**** (one-way ANOVA: p<0.0001; Tukey post-test: *p≤0.0149, ****p≤0.0001)]. Besides, a lower expression for BGL2 was quantified for C135** and Far*** *versus* vehicle (one-way ANOVA: p<0.0001; Tukey post-test: **p≤0.0016, ****p≤0.0001). A similar gene expression profile was observed for ERG11 (important for ergosterol synthesis, morphogenesis and virulence of *C. albicans* [16,18]) with statistical significative differences vs vehicle for C135 (one-way ANOVA: p<0.0001; Tukey post-test: p≤0.0404), C135+Far*** and C135+Far+F**** (one-way ANOVA: p<0.0001; Tukey post-test: ***p=0.0006; ****p≤0.0001). Also for FKS1** (essential beta-1,3-glucan synthase subunit) and XOG1* (Exo-1,3 β-glucanase) (important genes for the matrix construction [14]; there was a reduced gene expression for biofilms treated by C135+Far+F** (one-way ANOVA: p=0.0208; Tukey post-test: *p=0.0467; one-way ANOVA: p=0.0153; Tukey post-test: **p=0.0046). For CDR1 (a multidrug transporter of ABC superfamily gene), a lower gene expression was presented by C135+Far+F, with significant statistic differences *versus* C135* and Far**; and, between Far *versus* V* (one-way ANOVA: p=0.0018; Tukey post-test: *p≤0.0244, **p=0.0041).

For PHR1 (a cell surface glucosidase) the significant differences were observed between C135+Far+F *versus* C135 + F**, F** and *versus* vehicle* (one-way ANOVA: p=0.0012; Tukey test: *p=0.0248, **p=0.0060). While the expression of PHR2 (a glucosidase) for all treatment groups was higher than C135+Far+F, with significant statistical differences (one-way ANOVA: p<0.0001), except for C135+Far (Tukey test p=0.8966).

Besides, for SOD1 (a superoxide dismutase gene), all treatments containing C135 (C135**, C135+Far**, C135+F*, and C135+Far+F****) showed a lower gene expression than the vehicle; in addition, C135+Far+F presented a lower gene expression *versus* Far and F* (one-way ANOVA: p<0.0001; Tukey test: *p≤0.0450, **p≤0.0015, ****p<0.0001).

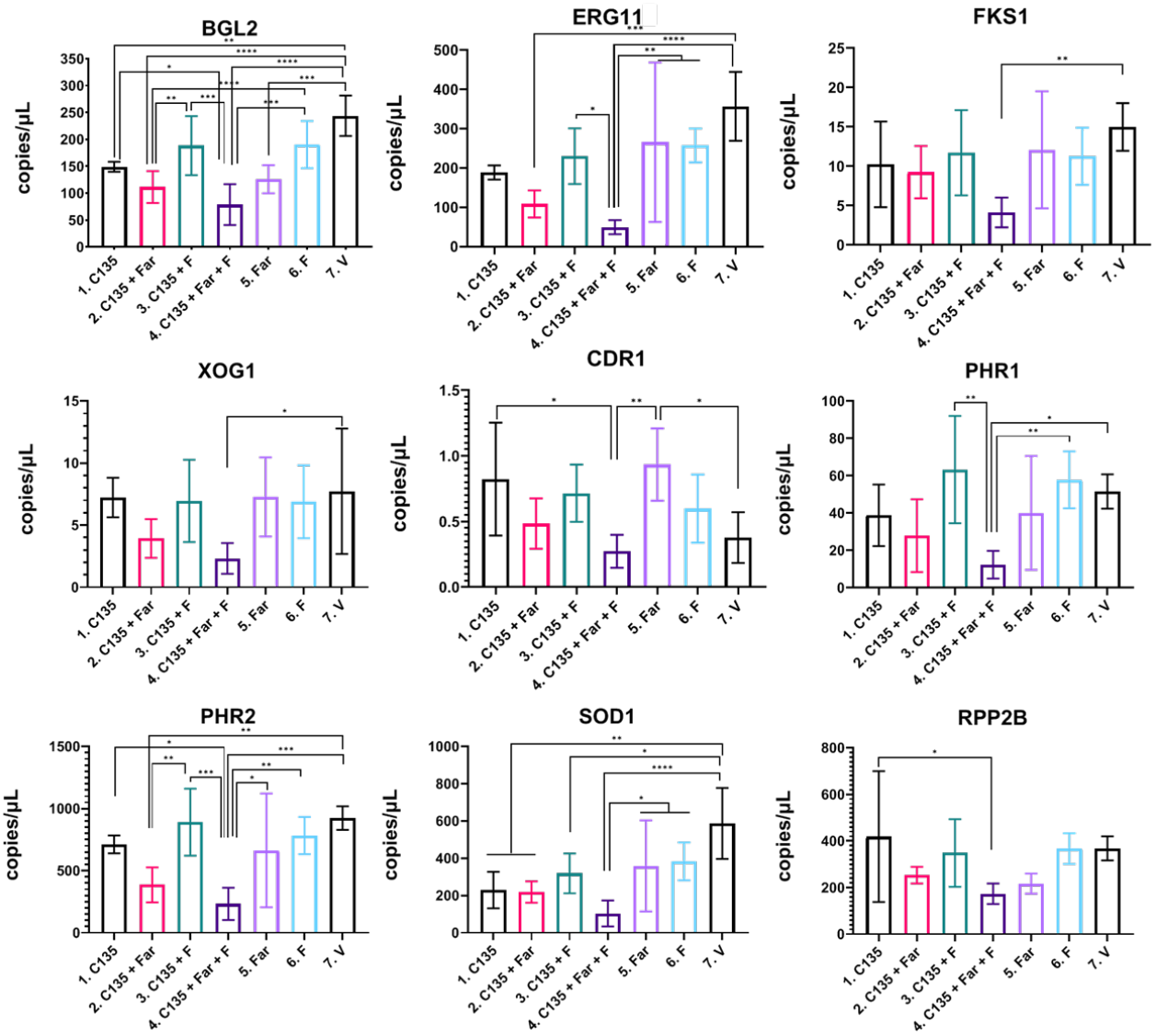


Figure 4. *C. albicans* gene expression of *S. mutans* and C. albicans biofilms with 28h. Biofilms were treated with combined compounds of C135, *tt*-farnesol (Far) and sodium fluoride (F) [1.C135; 2.C135 + Far; 3.C135 + F; 4.C135 + Far + F; 5.Far; 6.F and 7.Vehicle or V (EtOH + DMSO]. Data represent means and standard deviations (n = 3) from three different experiments). Differences are presented by *: [one-way ANOVA: p≤0.0341; Tukey post-test *(p≤0.0450), **(p≤0.0060), ***(p≤0.0006), and **** (p≤0.0001)]. Data were obtained using two cDNA from each experiment and the quantification of qPCR expression in duplicate (n = 3).

Microbial population

The viable microbial population after treatments was different between *S. mutans* and *C. albicans* (Figure 5). of *S. mutans* population presented significant statistic differences for C135 (*vs*. C135+F and F****), C135+Far (*vs*. C135+F, C135+Far+F and F****), Far (vs C135+Far+F, C135+F and F****) (one-way ANOVA: p<0.0001; Tukey post-test: ****p<0.0001). Biofilms treated with C135, Far, or both compounds combined (C135+Far) reduced microbial population than the formulations

containing fluoride. Besides, C135+Far** and Far*** yielded lower *S. mutans* CFU/mL counts with significative differences *versus* vehicle (Tukey test: **p=0.0012; ***p=0.001). Additionally, biofilms treated with C135 presents less bacterium quantification than C135+Far+F* (Tukey post-test: *p=0.0172). However, for *C. albicans* biofilms, the treatments containing C135+Far and C135+Far+F showed a lower CFU/mL count than other tested groups. Thus, significant statistical differences for *C. albicans* were observed for C135+Far (*vs*. C135+F, Far, F, and V****); C135+Far+F (*vs*. C135+Far and C135+F***; and *vs*. Far and F**) (one-way ANOVA: p<0.0001; Tukey post-test: **p≤0.0056; ***p≤0.001; ****p<0.0001).

Biomass (dry-weight) and proteins from the insoluble portion of biofilms

The biomass (dry-weight) and proteins from the insoluble portion of biofilms data are presented in Figure 5. The lower biomass of biofilms was presented by C135+Far with significant statistical differences (*vs.* C135 + F and Far**; and *vs.* C135 and V***) (one-way ANOVA: p<0.0001; Tukey test: **p≤0.0051; ***p≤0.0007). The same behavior was observed for biofilm proteins, where C135+Far also presents a lower quantification with significant statistical differences (*vs.* C135+F, F, and V*; and *vs,* C135***) (one-way ANOVA: p=0.0009; Tukey test: *p≤0.0388; ***p=0.0002).

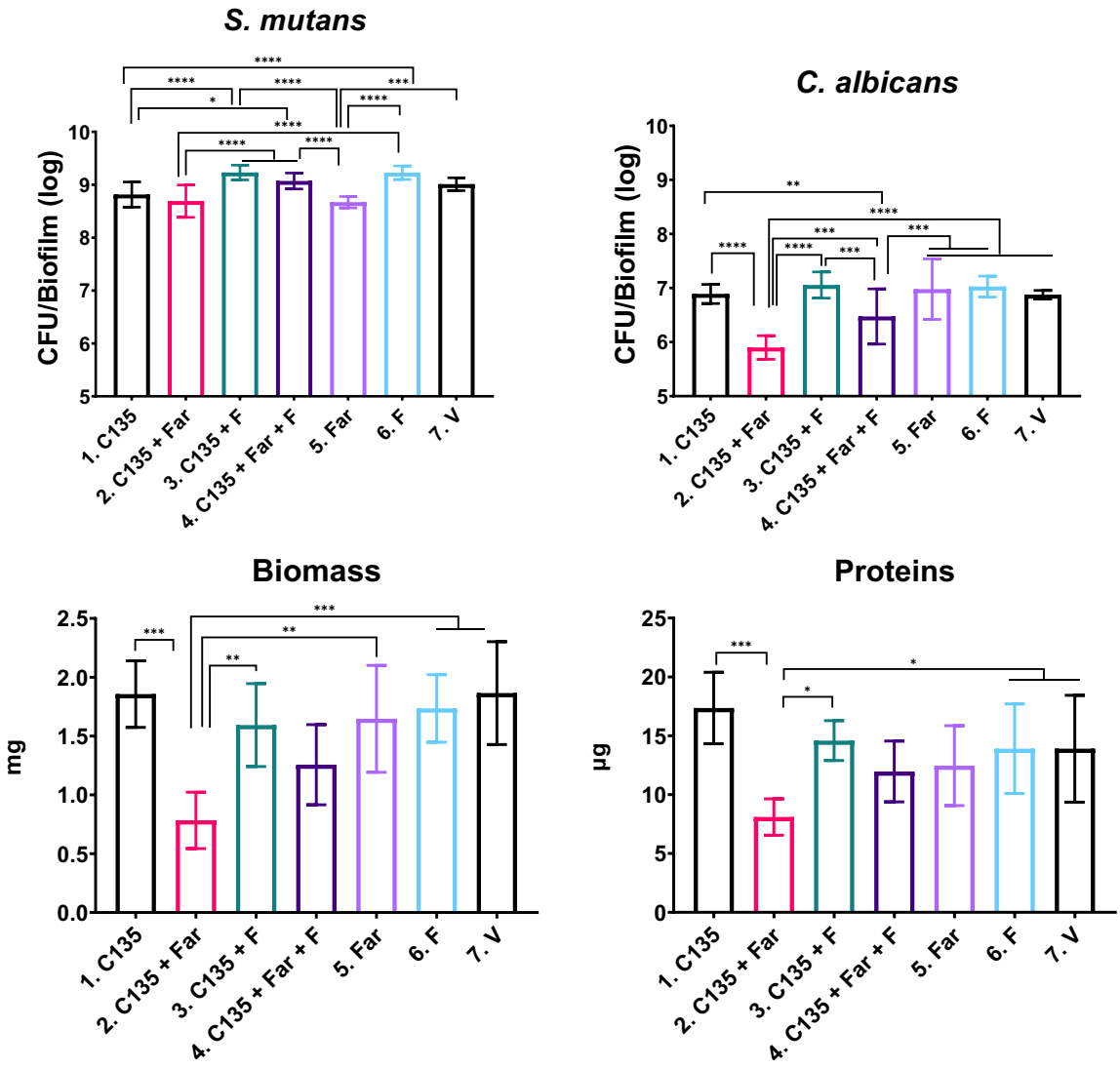


Figure 5: Overall biofilm features at 43 h. Microbial population, biomass (dry weight), and proteins (in the insoluble portion). Characteristics of biofilms formed by *S. mutans* and *C. albicans* treated with combined compounds of C135, *tt*-farnesol (Far) and sodium fluoride (F) [1.C135; 2.C135 + Far; 3.C135 + F; 4.C135 + Far + F; 5.Far; 6.F and 7.Vehicle or V (EtOH + DMSO)]. Data represent means and standard deviations (n = 6) of three independent experiments. Differences are represented by asterisks, considering (one-way ANOVA: p≤0,0009; Tukey post-test *p≤0.0396, **p≤0.0056, ***p≤0.001, and ****p≤0.0007).

Extracellular matrix components

The quantification of exopolysaccharides represented as ASP and WSP for each treatment group is depicted in Figure 6. The combination of C135+Far showed a lower ASP quantification *versus* Far and C135+F**; and C135, F, and V*** (one-way ANOVA: p<0.0001; Tukey post-test: **p≤0.0051; ***p≤0.0007); while no differences were observed *versus* C135 + Far + F (one-way ANOVA: p<0.0001; Tukey post-test:

p=0.2532). However, no significant differences between treatment groups were detected for WSP (one-way ANOVA: p=0.1408). Also, no differences were observed for proteins (soluble portion) (one-way ANOVA: p=0.1361) and eDNA (one-way ANOVA: p=0.1513) (Figure 6). Thus, the formulation C135+Far impacted more ASP composition, a virulence determinant of cariogenic biofilms [43], compared to the other matrix components. The reduced production of ASP results in less biomass (dry-weight; Figure 6), as these components are the major ones in the matrix of cariogenic biofilms.

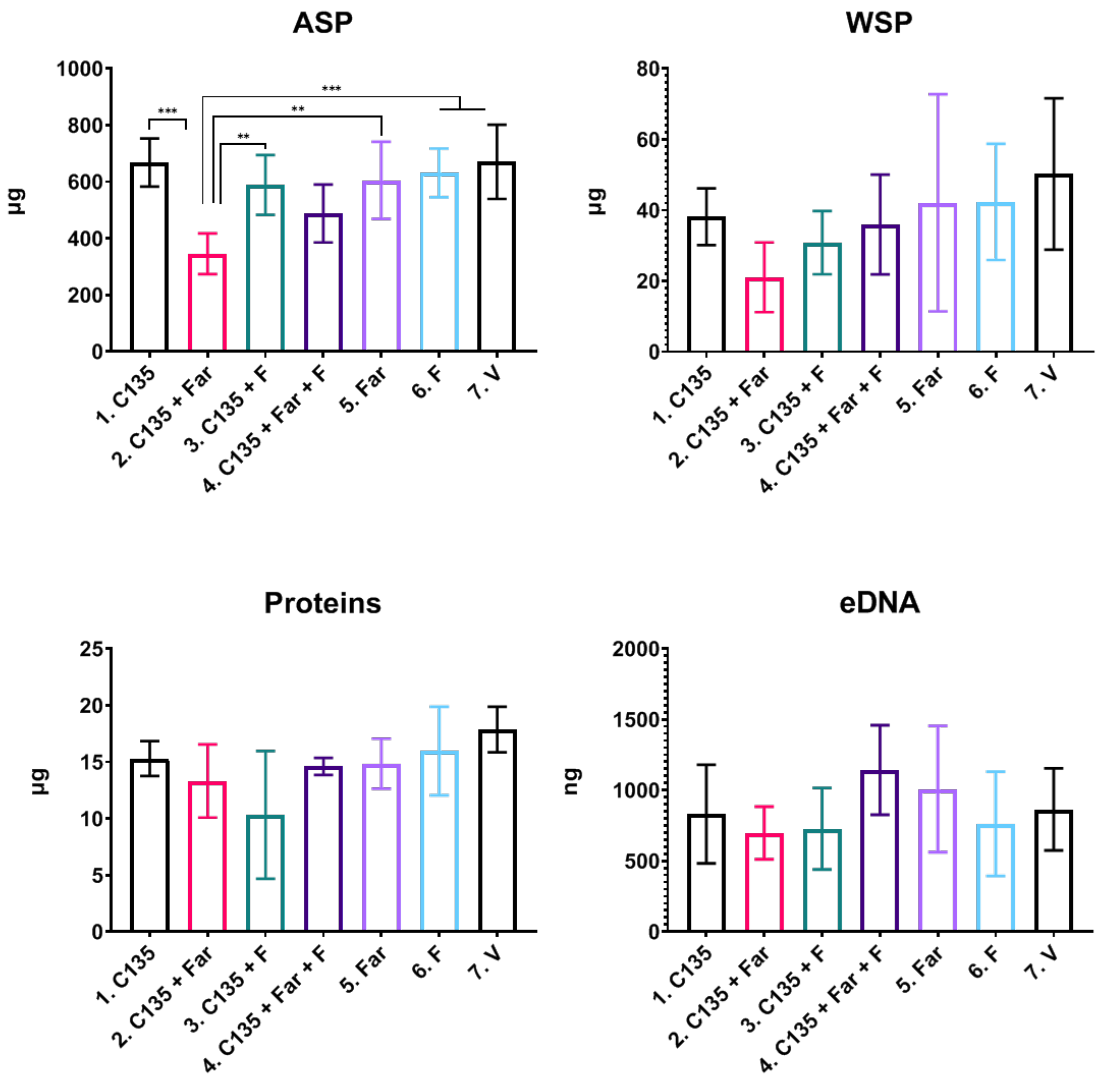


Figure 6. Data of the extracellular matrix components (ASP and WSP), proteins from the soluble portion, and eDNA of the *S. mutans* and *C. albicans* biofilms with 43h. Biofilms were treated with combined compounds of C135, *tt*-farnesol (Far) and sodium fluoride (F) [1.C135; 2.C135 + Far; 3.C135 + F; 4.C135 + Far + F; 5.Far; 6.F and 7.Vehicle or V (EtOH + DMSO)]. Data represents means and standard deviations (n = 6). Differences are represented by asterisks, considering one-way ANOVA: p<0,0001; Tukey post-test: **p≤0.0051 and ***p≤0.0007.

3D structure, biovolume, maximum thickness, and percentage are coverage of microorganisms and exopolysaccharides

The 3D structures of *S. mutans* and *C. albicans* biofilms were acquired at 43h and are represented in Figure 7. Biofilms treated with C135 showed thicker biofilms with large and organized clusters of bacterial cells (microcolonies), but visible fungal cells are sparse. However, biofilms treated with C135+F present a reduced thickness and spaced-out clusters of bacterial cells surrounded by *C. albicans* cells in yeast morphology. The biofilms treated by all compounds combined (C135+Far+F) are ticker and organized; however, the bacterial microcolonies were smaller when compared with vehicle, and no *C. albicans* cells were visible. In the biofilms treated with C135+F, many yeast cells were observed near the large *S. mutans* clusters. Biofilm treated with Far was smaller than F and vehicle; however, the fungi cells were visible in different morphologies.

In addition, quantification of fluorescence of biofilm components labeled with SYTO9 (both microorganisms) and Alexa Fluor 647 (bacterium derived exopolysaccharides) by COMSTAT2 included the biomass or biovolume ($\mu m^3/\mu m^2$; the volume occupied by the fluorescence signals) and maximum thickness (μm; distance from the interface disc/biofilm until interface biofilm/fluid or until no more fluorescence is detected), and the percentage coverage areas (coverage area from the interface disc/biofilm until no more fluorescence is detected). The biomass and maximum thickness data are shown in Figure 8, and the percentage coverage areas are depicted in Figure 9.

Regarding biomass (Figure 8A), mixed ANOVA analysis, comparing as factors formulation and biofilm component demonstrated that formulation presented difference ($p=0.0005$) and components also had difference ($p=0.0057$); but not for both formulations *versus* components ($p=0.1950$). Thus, for exopolysaccharides, the formulation C135+F presented less biovolume *versus* F and V (**$p \leq 0.0068$; Tukey post-test), followed by formulation C135+Far+F *versus* V (*$p=0.0289$). Similarly, for microorganisms, the formulation that yielded the lowest biovolume was C135+F *versus* F ($^{\#}p=0.0178$) and V ($^{\#\#\#}p=0.0006$), followed by formulation C135+Far+F *versus* V ($^{\#}p=0.0141$) and C135 *versus* V ($^{\#\#}p=0.0086$).

Concerning maximum thickness (Figure 8B), mixed ANOVA analysis, comparing as factors formulation and biofilm component demonstrated that formulation

presented difference (p=0.0003) and components did not have difference (p=0,0882); also, there was no difference for formulation *versus* components (p=0.2517). For exopolysaccharides and microorganisms, the lowest thickness was observed for C135+F *versus* C135+Far (*** and ### p=0.0036 for both components), *versus* F and V (** e ## $p \leq 0.0038$ for both components).

Lastly, regarding the coverage area by exopolysaccharides and microorganisms (Figure 9), five formulations presented similar patterns, with two exceptions: C135+F presented the lowest distance (which is coherent with the maximum thickness data), while V showed a higher percentage of microorganisms than exopolysaccharides in the most external layers (towards the biofilm/fluid interface).

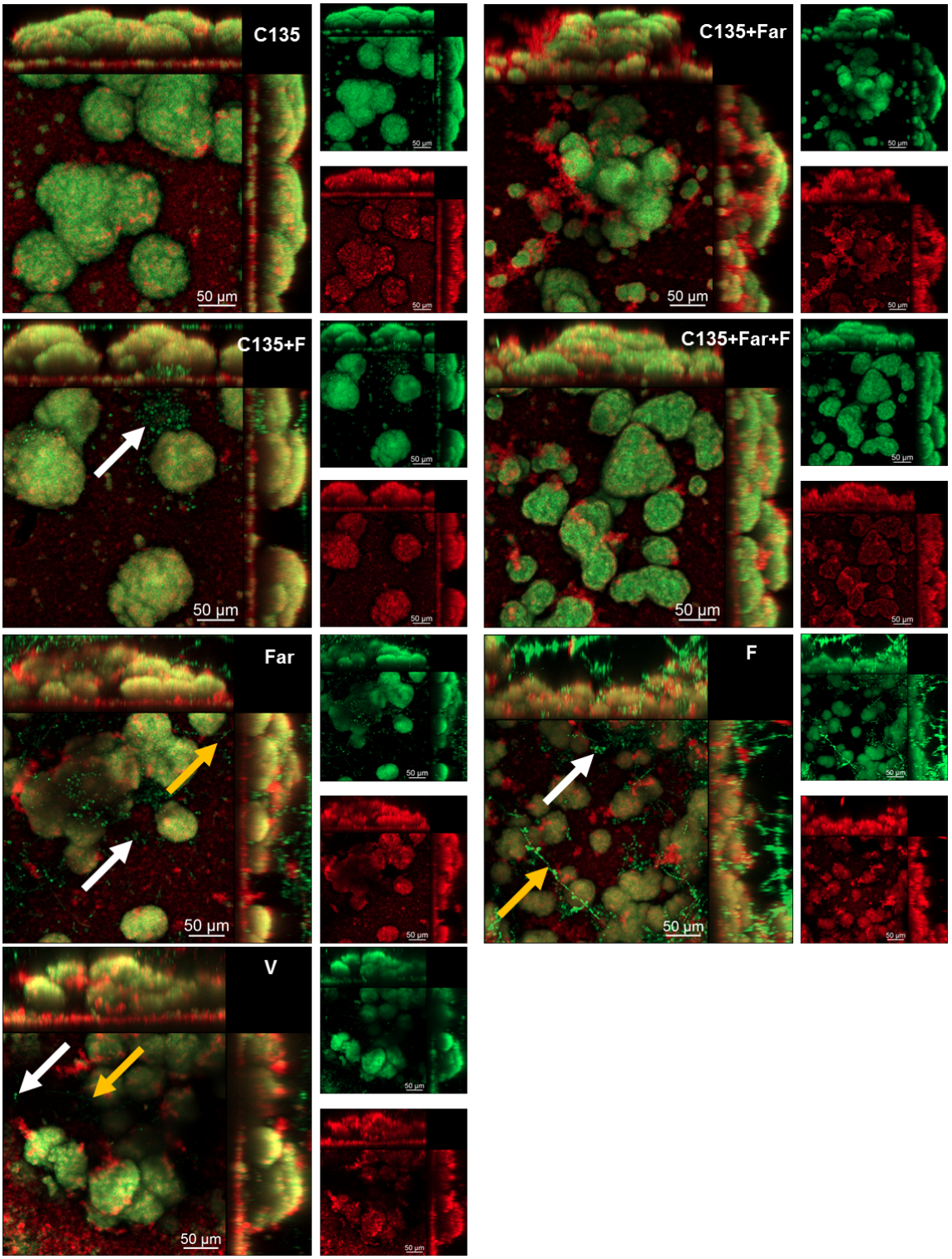


Figure 7: Representative confocal microscopy images of *S. mutans* and *C. albicans* biofilms with 43h. Biofilms were treated with combined compounds of C135, *tt*-farnesol (Far), and sodium fluoride (F) [C135, C135+Far, C135+F, C135+Far+F, Far, F], and Vehicle or V. The arrow shows *C. albicans* cells, yeast in white and hyphae in yellow. Representative images from three different experiments. The green color represents the microbial cells labeled with SYTO9, and the red color represents the exopolysaccharides in the extracellular matrix (labeled with Alexa Fluor 647). The larger images show the overlay of both components.

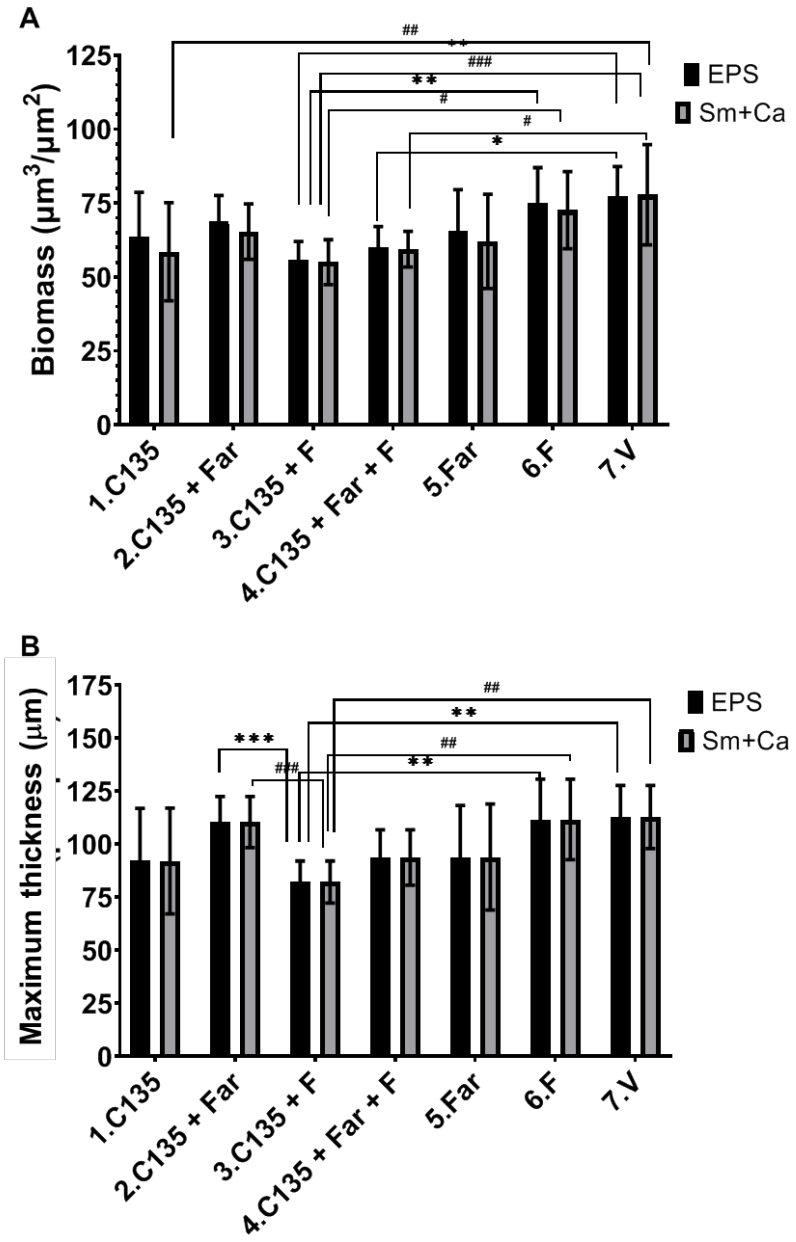


Figure 8: Data represents means and standard deviations of the exopolysaccharides from the biofilm matrix (EPS, represented in black) and microorganisms (*S. mutans*: Sm and *C. albicans*: Ca, represented in grey). Graph A represents the biomass or biovolume (top) Differences are represented by * for EPS or # for Sm+Ca. One-way ANOVA: p<0,1950; Tukey post-test: *p=0,0289; **p=0.0068; #p≤0.0178; ##p=0.0086 and ###p=0.0006. Graph B (bottom) represents the maximum thickness of the biofilm confocal images. One-way ANOVA: p≤0,2517; Tukey post-test: **p≤0,0038; ##p≤0.0038, ###p=0.0036. Biofilms were treated with combined compounds of C135, *tt*-farnesol (Far) and sodium fluoride (F) [1.C135; 2.C135 + Far; 3.C135 + F; 4.C135 + Far + F; 5.Far; 6.F and 7.Vehicle or V (EtOH + DMSO)].

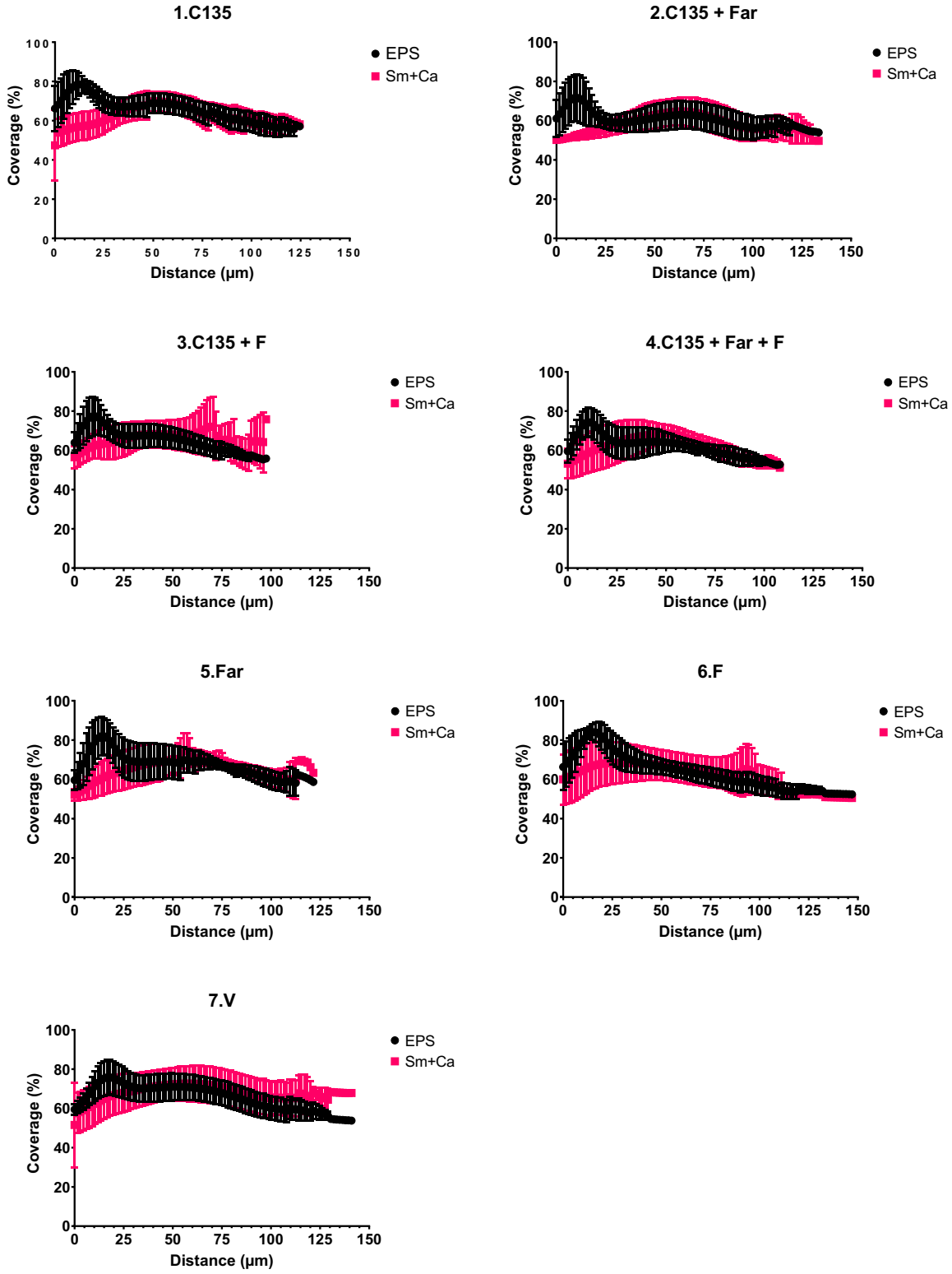


Figure 9: Data represents means and standard deviations of the coverage percentage exopolysaccharides of the biofilm matrix (EPS, represented in black color) and microorganisms (*S. mutans*: Sm and *C. albicans*: Ca, represented in pink color) on hydroxyapatite discs. Biofilms were treated with combined compounds of C135, *tt*-farnesol (Far) and sodium fluoride (F) [1.C135; 2.C135 + Far; 3.C135 + F; 4.C135 + Far + F; 5.Far; 6.F and 7.Vehicle or V (EtOH + DMSO)].

SEM

The **SEM** images are represented in Figure 10. Biofilms treated with C135+Far and C135+Far+F were smaller than the vehicle (V), with less microbial clusters and presented *C. albicans* only in yeast form (easily observed on 1,000 and 5,000X magnifications). Biofilms treated with Far, presented some *C. albicans* on the initial filamentary formation (clear on 5,000X), and others in yeast form above the *S. mutans* clusters. Biofilms treated with C135 and C135+F presented all morphologies of *C. albicans*, but *S. mutans* clusters were still smaller than the vehicle control (V) and F. For V and F, the hyphal cells were scattered throughout the disc and surrounding the bacterial cells clusters, especially for V.

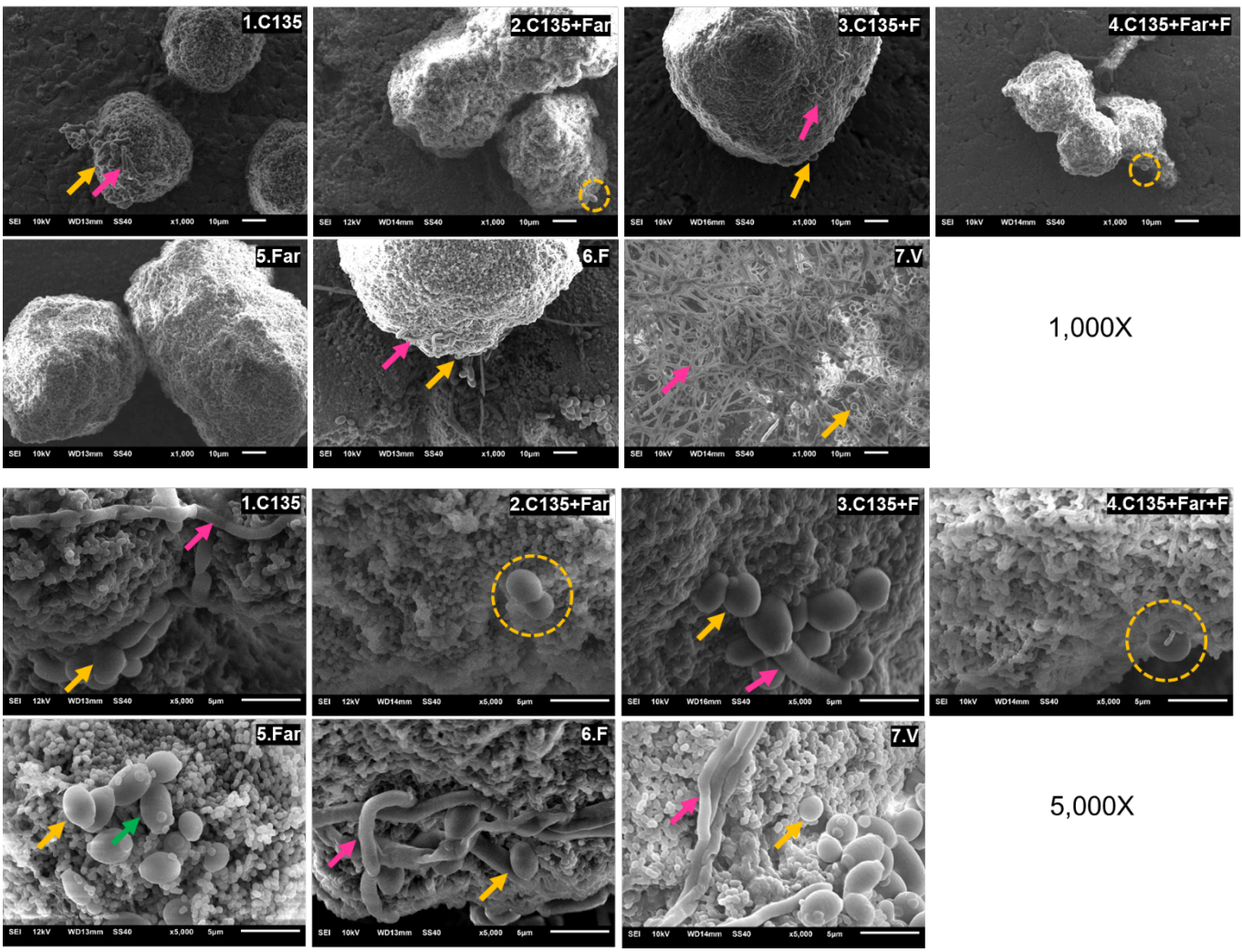


Figure 10: Representative images of scanning electronic microscopy of *S. mutans* and *C. albicans* biofilms with 43h. Biofilms were treated with combined compounds of C135, *tt*-farnesol (Far), and sodium fluoride (F) [C135, C135+Far, C135+F, C135+Far+F, Far, F], and Vehicle or V. The images were acquired on 1,000 and 5,000X; *C. albicans* in yeast form are identified by yellow; the initial filamentary morphology in green and the hyphal cells are in pink.

Cytotoxicity activity

The cytotoxic activity is presented in Figure 11. The formulations containing *tt*-farnesol (C135+Far, C135+Far+F, and Far) were highly cytotoxic with more than 75% of the cell viability reduction *versus* the live control, and the same behavior was observed for chlorhexidine digluconate 0.12% (with 30 min exposition) and dead control (Triton X-100. The formulations C135 and C135+F had moderated cytotoxicity, while F and V were not cytotoxic. The analysis was based on ISO 10993-5:2009 (Biological evaluation of medical devices – part 5: tests for in vitro cytotoxicity; (http://www.iso.org/standard/36406.html); were <25% not cytotoxic; between 25 and 50% slightly cytotoxic; between 50 and 75% cytotoxic; 75%< highly cytotoxic *versus* live control.

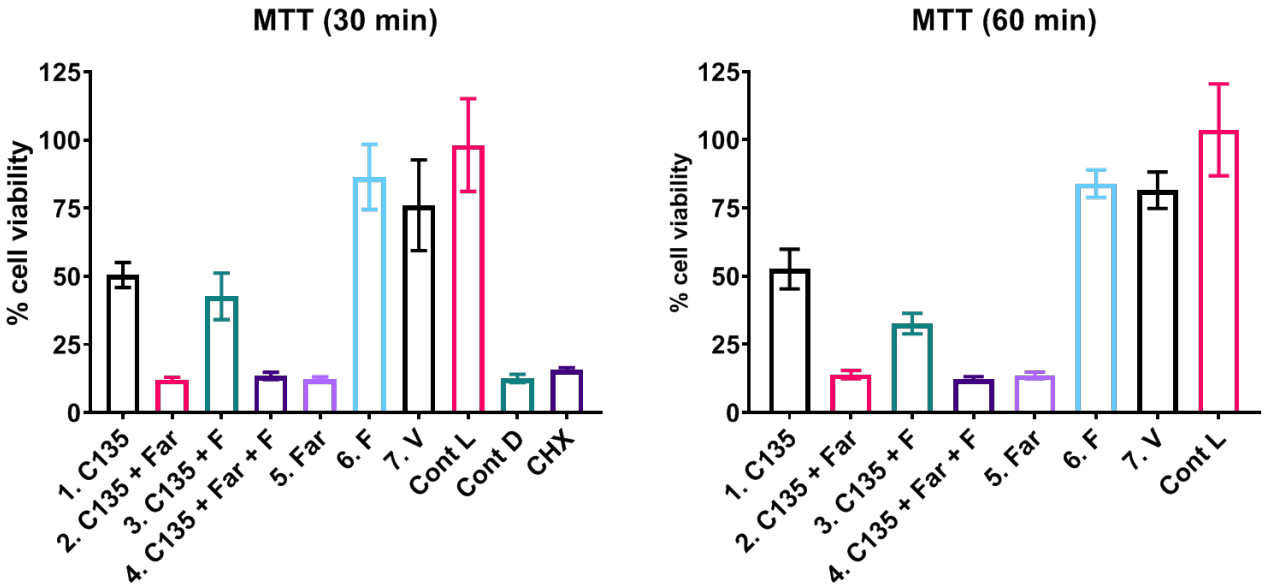


Figure 11. Cytotoxicity of formulations. Data represents percentage of living cells (cytotoxicity analysis) by MTT assays for formulations [1.C135; 2.C135 + Far; 3.C135 + F; 4.C135 + Far + F; 5.Far; 6.F and 7.Vehicle or V, and chlorhexidine digluconate (CHX)], and the live control (Cont. L) and dead control (Cont. D). A monolayer of NOK-Si cells was treated by 30 min (first graph) or 60 min (second graph). The means and standard deviations are related with the cell viability reduction *versus* control live (considered as 100%): <25% not cytotoxic; between 25 and 50% slightly cytotoxic; between 50 and 75% cytotoxic; 75%< highly cytotoxic; [30 min (n=16); 60 min (n=8)].

Discussion

The formulations applied in this study presented diverse effects against *C. albicans* and *S. mutans* dual-species biofilms. Furthermore, the *in vitro* model used

has extremely cariogenic conditions such as the combination of *C. albicans* and *S. mutans* growing the presence of sucrose [3]; hence, a strategy that can affect virulence parameters is desired because it could be an indication of further investigation to improve the strategy for clinical application.

Among the virulence parameters, the acidogenicity of biofilms reflects the metabolization of cariogenic sugar into acids that can cause demineralization. A previous study of the dual-species model used here demonstrated that the pH values of spent culture medium at 19 and 43 h are more acidic (≅4.2) than at 27 h (≅4.7); [5]. Here, a similar behavior was observed for biofilms treated with formulations without fluoride (C135, C135+Far, Far, and Vehicle) at 19 and 43 h, while increased pH was measured for formulations with fluoride (C135+F, C135+Far+F, and F) (more pronounced at 43h). These data suggest that formulations containing fluoride on composition can increase on pH of the culture medium, which can be related to the remineralization process necessary to maintain teeth enamel surface. Although all values were below the threshold for enamel demineralization (*i.e.,* pH of 5.5), at 27 h, formulations with C135 and Far present increased pH values than other groups, and these findings can be related to a possible reduction in the virulence potential of biofilms when both compounds are associated.

Biofilms formed by *S. mutans* and *C. albicans* are more complex, structured, and exhibit an organized extracellular matrix, making both species more tolerant to environmental stresses [5]. The gene expression of microbial species regulates the mechanisms for surviving and developing biofilm. For *S. mutans*, the gene expression for oxidative stress (*nox1*) was increased when biofilms were treated with C135+Far+F, while for *C. albicans,* all formulations with C135 led to a decrease SOD1 expression. These findings reveal that the combination of all compounds was more effective than other treatments, affecting the oxidative tolerance for the bacterium since a previous study [5] observed a reduced gene expression of *nox1* for dual-species biofilm of *S. mutans* and *C. albicans* with no treatment. Thus, the decreased gene expression for the treatments containing C135 for *C. albicans* can be related to the interference of the effect of chalcones in cell wall formation and in mitochondrial dysfunctions [24]. These data also confirm the antibiofilm effect of C135 for *C. albicans* observed in initial and pre-formed single- and dual-species biofilms after prolonged exposure to the agent (24h) [25].

The genes BGL2, FKS1, PHR1, and XOG1 are related with β-1,3 glucan and β-1,6 glucan (PHR1) synthesis and are essential for the extracellular matrix construction [12-14]. In response to specific signals, *C. albicans* cells reduce the exposure of β-1,3-glucan at their cell surface, causing impacts for the cell wall biosynthesis [11]. Here, BGL2 gene showed a reduced expression for all treatments containing C135, Far, or when both compounds were combined. In addition, for FKS1, PHR1, XOG1, and CDR1 (important for fungal resistance) genes, the impact on *C. albicans* cells with decreased gene expression was observed for C135+Far+F. Also, a reduced gene expression for acid stress and tolerance gene PHR2 occurred C135+Far and C135+Far+F; and the same behavior was observed for ERG11 gene expression (also for C135). The ERG11 gene is essential for the ergosterol biosynthesis that can influence membrane permeability and consequently influence fungal virulence [16,18]. In addition, previous study ERG11 interfered in hyphal elongation [18], corroborating with the confocal images (Figure 7; in which it was not possible to visualize the fungal cells for C135, C135+Far, and C135+Far+F) and with SEM images (Figure 10; C135+Far and C135+Far+F presented *C. albicans* cells only as yeast morphology). Thus, these findings reveal that the better effect against *C. albicans* in dual-species biofilms with *S. mutans* is observed using formulation combining compounds that affect the biofilm construction and virulence (acid and oxidative stresses, and antifungal resistance, extracellular matrix construction).

The viable microbial population was affected by some formulations, but the log reduction was more pronounced for the fungus. *S. mutans* counting was lower when biofilms were treated with C135, C135+Far, and Far. *C. albicans* counting was reduced by C135+Far with or without fluoride, corroborating with the gene expression of fungi. These findings for the biofilm treated with C135+Far are also related to the biomass (insoluble dry-weight), proteins (from insoluble portion), and exopolysaccharides in the matrix (mainly for ASP). Meanwhile, C135 and C135+F decrease biomass (biovolume), maximum thickness, and percentage area coverage via the quantitative confocal analysis.

Considering the ASP data, it was expected to observe the lowest exopolysaccharides biovolume for C135+Far, but the biovolume was lowest for C135+F, which presented a higher amount of ASP. A contradiction is apparent between quantifying exopolysaccharides via fluorescence (Biovolume data, Figure 8)

and biochemical assay for insoluble exopolysaccharides (ASP data, Figure 6). This contradiction could be because the biochemical assay is more sensitive and quantifies the total ASP in the biofilm sample (covering all discs surfaces) and exopolysaccharides produced by the bacterium and the fungus. In contrast, confocal analysis was performed by at least three images from one of the surfaces (*i.e.,* there is no quantification of signals in the entire biofilm sample grown on a disc), and the fluorescence signal is for exopolysaccharides produced by the bacterium. Another point is that the volume occupied by the fluorescence signal could present a distinct "density"; it could be that the fluorescence is spread out for bigger microcolonies observed for C135+Far *versus* the smallest microcolonies observed for C135+F.

Nevertheless, C135 could be acting primarily on bacterial exopolysaccharides production [23] and on fungal cells and their beta-glucans metabolism on the cell wall and for the matrix [12,13], and its combination with *tt*-farnesol that hinders fungal filamentation could further decrease the substrate available for bacterial Gtfs binding and activity. Thus, the net result would be a biofilm with the lowest ASP and consequent lowest insoluble dry-weight, as observed here.

Furthermore, on confocal images, it was challenging to observe *C. albicans* cells on biofilms treated with C135, C135+Far, and C135+Far+F; however, for *S. mutans,* organized clusters were present, and the fungi cells could have been immersed by the bacterial cells and extracellular matrix. In addition, yeast cells were observed for C135+F, suggesting that the formulation affects the fungal morphology and implicates reduced biomass, thickness, and coverage of HA discs; F may potentiate the effect of C135. In addition, host-relevant conditions, including hypoxia, shifts in ambient pH, influence cell wall architecture [24]. However, the mechanisms governing β-1,3 glucan masking in *C. albicans* have proven elusive [44].

The SEM images of biofilms confirm the effect of C135 and Far on the morphology of *C. albicans* cells, especially for treatments combining both agents (with or without F) with fewer *S. mutans* clusters and fungal cells only in yeast form. These data are in aggrement with the *C. albicans* population data and with the gene expression profile of BGL2, ERG11, PHR1, and SOD1, indicating the effect on the cellular membrane, the oxidative stresses adaptation, filamentary morphogenesis, and fungal virulence [13,14,15,18].

Lastly, the three formulations with *tt*-farnesol (C135+Far, C135+Far+ F and Far) were highly cytotoxic for oral keratinocytes NOK-Si, organized as a monolayer. The same outcome was observed for CHX (at 0.12%), which is the gold-standard oral antimicrobial formulation. However, higher concentrations of Far against distinct cell lineages, such as fibroblasts L929 [45] and SW480 [46], did not present cytotoxicity; so, the effect observed here could be because of cell type. Moreover, a previous animal study used formulations with *tt*-farnesol for topical application at higher concentrations than the one used here, and no oral mucosal damage was observed [47]. Thus, the current findings could be because of the monolayer of keratinocytes instead of a tridimensional tissue of oral mucosas.

In summary, C135 and *tt*-farnesol affected the *C. albicans* and *S. mutans* dual-species biofilms. Thus, their formulations with and without fluoride affected acidogenicity, exopolysaccharide production, and the hyphal morphogenesis of *C. albicans*. However, it is still necessary to further understand the virulence mechanisms triggered in biofilms when both species are together. In addition, saliva was used for pellicle formation on HA discs, but it was not continuously present as it occurs in the oral cavity for individuals without saliva production issues. The presence of saliva can modify the characteristics of teeth and microbial surfaces [19]. Also, saliva could complex the compounds, which could reduce or mask the effect of the formulation. Thus, an alternative could be the encapsulation of the compounds for controlled release and/or the use of delivery systems via nanotechnology [48] to circumvent the virulence mechanisms and improve the effectiveness of the compounds to prevent cariogenic biofilms development and caries occurrence.

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4 DISCUSSÃO

O estudo investigou compostos selecionados com alvos distintos nas células e vias metabólicas de *S. mutans* e *C. albicans* para identificar agentes seletivos antibiofilme que apresentassem aplicações potenciais na prevenção de biofilme cariogênico e na ocorrência de cárie.

A interação de *S. mutans* e *C. albicans* favorece uma ação cooperativa entre as duas espécies, que resulta na formação de biofilmes melhor organizados tridimensionalmente[39,40], mais virulentos[49] e com maior capacidade de resistência contra os agentes antimicrobianos e antibiofilmes. Portanto torna-se importante a investigação de alternativas que tenham alvos disdintos e que possam auxiliar no controle ou prevenção desses biofilmes.

Os seis compostos selecionados [*tt*-farnesol (Far), mirecetina (Myr), três hidroxichalconas: 2' (AGN e R815) e 4' (C135), e composto 1771 (1771)] foram testados para avaliar a atividade antimicrobiana das culturas planktônicas de cada microrganismo. Posteriormente foi avaliada a atividade antibiofilme das culturas simples e mistas das duas espécies (biofilmes inicialmente formados ou com 24 h de crescimento; e biofilmes pré-formados ou com 48 h de crescimento). Ainda, as concentrações mais promissoras dos compostos selecionados formam testadas e combinadas entre si, e com ou sem flúor (F) para aplicação sobre biofilmes de 43 h formados sobre discos de HA com película salivar em regime de tratamento tópico por 5 min; seguida de avaliação da citotoxicidade dos tratamentos (ou formulações) em modelo monocamada de queratinócitos orais (NOK-si).

Nos testes de atividade antimicrobiana, os compostos C135, Far, Myr e 1771 apresentaram efeito promissor contra bactéria e portanto foram selecionados e testados contra *C. albicans*. Então verificou-se que, C135 e Far inibiram 50% do crescimento microbiano para ambas espécies; Far eliminou totalmente a contagem de colônias e foi o único que teve efeito dose-dependente (apenas contra bactéria). Entretanto, alguns compostos precipitavam ao entrarem em contato com o meio de cultura, o que pode interferir no efeito dose-dependente do agente e na ausência de efeito dos compostos R815 e AGN. Porém, Myr e 1771 reduziram o crescimento microbiano em 50% da cultura de *S. mutans* e a maior concentração do composto 1771 (250 µg/mL) eliminou totalmente a contagem de colônias bacterianas.

Para a atividade antibiofilme, todos os compostos (C135, Far, Myr e 1771) inibiram o crescimento microbiano dos biofilmes inicialmente formados (24 h) de *S.*

mutans. Ainda, os compostos C135 e Far inibiram o crescimento dos biofilmes pré-formados (48 h de crescimento) de *S. mutans* e também inibiram crescimento dos biofilmes de 24 h de *C. albicans;* e apenas C135 inibiu o crescimento microbiano dos biofilmes de 48 h de *C. albicans* (ou com 48 h de crescimento). *tt*-farnesol apresentou o mesmo efeito contra os biofilmes de *S. mutans.* Assim, os dados mostraram que o efeito dos agentes em biofilmes pré-formados (com 48 h de crescimento) foi menor, pois tais biofilmes são mais difíceis de serem desorganizados e ou inibidos *versus* biofilmes inicialmente formados; e nesta análise os compostos pareceram mais eficazes contra *S. mutans*. Este resultado é interessante visto se tratar de um microrganismo importante para a construção da matriz extracelular[10] e portanto os alvos dos compostos podem auxiliar no controle desse tipo de biofilme. Uma alternativa para potencializar a ação dos compostos e ou aumentar os possíveis alvos terapêuticos, foi selecionar as concentrações efetivas nas culturas simples e combinar essas concentrações, com ou sem flúor para aplicação sobre os biofilmes mistos das duas espécies.

Assim, 16 combinações de tratamentos (ou formulações) foram testadas contra os biofilmes mistos de *C. albicans* e de *S. mutans* inicialmente formados (24 h) e pré-formados (48 h). Além de serem combinados entre si, os tratamentos também foram combinados com flúor (F). Todas as combinações de tratamentos (C135, C135+Far, C135+1771, C135+Myr, C135+Far+1771, C135+Far+Myr, C135+Far+1771+Myr, C135+1771+Myr, Far+1771, 1771+Myr, Far+1771+Myr C135+F, C135+Far+F, C135+1771+F, C135+Myr+F, C135+Far+1771+Myr+F) foram eficazes na inibição e erradicação do crescimento microbiano dos biofilmes mistos das duas espécies quando testadas em biofilmes inicialmente formados (24 h). Já para os biofilmes mistos pré-formados (48 h), algumas combinações foram eficazes apenas contra *S. mutans*, outras apenas contra *C. albicans*, e 3 combinações de tratamentos inibiram e erradicaram o crescimento microbianos das duas espécies microbianas C135+Far+Myr, C135+Far+F e C135+Far+1771+Myr+F. Os tratamentos contendo flúor apresentaram melhor eficácia contra os biofilmes mistos pré-formados, corroborrando com os resultados obtidos em modelo animal, onde os tratamentos contendo flúor na composição, apresentaram menos lesões de cárie e com menor virulência[34, 36].

Após avaliar os bofilmes mistos inicialmente formados e pré-formados, utilizando as mesmas concentrações para os compostos C135 e Far (125 μg/mL)

foram utilizadas combinadas entre si e com e sem flúor para aplicação tópica sobre os biofilmes mistos formados em discos de HA com película salivar. Os biofilmes de 43 h foram avaliados quanto ao pH do meio de cultura, expressão gênica, população microbiana, biomassa dos biofilmes, proteínas da porção insolúvel, componentes da matriz extracelular (ASP, WSP, proteínas na porção solúvel, eDNA), estrutura 3D e microscopia eletrônica de varredura dos biofilmes. Os tratamentos tópicos avaliados foram C135, C135+Far, C135+F, C135+Far+F, Far, F e Veículo.

O pH foi aferido em 19, 27, 28 e 43 h. Tratamentos com flúor, apresentaram maior pH em 19 e 43 h (considerados momentos com intervalos longos sem suplementação), sugerindo que o flúor além de ser agente remineralizador, pode contribuir com o aumento do pH, estando combinado com outros agentes ou isolado

A combinação dos compostos com flúor resultou em aumento na expressão do *nox1* (stress oxidativo e tolerência aos ambientes ácidos para *S. mutans*), e redução na expressão de todos os genes avaliados para *C. albicans*. Os dados sugerem que a combinação dos compostos C135+Far+F é eficaz contra *S. mutans* e tem como alvos, as vias que interferem na tolerância ao estresse oxidativo; entretanto, para o biofilme *de C. albicans,* pode-se pensar que a combinação dos tratamentos afeta diferentes alvos do biofilme. Estes achados, confirmam os dados do estudo anterior[47] onde foi avaliada a atividade antibiofilme dos biofilmes mistos de *C. albicans* e *S. mutans,* e a mesma combinação de tratamentos (C135+Far+F) inibiu e erradicou a contagem de UFC/mL de *C. albicans*.

Os tratamentos afetaram a população microbiana das duas espécies, entretanto para *C. albicans* houve maior redução, principalmente na combinação de C135+Far com e sem flúor, corroborando os dados de expressão gênica do fungo. Ainda, a redução na UFC/mL se correlaciona com a redução das proteínas, biomassa do biofilme e principalmente com a redução de ASP (que é o maior componente da matriz extracelular). Porém, a análise confocal, mostrou redução do biovolume, espessura máxima e porcentagem de cobertura nos biofilmes tratados com C135 e C135+flúor diferente do que se esperava pelos dados de ASP. Assim, pode-se pensar que ao processar o biofilme para uma análise de ASP, a amostra é utilizada no seu todo. Dessa forma, os discos de HA são submetidos a vários processos de processamento que garantem a remoção de todo o biofilme para análise (frente, verso e laterais do disco). No entanto, as imagens confocal são adquiridas em apenas uma das faces do disco, onde são feitas capturas de imagens representativas por disco

(geralmente 3 imagens), e em algumas situações os lasers podem apresentar limitações como a dificuldade de penetração em toda a espessura de biofilmes maduros que podem interferir na captura das imagens. Adicionalmente, neste estudo o fluoróforo Alexa Flúor 647 utilizado é específico para a marcação de exopolissacarídeos produzidos pela bactéria, portanto pode ser que as células fúngicas foram envoltas pelos exopolissacarídeos da bactéria e por isso torna-se difícil a sua visualização.

A combinação C135 e Far interferiu na morfologia de *C. albicans* (especificamente na transição de levedura em hifa), como observado nas imagens de MEV e corroborando com os dados redução da expressão gênica para os genes BGL2, ERG11, PHR2 e SOD1; afetando a interação com Gtfs de *S. mutans.* Dessa forma, uma vez reduzida a diferenciação morfológica fúngica, a predominância de levedura poderia explicar a redução de ASP, das proteinas e a biomassa do biofilme tratado com C135+Far. Ainda nas imagens dos biofilmes tratados por C135, C135+Far e C135+Far+F não se observam células fúngicas, e nos biofilmes tratados com C135+F é possível observar células fúngicas apenas no formato de levedura, sugerindo que C135 também interfere na morfologia de *C. albicans*, o que justificaria a redução do biovolume, espessura do biofilme e porcentagem de cobertura dos discos de HA.

Adicionalmente, todos os tratamentos contendo *tt*-farnesol, assim como a clorexidina à 0,12% (padrão ouro e porcentagem utilizada comercialmente em colutórios) foram altamente tóxicos para o modelo de queratinócitos orais NOK-si, organizados em monocamada; entretanto, estudos utilizando maiores concentrações de *tt*-farnesol em outras linhagens celulares como fibroblastos L929[50] e SW480[51], não foram citotóxicas, sugerindo que esse resultado seja induzido pelo tipo celular. Ainda, não se observou comprometimento da mucosa de animais que foram tratados com altas concentrações de *tt*-farnesol[52].

Portanto C135 e *tt*-farnesol afetaram as duas espécies dos biofilmes mistos de *C. albicans* e *S. mutans* e as suas formulações como ou sem flúor, afetaram a acidogenicidade, capacidade de resposta oxidativa, produção de exopolissacarídeos dos biofilmes, além de interferirem na morfologia filamentar de *C. albicans*. Entretanto, estudos sobre como os agentes afetam a virulência das duas espécies podem contribuir e direcionar as futuras formulações antibiofilme.

Por outro lado, pode-se pensar no encapsulamento dos compostos para liberação controlada e/ou o uso de sistemas de liberação via nanotecnologia para contornar os mecanismos de virulência e melhorar a eficácia dos compostos na prevenção do desenvolvimento de biofilmes cariogênicos e ocorrência de cárie.

5 CONCLUSÃO

Os compostos testados apresentaram eficácia antimicrobiana e antibiofilme contra diferentes alvos para *S. mutans* e *C. albicans*. Especificamente*:*

Os compostos C135, *tt*-farnesol, mirecetina e 1771 foram promissores e apresentaram efeito antimicrobiano contra *S. mutans* e os compostos C135 e *tt*-farnesol apresentaram potencial antimicrobiano contra *C. albicans*.

Os compostos C135, *tt*-farnesol, mirecetina e 1771 isolados, inibiram a formação de biofilmes inicais simples de *C. albicans* e de *S. mutans* (24h); C135 isolado eliminou os biofilmes pré-formados simples das duas espécies (48h) e *tt*-farnesol eliminou os biofilmes pré-formados de *S. mutans*. Ainda, a combinação dos agentes selecionados com e sem flúor, foi efetiva contra os biofilmes mistos iniciais e a combinação de todos os compostos com flúor, C135 + *tt*-farnesol + mirecetina e C135+*tt*-farnesol+flúor, inibiu o crescimento das duas espécies microbanas em biofilmes mistos pré-formados de 48h.

A aplicação tópica por 5 minutos de formulações contendo C135 + *tt*-farnesol (com ou sem flúor) afetou a acidogenicidade, capacidade de resposta oxidativa e a produção de exopolissacarídeos dos biofilmes misto de *S. mutans* e *C. albicans*, além de interferir na morfologia filamentar das células de *C. albicans.*

Os tratamentos (formulações) C135 e C135+F, apresentaram citotoxicidade moderada e todos os tratamentos contendo *tt*-farnesol foram altamente citotóxicos quando aplicados por 30 min sobre o modelo de monocamada de queratinócitos orais.

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* 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-normalizacao-atualizado.pdf

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ANEXO A – Autorização artigo 1

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ANEXO B – Comité de Ética

UNESP - FACULDADE DE ODONTOLOGIA - CAMPUS ARARAQUARA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Estratégia para controle de biofilme patogênico
Pesquisador: MARLISE INEZ KLEIN
Área Temática:
Versão: 2
CAAE: 08189119.7.0000.5416
Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP
Patrocinador Principal: CONSELHO NACIONAL DE DESENVOLVIMENTO CIENTIFICO E TECNOLOGICO-CNPQ

DADOS DO PARECER

Número do Parecer: 3.208.905

Apresentação do Projeto:
Análise da eficácia antimicrobiana e antibiofilme de seis compostos selecionados (um terpenóide, dois flavanóides, duas hidroxichalconas e um agente que modula a síntese de ácido lipoteicóico em bactérias Gram positivas) com possíveis alvos tanto na bactéria Streptococcus mutans quanto no fungo Candida albicans.

Objetivo da Pesquisa:
Objetivo Primário:
O objetivo geral do projeto é avaliar a eficácia de seis compostos selecionados com possíveis alvos tanto na bactéria Streptococcus mutans quanto no fungo Candida albicans: um terpenóide, dois flavanóides, duas hidroxichalconas e um agente que modula a síntese de ácido lipoteicóico em bactérias Gram positivas. Esses alvos incluem as células microbianas per se e também a produção de matriz extracelular e de metabólitos em biofilmes relacionados ao desenvolvimento de cárie.

Objetivo Secundário:
Os objetivos específicos são: (1) Avaliar o potencial antimicrobiano (antibacteriano e antifúngico) de cada composto para as duas espécies cultivadas isoladamente e em conjunto (pois o co-cultivo pode interferir na efetividade da atividade de um composto) via determinação de concentração inibitória mínima (CIM) e concentração bactericida e fungicida mínima (CBM/CFM). (2) Analisar o

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ANEXO C – Autorização artigo 2

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APÊNDICE A - MATERIAL E MÉTODOS (Artigo 1)

Desenho experimental

A atividade antimicrobiana foi realizada via análise das culturas planctônicas de *C. albicans* e *S. mutans* para a determinação da concentração inibititória mínima (CIM) e da concentração bactericida ou fungicida mínima (CBM ou CFM). Posteriomente, foi realizada a análise da atividade antibiofilme dos compostos sobre os biofilmes simples de cada espécie microbiana. Esta análise antibiofilme foi avaliada para biofilmes inicialmente formados ou biofilmes de 24 h e para os biofilmes pré-formados ou biofilmes de 48 h. Portanto, primeiro foram analisados os biofilmes simples de cada espécie e seguiu-se a análise dos biofilmes mistos das duas espécies. Nos biofilmes mistos foram testadas as combinações dos compostos selecionados com e sem flúor para os biofilmes de 24 e 48 h. Todas análises foram realizadas em microplacas de 96 poços para a determinação das unidades formadoras de colônias ou UFC. Em cada análise foram realizadas 3 ocasiões experimentais independentes utilizando um n = 9. Ainda, foi realizada a análise estatística descritiva e inferencial dos dados utilizado α = 5%. A hipótese considerou que os compostos ou as combinações dos compostos eliminassem ou reduzissem pelo menos 50% das células microbianas de cada espécie microbiana.

Agentes testados

Os compostos utilizados no estudo foram: tt-farnesol ou (E,E)-3,7,11-Trimetil-2,6,10-dodecatrieno-1-ol, trans,trans-3,7,11-Trimetil-2,6,10-dodecatrieno-1-ol (Sigma-Aldrich Co., St Louis, MO, USA; Cat.#46193; 96% pureza), mirecetina ou 3,5,7-trihidroxi-2-(3,4,5-trihidroxifenil)-4H-cromen-4-um (AK Scientific, Inc., Union City, CA, USA; Cat.#J10595; 95% purity), três hidroxichalconas [2'-hidroxichalcona ou 1-(2-hidroxifenil)-3-fenilprop-2-em-1-um (Angene, Hong Kong, China; Cat.#AGNPC-015IM; 95% pureza), 2'-hidroxichalcona ou (2E)-1-(2-hidroxifenil)-3-fenilprop-2-em-1-um (AK Scientific, Inc.; Cat.#R815; 98% pureza), 4'-hidroxichalcona ou (2E)-1-(4-hidroxifenil)-3-fenilprop-2-em-1-um (AK Scientific, Inc.; Cat.#C135; 98% pureza)], e composto 1771 [(5-fenil-1,3,4-oxadiazol-2-il)carbamoil]metil 2-{nafto[2,1-b]furan-1-il}acetato)] (UkrOrgSynthesis Ltd., Kiev, Ukraine; Cat.#PB25353228; pureza não descrita). Todos os compostos foram diluidos em 84,15% de etanol ou EtOH (Sigma-Aldrich; Cat.#E7023) e 15% de dimetil sulfóxido ou DMSO (Sigma-Aldrich; Cat.#D8418) para a otenção das soluções estoque à 15 mg/mL, exceto o composto 1771 que foi utlizado

na concentração de 2 mg/mL. A concentração do composto 1771 foi baseada em testes prévios onde se observaram dificuldades para a diluição do composto, portanto ao se utilizar 2 mg/mL verificou-se que o mesmo diluiu completamente. Adicionalmente, foi utilizado o fluoreto de sódio na concentração estoque de 5000 ppm (Sigma-Aldrich; Cat.#71519). Assim, para a atividade antimicrobiana, diferentes concentrações dos tratamentos foram testadas à partir das concentrações estoque 15 mg/mL: 1250, 1000, 500, 250, 125, 62.5, 31.25, 15.625 µg/mL; e para o composto 1771 à 2 mg/mL: 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, 0.977, 0.488, 0.244 µg/mL.

As concentrações promissoras na avaliação da atividade antimicrobiana, foram utilizadas para avaliar a atividade antibiofilme das culturas simples e à partir desta análise foram selecionadas as concentrações e as combinações dos compostos para a análise antibiofilme das duas espécies combinadas.

Cepas Microbianas e Crescimento Planctônico

Estoques das cepas *S. mutans* UA159 (serotipo c; ATCC 700610) e *C. albicans* SC5314 armazenados em freezer -80ºC foram reativados pelo método de semeadura por esgotamento em placas de ágar sangue (5% sangue de cordeiro – Laborclin, Pinhais, PR, Brasil) e incubadas por 48 h (37°C, 5% de CO_2; Steri-Cult™ Thermo Scientific, Waltham, MA, USA) para uso imediato e/ou armazenamento em geladeira 4ºC (7 dias para *C. albicans*, e 1 mês para *S. mutans*). Os pré-inóculos para cada cepa foram realizados pela transferência de 5 colônias individuais para tubos contendo 10 mL de meio líquido TYE [2,5% triptona e 1,5% extrato de levedura, pH 7.0; Becton, Dickinson and Company (BD), Sparks, MD, USA] com 1% de glicose (v/v) e incubação (37°C, 5% de CO_2). Após 16 h de crescimento do pré-inóculo, foram realizadas diluições de 1:20 para o inóculo (500 *µ*L de cada pré-inóculo para 9,5 mL de meio líquido TYE com 1% de glicose). Os tubos foram incubados até a fase de crescimento *mid-log*: *S. mutans* UA159 com Densidade óptica ou DO_{562nm} 0,500 (±0,100) e *C. albicans* SC5314 com DO_{562nm} 0,482 (±0,058) (leitor de ELISA; Biochrom Ez, Cambourne, UK). Após atingirem a DO desejada, foi verificada a pureza das culturas em microscópio óptico e para a análise da atividade antimicrobiana, essas culturas foram diluídas para obtenção do inóculo final à $2x10^6$ unidades formadoras de colônias por mililitro (UFC/mL) em meio TYE + 1% de glicose; entretanto, para a análise de atividade antibiofilme dos biofilmes simples, a mesma concentração de

inóculo ($2x10^6$ UFC/mL) final foi diluída em meio TYE + 1% de sacarose (v/v). Para os biofilmes mistos, o inóculo final de cada microrganismo foi diluído em TYE + 1% de sacarose, sendo $2x10^6$ UFC/mL para *S. mutans* e $2x10^4$ UFC/mL para *C. albicans*.

Atividade antimicrobiana

A atividade antimicrobiana foi determinada via concentração inibitória mínima (CIM) e concentração bactericida e fungicida mínima (CBM e CFM), utilizando microplacas de 96 poços, seguindo as instruções do *The Clinical & Laboratory Standards Institute* [31-33], com algumas modificações [34]. As análises de cada composto foram realizadas de acordo com as soluções estoques: se 15 mg/mL (soluções teste de 0 à 1250 µg/mL) e se 2 mg/mL (soluções teste de 0 à 250 µg/mL), sendo 0 µg/mL o controle negativo ou o controle de veículo para todas analises realizadas.

Geralmente a CIM é utilizada para analisar novos compostos, entretanto, em algumas situações podem ser realizadas algumas modificações, como quando existe a precipitação dos compostos e ocorre o comprometimento da análise visual e da densidade ótica [35]. Muitos dos compostos testados neste projeto, complexaram ao entrarem em contado com o meio de cultura, comprometendo assim a inspeção visual e as leituras da densidade óptica e a maioria deles não apresentou um efeito dose dependente. Neste caso, foi utilizada a concentração inibitória mínima do agente, com a abreviação IC_{50}, definida como a concentração mínima que inibiu o crescimento microbiano em 50% da quantificação de UFC/mL [34]; e a abreviação CIM não foi utilizada.

Todos os compostos selecionados (tt-farnesol, mirecetina, hidroxichalcona 2' (AGN), hidroxichalcona 2' (R815), hidroxichalcona 4' (C135), e o composto 1771 (1771) foram testados para avaliação da atividade antimicrobiana de *S. mutans*; entretanto, para a avaliação da atividade antimicrobiana de *C. albicans* apenas foram testados os compostos efetivos contra a bactéria (tt-farnesol, mirecetina, C135 e 1771). Esta estratégia foi baseada em estudos preliminares que confirmam que biofilmes formados pela bactéria e pelo fungo são mais robustos e organizados mediados pela presença de exopolissacarídeos produzidos pela bactéria em ambientes ricos em sacarose (Falsetta 2014).

Portanto, em uma placa de 96 poços contendo o meio de cultura TYE + 1% de glicose (83,33 µL), foram adicionadas as diferentes concentrações pré-determinadas

para cada composto (desde a menor à maior, 16,67 µL) e em seguida 100 µL do inóculo de cada cultura microbiana (bactéria ou fungo) para a obtenção do volume final de 200 µL por poço. As placas de cada ocasião experimental, tinham como seus controles: poços contendo apenas o meio de cultura (200 µL), apenas o inóculo da ocasião (100 µL do meio + 100 µL do inóculo), apenas o veículo (concentração final de 7% EtOH + 1.25% DMSO: 183,33 µL do meio + 16,67 do veículo) e o veículo com o inóculo (83,33 µL do meio + 16,67 do veículo + 100 µL do inóculo), para descartar a possibilidade de algum efeito do veículo sobre a cultura de crescimento. Após a montagem, as placas foram incubadas por 24 h (37ºC, 5% CO_2), e seguiu-se a inspeção visual dos poços, onde poços turvos significaram crescimento microbiano e poços límpidos, significaram ausência de crescimento microbiana. Em seguida a placa foi novamente incubada para a homogeneização da cultura (5 min, 37ºC, 75 rpm) (Quimis, São Paulo, Brazil) e posteriormente 100 µL da cultura homogeneizada foram retirados para diluição seriada em solução de NaCl 0,89% (Synth, Diadema, SP, Brazil), plaqueamento (dede a cultura não diluída, 10^{-1} à 10^{-5}) e incubação por 48h (37ºC, 5% CO_2) para posterior contagem de UFC/mL e determinação da concentração inibitória (IC_{50}) dos compostos para cada microrganismo.

Os dados de MBC e MFC também foram determinados pela quantificação da UFC/mL, sendo definidos como mínimas concentrações de cada composto capazes de inibir o crescimento microbiano (ou ausência de crescimento microbiano na placa de ágar). Entretanto, para alguns compostos que podem ter como alvos a produção da matriz extraçelular dos biofilmes, as concentrações dos compostos que inibiram pelo menos 50% do crescimento microbiano (redução da UFC/mL em 50% vs veículo), também foram consideradas promissoras [34,35]. Ainda, a cultura remanescente na placa de 96 poços foi submetida a leituras de DO_{562nm} (Leitor ELISA).

Atividade antibiofilme

A análise antibiofilme foi realizada após a determinação da atividade antimicrobiana dos compostos. Assim, foi avaliada a atividade antimicrobiana dos compostos sobre os biofilmes simples de cada microrganismo e posteriormente contra biofilmes mistos de *S. mutans* e *C. albicans*. Ainda, os compostos foram testados contra biofilmes inicialmente formados ou biofilmes de 24 h e contra biofilmes pré-formados ou biofilmes de 48h. Os biofilmes inicialmente formados (24 h) foram tratados desde a hora 0 durante montagem da placa, e analisados após 24 h de

desenvolvimento. Entretanto, para a avaliação dos biofilmes pré-formados (48 h), biofilmes não tratados eram crescidos em placa de 96 poços, e após 24 h de crescimento eram tratados e incubados por mais 24 h para posterior análise em 48 h. Portanto, foi avaliada a inibição de biofilmes inicialmente formados em 24 h e a erradicação dos biofilmes crescidos por 48 h ou biofilmes pré-formados. Assim, foram consideradas efetivas as concentrações dos compostos que reduziram 50% da quantificação de UFC/mL dos biofilmes vs veículo [34,36].

O crescimento inicial das culturas obedeceu a mesma metodologia acima descrita para a atividade antimicrobiana. Entretanto, o meio de cultura e o inóculo final para a atividade antibiofilme foram preparados utilizando TYE + 1% sacarose.

A seleção dos compostos e suas concentrações testadas, foram determinadas após dados da atividade antimicrobiana: C135 (de 62,5 à 1250 µg/mL), mirecetina (de 125 à 1250 µg/mL) *tt*-farnesol (de 31,25 à 1250 µg/mL) e composto 1771 (de 1,953 à 250 µg/mL). Ainda, estudos anteriores avaliaram a atividade antibiofilme de mirecetina, *tt*-farnesol e do composto 1771 para *S. mutans* [18,19,30], entretanto neste projeto, foram avaliadas diferentes concentrações desses compostos contra as culturas simples e combinadas de *S. mutans* e *C. albicans*. Adicionalmente, os dados do composto 1771 para *C. albicans* e de C135 para os dois microrganismos foram apresentados aqui pela primeira vez.

Inicialmente, foi realizada a análise de biofilmes simples inicialmente formados (24 h). Em uma placa de 96 poços contendo 83,33 µL do meio de cultura TYE + 1% sacarose, foram adicionados 100 µL do inóculo final para cada microrganismo na concentração de 2 x 10^6 UFC/mL e as respetivas concentrações de tratamento para cada composto testado (16,67 µL). O volume final de cada era de 200 µL e a placa foi incubada contendo a concentração microbiana de 1 x 10^6 UFC/mL. Nessa placa, também foram montados poços contendo os controles para cada ocasião experimental como descrito para a atividade antimicrobiana (poços contendo apenas o meio de cultura, inóculo, veículo e veículo mais o inóculo). A placa foi incubada por 24 h (37ºC, 5% CO_2), submetida a inspeção visual e incubação orbital por 5 min (37ºC, 75 rpm) para homogeneização da cultura. Então, o meio de cultura foi transferido para uma nova placa de 96 poços para leitura da DO_{562nm}, e os biofilmes do fundo da placa inicial foram lavados 3x com 200 µL de solução de NaCl 0,89%. Após lavados, os biofilmes foram raspados com o auxílio de uma ponteira acoplada à uma pipeta por 5x com 200 µL solução de NaCl 0,89%, totalizando 1 mL da suspenção do biofilme.

Então, a suspenção do biofilme foi submetida a diluições seriadas (10^{-1} à 10^{-5}), plaqueamento de todas as diluições (incluindo a solução não diluída) e incubação por 48 h (37ºC, 5% CO_2) para quantificação de UFC/mL. Depois, a inibição de crescimento das concentrações de cada composto foi comparada ao controle de veículo.

Posteriormente, foi avaliada a atividade antibiofilme dos compostos sobre os biofilmes pré-formados (48 h), onde em uma placa de 96 poços contendo 100 µL do meio de cultura TYE + 1% sacarose, foram adicionados 100 µL do inóculo final para cada microrganismo na concentração de 2 x 10^6 UFC/mL. Todos os poços contendo o inóculo final ficaram com a concentração microbiana final de 1 x 10^6 UFC/mL, entretanto alguns poços continham apenas 200 µL do meio de cultura (que serviram de controle do experimento). A placa sem tratamentos foi incubada por 24 h (37ºC, 5% CO_2), e após a incubação os poços foram inspecionados visualmente e em seguida o meio de cultura foi descartado. Os poços contendo os biofilmes no fundo da placa foram lavados 3x com 200 µL de solução de NaCl 0,89%. Em seguida foram adicionados 183,33 µL do meio de cultura fresco (TYE + 1% sacarose) e as respetivas concentrações dos agentes. Ainda os poços que serviram de controle do experimento, continham 200 µL de TYE + 1% sacarose (controle do meio de cultura) e 183,33 µL de TYE + 1% sacarose e 16,67 de veículo (controle do veículo). A placa foi novamente incubada por 24 h (37ºC, 5% CO_2) e após incubação (quando os biofilmes tinham 48 h de crescimento), seguiu-se o mesmo protocolo utilizado para os biofilmes inicialmente formados (24 h): a placa foi submetida a inspeção visual e incubação orbital por 5 min (37ºC, 75 rpm) para homogeneização da cultura. Então, o meio de cultura foi transferido para nova placa de 96 poços para leitura da DO_{562nm}, e os biofilmes da placa inicial foram lavados (3x com 200 µL NaCl 0,89%) e raspados utilizando pipeta de 200 µL de NaCl 0,89% por 5x, totalizando 1 mL da suspenção do biofilme. A suspenção do biofilme foi sonicada (30 s, 7 w, Sonicadora QSonica, Q125), e submetida a diluição seriada (10^{-1} à 10^{-5}), plaqueamento de todas as diluições (incluindo a solução não diluída) e incubação por 48 h (37ºC, 5% CO_2) para quantificação de UFC/mL. Posteriormente a quantificação de UFC/mL para cada composto, foi comparada ao controle de veículo e as concentrações mais promissoras foram selecionadas para os testes contra os biofilmes combinados das duas espécies microbianas.

Assim, as concentrações dos compostos mais promissoras contra os biofilmes simples das duas espécies microbianas foram: C135 e *tt*-farnesol (125 µg/mL),

mirecetina (500 µg/mL) e 1771 (3.906 µg/mL). Estas concentrações foram selecionadas e utilizadas contra os biofilmes mistos de *S. mutans* e *C. albicans*. C135 foi o composto mais promissor do estudo e, portanto, foi testado na condição simples e combinado com os outros compostos contra os biofilmes mistos. Ainda, os compostos foram combinados entre si e com ou sem fluoreto de sódio (flúor à 250 ppm), totalizando 16 grupos teste. A concentração do flúor foi baseada na concentração encontrada nas soluções para bochechos disponíveis comercialmente [19,37]. Os grupos teste sem flúor foram: C135, C135+*tt*-farnesol, C135+1771, C135+mirecetina, C135+*tt*-farnesol+1771, C135+*tt*-farnesol+mirecetina, C135+*tt*-farnesol+mirecetina+1771, C135+1771+mirecetina, *tt*-farnesol+mirecetina, 1771+mirecetina, *tt*-farnesol+1771+mirecetina; e os grupos teste contendo flúor à 250 ppm foram: C135+flúor, C135+*tt*-farnesol+flúor, C135+1771+flúor, C135+mirecetina+flúor, C135+*tt*-farnesol+1771+mirecetina+flúor.

Portanto, a atividade antibiofilme dos compostos contra os biofilmes mistos de *C. albicans* e *S. mutans* foi avaliada em 24 e 48 h testando 16 combinações de tratamentos. Assim, os inóculos utilizados foram preparados com $2x10^6$ UFC/mL para *S. mutans* e $2x10^4$ UFC/mL para *C. albicans* [3]. Portanto, a cultura final do experimento, contendo o meio de cultura TYE + 1% de sacarose e o inóculo das duas espécies microbianas, ficava na concentração final de $1x10^6$ UFC/mL para *S. mutans* e $1x10^4$ UFC/mL para *C. albicans*. Posteriormente, a sequência de processamento dos biofilmes mistos, seguiu a mesma metodologia descrita para os biofilmes simples de cada espécie microbiana em 24 e 48 h.

Análise estatística

A análise estatística dos valores de UFC/mL foi realizada utilizando a estatística descritiva e inferencial. A normalidade foi avaliada pelo teste de Shapiro-Wilk, utilizando o nível de significância de 5%. Todos os dados foram não paramétricos, portanto, foram avaliados com o teste Kruskal-Wallis, seguido do pós-teste de Dunn's (Prisma 9 software, GraphPad Software, Inc. 2021). Os dados de UFC/mL foram transformados em $\log_{10}$ para verificar a log redução, e a inibição do crescimento microbiano de cada agente e as concentrações, foram comparadas ao controle de veículo.

APÊNDICE B – Supplementary Materials

Material available online at https://www.mdpi.com/article/10.3390/jof7050340/s1.

Compounds with distinct targets present diverse antimicrobial and antibiofilm efficacy against *Candida albicans* and *Streptococcus mutans*, and combinations of compounds potentiate their effect

Supplementary Information

Tables with tabulated results are presented for Figures depicting graphs in the main document.

Table S1. Antimicrobial activity of *S. mutans* using six compounds: C135, R815, AGN, *tt*-farnesol, myricetin, and 1771. Data shown in Figure 1 as CFU/mL.

C135

	1250	1000	500	250	125	62.5	31.25	15.625	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.00E+02	0.00E+00	0.00E+00	1.00E+07	1.00E+07
25% Percentile	7.50E+01	2.50E+01	0.00E+00	0.00E+00	0.00E+00	7.75E+02	0.00E+00	1.38E+07	1.83E+07	1.78E+07
Median	2.00E+07	1.50E+02	0.00E+00	0.00E+00	1.50E+02	1.15E+03	5.75E+04	1.85E+07	2.85E+07	2.75E+07
75% Percentile	5.05E+07	6.53E+03	1.00E+02	1.00E+02	2.40E+03	1.70E+03	2.75E+07	4.00E+07	3.60E+07	4.23E+07
Maximum	6.40E+07	2.00E+06	3.00E+02	3.00E+02	3.00E+03	4.00E+03	5.30E+07	6.20E+07	4.80E+07	5.80E+07
Range	6.40E+07	2.00E+06	3.00E+02	3.00E+02	3.00E+03	3.80E+03	5.30E+07	6.20E+07	3.80E+07	4.80E+07

R815

	1250	1000	500	250	125	62.5	31.25	15.625	0 (V)	S. mutans
Minimum	1.40E+06	0.00E+00	5.00E+04	6.40E+04	5.90E+04	0.00E+00	4.00E+05	1.40E+07	1.20E+07	2.60E+07
25% Percentile	3.00E+07	1.68E+05	6.25E+04	6.50E+04	6.28E+04	9.00E+04	4.88E+05	2.73E+07	1.50E+07	2.85E+07
Median	1.65E+08	1.14E+06	8.50E+04	7.20E+04	1.00E+05	2.85E+05	2.40E+06	3.20E+07	2.20E+07	3.60E+07
75% Percentile	2.23E+08	5.20E+06	1.08E+05	1.45E+05	2.59E+06	1.98E+06	1.55E+07	3.68E+07	3.78E+07	4.08E+07
Maximum	2.40E+08	1.00E+07	1.60E+05	2.00E+05	1.03E+07	8.00E+06	3.10E+07	4.40E+07	6.90E+07	6.00E+07
Range	2.39E+08	1.00E+07	1.10E+05	1.36E+05	1.02E+07	8.00E+06	3.06E+07	3.00E+07	5.70E+07	3.40E+07

AGN

	1250	1000	500	250	125	62.5	31.25	15.625	0 (V)	S. mutans
Minimum	4.00E+06	4.50E+07	3.00E+06	6.40E+06	7.00E+06	6.00E+06	1.10E+07	6.00E+06	1.70E+07	1.00E+07
25% Percentile	8.25E+06	5.35E+07	4.55E+06	1.53E+07	3.50E+07	1.53E+07	1.70E+07	1.63E+07	3.05E+07	1.70E+07
Median	8.10E+07	7.50E+07	6.15E+07	2.55E+07	3.95E+07	2.70E+07	2.40E+07	1.95E+07	4.20E+07	2.05E+07
75% Percentile	1.02E+08	9.88E+07	9.65E+07	4.03E+07	4.25E+07	2.78E+07	3.33E+07	2.73E+07	7.63E+07	2.83E+07
Maximum	1.70E+08	1.70E+08	1.21E+08	8.40E+07	5.00E+07	7.40E+07	5.10E+07	3.90E+07	8.00E+07	4.00E+07
Range	1.66E+08	1.25E+08	1.18E+08	7.76E+07	4.30E+07	6.80E+07	4.00E+07	3.30E+07	6.30E+07	3.00E+07

tt-farnesol

	1250	1000	500	250	125	62.5	31.25	15.625	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+02	1.00E+07	1.00E+07
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.75E+02	1.40E+07	1.40E+07
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.45E+03	1.80E+07	1.50E+07
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.75E+02	5.70E+06	2.40E+07	1.60E+07
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+03	7.10E+06	2.70E+07	2.00E+07
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+03	7.10E+06	1.70E+07	1.00E+07

Myricetin

	1000	500	250	125	62.5	0 (V)	*S. mutans*
Minimum	1.00E+02	0.00E+00	1.00E+04	2.90E+05	9.00E+07	6.00E+06	2.40E+08
25% Percentile	1.75E+02	1.25E+02	2.08E+04	2.10E+08	1.40E+08	3.38E+08	2.90E+08
Median	1.05E+03	1.00E+03	3.55E+04	2.85E+08	2.80E+08	4.80E+08	3.20E+08
75% Percentile	3.30E+03	1.68E+03	1.25E+05	3.63E+08	3.80E+08	5.95E+08	3.80E+08
Maximum	3.30E+03	2.70E+03	2.40E+05	4.30E+08	5.60E+08	8.10E+08	4.20E+08
Range	3.20E+03	2.70E+03	2.30E+05	4.30E+08	4.70E+08	8.04E+08	1.80E+08

Compound 1771

	250	125	62.5	31.25	15.625	7.813	3.906	1.953	0.977	0.488	0.244	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+02	8.00E+02	1.30E+04	2.00E+04	2.50E+05	1.80E+08	1.80E+08	1.60E+07	5.00E+06
25% Percentile	0.00E+00	7.50E+02	0.00E+00	7.50E+01	1.75E+02	9.50E+02	1.83E+04	2.00E+04	2.50E+05	1.88E+08	1.88E+08	2.13E+07	5.75E+06
Median	0.00E+00	1.50E+03	1.50E+03	5.50E+02	6.00E+02	1.40E+03	2.25E+04	4.00E+04	5.60E+06	2.05E+08	2.05E+08	2.05E+08	7.50E+06
75% Percentile	0.00E+00	5.00E+03	3.25E+03	1.25E+03	8.00E+02	2.20E+03	4.08E+04	5.50E+04	2.55E+08	3.75E+08	3.75E+08	3.88E+08	1.08E+07
Maximum	0.00E+00	8.00E+03	4.00E+03	2.00E+03	8.00E+02	2.20E+03	5.20E+04	7.00E+04	3.00E+08	4.50E+08	4.50E+08	4.50E+08	1.30E+07
Range	0.00E+00	8.00E+03	4.00E+03	2.00E+03	7.00E+02	1.40E+03	3.90E+04	5.00E+04	3.00E+08	2.70E+08	2.70E+08	4.34E+08	8.00E+06

Table S2. Antimicrobial activity of *C. albicans* with compounds: C135, *tt*-farnesol, myricetin, and 1771. Data shown in Figure 2 as CFU/mL.

C135

	1250	1000	500	250	125	62.5	31.25	15.625	0 (V)	*C. albicans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+04	1.20E+07
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.20E+03	5.73E+04	1.65E+07
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.00E+02	1.90E+04	1.45E+05	2.05E+07
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	5.35E+05	5.75E+04	6.33E+05	2.65E+07
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	7.10E+05	1.20E+05	1.30E+06	3.30E+07
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	7.10E+05	1.20E+05	1.29E+06	2.10E+07

***tt*-farnesol**

	1250	1000	500	250	125	62.5	31.25	15.625	7.813	0 (V)	*C. albicans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+03	8.00E+03	1.20E+04	1.30E+04	6.00E+04	1.60E+07
25% Percentile	0.00E+00	0.00E+00	0.00E+00	2.00E+02	0.00E+00	3.00E+03	1.60E+04	1.60E+04	2.18E+04	1.00E+06	2.00E+07
Median	0.00E+00	0.00E+00	0.00E+00	6.50E+02	7.00E+02	9.00E+03	1.90E+04	2.30E+04	4.10E+04	6.40E+06	2.35E+07
75% Percentile	0.00E+00	0.00E+00	1.00E+02	1.45E+03	4.63E+03	2.13E+04	2.35E+04	2.93E+04	1.33E+05	1.38E+07	2.88E+07
Maximum	0.00E+00	0.00E+00	2.00E+02	3.00E+03	1.20E+04	2.50E+04	3.00E+04	4.60E+04	5.00E+05	2.90E+07	3.30E+07
Range	0.00E+00	0.00E+00	2.00E+02	3.00E+03	1.20E+04	2.40E+04	2.20E+04	3.40E+04	4.87E+05	2.89E+07	1.70E+07

Myricetin

	1000	500	250	125	0 (V)	*C. albicans*
Minimum	2.10E+03	5.00E+03	1.00E+04	5.00E+03	7.00E+04	1.00E+07
25% Percentile	2.55E+03	1.00E+04	1.50E+04	1.60E+04	1.00E+05	1.40E+07
Median	9.00E+03	1.80E+04	2.00E+04	5.90E+04	4.20E+05	1.85E+07
75% Percentile	1.43E+04	3.00E+04	3.35E+04	2.83E+05	2.83E+06	2.20E+07
Maximum	4.50E+04	5.00E+04	4.20E+04	3.10E+05	5.00E+06	2.90E+07
Range	4.29E+04	4.50E+04	3.20E+04	3.05E+05	4.93E+06	1.90E+07

Compound 1771

	250	125	62.5	31.25	15.625	7.813	3.906	1.953	0.977	0.488	0.244	0 (V)	*C. albicans*
Minimum	7.00E+03	1.10E+05	6.00E+04	4.20E+04	3.20E+04	1.90E+04	8.00E+03	8.00E+03	3.90E+03	3.80E+03	6.00E+03	6.00E+04	1.00E+07
25% Percentile	8.00E+03	1.45E+05	1.05E+05	9.25E+04	3.28E+04	2.15E+04	1.33E+04	1.08E+04	4.48E+03	4.25E+03	1.60E+04	1.43E+05	1.35E+07
Median	1.00E+04	1.75E+05	1.60E+05	1.15E+05	4.00E+04	2.70E+04	1.80E+04	1.30E+04	1.50E+04	1.55E+04	2.20E+04	3.00E+05	1.60E+07
75% Percentile	1.38E+04	2.63E+05	2.63E+05	1.90E+05	5.13E+04	3.23E+04	2.00E+04	1.48E+04	1.73E+04	2.15E+04	2.60E+05	9.75E+05	2.08E+07
Maximum	2.70E+04	3.10E+05	4.10E+05	2.40E+05	5.80E+04	3.70E+04	2.60E+04	1.80E+04	2.40E+04	2.40E+04	5.70E+05	2.30E+06	2.50E+07
Range	2.00E+04	2.00E+05	3.50E+05	1.98E+05	2.60E+04	1.80E+04	1.80E+04	1.00E+04	2.01E+04	2.02E+04	5.64E+05	2.24E+06	1.50E+07

Table S3. Antibiofilm activity of *S. mutans* with compounds: C135, *tt*-farnesol, myricetin, and 1771 of 24h biofilm (initial biofilm formation) and pre-formed biofilms (48h). Data shown in Figure 3 as CFU/mL.

C135 (24 h)

	1250	1000	500	250	125	62.5	0 (V)	*S. mutans*
Minimum	1.00E+02	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.50E+06	1.30E+06
25% Percentile	8.00E+02	9.25E+02	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.33E+06	1.98E+06
Median	2.35E+03	4.00E+03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.00E+06	2.50E+06
75% Percentile	4.53E+03	3.83E+04	2.70E+03	0.00E+00	0.00E+00	0.00E+00	1.68E+07	4.60E+06
Maximum	1.60E+04	6.00E+04	9.00E+03	0.00E+00	0.00E+00	0.00E+00	2.40E+07	6.40E+06
Range	1.59E+04	6.00E+04	9.00E+03	0.00E+00	0.00E+00	0.00E+00	2.25E+07	5.10E+06

***tt*-farnesol (24 h)**

	1250	1000	500	250	125	62.5	31.25	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.00E+06	4.00E+05
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.00E+06	5.00E+05
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.00E+06	1.15E+06
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	7.25E+06	6.80E+06
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	8.60E+06	8.00E+06
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	6.60E+06	7.60E+06

Myricetin (24 h)

	1250	1000	500	250	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.20E+06	8.40E+05
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.63E+06	1.44E+06
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.15E+07	1.70E+06
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.43E+07	2.03E+06
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.80E+07	2.20E+06
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.58E+07	1.36E+06

Compound 1771 (24 h)

	250	125	62.5	31.25	15.625	7.813	3.906	1.953	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.00E+03	1.40E+05
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.08E+05	1.83E+05
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	5.70E+05	2.60E+05
75% Percentile	0.00E+00	0.00E+00	0.00E+00	1.50E+02	0.00E+00	0.00E+00	0.00E+00	0.00E+00	8.58E+06	3.50E+05
Maximum	0.00E+00	0.00E+00	0.00E+00	1.30E+03	0.00E+00	0.00E+00	0.00E+00	3.00E+00	2.20E+07	4.50E+05
Range	0.00E+00	0.00E+00	0.00E+00	1.30E+03	0.00E+00	0.00E+00	0.00E+00	3.00E+00	2.20E+07	3.10E+05

C135 (48 h)

	1250	1000	500	250	125	62.5	0 (V)	*S. mutans*
Minimum	7.00E+05	2.00E+05	5.50E+05	1.70E+05	0.00E+00	0.00E+00	2.70E+04	5.00E+04
25% Percentile	2.18E+06	1.25E+06	1.09E+06	4.63E+05	0.00E+00	2.00E+03	7.68E+04	9.48E+04
Median	2.85E+06	1.90E+06	2.95E+06	9.75E+05	0.00E+00	5.50E+03	1.04E+05	1.16E+05
75% Percentile	1.50E+07	3.65E+06	5.05E+06	2.13E+06	0.00E+00	2.03E+04	2.13E+06	3.65E+06
Maximum	2.60E+07	9.00E+06	6.90E+06	5.10E+06	0.00E+00	4.00E+04	3.10E+06	5.30E+06
Range	2.53E+07	8.80E+06	6.35E+06	4.93E+06	0.00E+00	4.00E+04	3.07E+06	5.25E+06

***tt*-farnesol (48 h)**

	1250	1000	500	250	125	62.5	31.25	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.30E+05	1.30E+05
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.85E+05	1.83E+05
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.00E+02	2.75E+05	3.10E+05
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	7.75E+02	1.00E+06	6.15E+05
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.10E+03	2.00E+06	2.30E+06
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.10E+03	1.87E+06	2.17E+06

Myricetin (48 h)

	1250	1000	500	250	0 (V)	*S. mutans*
Minimum	2.00E+03	1.80E+03	1.00E+03	2.70E+03	1.50E+04	1.50E+04
25% Percentile	4.83E+03	3.85E+03	1.25E+03	1.98E+04	3.00E+04	2.75E+04
Median	6.00E+03	5.05E+03	3.20E+03	3.10E+04	1.25E+05	1.85E+05
75% Percentile	9.50E+03	8.35E+03	7.00E+03	5.93E+04	1.80E+05	2.30E+05
Maximum	1.40E+04	9.50E+03	1.30E+04	7.00E+04	2.10E+05	2.60E+05
Range	1.20E+04	7.70E+03	1.20E+04	6.73E+04	1.95E+05	2.45E+05

Compound 1771 (48 h)

	250	125	62.5	31.25	15.625	7.813	3.906	1.953	0 (V)	*S. mutans*
Minimum	1.90E+03	1.10E+03	1.30E+03	1.00E+03	7.60E+03	3.20E+03	4.00E+03	8.30E+03	5.60E+04	1.30E+05
25% Percentile	5.58E+03	5.18E+03	1.75E+03	1.50E+03	1.03E+04	1.63E+04	2.10E+04	3.95E+04	8.90E+04	2.03E+05
Median	8.10E+03	1.10E+04	1.23E+04	1.25E+04	3.50E+04	2.90E+04	2.80E+04	6.30E+04	1.52E+05	2.70E+05
75% Percentile	1.38E+04	1.78E+04	2.38E+04	3.00E+04	5.18E+04	5.58E+04	5.00E+04	7.68E+04	2.35E+05	3.68E+05
Maximum	2.70E+04	3.40E+04	3.90E+04	6.00E+04	7.60E+04	1.06E+05	8.00E+04	9.80E+04	3.30E+05	4.80E+05
Range	2.51E+04	3.29E+04	3.77E+04	5.90E+04	6.84E+04	1.03E+05	7.60E+04	8.97E+04	2.74E+05	3.50E+05

Table S4. Antibiofilm activity of *C. albicans* with compounds: C135, *tt*-farnesol, myricetin, and 1771 of 24h biofilm (initial biofilm formation) and pre-formed biofilms (48h). Data shown in Figure 4 as CFU/mL.

C135 (24 h)

	1250	1000	500	250	125	62.5	31.25	15.625	0 (V)	*C. albicans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+04	7.00E+04
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.58E+04	2.40E+05
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.05E+05	2.90E+05
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.58E+05	4.85E+05
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.30E+05	7.40E+05
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.20E+05	6.70E+05

***tt*-farnesol (24 h)**

	1250	1000	500	250	125	62.5	31.25	0 (V)	*C. albicans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+05	2.40E+05
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.03E+05	2.65E+05
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.25E+05	3.20E+05
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+02	1.40E+05	4.28E+05
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+02	1.80E+05	6.70E+05
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+02	8.00E+04	4.30E+05

Myricetin (24 h)

	1250	1000	500	250	125	0 (V)	*C. albicans*
Minimum	3.30E+03	1.20E+03	1.00E+02	0.00E+00	0.00E+00	1.50E+03	3.70E+04
25% Percentile	4.60E+03	2.25E+03	5.25E+02	2.50E+02	2.50E+02	4.00E+04	1.18E+05
Median	1.50E+04	4.10E+03	1.10E+03	8.50E+02	8.00E+02	1.90E+05	4.00E+05
75% Percentile	4.60E+04	1.85E+04	2.53E+03	2.33E+03	1.45E+03	2.75E+05	1.03E+06
Maximum	1.40E+05	4.40E+04	6.40E+03	4.10E+03	3.80E+03	3.80E+05	1.20E+06
Range	1.37E+05	4.28E+04	6.30E+03	4.10E+03	3.80E+03	3.79E+05	1.16E+06

Compound 1771 (24 h)

	250	125	62.5	31.25	15.625	7.813	3.906	1.953	0 (V)	*C. albicans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	9.00E+03	1.00E+04
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.50E+02	1.33E+04	2.65E+05
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.00E+02	0.00E+00	1.00E+02	8.50E+02	6.05E+04	3.85E+05
75% Percentile	0.00E+00	1.33E+04	0.00E+00	1.65E+03	7.00E+02	1.25E+03	3.70E+03	2.58E+03	3.98E+05	5.08E+05
Maximum	1.00E+02	1.90E+04	4.00E+02	4.00E+03	1.70E+03	2.60E+03	7.00E+03	4.60E+03	5.20E+05	5.60E+05
Range	1.00E+02	1.90E+04	4.00E+02	4.00E+03	1.70E+03	2.60E+03	7.00E+03	4.60E+03	5.11E+05	5.50E+05

C135 (48 h)

	1250	1000	500	250	125	62.5	31.25	15.625	0 (V)	*C. albicans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	5.00E+02	2.20E+06	2.10E+06	3.30E+06
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.90E+04	2.35E+06	2.68E+06	3.70E+06
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.30E+06	2.55E+06	3.10E+06	4.10E+06
75% Percentile	0.00E+00	1.00E+02	0.00E+00	2.50E+01	0.00E+00	3.38E+03	2.00E+06	2.85E+06	3.35E+06	4.75E+06
Maximum	0.00E+00	2.00E+02	0.00E+00	8.00E+02	0.00E+00	3.10E+04	2.40E+06	3.30E+06	4.70E+06	5.80E+06
Range	0.00E+00	2.00E+02	0.00E+00	8.00E+02	0.00E+00	3.10E+04	2.40E+06	1.10E+06	2.60E+06	2.50E+06

***tt*-farnesol (48 h)**

	1250	1000	500	250	125	62.5	31.25	0 (V)	*C. albicans*
Minimum	0.00E+00	0.00E+00	1.20E+03	1.00E+02	0.00E+00	1.20E+05	1.00E+06	1.80E+06	2.90E+06
25% Percentile	1.05E+04	6.00E+03	3.93E+03	4.40E+03	2.00E+05	3.33E+05	1.58E+06	3.18E+06	3.50E+06
Median	3.65E+05	2.00E+04	3.00E+04	1.45E+04	3.00E+05	1.15E+06	2.05E+06	3.70E+06	4.00E+06
75% Percentile	1.73E+06	2.95E+05	1.20E+05	2.60E+04	1.10E+06	1.95E+06	3.18E+06	4.45E+06	4.85E+06
Maximum	2.90E+06	8.00E+05	2.90E+05	4.20E+04	1.80E+06	2.50E+06	3.90E+06	6.00E+06	5.60E+06
Range	2.90E+06	8.00E+05	2.89E+05	4.19E+04	1.80E+06	2.38E+06	2.90E+06	4.20E+06	2.70E+06

Myricetin (48 h)

	1250	1000	500	250	125	0 (V)	*C. albicans*
Minimum	1.90E+06	2.20E+06	2.40E+06	2.20E+06	2.30E+06	1.60E+06	2.30E+06
25% Percentile	2.63E+06	3.03E+06	2.93E+06	2.85E+06	2.75E+06	2.18E+06	3.40E+06
Median	2.90E+06	3.35E+06	3.35E+06	3.10E+06	3.10E+06	2.60E+06	3.50E+06
75% Percentile	3.08E+06	3.93E+06	4.20E+06	3.58E+06	3.28E+06	2.78E+06	3.95E+06
Maximum	4.60E+06	4.60E+06	4.90E+06	4.20E+06	3.40E+06	3.50E+06	4.10E+06
Range	2.70E+06	2.40E+06	2.50E+06	2.00E+06	1.10E+06	1.90E+06	1.80E+06

Compound 1771 (48 h)

	250	125	62.5	31.25	15.625	7.813	3.906	1.953	0 (V)	*C. albicans*
Minimum	0.00E+00	1.20E+06	9.00E+03	3.20E+04	1.10E+05	1.90E+06	1.50E+06	1.70E+05	1.10E+06	2.70E+06
25% Percentile	0.00E+00	2.00E+06	4.25E+05	5.40E+04	1.55E+06	2.00E+06	2.45E+06	8.35E+05	1.73E+06	3.48E+06
Median	3.20E+04	2.30E+06	1.60E+06	1.50E+06	2.70E+06	2.35E+06	3.40E+06	3.15E+06	2.15E+06	4.50E+06
75% Percentile	5.73E+05	2.68E+06	2.13E+06	2.48E+06	4.00E+06	2.88E+06	3.73E+06	4.43E+06	3.38E+06	5.83E+06
Maximum	1.40E+06	3.20E+06	2.90E+06	3.10E+06	5.00E+06	3.60E+06	5.30E+06	5.70E+06	5.10E+06	7.40E+06
Range	1.40E+06	2.00E+06	2.89E+06	3.07E+06	4.89E+06	1.70E+06	3.80E+06	5.53E+06	4.00E+06	4.70E+06

Table S5. Antibiofilm activity of dual-species *S. mutans* and *C. albicans* biofilms with combined compounds (with and without sodium fluoride): C135 (C), *tt*-farnesol (Far), myricetin (Myr), 1771, and sodium fluoride (F). Data shown in Figure 5 as CFU/mL.

***S. mutans* (24 h)**

	C	C+Far	C+1771	C+Mir	C+Far+1771	C+Far+Mir	C+Far+1771+Mir	C+1771+Mir	Far+1771	1771+Mir	Far+1771+Mir	C+F	C+Far+F	C+1771+F	C+Mir+F	C+Far+1771+Mir+F	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.40E+05	3.40E+06
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	4.43E+06	4.00E+06
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	5.75E+06	4.90E+06
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.16E+07	5.53E+06
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.40E+07	5.90E+06
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.37E+07	2.50E+06

***S. mutans* (48 h)**

	C	C+Far	C+1771	C+Mir	C+Far+1771	C+Far+Mir	C+Far+1771+Mir	C+1771+Mir	Far+1771	1771+Mir	Far+1771+Mir	C+F	C+Far+F	C+1771+F	C+Mir+F	C+Far+1771+Mir+F	0 (V)	*S. mutans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+05	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.20E+06	1.00E+07
25% Percentile	1.88E+03	0.00E+00	1.33E+03	1.25E+02	0.00E+00	0.00E+00	0.00E+00	7.50E+01	4.75E+03	4.75E+05	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.33E+06	1.60E+07
Median	7.95E+03	0.00E+00	2.15E+03	2.50E+02	0.00E+00	0.00E+00	0.00E+00	2.50E+03	2.10E+04	1.90E+06	2.00E+04	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.85E+06	1.95E+07
75% Percentile	2.25E+04	1.50E+02	6.78E+03	1.28E+04	7.50E+01	0.00E+00	0.00E+00	1.03E+04	9.00E+04	9.13E+06	4.00E+04	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+07	2.20E+07
Maximum	5.20E+04	2.00E+02	1.30E+04	2.30E+04	2.00E+02	0.00E+00	0.00E+00	2.00E+04	1.30E+05	1.10E+07	1.00E+05	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.70E+07	3.60E+07
Range	5.20E+04	2.00E+02	1.30E+04	2.30E+04	2.00E+02	0.00E+00	0.00E+00	2.00E+04	1.30E+05	1.09E+07	1.00E+05	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.48E+07	2.60E+07

***C. albicans* (24 h)**

	C	C+Far	C+1771	C+Mir	C+Far+1771	C+Far+Mir	C+Far+1771+Mir	C+1771+Mir	Far+1771	1771+Mir	Far+1771+Mir	C+F	C+Far+F	C+1771+F	C+Mir+F	C+Far+1771+Mir+F	0 (V)	*C. albicans*
Minimum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.20E+04	5.00E+05
25% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.85E+05	1.83E+06
Median	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.90E+05	2.05E+06
75% Percentile	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	3.95E+05	2.50E+06
Maximum	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.20E+06	3.40E+06
Range	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.18E+06	2.90E+06

APÊNDICE C – MATERIAIS E MÉTODOS (Artigo 2)

Compostos e suas combinações (formulações testadas)

Previamente o nosso grupo de pesquisa selecionou e combinou compostos contra biofilmes simples e mistos de *C. albicans* e S. *mutans* crescidos em placa de 96 poços (Lobo et al., 2021). Portanto, as combinações mais promissoras dos compostos na publicação 1, foram testadas no modelo de biofilmes formados sobre discos de hidroxiapatita (HA) com película salivar). Os compostos testados também foram diluidos em 84,15% EtOH e 15% de DMSO, e as soluções estoque ficaram à 15 mg/mL, exceto para o composto 1771 (2 mg/mL). Os compostos foram testados nas concentrações: 125 µg/mL - C135 e *tt*-farnesol (Far); 500 µg/mL – mirecetina; 3.906 µg/mL composto 1771 (1771) (Lobo et al., 2021) e fluoreto de sódio (F) (250 ppm) (Zero et al., 2006). Assim, combinações inicialmente testadas foram: C135, C135+Far, C135+1771, C135+mirecetina, C135+Far+mirecetina, C135+F, C135+Far+F, C135+mirecetina+F, C135+Far+1771+mirecetina+F. Em seguida, foram testadas as combinações: C135, C135+Far, C135+mirecetina, C135+F, C135+Far+F, C135+mirecetina+F. Ainda, foram realizados outros testes utilizando combinações com as concentrações do composto C135 à 125 µg/mL e as mesmas combinações com o composto C135 à 250 µg/mL: C135, C135+Far, C135+F, C135+Far+F e Far. Considerando os dados obtidos na publicação 1 e após todas as avaliações anteriores, os grupos teste para a analisar os biofilmes formados sobre HA com película foram: 1. C135, 2. C135+Far, 3. C135+F, 4. C135+Far+F, 5. Far, 6. F, e 7. veículo (ou grupo controle: solução utilizada para diluir os compostos e suas concentrações de trabalho: 7% EtOH + 1.25% DMSO). Ainda, para as análies de citotoxicidade, foi utilizada a solução de digluconato de clorexidina à 0.12%.

Cultivo das cepas e formação do biofilme

As cepas *S. mutans* UA159 (serotipo c; ATCC 700610) e *C. albicans* SC5314 armazenados em freezer -80ºC foram reativadas em placas de ágar sangue (5% sangue de cordeiro – Laborclin, Pinhais, PR, Brasil) e incubadas por 48 h (37°C, 5% de CO_2; Steri-Cult™ Thermo Scientific, Waltham, MA, USA). Então, 5 colônias de cada espécie microbiana foram transferidas para tubos individuais contendo 10 mL de meio líquido [9,5 mL (2,5% triptona e 1,5% extrato de levedura ou TYE, pH 7.0; Becton, Dickinson and Company - BD, Sparks, MD, USA) suplementado com 0,5 mL (20% glicose v/v): TYE + 1% glicose]. Os tubos foram incubados por 16 h (37°C, 5% de

CO_2), e em seguida diluídos (1:20) para o crescimento do inóculo (500 μL de cada pré-inóculo + 9,5 mL do mesmo meio líquido TYE com 1% de glicose). O inóculo foi incubado (37°C, 5% de CO_2) e cada cultura foi utilizada na sua fase de crescimento *mid-log*: *S. mutans* UA159 DO_{562nm} 0,500 (±0,100) e *C. albicans* SC5314 DO_{562nm} 0,482 (±0,058) (leitor de ELISA; Biochrom Ez, Cambourne, UK). A cultura final para a formação do biofilme contendo as duas espécies microbianas, foi preparada utilizando o meio TYE + 1% de sacarose e o inóculo final à $2x10^6$ unidades formadoras de colônias por mililitro (UFC/mL) para *S. mutans* e $2x10^4$ CFU/mL para *C. albicans* (Falsetta et al., 2014).

Os biofilmes foram formados sobre discos de hidroxiapatita com área de superfície de 2,7 ± 0,2 cm^2 (Clarkson Chromatography Products, Inc. South Williamsport, PA). Aparatos metálicos foram utilizados para o posicionamento vertical dos discos de HA nas placas de 24 poços (KASVI, China). Previamente a formação dos biofilmes, foi formada uma película salivar sobre os discos. Para tal, a saliva foi doada por três doadores saudáveis (aprovado pelo Comitê de Ética Institucional CAAE: 08189119.7.0000.5416), esterilizada por filtração (unidade de filtração de 500 mL, 0.2 µm aPES membrana, 75mm dia, Mexico), e preparada seguindo o protocolo prévio (Lemos et al., 2010). Para a formação da película salivar, os discos foram hidratados com água MiliQ por 20 min e posteriormente incubados na saliva (1h, 37ºC, 75 rpm; Quimis, São Paulo, BR). Após incubação e formação da película salivar, os discos foram lavados em solução tampão de absorção (AB) [(50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, 1 mM$MgCl_2$, 0.1 mM PMSF (fluoreto de fenilmetilsulfonil – Sigma), diluido em H_2O, pH 6.5)] para a remoção do excesso de saliva e então os discos foram tratados (como está descrito em regime de tratamento) e transferidos para a placa contendo o inóculo final para a formação do biofilme. Ainda, os biofilmes foram tratados em 6, 21 e 29 h.

Em 19 e 27 h do experimento, os biofilmes, foram transferidos para nova placa de 24 poços, contendo meio de cultura fresco (TYE + 1% de sacarose) e incubados novamente. Após 2 h de incubação (21 e 29 h) os biofilmes foram tratados como será descrito abaixo. O meio de cultura trocado (19, 27, 28 e 43 h) foi utilizado para aferir o pH. Os biofilmes foram crescidos até as 28 h para a análise de expressão gênica e 43 h para a população microbiana, biomassa, bioquímicas [componentes da matriz-exopolissacarídeos, proteínas e DNA extracelular (eDNA)] e organização estrutural.

Regime de tratamento (aplicação tópica)

As formulações foram aplicadas em 0, 6, 21 e 29 h. Inicialmente as aplicações foram aplicadas em regime de gotejamento por 1:30 min, entretanto o efeito observado não condizia com os dados do estudo prévio. Assim, foram realizados testes tratando os biofilmes pelo regime de submersão, onde os discos de HA ficavam totalmente submersos às formulações. Este regime de aplicação foi testado por 1:30 e 5:00 min. Então, verificou-se que o melhor efeito das aplicações e combinações das formulações contra biofilmes tratados por submersão foi observado após 5:00 min de exposição à formulação. Portanto, para a aplicação tópica, 2 mL de cada formulação ou (controle), para 5:00 foi preparada nas placas de 24 poços. Assim, os discos (com película formada em 0 h, e com biofilmes de 6, 21 e 29 h foram lavados por submersão com tampão AB (para a remoção do excesso de saliva após a formação da película) ou solução de 0,89% NaCl (durante as trocas de meio para o crescimento do biofilme) do meio de cultura (em 6, 21 e 29 h), transferidos para a placa contendo o inóculo do experimento, seguida de incubação (37ºC, 5% CO_2).

Expressão gênica

A metodologia RT-qPCR (*reverse transcription-quantitative polymerase chain reaction*) incluiu o isolamento de RNA, síntese de cDNA e qPCR, e foi utilizada para avaliar o perfil de expressão dos genes de *S. mutans* (*atpD*, *gtfB*, *nox1*, e *16S rRNA* - normalizador) e *C. albicans* (BGL2, CDR1, ERG11, PHR1, PHR2, FKS1, SOD1, XOG1, RPP2B-normalizador.

Os biofilmes foram processados em 28h. Foram utilizados 4 discos de HA para cada formulação, em três ocasiões experimentais distintas. Assim, após 28 h de incubação os biofilmes foram lavados por submersão em placa contendo 0,89% de NaCl. Em seguida, cada disco foi transferido para tubo individual, contendo 1 mL de RNAlater (Ambion – Molecular Probes). Os tubos passaram pelo banho ultrasônico (Bransonic 3800, Emerson, Mexico) por 10 min e em seguida foram raspados utilizando uma espátula metálica esterilizada para remoção do biofilme remanescente aderido sobre os discos de HA). Então, as suspensões dos biofilmes tratados com a mesma formulação foram agrupadas, e os respectivos tubos de vidro foram novamente lavados com 1 mL de RNAlater e esse volume foi adicionado ao tubo Falcon inicial (totalizando 4 mL da suspensão de biofilme). As suspensões foram concervadas em freezer -80ºC até serem utilizadas.

Isolamento de RNA

O RNA foi isolado conforme a metodologia optimizada para biofilmes (Cury & Koo, 2007) com algumas modificações (Lobo et al., 2019). Primeiro, o RNA foi extraído utilizando o método de separação fenol-clorofórmio, e purificado via tratamento com DNAse em coluna (RNeasy Micro Qiagen, Germany) e em solução (TURBO DNase, Ambion). A DNAse foi removida usando o RNeasy MinElute clean-up kit (Qiagen) e a integridade do RNA purificado foi determinada por eletroforese de gel de agarose (1%) (Ultra-Pure ™ Invitrogen). Então, a quantidade (DO 260nm) e a pureza (razão DO 260/280) do RNA total foram avaliadas por espectrofotometria (Nano-spectrophotometer DS-11+, Denovix).

Síntese de cDNA

O cDNA foi sintetizado utilizando o iScript kit (Cat.#1708891; BioRad). Assim, para preparar as amostras de +RT+, 1 *μ*g de RNA total (correspondente a 5 *μ*g de RNA a 200 ηg/μL) foi misturado com 4 *μ*L do tampão 5X iScript, 1 *μ*L da enzima transcriptase reversa, e água de grade molecular *q.s.p.* para completar 20 *μ*L (10 μL; *i.e.*, amostras +RT, ou seja, com a enzima transcriptase reversa). A mesma reação, sem utilizar a enzima transcriptase reversa, foi utilizada para as amostras de -RT (grupo controle) (Lobo et al., 2019). As reações foram incubadas (25ºC / 5 min, 42ºC / 30 min, 85ºC / 5 min, finalizado com 4ºC; CFX96 thermocycler- BioRad) e armazenadas em -20ºC até o uso para quantificação da expressão gênica (qPCR).

Quantificação da expressão gênica via RT-qPCR

As amostras de cDNA foram diluídas (+RT à 1:5 para todos os genes, com exceção do 16SrRNA, que foi diluído à 1:1000; e -RT que foi utilizado sem diluição para garantir a ausência da contaminação do DNA), e amplificadas com *primers* específicos seguindo protocolos padronizados (Klein et al., 2012). Para as reações do qPCR, 5 *μ*L de cDNA foram amplificados com 0,5 *μ*L dos *primers* específicos (10 *μ*M, *forward* e *reverse* – Tabela 1) e 12,5 *μ*L de 2X SYBR Green Supermix (BioRad). As reações seguiram a concentração ótima e temperatura de cada primer utilizando o CFX96 (BioRad) [passo 1 (1X) 95ºC / 3 min; passo 2 (35X) 95ºC / 15 seg, 58ºC / 30 seg, 68ºC / 15 seg, onde ocorrerão a coleta de dados e as análises quantitativas; passo 3 (1X) 95ºC / 1 min; passo 4 (1X) 55ºC / 1 min; passo 4 (80X) 55ºC / 1 min, para

análise da *melting curve*e] e o equipamento StepOnePlus Real-time PCR System (Applied Biosystems). Curvas padrão baseadas no produto de PCR foram utilizadas para cada *primer* como descrito anteriormente (Yin et al., 2001), respeitando $R^2 \cong 1$, a eficiência da reação entre 90-100% e o slope de -3,3. A expressão relativa dos genes de *S. mutans* foi calculada através da normalização de cada gene de interesse pelo gene 16S rRNA de *S. mutans* (Klein et al., 2012), enquanto que para *C. albicans* o gene RPP2B (constituinte da estrutura ribossomal) foi selecionado (Nailis 2006). Entretanto, para o presente desenho experimental este gene não funcionou como normalizador, como foi observado anteriormente (Lobo et al., 2019).

As amostras do biofilme foram obtidas pelo pool de 4 replicatas por tratamento em três experimentos independentes (o isolamento do RNA, a junção das soluções do biofilme resultante em duas amostras de cDNA, cada uma em duplicata, n=3).

Processamento do biofilme e análises para determinação da viabilidade celular, biomassa, proteínas e composição da matriz extracelular

Em 43h de desenvolvimento, os biofilmes foram processados para quantificar a viabilidade das células bacterianas e fúngicas, biomassa (peso seco insolúvel), proteínas (das porções solúvel e insolúvel do biofilme) exopolissacarídeos da matriz [polissacarídeos solúveis em água (WSP) eplissacarídeos solúveis em álcali (ASP); Dubois et al., 1956] (Lemos et al., 2010; Klein et al., 2011) e eDNA (Castillo Pedraza et al., 2017).

Para tanto, cada disco contendo os biofilmes foi lavado por submersão 3 vezes com NaCl 0,89% (em placa de 24 poços) e transferidos para tubos de vidro contendo 2 mL de NaCl 0,89%. Os tubos foram sonicados por 10 min (Bransonic 3800, Emerson - Mexico) e raspados utilizando uma espátula metálica esterilizada (para remoção de qualquer resquício de biofilme que continuou aderido ao disco de HA). Cada suspenção de biofilme foi transferida para um tubo Falcon de 15 mL, e os tubos de vidro foram lavados novamente com 3 mL NaCl 0,89% e a suspensão resultante foi adicionada ao tubo Falcon inicial, totalizando 5 mL da suspensão de biofilme. essa suspenção de biofilme foi sonicada (30 s, 7W; Sonicator QSonica, Q125, Newtown CT, USA). Em seguida, 0,1 mL foram separados para diluição seriada e plaqueamento (semeadura em placa de ágar sangue ou placa de soro fetal bovino (BHI), seguida de incubação por 48 h à 37°C e 5% de CO_2), para contagem de UFC. O restante do volume (4,9 mL) foi homogeneizado e centrifugado (4000 rpm / 10 min / 4ºC;

Eppendorf, Centrifuge 5810R). O sobrenadante foi transferido para novo tubo Falcon de 50 mL e o *pellet* (precipitado celular com os componentes insolúveis da MEC) foi lavado com 2,6 mL de água MilliQ esterilizada, e novamente homogeneizado e centrifugado (4000 rpm / 10 min / 4ºC). O sobrenadante resultante, foi adicionado ao tubo Falcon de 50 mL e o *pellet* foi lavado mais uma vez (2,5 mL de água MilliQ), homogeneizado e centrifugado (4000 rpm / 10 min / 4ºC). O sobrenadante resultante, foi adicionado ao tubo Falcon de 50 mL inicial, totalizando 10 mL da suspensão do biofilme.

Dos 10 mL da suspensão do biofilme: 0,5 mL foram utilizados para análise de eDNA, 0,5 mL para as proteínas da porção solúvel (método Bradford) e 6 mL foram precipitados com três volumes de etanol 99% (18 h, −20°C) para a análise de WSP (Dubois et al., 1956).

O *pellet* foi ressuspenso com 1 mL de água MilliQ esterilizada, dos quais 0,05 mL foram utilizados para quantificar as proteínas totais (porção insolúvel) (método Bradford) e a suspensão remanescente (0,995 mL) foi transferida para microtubos pré-pesados. Os microtubos foram homogeneizados e centrifugados (12000 rpm / 10 min / 4ºC; Eppendorf, Centrifuge 5430R), o sobrenadante foi descartado e o *pellet* foi secado por 270 minutos (Speed Vac Concentrator RVC 2–18 CD Plus, Christ). Após a secagem, os microtubos foram novamente pesados e o cálculo da diferença entre o peso final e o inicial foi considerado como biomassa do biofilme (peso seco). O mesmo *pellet* foi utilizado para a extração do ASP. Para tal, 1 M de NaOH (0,3 mL à 1 M NaOH por 1 mg do peso seco). A solução extraida foi precipitada com etanol, seguida da quantificação dos carbohidratos (Dubois et al., 1956), como foi detalhado acíma (Lemos et al., 2010).

Todos os testes foram realizados em 3 ocasiões experimentais independentes, onde cada grupo teve e discos por formulação de tratamento (n = 6).

Microscopia de fluorescência (confocal)

Nesta fase foram analisados biofilmes com 43 h. Todos os procedimentos para a formação dos biofilmes, seguiu o que foi descrito acima, com exceção da adição de 13,4 µL do fluoróforo Alexa Flúor 647 (emissão de fluorescência máxima de 647/668 nm; Molecular Probes, Carlsbad, CA, EUA) para a marcação da matriz extracelular do biofilme. O Alexa flúor foi adicionado desde o início da formação do biofilme (0 h) e após cada troca de meio de cultura para meio fresco (19 e 27 h); e no final do

experimento, em 43 h, os biofilmes foram lavados por submersão com 0,89% de NaCl em placa de 24 poços, e incubados (30 min) em nova placa de 24 poços contento 0,89 % NaCl + 1.5 µL do fluoróforo SYTO9 (485/498 nm; Molecular Probes) para a marcação das células microbianas. Após a incubação com SYTO 9, os biofilmes foram lavados em nova placa contendo 0,89% de NaCl e então estavam prontos para serem analisados.

As imagens foram adquiridas com um microscópio de fluorescência confocal (Carls Zeiss LSM 800 with Airyscan with GaAsp detector, Germany), com a objetiva ECde Plan-Neofluar 20x/0.50 Oil DIC M27, comprimentos de onda de 488 nm: 2.10% e 561 nm: 1.81%), configuração de 1024x1024 em incrementos de 1,5 μm. As imagens foram posteriormente analisadas utilizando o software ZEN Blue 2.3 (reconstrução 3D) e COMSTAT2 (quantificação dos componentes do biofilme: biomassa, espessura máxima e porcentagem de cobertura).

Foram realizadas três ocasiões experimentais, onde foram utilizados 2 discos por formulação de tratamento e foram obtidas três imagens representativas de cada disco (n = 6). Os arquivos dos dados foram utilizados para as análises quantitativas as imagens de cada grupo de tratamento foram selecionadas e ilustradas para análise descritiva e qualitativa.

Microscopia eletrônica de varredura (MEV)

Os biofilms tratados e crescidos até 43 h foram processados preparados para a análise por microscopia eletrônica de varredura. Para tanto com 43 h os discos foram lavados com 0,89% NaCl e posteriormente fixados com solução de glutaraldeido à 2,5% (por 1 h a temperatura ambiente). Em seguida, as amostras foram lavadas 3x com 0,89% NaCl e desidratadas com soluções crescentes de etanol [70% (1x / 1h de incubação), 90% (1x / 1h de incubação) e 99% (5x / 30 min de incubação)]. Em seguida, os discos foram passados para uma placa nova vazia posicionados para secagem no dissecador de sílica à vácuo por 7 dias. Posteriormente, os biofilmes foram metalizados com ouro (Denton Vaccum, Desc V) e as analyses foram adquiridas no microscopio eletrônico de varredura (JEOL JSM-6610LV) utilizando as ampliações em 1,000 e 5,000X.

Each treatment group (formulation) was represented by 2 discs and at least three images of each amplification were acquired per disc.

Atividade citotóxica

Células e Cultura celular

Cultura de células de queratinócitos da linhagem NOK-si (Castilho et al., 2010) foram cultivadas em meio Dulbecco´s Modified Eagle´s Medium (DMEM, GIBCO, Grand Island, NY, EUA) com 2,0 mmol/L de glutamina; contendo 10% de soro fetal bovino (SFB, GIBCO, Grand Island, NY, EUA), penicilina G (10,000 µg/mL), estreptomicina (10,000 µg/mL) e anfotericina (25 µg/mL) (Invitrogen) em incubadora (CO_2 / 5% / 37ºC) (Midi 40™Thermo Scientific, EUA). As células foram cultivadas até atingirem a confluência (90%); então foram lavadas com tampão fosfato PBS 1X pH 7.2 (NaCl 140 mmol/L, KCl 3,0 mmol/L, Na_2HPO_4 4,30 mmol/L, KH_2PO_4 1,40 mmol/L), removidas com solução de tripsina (0,05%) / EDTA (0,53 mmol/L) (Invitrogen) e centrifugadas (400 xg / 5 min). As células foram ressuspendidas em meio de cultura fresco (DMEM) e replaqueadas. O meio de cultura foi trocado a cada dois e os experimentos foram realizados quando as células estavam entre a 3ª e 4ª passagem. Portanto, as células foram contadas utilizando a câmara de Neubauer e plaqueadas em placa de 96 poços (20,000 mil células/poço).

Ensaio de viabilidade celular – MTT

Todas as formulações utilizadas contra os biofilmes foram testadas para a citotoxicidade pelo ensaio colorimétrico de viabilidade celular MTT [3-(4,5-dimetiltiazol-2-yl)2,5-difeniltetrazolio brometo] (Sigma). O ensaio de MTT consiste na detecção da viabilidade celular, de acordo com a capacidade de conversão do sal tetrazolio MTT em cristais de formazan, formados pelas células viva que determinam a atividade mitocondrial. Assim, ocorre a formação de um produto de coloração roxa que pode ser medido em espectrofotômetro. O princípio desse método consiste no fato de que as células viáveis possuem mitocôndrias competentes, que promovem a respiração celular e liberam no meio espécies redutoras provenientes da cadeia respiratória celular, o que promove a redução do sal MTT amarelo no produto corado, roxo.

Assim, uma cultura de células em monocamada foi incubada (37ºC / 5% CO_2) por 30 ou 60 min com 200 µL dos tratamentos (C135; C135+Far; C135+F; C135+Far+F; Far; F; e digluconato de clorexidina à 0,12%) ou controles [o veículo dos tratamentos (EtOH + DMSO); o meio de cultura celular (DMEM) e o triton X-100 (controle de morte celular)]. Após a incubação, os tratamentos ou controles foram

descartados e 100 µL da solução de MTT (3.0 mg/mL) diluida no RPMI sem fenol foram adicionados aos poços da placa e incubados por 4 h (37ºC / 5% CO_2). Em seguida, a solução de MTT foi substituida por 100 µL de 2-propanol para a solubilização dos cristais formados pela reação do MTT. A placa contendo 2-propanol foi sonicada por 15 min (Limp Sonic, modelo LS-0,8D-3/X) e seguiu-se a leitura das medidas espectrofotométricas utilizando comprimento de onda de 562 nm (Reader 400 EZ – Biochrom).

Análise estatística

Os dados quantitativos foram analisados utilizando estatística descritiva e inferencial. A normalidade foi avaliada pelo teste de Shapiro-Wilk, utilizando o nível de significância de 5% e utilizou-se o teste de Brown-Forsythe para a homogeneidade das variâncias. Os testes de ANOVA a um fator ou mista foram aplicados, seguidos de pós-teste de Tukey (Prism 9 software, GraphPad Software, Inc. 2021). Adicionalmente, foram realizadas análises qualitativas e descritivas para as imagens de confocal e da microscopia eletrônica de varredura.

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

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