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CAROLINA SCHÄFFER MORSCH

EFFECT OF ANTIBIOFILM COMPOUNDS ON CELL VIABILITY AND BIOFILM FORMATION SINGLE AND MULTI-SPECIES ON HYDROXYAPATITE AND PEEK DISCS

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2019

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Tese submetida ao Programa de Pós-Graduação em Odontologia da Universidade Federal de Santa Catarina para a obtenção do título de Doutora em Odontologia.
Orientador: Prof. Dr. César Augusto Magalhães Befatti
Co-orientador: Prof. Dr. Wim Teughels

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

Prof. Dr. Ricardo de Souza Magini
Universidade Federal de Santa Catarina

Prof. Dr. Felipe Daltoé
Universidade Federal de Santa Catarina

Prof. Dr. Wim Teughels
KU Leuven

Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de doutor em Odontologia, área de concentração Implantodontia.

Elena Riet Correa Rivero:69108390053
Assinado de forma digital por Elena Riet Correa Rivero:69108390053
Dados: 2019.10.10 17:28:56 -03'00'

Prof. Dra. Elena Riet Correa Rivero
Coordenadora do Programa de Pós-Graduação

Cesar Augusto Magalhaes Benfatti:27819733861
Assinado de forma digital por Cesar Augusto Magalhaes Benfatti:27819733861
Dados: 2019.10.10 13:55:17 -03'00'

Prof. Dr. César Augusto Magalhães Benfatti
Orientador

Florianópolis, 2019.

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RESUMO

Objetivo: Avaliar a citotoxicidade da furanona C-30 e das lactamas U11, U12 e U26 e seu efeito inibitório na formação de biofilme oral mono e multi-espécies.

Materiais e métodos: O efeito da furanona C-30 e lactamas na viabilidade celular de queratinócitos orais humanos (HOK-18A), células da polpa dental humana (HDPC's) e fibroblastos L929 foi avaliado por XTT e MTS. O efeito da furanona C-30 na formação de biofilme mono-espécie foi analisado por cristal violeta, enquanto o efeito da furanona C-30 e das lactamas solubilizadas em meio líquido ou incorporadas em discos de sPEEK na formação do biofilme multi-espécies foi avaliado por PCR quantitativo. Análise estatística foi realizada por comparações entre as condições de crescimento e o grupo controle pelo teste de hipóteses múltiplas (Sidak).

Resultados: 100% de viabilidade de HOK-18A foi observada quando expostas a concentrações de 0.5 a 10 µg/mL de furanona C-30, 90% de HDPC's a 10 µg/mL em contraste à concentração de 128 µg/mL que apresentou um efeito citotóxico. Somente o grupo sP3LF128 após 7 dias de exposição demonstrou um efeito citotóxico em fibroblastos L929 resultando em 55% de células viáveis. O crescimento bacteriano planctônico e a formação do biofilme pelos periodontopatógenos *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* e *Porphyromonas gingivalis* foram inibidos em >20% e >50%, respectivamente pela furanona nas concentrações de 8 a 128 µg/mL nos experimentos mono-espécie. Nos experimentos multi-espécie a furanona C-30 solubilizada em meio líquido nas concentrações de 10 e 128 µg/mL alterou a composição do biofilme em comparação ao controle reduzindo a concentração bacteriana de *A. actinomycetemcomitans* e *P. intermedia e de P. gingivalis* na concentração de 128 µg/mL. A associação da furanona C-30 com lactamas na concentração de 128 µg/mL inibiu significativamente a formação do biofilme de *F. nucleatum, P. gingivalis* e *P. intermedia* alterando a composição da comunidade multi-espécies para uma condição mais benéfica e menos patogênica. Quando os compostos antibiofilme foram incorporados em sPEEK a sua atividade inibitória diminuiu consistentemente, apresentando um estímulo na formação do biofilme de *S. sobrinus* e *S. gordonii*.

Conclusão: O efeito da furanona C-30 e das lactamas na viabilidade celular de HOK-18A, HDPC's e L929, assim como na formação do biofilme mono e multi-espécie foi dose dependente, inibindo principalmente periodontopatógenos Gram-negativos, enquanto a maioria das bactérias benéficas praticamente não foram afetadas pelos compostos. Quando os compostos antibiofilme foram incorporados nos discos de sPEEK o seu efeito inibidor na formação de biofilme multi-espécies foi perdido.

Palavras-chave: Biofilme, Periodontopatógenos, Furanona C-30, Lactama

ABSTRACT

Aim: To evaluate the cytotoxic effect of furanone C-30 and lactams U11, U12 and U26 and the inhibitory activity on single and multi-species oral biofilm formation.

Material and methods: The effect of furanone C-30 and lactams on cell viability of Human Oral Keratinocytes (HOK-18A), Human Dental Pulp Cells (HDPC's) and L929 fibroblasts was evaluated by XTT and MTS assays. The effect of furanone C-30 on single-species biofilm formation was analysed by crystal violet assay, while the effect of furanone C-30 and lactams solubilized in liquid medium or incorporated in sPEEK discs on multi-species biofilms formation was evaluated by quantitative PCR. Statistical analysis were conducted by comparisons between growing conditions and the control group by multiple hypothesis testing (Sidak).

Results: 100% viability of HOK-18A was observed when were exposed to 0.5 to 10 µg/mL of furanone C-30, 90% of HDPC's to 10 µg/mL in contrast to 128 µg/mL in which a cytotoxic effect was found. Only group sP/3LF128 at the 7th day of exposure showed a cytotoxic effect on L929 fibroblasts resulting in 55% of viable cells. Planktonic bacterial growth and biofilm formation of the periodontopathogens *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia and Porphyromonas gingivalis* was inhibited by >20% and >50%, respectively at furanone C-30 concentrations of 8 to 128 µg/mL in single-species experiments. In multi-species experiments at 10 µg/mL and 128 µg/mL furanone C-30 solubilized in liquid medium changed the biofilm composition in comparison to the control by reducing the bacterial concentration of *A. actinomycetemcomitans* and *P. intermedia* and of *P. gingivalis* at 128 µg/mL. The association of furanone C-30 and lactams at 128 µg/mL significantly inhibited biofilm formation of *F. nucleatum, P. gingivalis* and *P. intermedia* shifting the multi-species community composition to a more beneficial and less pathogenic state. When incorporated in sPEEK discs the inhibitory activity of antibiofilm agents on multi-species biofilms formation decreased consistently, showing an increase in the biofilm formation of *S. sobrinus* and of *S. gordonii*.

Conclusion: The effect of furanone C-30 and lactams on cell viability of HOK-18A, HDPC's and L929, as well as on single e multi-species biofilm formation was dose dependent, inhibiting mainly Gram-negative periodontopathogens, while most beneficial bacteria were relatively unaffected by the compounds. When antibiofilm compounds were incorporated in sPEEK their inhibitory activity on multi-species biofilm formation has been lost.

Keywords: Biofilm inhibition, Periodontopathogens, Furanone C-30, Lactam

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Introdução e Revisão de Literatura

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LISTA DE ABREVIATURAS E SIGLAS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
AI	autoinducers
A. naeslundii	*Actinomyces naeslundii*
A. viscosus	*Actinomyces viscosus*
BHI	brain heart infusion
CFU	colony forming unit
CHX	chlorhexidine
D. pulchra	*Delisea pulchra*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
Furanone C-30	(Z-)-4-Bromo-5-(bromomethylene)-2(5H)-furanone
F. nucleatum	*Fusobacterium nucleatum*
HGFs	human gingival fibroblasts
HOK-18A	Human Oral Keratinocytes
HDPC's	Human Dental Pulp Cells
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P. aeruginosa	*Pseudomonas aeruginosa*
PEEK	poly-ether-ether-ketone
P. gingivalis	*Porphyromonas gingivalis*
P. intermedia	*Prevotella intermedia*
PLLA	poly (L-lactic acid)
PMS	Phenazine methyl sulphate
qPCR	quantitative polymerase chain reaction
QS	quorum sensing
QSI	quorum sensing inhibitor
REML	restricted maximum likelihood
RT	room temperature

SEM	scanning electron microscope
S. aureus	*Staphylococcus aureus*
S. gordonii	*Streptococcus gordonii*
S. mitis	*Streptococcus mitis*
S. mutans	*Streptococcus mutans*
S. oralis	*Streptococcus oralis*
sPEEK	sulfonated PEEK
S. salivarius	*Streptococcus salivarius*
S. sanguinis	*Streptococcus sanguinis*
S. sobrinus	*Streptococcus sobrinus*
THP-1	acute monocytic leukemia cells
V. parvula	*Veillonella parvula*

SUMÁRIO

CAPÍTULO I

1.1 Introdução e Revisão de Literatura

Diferentes espécies bacterianas agregadas em micro colônias interagem com a película adquirida formada sobre os tecidos duros e moles da boca e sobre outras bactérias originando o biofilme (Marsh, 1992). Biofilme é uma comunidade microbiológica complexa embebida em uma matriz extracelular composta por exopolissacarídeos, proteínas, sais e água (Costerton *et al*., 1999; Marsh and Martin, 1999; Hogan & Kolter, 2002). Inicialmente, colonizadores iniciais são transportados para a superfície sobre a qual se aderem, multiplicam e formam microcolônias. A medida que as condições do meio mudam no biofilme imaturo e a superfície fica coberta por bactérias, colonizadores secundários podem então aderir aos colonizadores iniciais, assim o biofilme começa a desenvolver uma comunidade multi-espécies. Estas espécies se multiplicam promovendo a estruturação e maturação do biofilme. A nutrição dessa comunidade se dá por meio de canais de água que permitem a passagem de nutrientes ao longo do biofilme, assim como a remoção de restos de produtos metabólicos (Costerton *et al*., 1999; Marsh & Martin, 1999; Davies, 2003; Socransky & Haffajee, 2002).

A comunicação célula-célula se dá por meio de um sistema conhecido como *Quorum sensing* (QS). Processo químico que controla a expressão gênica de fatores de virulência por meio do acúmulo de moléculas sinalizadoras, chamadas auto indutoras (AI) (Kalaiarasan *et al*., 2017; Prosser *et al*., 1999; Pereira *et al*., 2014). O mesmo possibilita que as bactérias mudem de comportamento em resposta à alterações na densidade celular e composição das espécies microbianas da comunidade (Papenfort & Bassler, 2016), por meio de atividades como bioluminescência, produção de fatores de virulência e de proteínas, assim como pela formação de biofilme (Schuster *et al*., 2013; Ren *et al*., 2004). Diferentes espécies usam moléculas sinalizadoras diferentes, a exemplo da homoserina lactona acilada (AHLs ou AI-1), regulada pelos sistemas LuxI/LuxR e secretada pelas bactérias Gram-negativas e AI-2, regulada pelo sitema LuxS e secretada tanto por bactérias Gram-negativas quanto por Gram-positivas (Ren *et al*., 2004).

As células microbianas, quando organizadas em biofilmes, demonstram um fenótipo completamente diferente daquele encontrado quando isoladas em estado planctônico (Liu *et al*., 2011; Yoshida *et al*., 2005; Merritt *et al*., 2005). Tornam-se

mais difíceis de serem eliminadas, sendo capazes de resistir a determinados antibióticos em concentrações até 1000 vezes maiores do que as utilizadas para eliminar as mesmas espécies em estado planctônico (Costerton, 1999; Olson *et al*., 2002; Davies, 2003).

Biofilmes são conhecidos como responsáveis por infecções bacterianas, contaminação de dispositivos médicos e doenças periodontais e periimplantares (Donlan & Costerton, 2002). As doenças periodontais e periimplantares se iniciam com um processo inflamatório nos tecidos moles (gengiva e mucosa periimplantar) devido ao acúmulo de biofilme oral nas superfícies dos dentes e dos implantes dentais. Quando não há controle e remoção do biofilme oral; as condições locais favorecem a colonização por espécies patogênicas que cronificam a inflamação dos tecidos moles, respectivamente denominadas gengivite e mucosite. Em indivíduos susceptíveis, a evolução desta inflamação leva a perdas progressivas dos tecidos de sustentação, denominadas periodontite e periimplantite, respectivamente (Tonetti *et al*., 2015). Mais de 50% da população adulta é afetada pela periodontite, sendo 43% a prevalência de mucosite e 22% de periimplantite (Derks & Tomasi, 2015). Periimplantite, assim como a periodontite, é uma doença multifatorial que está associada com o pobre controle de placa e com histórico de doença periodontal. A microbiota em dentes e implantes comprometidos periodontalmente é composta em sua maioria por bactérias anaeróbicas. Bactérias patogênicas associadas à periodontite foram isoladas de sítios saudáveis ao redor de implantes assim como de sítios com doença periimplantar (Renvert & Quirynen, 2015), porém em quantidades maiores (Schwartz *et al*., 2018).

Uma das alternativas de tratamento não cirúrgico das doenças periodontais e periimplantares é a associação do debridamento mecânico do biofilme subgengival com o uso concomitante de terapia antibiótica, reduzindo a profundidade de sondagem e aumentando o nível clínico de inserção (Sanz *et al*., 2008; Graziani *et al*., 2017).

O uso indiscriminado de antibióticos devido ao fácil acesso, prescrições desnecessárias, uso incorreto levou ao surgimento do que chamamos de resistência bacteriana (Hwang & Gums, 2016). Por este motivo o uso de antibióticos no tratamento de doenças periodontais deve ser restrito a condições mais agressivas e severas (Sanz *et al*., 2008). Uma nova estratégia de tratamento se faz necessária com o desenvolvimento de drogas antimicrobianas que controlem as etapas inicias

da formação do biofilme, interferindo na comunicação intercelular, o *Quorum Sensing* (Davies, 2003). A aplicação de inibidores do *Quorum Sensing* (QSIs) é uma das técnicas possíveis.

O primeiro exemplo de QSIs é a furanona bromada produzida pela alga marinha vermelha *Delisea pulchra* (Manefield *et al*. 2002; He *et al*. 2012). Esta alga identificada na Australia chamou a atenção de pesquisadores por não apresentar colonização de sua superfície por bactérias e epífitas como nas demais espécies existentes no mesmo meio ambiente (Pettus *et al*., 1977; Nys *et al*., 1993; Hentzer & Givskov, 2003). A mesma é capaz de produzir mais de 30 metabólitos secundários (furanona halogenada) bioativos únicos, porém com estruturas semelhantes, (Hentzer & Givskov, 2003; Nys *et al*. 1993; Maximilien, R. *et al*., 1998) capazes de inibir o crescimento bacteriano de *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa* (Pettus *et al*., 1977; Nys *et al*., 1993; Nys *et al*., 1995; Ren *et al*. 2001; Ren *et al*. 2002; Hentzer *et al*. 2002). Desta gama de compostos, 4 constituem a maioria (95%), sendo os compostos 3 e 4 os mais abundantes (Figura 1) (Nys *et al*., 2006).

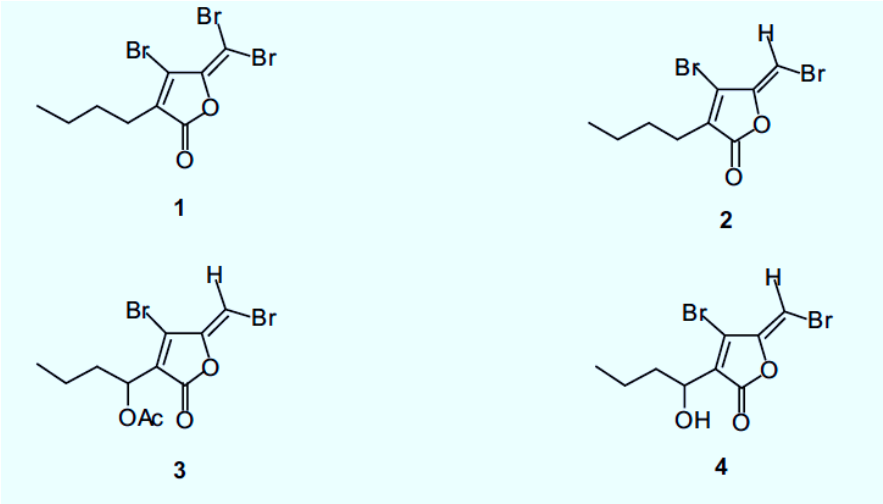

Figura 1. Estrutura das 4 furanonas naturais mais abundantes (removido de Nys *et al*., 2006)

As furanonas são compostos que não matam as bactérias diretamente, mas atrapalham a população bacteriana resistente interferindo na organização do biofilme, evitando o desenvolvimento de resistência bacteriana (Pereira *et al*., 2014). São substâncias que mimetizam as atividades das moléculas sinalizadoras AHL e competem pelo receptor LuxR afetando o *quorum-sensing* das bactérias associadas a este sistema de regulação (Hentzer & Givskov, 2003).

A partir desta descoberta, uma série de compostos com estruturas similares à furanona halogenada foram sintetizados, dentre eles, a furanona C-30 (*Z*-)-4-Bromo-5-(bromometileno)-2(5*H*)-furanona e as lactamas (Figura 2) (Manefield *et al*., 2002; He *et al*., 2012) .

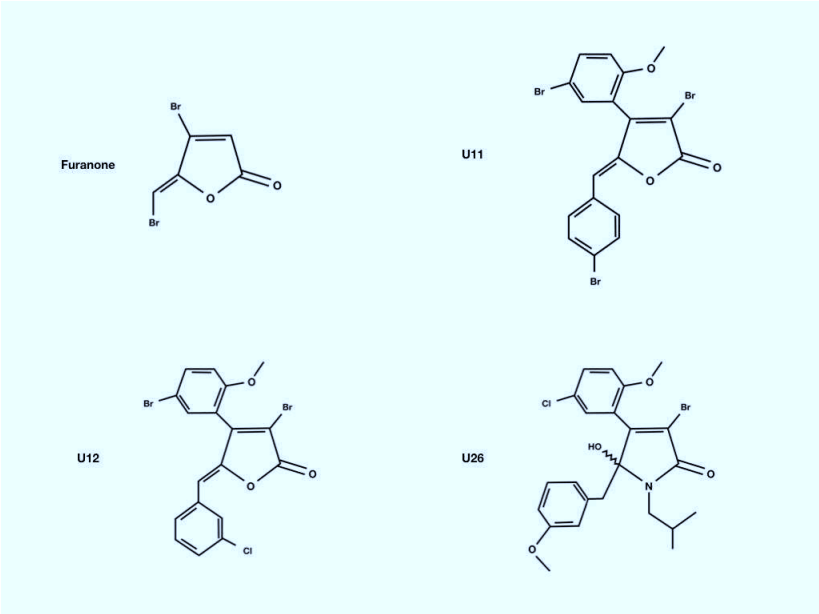

Figura 2. A) Estrutura da furanona C-30 sintética (Sigma Aldrich); B) Estruturas químicas das lactamas U11, U12 e U26.

A furanona C-30 em concentrações de 2 e 4 µg/ml diminuiu significativamente a quantidade de biofilme formado *in vitro* por *S. mutans*, sem afetar a proliferação celular (He *et al*., 2012). Já em uma concentração de 60 µg/ml inibiu a formação do biofilme de *E. coli* e diminuiu a espessura do biofilme em 55%, reduziu o número de canais de água e o percentual de células vivas em 87% (Ren *et al*., 2001).

Os rubrolídeos apresentam algumas características estruturais parecidas as do composto produzido pela alga marinha *Delisea pulchra* e da furanona sintética C-30 e todos correspondem com γ-alkilidine-γ-lactonas. Então γ-alkilidine-γ-lactonas quando convertidas nas lactamas correspondentes γ-hidroxi-γ-lactamas e γ-alkilidine-γ-lactamas foram capazes de inibir o biofilme formado por *Enterococcus faecalis* (Pereira *et al*., 2014a), *Staphylococcus epidermidis* e *P. aeruginosa* em concentração de 0.6 µg/mL (Pereira *et al*., 2014b).

Com o intuito de ampliar o efeito inibitório dos compostos, levando esse efeito diretamente no local desejado, compostos antimicrobianos vem sendo incorporados em materiais de uso na odontologia. Furanona incorporada em resina composta

demonstrou atividade antibacteriana significante sobre *S. mutans* sem diminuir a força mecânica do material (Weng *et al*., 2012a). O mesmo grupo de pesquisadores incorporou novamente 0.5 mol de furanona em cimento ionômero de vidro e mostrou a atividade antibacteriana do composto sobre *S. mutans* e *Lactobacillus sp,* assim como a biocompatibilidade do cimento modificado com furanona sobre células da polpa dental (Weng *et al*., 2012b). A lactama U27 em uma concentração de 87.5 µg/mL foi capaz de reduzir a formação do biofilme produzido por *S. mutans* sobre superfícies funcionalizadas de titânio (Ti6Al4V) (Xavier *et al*., 2016).

Apesar de o titânio e suas ligas, por suas excelentes propriedades mecânicas, ser o material de eleição para os sistemas de implantes, este material, quando em contato com substâncias corrosivas (flúor, agentes clareadores), ou quando submetido à desgaste, perde o fino filme protetor constituído basicamente de TiO_2 liberando partículas metálicas que apresentam potencial tóxico para os tecidos, consequentemente podendo induzir inflamações crônicas dos tecidos peri-implantares, perda óssea e até perda do implante (Souza *et al*., 2015; Juanito *et al*., 2015). Para superar estas limitações e minimizar estas reações biológicas negativas outros materiais estão sendo pesquisados. Uma alternativa é a poli-éter-éter-cetona (PEEK), um polímero que apresenta baixo módulo de elasticidade, boa resistência química, é bioinerte, biocompatível e não reabsorvível. Pode ser repetidamente esterilizável e conformado por usinagem e aquecimento (Zhao *et al*., 2013). Além de poder ser utilizado para a incorporação de compostos com capacidade de inibir a formação do biofilme. Como demonstrado no trabalho de Montero *et al*., 2016 onde a lactama U27 foi incorporada em filme de poli-éter-éter-cetona sulfonada (sPEEK) em uma concentração de 87.5 µg/ml e a sua capacidade de inibir a formação do biofilme mono-espécie produzido por *S. mutans* foi novamente evidenciada.

Estudos relacionados com a formação do biofilme focam em biofilmes constituídos por uma ou poucas espécies bacterianas como o conduzido por de Almeida e colaboradores que avaliaram o efeito de 3 γ-alkilidine-γ-lactamas na formação de biofilme multi-espécies de *S. mutans*, *E. faecalis* e *Candida glabrata* e obtiveram resultados que mostram a inibição da formação do biofilme multi-espécies pelas 3 lactamas testadas devido a habilidade de reduzir a quantidade de proteína e exopolissacarídeos (de Almeida *et al*., 2018). Como a placa dental é um biofilme complexo no qual mais de 700 espécies microbianas diferentes podem residir (Aas

et al., 2005), neste estudo foi desenvolvido em laboratório um modelo de biofilme multi-espécies se assemelhando mais com a realidade do meio bucal.

1.2 Objetivos

Objetivo principal: estudar os efeitos de compostos sintéticos na formação de biofilme bacteriano sobre superfícies poliméricas (PEEK) e discos de hidroxiapatita.

Objetivos específicos:

a) Avaliar o efeito da Furanona C-30 sobre a viabilidade celular de queratinócitos orais humanos (HOK-18A) e células da polpa dental humana (HDPC's).

b) Avaliar o efeito da Furanona C-30 na formação de biofilme mono-espécie envolvendo 14 espécies de bactérias orais.

c) Avaliar o efeito de compostos anti-biofilme incorporados em discos de poli-éter-éter-cetona (sPEEK) sobre a viabilidade celular de fibroblastos (L929).

d) Avaliar o efeito de compostos anti-biofilme solubilizados em meio líquido sobre discos de hidroxiapatita e incorporados em discos de sPEEK na formação de biofilme multi-espécies.

2.1 Manuscript 1

Effect of furanone C-30 on cell viability, single and multi-species oral biofilm formation

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Carolina Schäffer Morsch[a]
Esteban Rodriguez Herrero[b]
Vera Slomka[b]
Andréa de Lima Pimenta[c]
Júlio César Matias de Souza[a]
Wim Teughels[b]
Cesar Augusto Magalhães Benfatti[a]

[a] Center for Education and Research on Dental Implants (CEPID), Post-Graduate Program in Dentistry (PPGO), Department of Dentistry (ODT), Federal University of Santa Catarina (UFSC), Florianópolis/SC, 88040-900, Brazil
[b] Department of Oral Health Sciences, KU Leuven, Kapucijnenvoer 33, 3000 Leuven, Belgium
[c] Department of Biology, Université de Cergy Pontoise, 2 Av. Adolphe Chauvin 95302 Cergy Pontoise, France

Corresponding author:
Carolina Schäffer Morsch
dracarolmorsch@gmail.com
Federal University of Santa Catarina
Center for Education and Research on Dental Implants (CEPID)
Campus Trindade, Florianópolis, 88040-900, Brazil
Phone/Fax: +55 48 3721-9000

Abstract

Aim: To evaluate the effect of furanone C-30 on cell viability of Human Oral Keratinocytes (HOK-18A) and Human Dental Pulp Cells (HDPC's) and on single and multi-species oral biofilm formation.

Material and methods: The effect of furanone C-30 on cell viability was evaluated by XTT cell proliferation assay. Single-species biofilm experiments were analysed by crystal violet assay, while the effect of furanone C-30 on multi-species biofilms was evaluated by quantitative PCR.

Results: 100% viability of HOK-18A was observed when were exposed to 0.5 to 10 µg/mL of furanone C-30, 90% of HDPC's to 10 µg/mL in contrast to 128 µg/mL in which a cytotoxic effect was found. Planktonic bacterial growth and biofilm formation of the periodontopathogens *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia and Porphyromonas gingivalis* was inhibited by >20% and >50%, respectively at furanone C-30 concentrations from 8 to 128 µg/mL in single-species experiments. In multi-species experiments at 10 µg/mL and 128 µg/mL furanone C-30 changed the biofilm composition in comparison to the control by reducing the bacterial concentration of *A. actinomycetemcomitans* with 0.73 log (±0.72) and 0.78 log (±0.83) and *P. intermedia* with 1.52 log (±1.28) and 2.41 log (± 0.59), respectively and of *P. gingivalis* with 1.49 log (± 0.78) at 128 µg/mL, while changes in the concentrations of the other species were not statistically significant.

Conclusion: The effect of furanone C-30 on cell viability of HOK-18A and HDPC's, as well as on single and multi-species biofilm formation was dose dependent, inhibiting mainly Gram-negative periodontopathogens, while most beneficial bacteria were relatively unaffected by the compound.

Keywords: Biofilm inhibition, Periodontopathogens, Furanone C-30

1. INTRODUCTION

The oral cavity is a habitat of multiple bacterial species organized in complex communities called biofilms. These organized microbial communities are surrounded by an extracellular matrix composed of polysaccharides, nucleic acids and proteins, forming an intricate network of water channels that allows the penetration of nutrients and the release of metabolites [1,2,3]. When organized in biofilms, bacteria demonstrate a radically different phenotype from those in the planktonic state [4,5,6]. As such, they are able to resist antibiotics at concentrations up to a thousand times higher than concentrations that show to kill these species in planktonic state [7,8]. Biofilm formation is mediated by an intercellular communication system known as quorum sensing (QS). QS involves the regulation of virulence-related gene expression through the accumulation of signaling molecules called autoinducers (AI), when the bacterial population reaches a certain density [9,10].

The development of a new generation of antimicrobials that control the early steps of biofilm formation by disrupting the bacterial QS has been proposed as a new antibiofilm strategy as the easy access and misuse of antimicrobials drugs led to the increment of bacterial resistance [10]. The application of quorum sensing inhibitors (QSIs) is one of the promising techniques for disrupting QS. The prime example of such QSIs are the bromated furanones produced by the marine red algae *Delisea pulchra* [11,12], which were shown to inhibit biofilm formation of *Escherichia coli, Bacillus subtilis* and *Pseudomonas aeruginosa* [13,14,15,16]. These finding lead to the development of a series of synthetic halogeno-furanone compounds whose structure were similar to those found in *D. pulchra* such as furanone C-30 ((*Z*-00)-4-Bromo-5-(bromomethylene)-2(5*H*)-furanone) and lactams [11,12]. Such compounds do not kill the pathogens directly but disrupt the biofilm formation avoiding the

development of bacterial resistance [10]. To be suitable for clinical use in the oral cavity, these compounds must interfere on biofilm formation without having a cytotoxic effect on eukaryotic cells.

This study aimed to evaluate the cytotoxic effect of furanone C-30 on cell viability of Human Oral Keratinocytes and Human Dental Pulp Cells and its inhibitory effect on single and multi-species oral biofilm formation.

2. MATERIALS AND METHODS

Furanone C-30

The synthetic furanone C-30 (Fig. 1), was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution was made by dissolving 10 mg of furanone C-30 in 1 mL of ethanol 95% and stored at -20ºC. The final working concentrations were reached by diluting it in keratinocyte growth medium (KSFM, Gibco®, Life Technologies Ltd., Paisley, Scotland) and Dulbecco's Modified Eagle Medium (DMEM, Gibco) to perform the cell viability assay. To test single-species biofilm inhibition it was diluted in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, USA), while the multi-species in modified BHI (BHI_2) [17].

The effect of the ethanol on bacterial and cell cultures was also tested, by making the same dilution as for the highest final concentration of furanone C-30, but without the compound.

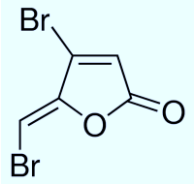

Figure 1. Structure of furanone C-30 ((*Z*-)-4-Bromo-5-(bromomethylene)-2(5*H*)-furanone)

Cell culture: Immortalized Human Oral Keratinocytes (HOK-18A) were seeded in cell culture flasks (Costar, Cambridge, MA, USA) with KSFM, supplemented according to the manufacturer's instructions. Fibroblast human dental pulp cells (HDPC's) were cultured from human third molars, which were extracted for orthodontic reasons [18,19]. HDPC's were grown in DMEM, supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies Ltd., Paisley, Scotland). The culture medium were refreshed 3 times a week and cells were incubated at 37°C and 5% CO_2. When HOK-18A and HDPC´s reached 80% confluence, cells were harvested with 0.25% Trypsin/EDTA (Life Technologies, Paisley, UK), centrifuged at 1100 rpm for 5 min at room temperature and resuspended in growth medium.

Bacterial strains and culture conditions

Six oral pathogenic strains (*Streptococcus mutans* (ATCC 20523), *Prevotella intermedia* (ATCC 25611), *Porphyromonas gingivalis* (ATCC 33277), *Fusobacterium nucleatum* (ATCC 20482), *Aggregatibacter actinomycetemcomitans* (ATCC 43718), *Streptococcus sobrinus* (ATCC 20742)) and eight commensal, potentially beneficial species (*Streptococcus gordonii* (ATCC 49818), *Actinomyces naeslundii* (ATCC 51655), *Streptococcus mitis* (DSM 12643), *Streptococcus salivarius* (TOVE-R), *Streptococcus oralis* (DSM 20627), *Streptococcus sanguinis* (LMG 14657), *Actinomyces viscosus* (ATCC 43327) and *Veilonella parvula* (DSM 2008)) were included in this study.

All bacterial strains were maintained on blood agar plates (Oxoid, Basingstoke, UK) supplemented with 5 µg/mL hemin (Sigma, St. Louis, USA), 1 µg/mL menadione (Calbiochem-Novabiochem, La Jolla, USA) and 5% sterile horse blood (E&O Laboratories, Bonnybridge, Scotland). *A. actinomycetemcomitans, S. gordonii, S. mitis, S. mutans, S. salivarius, S. sanguinis* and *S. sobrinus* were incubated at 37ºC

in 5% CO_2. *A. naeslundii*, *A. viscosus*, *F. nucleatum*, *P. gingivalis*, *P. intermedia* and *V. parvula* were incubated at 37ºC under anaerobic conditions (10% Co_2, 10% H_2, 80% N_2). Overnight liquid cultures were prepared in 10 mL BHI and incubated at the same conditions.

Bioreactor derived multi-species community

To establish the multi-species biofilm, all strains were cultivated individually as described above. Before inoculating the bioreactor, the turbidity of each bacterial suspension was adjusted to an optical density (OD) at 600 nm (GeneQuant 100, GE Healthcare, Buckinghamshire, UK) of 1.4 with BHI_2.

For the establishment of a multi-species community, 750 mL of BHI_2 supplemented with 5 mg/mL hemin, 1 mg/mL menadione, were added to a Biostat B Twin 1L bioreactor (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The bioreactor conditions were 37°C, 0% O_2, 5% CO_2, pH 6.7 ± 0.1, stirring at 300 rpm and 200 µl/L of Antifoam Y-30 (Sigma, St. Louis, USA). Bacterial inoculation of the bioreactor started with 750 µl of *S. mitis* at OD_{600} 1.4; which grew until late exponential phase, after which the remaining 13 microbial species were added at OD_{600} 1.4. The chemostat culture was left to stabilize for 48h, after that the medium was replaced at a rate of 200 mL/24h and kept for 5 days [20].

2.1 Cell Viability assay

Cells were seeded in 96-well plates (Greiner Bio-One, Wemmel, Belgium) at a concentration of 20.000 cell/well. After 24h culture medium was refreshed and HOK-18A cells were exposed to furanone C-30 at 128, 64, 32, 30, 25, 20, 16, 15, 10, 8, 4, 2, 1, and 0.5 µg/mL. HDPC´s were exposed to the compound at 128 and 10 µg/mL. After 20 hours of exposure, the supernatant was removed and 50 µl of XTT dissolved in RPMI 1640 medium (Life Technologies, Gent, Belgium) at a concentration of 1

mg/mL and 1µl of Phenazine methyl sulphate (PMS) (AppliChem, Darmstadt, Germany) dissolved in phosphate buffered saline (PBS) at a concentration of 0.383 mg/mL were added to each well. Plates were incubated at the same conditions mentioned before for 4h. The supernatant was used for quantification of formazan release photospectrometrically at 450 nm using a microplate reader (Multiskan Ascent; Thermo Scientific, Waltham, MA, USA). Experiments were conducted in triplicate and repeated on 3 different days.

Cell viability is expressed as percentage in comparition to the control cells, which were not exposed to the compound.

2.2 Biofilm inhibition

2.2.1 Single-species biofilm

To examine the effect of furanone C-30 on single-species biofilm formation, 100 µL of BHI were added at each well of polystyrene 96-well microtiter plates (Grenier Bio-One, Frickenhausen, Germany). Furanone C-30 from the stock solution was diluted in BHI to reach a concentration of 512 µg/mL, 100 µL were dispensed at the first well and diluted in a serial two-fold dilutions to reach a final concentration of 1 µg/mL. Overnight bacterial cultures were diluted to adjust the bacterial concentration to an OD_{600} 0.5 (GeneQuant 100, GE Healthcare, Buckinghamshire, UK) and 100 µL were inoculated on each well. Final concentrations of furanone C-30 were 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 µg/mL. OD_{630nm} was measured at baseline (Thermo Scientific, Eletron Corporation, Multiskan Ascent, Ascent Software Version 2.6) and plates were then incubated at 37ºC for 24h in either aerobic or anaerobic conditions. After 24h of incubation, planktonic bacterial growth was measured at OD_{630nnm}. After discarding the supernatants wells were washed twice with sterilized PBS and the formed biofilm at the bottom of the wells was stained with 0.1% crystal violet (Sigma, St. Louis, Mo,

USA) for 15 min, washed five times with distilled water, dried, and solubilized with 5% acetic acid for 45 min and OD_{630nm} was measured. Experiments were performed in triplicate and repeated on 3 different days. The inhibitory effect of furanone C-30 was determined by the percent reduction in the OD in relation to the control in the absence of the compound.

2.2.2 Multi species biofilm

Samples were taken from the chemostat culture, diluted 1:10 in fresh BHI_2 medium and 100 µL were added to each well of a 24-well plate containing a Calcium Deficient Hydroxyapatite disc (Hitemco Medical, Old Bethpage, NY, USA). Furanone C-30 from the stock solution was diluted in BHI_2 added to reach final concentrations of 10 µg/mL and 128 µg/mL (concentrations choosen based on the cell viability assay results) at a final volume of 1 mL. Plates were incubated for 24h at 37°C, under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) stirring at 170 rpm.

Discs were washed three times in PBS after which, the biofilms were detached by the addition of 0.05% Trypsin-EDTA. After incubation for 45 min at 37ºC and stirring at 230 rpm, bacterial cells were harvested by centrifugation for 5 minutes at 8.000 rpm and resuspended in 500 µl of PBS.

Quantification by q-PCR

DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A quantitative PCR (qPCR) assay was performed to detect and quantify bacterial DNA with a CFX96 Real-Time System (Biorad, Hercules, USA) using the Taqman 5' nuclease assay PCR method. The Taqman reaction contained 12.5 µl Mastermix (Eurogentec, Seraing, Belgium), 4.5 µl sterile distilled water, 1 µl of species-specific primer and probe and 5 µl template DNA [21]. Assay conditions for all primer/probe sets consisted of an initial 2

min at 50°C, followed by a denaturation step for 10 min at 95°C, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. Quantification was based on a plasmid standard curve [20]. Experiments were performed in triplicate and repeated on three different days.

2.3 Scanning Electron Microscopy

Multi-species biofilms grown on the hydroxyapatite discs were carefully rinsed 3 times with PBS, biofilm was fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer, pH 7.4 at RT for 2h. Discs were rinsed 3 times in 0.1 M sodium cacodylate buffer for 10 minutes, postfixed in the dark with 1% osmiumtetroxide at 4ºC for 2h and washed 2 times with distilled water for 10 min. Samples were dehydrated through a series of 50, 70, 90 and 100% ethanol solutions and dried with hexamethyldisilazan (Acros Organics, Geel, Belgium). Discs were then mounted in support stubs, sputter coated with gold and analyzed with SEM (JSM-6610LV, JEOL, Tokyo, Japan).

2.4 Statistical analysis

Linear mixed models using compound concentrations as fixed factor and experiment runs as random factor were separately conducted for the cell viability assay and for each species on the planktonic bacterial growth, single and multi-species competition assays. The restricted maximum likelihood (REML) estimation approach was used. Fitted models were evaluated according to the assumptions of homoscedasticity and residuals normality. Outcome variables were logarithmic or squared transformed when residuals were skewed distributed. The multi-species biofilm formation data were Log_{10}-transformed from CFU/mL. Due to the presence of outliers among specific species, a bootstrapping procedure was conducted to obtain corrected standard errors (1.500 resamples). Comparisons between growing conditions and

the control group were adjusted for multiple hypothesis testing (Sidak). Cell viability and biofilm formation results were presented, respectively, as percentage relative to control group and percentage of inhibition.

3. RESULTS

3.1 Cell Viability

In order to achieve the optimal furanone C-30 concentration to obtain 100% of cell viability, the tested concentration range was extended to 14 concentrations including the 9 serial two-fold dilutions described before.

As shown in Fig. 2A, the highest furanone C-30 concentration showing 100% cell viability was 10 µg/mL. Higher concentrations of furanone C-30 (30 µg/mL to 128 µg/mL) significantly decreased cell viability of HOK-18A of more than 30% when compared to control. The cytotoxicity of furanone C-30 on HDPC's was tested in two concentrations, 10 µg/mL as the highest concentration that provided 100% of cell viability in the cell viability assay with HOK-18A and 128 µg/mL (the highest concentration tested). Similar effects were observed. More than 90% cell viability was retained at 10 µg/mL and a cytotoxic effect was observed at 128 µg/mL, (Fig 2B).

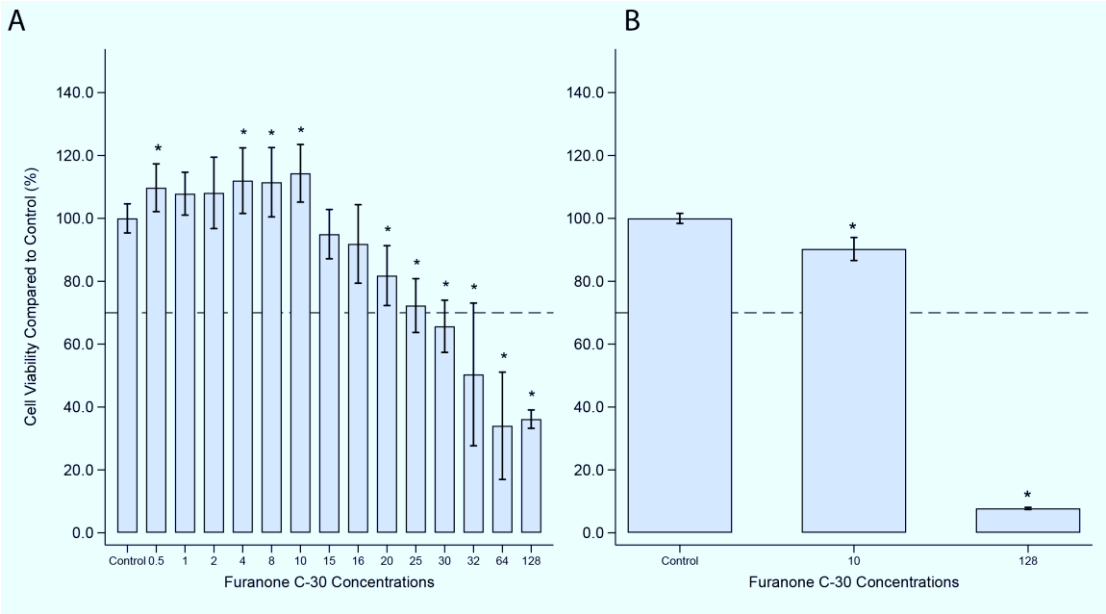

Figure 2. Percentage of cell viability A) for HOK-18A cell line and B) for HDPC's cell line in comparison to the control according to furanone C-30 concentrations. *Significant statistical difference in comparison to control group [%] ($p<0.05$); dashed lines denotes cytotoxicity threshold (30% inhibition).

3.2 Biofilm quantification

3.2.1 Planktonic Growth

The inhibitory effect of furanone C-30 on planktonic growth of selected bacteria is shown in table 1. Ten species presented above 20% inhibition when compared to the control and this inhibition was concentration dependent. Four of the 14 species tested (*S. sobrinus*, *S. sanguinis*, *F. nucleatum* and *V. parvula*), furanone C-30 inhibit planktonic bacterial growth less than 20%.

Please insert Table 1 here

3.2.2 Single-species biofilms

As shown in table 2, furanone C-30 at concentrations of 8 µg/mL to 128 µg/mL significantly reduced the biofilm formation of the periodontopathogens *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*. At 64 µg/mL and 128 µg/mL single-species biofilm formation of *S. mutans* and at 128 µg/mL of *F. nucleatum* was significantly inhibited (>50%) compared to the control. In regards to the six pathogenic oral strains tested, only the formation of the single-species biofilm of *S. sobrinus* was not significantly inhibited. Biofilm formation of the commensal, potentially beneficial bacteria was significantly inhibited in the case of *A. naeslundii* at concentrations from 8 µg/mL to 128 µg/mL, *A. viscosus* from 32 µg/mL to 128 µg/mL, *S. gordonii* from 4, 16 to 128 µg/mL, *S. oralis* from 4 µg/mL up to 128 µg/mL and *V.*

parvula at 64 µg/mL and 128 µg/mL for at least 20%. Results from the other species were not statistically significant.

Please insert Table 2 here

3.2.3 Multi-species biofilms

On multi-species biofilms, furanone C-30 was able to modulate the microbial community composition. Overall Gram-negative periodontopathogens were the most affected by furanone, while no significant change in the proportion of most health associated commensal bacteria could be observed. Furanone C-30 significantly inhibited at 10 µg/mL and 128 µg/mL *A. actinomycetemcomitans* with 0.73 Log_{10} CFU/mL (±0.72) and 0.78 Log_{10} CFU/mL (±0.83) and *P. intermedia* with 1.52 Log_{10} CFU/mL (±1.28) and 2.41 Log_{10} CFU/mL (± 0.59), respectively. Whereas, *P. gingivalis* biofilm formation was significantly inhibited at 128 µg/mL with 1.49 Log_{10} CFU/mL (± 0.78), as shown in table 3. No significant quantitative differences were found for the other bacterial species. In terms of community composition, the control biofilms consisted of 72.5% (± 20.7%) of commensal bacteria, and 27.5% (± 20.7%) of pathogens. At 128 µg/mL, furanone C-30 shifted significantly the biofilm composition to 95.6% (± 3.3%) of commensals and 4.4% (± 3.3%) of pathogenic bacteria, as shown in figure 3.

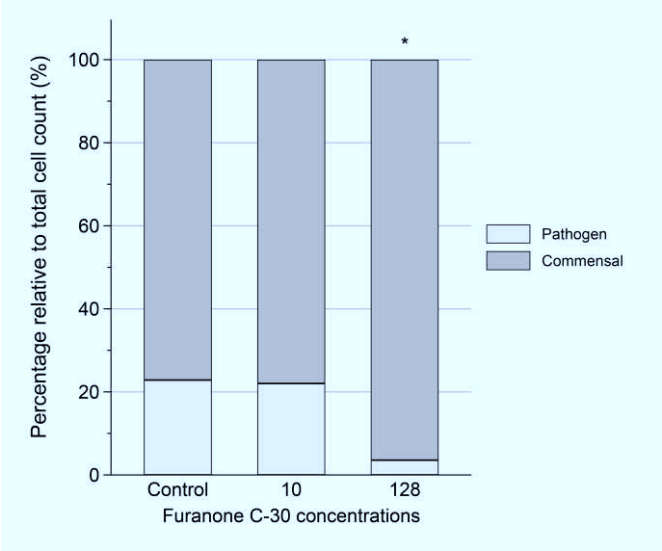

Figure 3. Effect of furanone C-30 at concentrations of 10 and 128 µg/mL on multi-species biofilm composition (CFU/mL) in comparison to control group. Note: *Significant statistical difference in comparison to control group [%] ($p<0.05$).

Please insert Table 3 here

3.2.4 Scanning Electron Microscopy

Quantitative inhibition of biofilm biomass could not be confirmed on the SEM images, however qualitatively changing in the multi-species biofilm composition could be observed in the images, specially in the group where the furanone C-30 was applied at 128 µg/mL as shown in Fig. 4. These images corroborate the results of the qPCR technique.

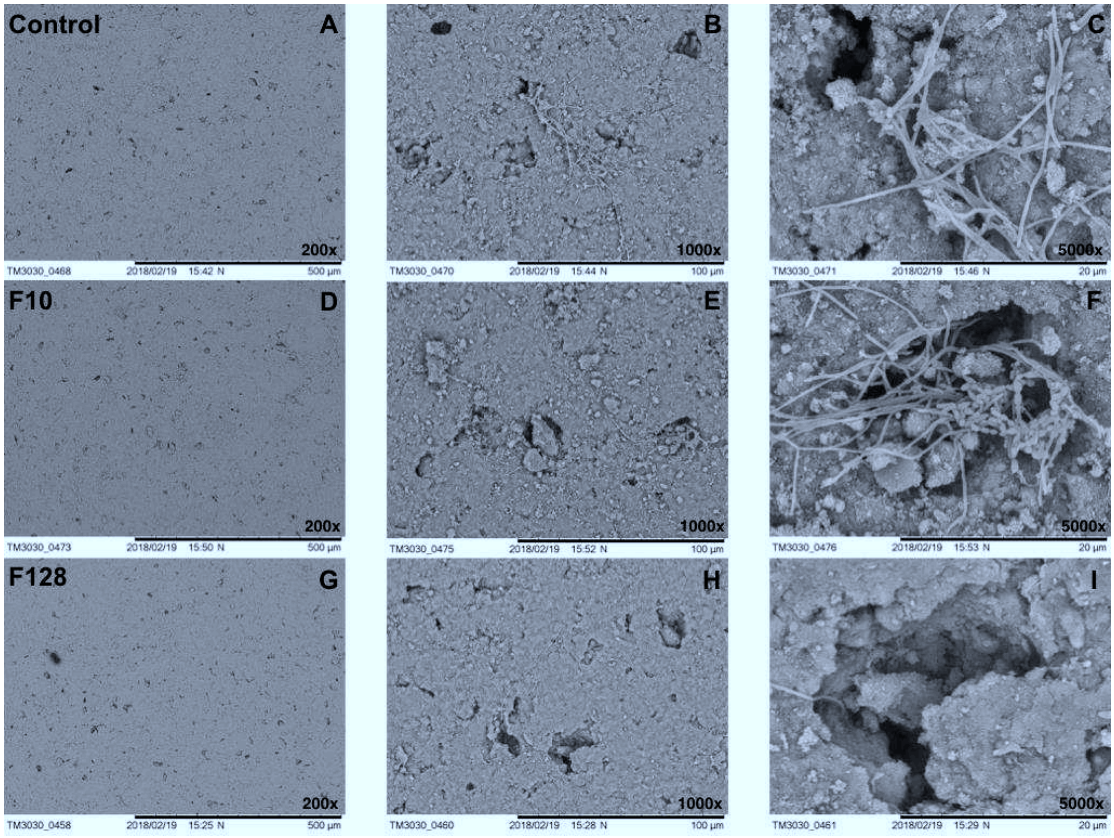

Figure 4. SEM images of multi-species biofilms on hydroxyapatite discs after 24h of growth without furanone C-30 (control: A, B, C), with the compound at 10 µg/mL (F10: D, E, F) and at 128 µg/mL (F128: G, H, I).

4. DISCUSSION

In this *in vitro* study the effect of the synthetic furanone C-30 on 14 oral bacterial species was tested. None of the 9 concentrations of furanone C-30 tested in these assays caused more than 20% inhibition of planktonic bacterial growth of *S. sobrinus*, *S. sanguinis*, *F. nucleatum* and *V. parvula*. An effect only on biofilm formation can be observed when less than 20% inhibition on planktonic bacterial growth is induced. This was limit established on the study of Pereira UA et al., 2014 [22]. These results indicate that the activity of the compound is primarily inhibition of biofilm for these 4 bacterial species, while for the other species the effect of furanone C-30 on planktonic growth was concentration-dependent, suggesting a possible antimicrobial activity of this compound.

The single-species planktonic bacterial growth of *S. mutans* was not significantly inhibited in more than 20% by furanone at 0.5 µg/mL to 16 µg/mL similarly to He et al., 2012, whom observed the same effect at 2 µg/mL and 4 µg/mL [12]. At these concentrations, the single-species biofilm formation was significantly inhibited with furanone C-30 [12], another similar synthetic furanone also showed to inhibit biofilm formation of *S. mutans* [23]. However, in the present study single-species biofilm inhibition was only observed at concentrations of 64 µg/mL and 128 µg/mL.

Furanone C-30, inhibited planktonic bacterial growth by more than 20% and single-species biofilm formation by more than 50% of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* at concentrations ranging from 8 µg/mL to 128 µg/mL. In multi-species experiments, colonization of *A. actinomycetemcomitans* and *P. intermedia* was significantly inhibited at both concentrations, while *P. gingivalis* only at 128 µg/mL. The topical inoculation of a similar synthetic furanone [(5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone] significantly interfere with the biofilm growth of *P. gingivalis* in mice infection model showing a reduction of bone destruction and a decrease in the number of bacteria *in vivo,* suggesting that QSIs can be a new approach to prevention and treatment of periodontitis [24].

Biofilm formation of *S. mitis*, *S. salivarius* and *S. sanguinis* was not significantly inhibited in single-species experiments, while in those 3 species and also *A. naeslundii*, *A. viscosus*, *S. gordonii*, *S. oralis* and *V. parvula* inhibition activity was not observed in the multi-species assays ether.

In this study, toxicity tests were conducted on immortalized human gingival epithelial cells (HOK 18-A), which are the first line of defense against oral bacterial infections [25]. Additionally, human fibroblasts (HDPC's) were used since they are the most common kind of cells on the connective tissue and are responsible for the healing

process. The viability of the HOK-18A cells was fully maintained at furanone C-30 concentrations between 0.5 µg/mL and 10 µg/mL, and for 90% for HDPC's at 10 µg/mL. On the other hand, furanone C-30 at the highest concentration (128 µg/mL) showed to be cytotoxic to both cells types. Furanones in general are described in the literature as too reactive, which make them presumably toxic for therapeutic use [26-28]. However, results from the study of Park et al., 2017 showed that (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone, which is similar to furanone C-30, maintained 100% of viable cells of human gingival fibroblasts (HGFs) and more than 90% of HOK-16B at 2 µM [29]. Yang YJ et al., 2013 showed that 0.04, 0.4 and 4 mM compound did not stimulate proinflammatory cytokines and did not affect the viability of human monocytic cell line and human gingival fibroblasts (HGFs) when the dehydrogenase activity of living cells was analyzed [30]. Also, no irritative or genotoxic effects in mice after 3 or 21 days of exposure were seen [31]. In contrast to that, the antimicrobial agent chlorhexidine, which is considered a gold standard in the treatment of oral diseases, is cytotoxic to human fibroblasts and keratinocytes [32], and also showed significantly less metabolic activity of fibroblasts, after 24h of incubation when compared to treatment with lactams [33].

In general, furanone C-30 shifted the biofilm community composition to a more beneficial and less pathogenic state. The reduction of pathogenic bacteria consequently enriched the amount of health-associated bacteria suggesting a selective effect of the compound.

Furanone C-30 showed to be suitable for the development of new antibiofilm drugs for therapeutic purposes. Although promising results, the oral environment is much more complex than those created in the present study, therefore the compound needs to be tested *in vivo*.

5. CONCLUSION

The effect of furanone C-30 on cell viability of HOK-18A and HDPC's as well as on single e multi-species biofilm formation was concentration dependent. Furanone C-30 inhibited mainly Gram-negative bacteria, associated with periodontal diseases, without affecting the commensal, potentially beneficial bacteria.

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Tables

Table 1. Percentage of planktonic bacterial growth in comparison to control group according to furanone C-30 concentrations.

Variables	Furanone C-30 concentrations									
	Control	0.5 µg/mL	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	16 µg/mL	32 µg/mL	64 µg/mL	128 µg/mL
	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)
Pathogenic										
A. actinomycetemcomitans	100.0 (10.4)	-1.8 (1.2)	-6.4 (5.0)	-16.0 (7.9)	**-33.6 (19.0)***	**-53.4 (21.8)***	**-66.9 (1.7)***	**-66.5 (1.8)***	**-66.2 (1.4)***	**-65.8 (1.3)***
F. nucleatum	100.0 (11.7)	-1.7 (6.5)	-3.7 (5.6)	-7.3 (2.0)	-7.4 (1.0)	-4.5 (2.6)	-2.5 (6.9)	6.0 (10.6)	-0.3 (12.5)	7.4 (66.8)
P. gingivalis	100.0 (1.4)	2.1 (6.4)	2.0 (12.9)	-5.7 (9.6)	**-38.0 (6.9)***	**-59.5 (0.1)***	**-59.7 (2.1)***	**-57.9 (1.9)***	**-55.6 (2.0)***	**-52.9 (1.8)***
P. intermedia	100.0 (29.8)	-6.8 (29.0)	-9.9 (33.1)	-1.7 (28.7)	-16.1 (41.6)	-30.3 (38.2)	-38.4 (48.7)	**-79.0 (15.2)***	**-77.2 (17.6)***	**-75.0 (19.9)***
S. mutans	100.0 (12.2)	-1.9 (12.0)	-2.2 (10.3)	-5.7 (8.8)	-10.7 (8.4)	-14.7 (7.0)	-22.8 (10.8)	**-31.8 (13.9)***	**-46.9 (21.8)***	**-60.1 (14.5)***
S. sobrinus	100.0 (9.4)	-3.1 (10.3)	-8.4 (6.7)	-12.5 (6.4)	-8.2 (5.3)	-4.7 (9.3)	-6.3 (6.5)	-6.6 (14.1)	-5.5 (28.0)	-3.1 (29.5)
Commensal										
A. naeslundii	100.0 (17.8)	-4.7 (17.3)	-9.9 (17.5)	-14.8 (25.4)	**-26.7 (27.9)***	**-46.9 (25.1)***	**-58.1 (22.5)***	**-65.9 (7.6)***	**-65.3 (8.2)***	**-61.0 (7.9)***
A. viscosus	100.0 (7.7)	-3.2 (29.2)	-10.1 (18.5)	-28.7 (11.7)	**-45.0 (8.8)***	**-41.0 (21.4)***	**-55.1 (12.1)***	**-59.5 (10.0)***	**-59.1 (16.5)***	**-54.4 (25.6)***
S. gordonii	100.0 (2.1)	1.7 (3.4)	-4.1 (3.0)	-7.6 (3.4)	-18.8 (3.8)*	-7.9 (6.4)	8.5 (4.2)	-9.8 (34.2)	-33.1 (28.0)	**-62.4 (0.6)***
S. mitis	100.0 (6.7)	-7.0 (6.0)	-8.3 (5.2)	4.4 (6.6)	-6.9 (4.2)	-10.7 (4.2)	-4.2 (5.8)	**-24.4 (6.3)***	**-34.3 (6.2)***	**-40.1 (14.1)***
S. oralis	100.0 (5.2)	2.2 (8.7)	-5.7 (7.2)	-4.9 (7.9)	**-21.2 (10.1)***	**-37.4 (18.1)***	**-73.0 (3.6)***	**-77.5 (0.9)***	**-77.4 (2.0)***	**-76.9 (2.7)***
S. salivarius	100.0 (19.3)	-15.3 (17.2)	-8.0 (13.6)	-8.9 (17.8)	-13.0 (11.3)	-25.1 (11.9)	-26.6 (16.1)	**-36.9 (13.7)***	**-39.3 (7.7)***	**-37.2 (16.1)***
S. sanguinis	100.0 (5.3)	1.1 (11.2)	2.6 (8.5)	4.2 (6.0)	-3.6 (13.7)	-7.4 (19.6)	-6.0 (17.0)	0.2 (15.5)	-10.7 (29.2)	-10.7 (56.0)
V. parvula	100.0 (6.9)	-2.9 (6.2)	-1.2 (7.5)	-1.1 (11.7)	-5.4 (13.1)	-7.4 (13.9)	-8.9 (15.0)	-10.6 (13.4)	-11.0 (16.7)	6.0 (30.5)

Note: *Significant statistical difference in comparison to control group [%] ($p<0.05$); negative values denotes planktonic inhibition; bold values highlight 20% mean inhibition.

Table 2. Percentage of single-species biofilm formation in comparison to control group according to furanone C-30 concentrations.

Variables	Furanone C-30 concentrations									
	Control	0.5 µg/mL	1 µg/mL	2 µg/mL	4 µg/mL	8 µg/mL	16 µg/mL	32 µg/mL	64 µg/mL	128 µg/mL
	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)	%(±sd)
Pathogenic										
A. actinomycetemcomitans	100.0 (12.1)	-18.7 (43.6)	-37.6 (35.7)	-39.9 (20.8)	-50.7 (26.5)*	-70.1 (29.4)*	-87.6 (4.5)*	-88.6 (3.1)*	-86.9 (1.2)*	-86.0 (1.3)*
F. nucleatum	100.0 (32.2)	-0.8 (32.4)	-8.2 (7.4)	0.6 (10.2)	9.2 (36.6)	4.3 (43.8)	12.9 (38.4)	25.3 (21.2)	1.9 (21.3)	-53.7 (26.7)*
P. gingivalis	100.0 (14.3)	19.3 (27.9)	-12.6 (9.6)	-7.4 (30.2)	-21.5 (24.4)	-63.4 (5.1)*	-62.8 (8.0)*	-65.6 (2.5)*	-61.6 (2.5)*	-57.5 (6.4)*
P. intermedia	100.0 (34.0)	-12.5 (6.4)	-19.1 (8.5)	-24.4 (17.3)	-23.1 (24.3)	-50.8 (23.5)*	-60.8 (21.8)*	-84.6 (12.3)*	-86.9 (9.4)*	-88.8 (6.1)*
S. mutans	100.0 (35.2)	-23.3 (21.0)	-9.4 (38.8)	-20.8 (16.6)	-10.0 (53.8)	-16.0 (21.4)	-44.5 (12.9)	-43.6 (9.3)	-61.6 (22.1)*	-61.4 (20.7)*
S. sobrinus	100.0 (14.3)	-18.4 (5.9)	-9.3 (17.6)	-20.3 (6.6)	-23.7 (4.9)	-12.3 (17.6)	-26.9 (6.0)	-20.8 (43.8)	10.7 (37.2)	28.2 (24.8)
Commensal										
A. naeslundii	100.0 (38.0)	-8.5 (29.0)	-14.7 (33.8)	-14.3 (42.0)	-29.2 (40.2)	-55.3 (29.8)*	-79.6 (23.4)*	-91.1 (6.2)*	-93.6 (3.3)*	-93.9 (2.2)*
A. viscosus	100.0 (7.9)	-8.3 (17.5)	-11.7 (14.0)	-24.2 (5.8)	-14.3 (9.5)	-27.2 (5.4)	-25.3 (18.4)	-39.8 (9.3)*	-63.5 (23.0)*	-78.8 (15.7)*
S. gordonii	100.0 (4.7)	-12.3 (11.7)	-3.3 (14.2)	-12.8 (11.3)	-28.6 (13.1)*	-22.5 (2.4)	-33.0 (5.6)*	-54.1 (20.4)*	-54.9 (20.1)*	-78.0 (1.5)*
S. mitis	100.0 (12.5)	53.0 (31.3)	48.0 (60.0)	76.8 (69.1)	32.9 (63.7)	23.3 (29.8)	-2.6 (15.4)	-10.4 (31.5)	-26.3 (24.0)	-22.7 (31.9)
S. oralis	100.0 (14.7)	-3.5 (20.4)	-15.1 (13.8)	-19.1 (11.5)	-33.1 (7.2)*	-39.0 (21.4)*	-67.5 (11.2)*	-78.7 (4.7)*	-82.1 (6.7)*	-82.8 (3.6)*
S. salivarius	100.0 (18.1)	-6.0 (36.9)	-0.7 (51.9)	-13.1 (39.1)	-14.5 (34.9)	-32.1 (14.2)	-39.1 (14.1)	-43.6 (12.8)	-60.8 (8.3)	-55.7 (29.7)
S. sanguinis	100.0 (6.9)	2.0 (32.6)	1.0 (26.4)	24.2 (55.0)	3.4 (32.3)	-11.8 (3.7)	9.0 (4.9)	-8.5 (44.6)	-18.7 (44.4)	-25.0 (18.1)
V. parvula	100.0 (24.9)	-10.0 (18.2)	5.3 (25.9)	-3.8 (28.8)	-10.3 (28.1)	-13.1 (33.8)	-12.3 (37.3)	-21.6 (36.9)	-35.5 (34.3)*	-35.1 (35.7)*

Note: *Significant statistical difference in comparison to control group [%] ($p<0.05$); negative values denotes biofilm inhibition.

Table 3. Multi-species biofilm formation (Log10 (CFU/mL) in comparison to control group according to Furanone C-30 concentrations.

Variables	Furanone C-30 concentrations	
	10 µg/mL	128 µg/mL
	mean (±sd)	mean (±sd)
Pathogenic		
A. actinomycetemcomitans	-0.73 (0.72)*	-0.78 (0.83)*
F. nucleatum	-0.56 (1.17)	-1.19 (1.15)
P. gingivalis	-0.59 (0.52)	-1.49 (0.78)*
P. intermedia	-1.52 (1.28)*	-2.41 (0.59)*
S. mutans	-0.34 (0.68)	0.13 (1.08)
S. sobrinus	0.03 (0.73)	-0.24 (1.37)
Commensal		
A. naeslundii	-0.15 (0.77)	-0.06 (1.01)
A. viscosus	-0.18 (0.90)	-0.01 (1.12)
S. gordonii	-0.41 (0.93)	-0.06 (1.15)
S. mitis	0.32 (0.35)	0.53 (0.62)
S. oralis	0.75 (2.02)	1.32 (1.64)
S. salivarius	0.19 (2.14)	1.16 (1.90)
S. sanguinis	-0.24 (0.65)	0.03 (0.97)
V. parvula	-0.39 (0.95)	-0.03 (0.98)
Summary of Species		
Pathogenic	-0.47 (0.78)	-1.15 (0.92)*
Commensal	-0.39 (0.95)	-0.03 (0.99)

Note: *Significant statistical difference in comparison to control group ($p<0.05$), [a] prevalence relative to total biofilm growth.

2.2 Manuscript 2

Effect on multi-species biofilm formation of furanone and lactams incorporated or not on hydroxyapatite and PEEK discs

Carolina Schäffer Morsch[a]
Esteban Rodriguez Herrero[b]
Vera Slomka[b]
Juan Felipe Dumes Montero[a]
Andréa de Lima Pimenta[c]
Luiz Cláudio. A. Barbosa[d]
Wim Teughels[b]
Cesar Augusto Magalhães Benfatti[a]
[a]Center for Education and Research on Dental Implants (CEPID), Post-Graduate Program in Dentistry (PPGO), Department of Dentistry (ODT), Federal University of Santa Catarina (UFSC), Florianópolis/SC, 88040-900, Brazil[1]
[b]Department of Oral Health Sciences, KU Leuven, Kapucijnenvoer 33, 3000 Leuven, Belgium
[c] Maison Internationale de la Recherche, University of Cergy Pontoise, Laboratoire ERRMECe, 95.000 Neuville sur Oise Cedex, France
[d] Department of Chemistry, Universidade Federal de Minas Gerais, Av. Pres. Antônio Carlos, 6627, Campus Pampulha, CEP 31270-901, Belo Horizonte, MG, Brazil

[1] Present address
[2] Permanent address

Corresponding author:
Carolina Schäffer Morsch
E-mail: dracarolmorsch@gmail.com
Federal University of Santa Catarina
Center for Education and Research on Dental Implants (CEPID)
Campus Trindade, 88040-900, Florianópolis/SC, Brazil
Phone/Fax: +55 48 3721-9000

Abstract

Aim: To evaluate the effect of furanone C-30 and lactams U11, U12 and U26 solubilized in liquid medium and incorporated to sPEEK discs on multi-species biofilm formation and the cytotoxicity of modified sPEEK discs on L929 murine fibroblasts.

Material and methods: PEEK was modified through sulfonation process (sPEEK) and functionalized to incorporate furanone C-30 and lactams. The effect of antibiofilm compounds solubilized in liquid medium and incorporated to sPEEK discs on multi-species biofilm formation was evaluated by quantitative PCR, while the cytotoxicity of sPEEK containing antibiofilm agents was evaluated by MTS assay.

Results: The association of lactams and furanone C-30 at 128 μg/mL significantly inhibited biofilm formation of *F. nucleatum, P. gingivalis and P. intermedia* shifting the multi-species community composition to a more beneficial and less pathogenic state. When incorporated to sPEEK discs the inhibitory activity of antibiofilm agents on multi-species biofilms formation decreased consistently, showing rather then inhibition an increase in the biofilm formation of *S. sobrinus* and of *S. gordonii*. Only group sP/3LF128 after 7 days of exposure showed a cytotoxic effect on L929 fibroblasts resulting in 55% of viable cells.

Conclusion: The association of furanone C-30 and lactams solubilized in liquid medium is more active against periodontopathogens than commensal potentially beneficial bacteria. When incorporated to sPEEK their inhibitory activity on multi-species biofilm formation was lost. A cytotoxic effect on L929 murine fibroblasts was observed only at the longer period of exposure to the group sP/3LF128.

Keywords: Biofilm inhibition, Periodontopathogens, Furanone C-30, Lactams

1. INTRODUCTION

Biofilms are a complex microbial communites embedded in an extracellular polymeric matrix that are present on intra-oral surfaces attached to hard and soft tissues [1,2]. These well-organized three-dimensional structures have been recognized as responsible for the development of many diseases. In the oral cavity, biofilms have a role in the development of dental caries and periodontal diseases. Both are among the most prevalent diseases in humans [3].

When grown in biofilms bacteria change their phenotypic behavior. They become less susceptible to antimicrobials, by wjich they are able to resist concentrations up to a thousand times higher than concentrations that show to eradicate the same species in a planktonic state [4,5]. Microorganisms can communicate by using chemical signals and cooperate for the biofilm formation by an intercellular communication system called quorum sensing (QS) [6]. QS coordinates gene expression, which regulates virulence, adhesion and biofilm formation, through the accumulation of signaling molecules, referred to as autoinducers (AI) [7].

The development of a new generation of antimicrobials with substances that interfere with the QS, and by thus inhibit the biofilm formation has been proposed as a promising strategy [8,9]. Among the QS inhibitors (QSI) halogeno-furanones and lactams, analogous to rubrolides, were produced synthetically. They have a similar structure as the bromated furanone produced by the marine algae *Delisea pulchra* [10,11] that show to inhibit biofilm formation of *Escherichia coli, Bacillus subtilis* and *Pseudomonas aeruginosa* [12,13,14,15]. Furanones and lactams are able to modulate biofilm formation of a range of Gram-positive and Gram-negative bacteria, inhibiting single and multi-species biofilm formation of *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Porphyromonas gingivalis, Streptococcus mutans*, *Enterococcus faecalis* and *Candida glabrata* [8,9,11,16,17,18].

In order to increase the inhibitory effect of the antibiofilm compounds by inducing this effect directly on the intended place of action, QSI have been incorporated in biomaterials applied in dentistry. Furanone incorporated to dental glass ionomer cement showed an antibacterial effect against *S. mutans* and *Lactobacillus sp,* without interfering with the viability of human dental pulp cells [19]. The same effect of a furanone incorporated in a modified resin composite against biofilm formation of

S. mutans was noticed, without substantially decreasing the mechanical strengths of the composite [20].

To overcome inflammatory reactions, such as mucositis and periimplantitis, induced by biofilm accumulation on implant surfaces, furanone C-30 ((Z-)-4-bromo-5-(bromomethylene)-2(5H)-furanone) was loaded on poly (L-lactic acid) (PLLA) coating titanium surfaces. This showed a relatively long lasting antibactericidal effect against *S. aureus* and an excellent biological performance on osteoblasts *in vitro* [21]. Also, synthetic lactams proved to be useful to reduce biofilm formation of *S. mutans* on titanium surfaces [22]. These novel lactam-based antibiofilm compounds were also incorporated in poly-ether-ether-ketone (PEEK), a biocompatible polymer that is being used in implantology to produce implants, abutments and healing abutments, by a sulfonation process (sPEEK). The antibiofilm activity of those compounds was maintained after incorporation into sPEEK. They inhibited the biofilm formation of *S. mutans,* without interfering in the planktonic growth [23]. Therefore, the aim of the present study was to evaluate the effect of furanone C-30 and lactams U11, U12 and U26 on multi-species biofilm formation on hydroxyapatite discs or on incorporated PEEK discs and on cell viability of L929 murine fibroblasts.

2. MATERIALS AND METHODS

Antibiofilm Compounds

Synthetic furanone C-30 ((Z-)-4-Bromo-5-(bromomethylene)-2(5H)-furanone) was acquired from Sigma-Aldrich (St. Louis, MO, USA). Stock solution was prepared by diluting 10 mg in 1 mL 95% ethanol and stored at -20ºC. Lactams U11, U12 and U26 were prepared by a synthetic route starting from mucobromic acid (Sigma-Aldrich, Milwaukee, Wisconsin, USA) reduction with sodium borohydride. The product was submitted to a Suzuki cross-coupling reaction. Alkylidenation reaction allowed preparation of compounds used in this study, as previously described (fig 1) [8]. Lactams were diluted in dimethyl sulphoxide (DMSO; Sigma-Aldrich) to prepare a stock solution. Furanone was tested at final concentrations of 10 and 128 µg/mL, while lactams at 128 µg/mL. For the experiments where antibiofilm compounds were solubilized in liquid medium, compounds were diluted to reach the final concentrations in modified brain heart infusion broth (BHI_2) (Difco Laboratories,

Detroit, USA) [24]. For the experiments where the compounds were incorporated in the sPEEK, the final concentrations were reached during the sulfonation process.

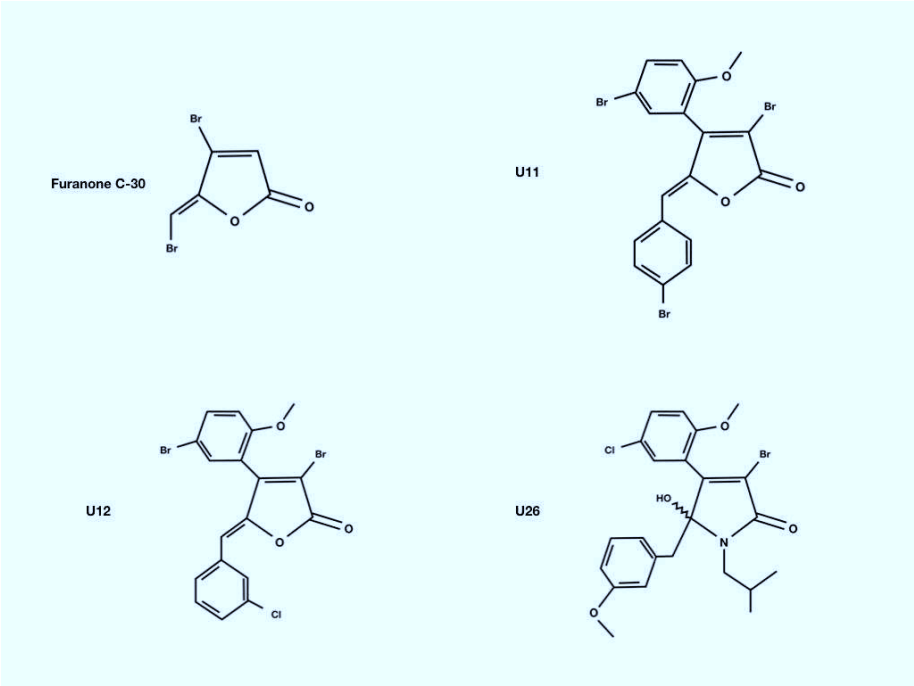

Figure 1. Chemical structures of antibiofilm compounds

PEEK functionalization and incorporation of antibiofilm compounds

Functionalization of PEEK (D0602/Optima 381G, grain shape, Victrex; England) was performed by sulfonation as previously described with minor modifications [23]. Granulated PEEK was solubilized in 98% sulfuric acid (Synth®, Brazil) under constant agitation at room temperature for 18 h. The mixture was heated up and kept at 50°C under mechanical stirring for 1h. Afterwards it was gradually trickled in 400 mL ice-cold distilled water under constant stirring, for the precipitation of the sPEEK. This preciptate was then filtered, washed with phosphate-buffered saline (PBS) until reach pH 7 and dried at 70°C for 24 h. After functionalization, the polymer was dissolved in DMSO, the antibiofilm compounds were added to the mixture and were left stirring for 1h at room temperature. Furanone C-30 and lactams from the stock solution were incorporated to sPEEK to reach final concentrations mentioned before obtaining the following groups: sP, sP/U11, sP/U12, sP/U26, sP/3L (U11, U12 and U26), sP/F10, sP/F128, sP/3LF10 (U11, U12, U26 and F10) and sP/3LF128 (U11, U12, U26 and F128).

Polystyrene discs [14mm diameter and 2mm thickness (for biofilm formation) and 5mm x 2mm (for cell viability assay)] were coated with a thin film of sPEEK with the

compounds incorporated by *dip coating* technique as described before [23]. Samples were then sterilized with ethylene oxide at 38ºC.

Bacterial Growth conditions

Both, pathogens and commensal bacteria were used in this study. As representative pathogens: *Streptococcus mutans* (ATCC 20523), *Prevotella intermedia* (ATCC 25611), *Porphyromonas gingivalis* (ATCC 33277), *Fusobacterium nucleatum* (ATCC 20482), *Aggregatibacter actinomycetemcomitans* (ATCC 43718), *Streptococcus sobrinus* (ATCC 20742). Commensal potentially beneficial species were: *Streptococcus gordonii* (ATCC 49818), *Actinomyces naeslundii* (ATCC 51655), *Streptococcus mitis* (DSM 12643), *Streptococcus salivarius* (TOVE-R), *Streptococcus oralis* (DSM 20627), *Streptococcus sanguinis* (LMG 14657), *Actinomyces viscosus* (ATCC 43327) and *Veilonella parvula* (DSM 2008). Bacterial strains were grown on blood agar plates (Oxoid, Basingstoke, UK) supplemented with 5 µg/mL hemin (Sigma-Aldrich Co, St. Louis, USA), 1 µg/mL menadione (Calbiochem-Novabiochem, La Jolla, USA) and 5% sterile horse blood (E&O Laboratories, Bonnybridge, Scotland). *A. actinomycetemcomitans, S. gordonii, S. mitis, S. mutans, S. oralis S. salivarius, S. sanguinis* and *S. sobrinus* were incubated at 37ºC under aerobic conditions (5% CO_2) while *A. naeslundii*, *A. viscosus*, *F. nucleatum*, *P. gingivalis*, *P. intermedia* and *V. parvula* under anaerobic conditions (10% Co_2, 10% H_2 and 80% N_2). Overnight liquid cultures were prepared in 10 mL BHI and incubated under the same conditions. The turbidity of each bacterial suspension was adjusted to an optical density (OD) at 600nm (GeneQuant 100, GE Healthcare, Buckinghamshire, UK) of 1.4 with fresh BHI_2.

Bioreactor derived multi-species community

The establishment of a multi-species community was set as previously described which include the use of 750 mL of BHI_2 supplemented with 5 mg/mL hemin, 1 mg/mL menadione, were added to a Biostat B Twin 1L bioreactor (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and sterilized in an autoclave [25]. The bioreactor growth conditions were stabilized (37°C, 0% O_2, 5% CO_2, pH 6.7 ± 0.1, stirring at 300rpm) and 200 µl/L of Antifoam Y-30 (Sigma, St. Louis, USA) were added. Bacterial inoculation started with 750 µl of *S. mitis* at OD_{600} 1.4; which were

let grow until late exponential phase (as determined photospectrometrically) before the remaining 13 microbial suspensions were added at OD_{600} 1.4. The chemostat culture was left to stabilize for 48h, before the medium was replaced at a rate of 200 mL/24h and kept for 5 days.

Cell Culture Protocol

L929 immortalized murine fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Brazil) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicilin/streptomycin (Gibco, USA). Culture medium were refreshed 3 times a week and cells were incubated at 37°C and 5% CO_2.

2.1 Multi-species Biofilm inhibition

Multi-species biofilms were grown on hydroxyapatite discs with compounds solubilized in liquid medium as following groups U11, U12, U26, 3L, 3LF10 and 3LF128 and also over sPEEK discs with compounds incorporated, as previously mentioned.

Hydroxyapatite and sPEEK discs were placed into 24-well plates (Grenier Bio-One, Frickenhausen, Germany). Samples were taken from the chemostat culture, diluted 1:10 in modified BHI medium and added to each well together with the compounds. The plates were incubated for 24h at 37°C, under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) stirring at 170 rpm. Discs were carefully washed with PBS to detach non-adherent cells and the remaining biofilms were disrupted by addition of 0.05% Trypsin-EDTA for 45 min under anaerobic conditions at 37ºC, at 230 rpm. Bacterial cells were harvested by centrifugation for 5 minutes at 8.000 rpm and resuspended in 500 µl of PBS.

Quantification by q-PCR

DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A quantitative PCR (qPCR) assay was performed with CFX96 Real-Time System (Biorad, Hercules, USA) using the Taqman 5' nuclease assay PCR method for detection and quantification of bacterial DNA. Taqman reaction contained 12.5 µl Mastermix (Eurogentec, Seraing, Belgium), 4.5 µl sterile distilled water, 1 µl of species-specific primer and probe and 5 µl template DNA [26]. Assay conditions for all primer/probe sets consisted of an initial 2 min at 50°C, followed by a denaturation step for 10 min at 95°C, followed by 45

cycles of 95°C for 15 sec and 60°C for 60 sec. Quantification was based on a plasmid standard curves. Experiments were performed in triplicate and repeated in three different days.

2.2 Cell Viability assay

Fibroblasts were seeded at a density of $4x10^4$ cell/cm^2 in 24-well plates. After 24h, the culture medium was refreshed and sPEEK discs with antibiofilm compounds or not (control group) were added to each well. The discs were in direct contact with cells for 1, 3 and 7 days. For metabolic activity evaluation, after each time point, the discs and medium were removed. The wells were rinsed carefully with PBS and 300 µL of culture medium and 60 µL of MTS reagent were added to each well. Plates were then incubated under the same conditions mentioned before for 2h and 30min. Contents were then homogenized and 100 µL were transfered to 96-well plates. The optical density was measured spectrophotometrically at 490 nm in a microplate reader (Molecular, Leica, EUA). Experiments were performed in technical replication triplicate.

2.3 Scanning Electron Microscopy

Hydroxyapatite and sPEEK discs with attached multi-species biofilm were rinsed carefully 3 times with PBS and fixed in 2,5% glutaraldehyde with 0,1 M sodium cacodylate buffer. Discs were rinsed 3 times in 0,1 M sodium cacodylate buffer, postfixed with 1% osmiumtetroxide and washed 2 times with distilled water. Samples were dehydrated through a series of 50, 70, 90 and 100% ethanol solutions and dried with hexamethyldisilazane (Acros Organics, Geel, Belgium). Discs were then mounted on support stubs, sputter-coated with gold and analyzed by SEM (JSM-6610LV, JEOL, Tokyo, Japan).

2.4 Statistical analysis

A two-level mixed model was separately conducted for the first, third and seventh day of the cell viability assay, and for each species on the multi-species competition assays. The models included compound and run as fixed and random factors, respectively. The restricted maximum likelihood (REML) estimation approach was used. Fitted models were evaluated according to the assumptions of homoscedasticity and residuals normality. Cell viability data were logarithmic or

squared transformed when residuals were skewed distributed. The multi-species biofilm formation data were Log_{10}-transformed from CFU/mL. Due to the presence of outliers among specific species, a bootstrapping procedure was conducted to obtain corrected standard errors (1.500 resamples). Comparisons between growing conditions and the control group were adjusted for multiple hypothesis testing (Sidak). Cell viability and biofilm results were presented, respectively, as percentage relative to the control group and Log_{10} mean difference.

3.RESULTS

3.1 Multi-species Biofilm inhibition

3.1.1 Multi-species biofilm formation on hydroxyapatite discs - antibiofilm compounds solubilized in liquid medium

As shown in figure 2, the community composition of the control group resulted in 22.9% (±1.7) of pathogens and 77.1% (±1.7) of commensal bacteria. The association of the 3 lactams and furanone C-30 at a concentration of 128 µg/mL (3LF128) significantly lowered the percentage of the sum of the six pathogenic species tested with 77.6% (±18.3), shifting the biofilm composition to 3.6% (±2.3) of pathogens and 96.4% (±2.3) of commensal bacteria. The concentration of *F. nucleatum* was reduced with 0.88 Log_{10} CFU/mL (±0.82) and of *P. gingivalis* with 0.75 Log_{10} CFU/mL (±0.16), as shown in table 1. Groups 3LF10 and 3LF128 inhibited the biofilm concentration of *P. intermedia* with 2.18 Log_{10} CFU/mL (±0.81) and with 2.62 Log_{10} CFU/mL (±0.49), respectively. In group 3LF128 *S. salivarius* was also inhibited with 1.39 Log_{10} CFU/mL (±0.16). Lactam U12 significantly inhibited *S. mitis* with 0.86 Log_{10} CFU/mL (±0.42), while lactam U26 inhibited *S. sanguinis* with 0.52 Log_{10} CFU/mL (±0.15). No significant quantitative differences were found for the other bacterial species.

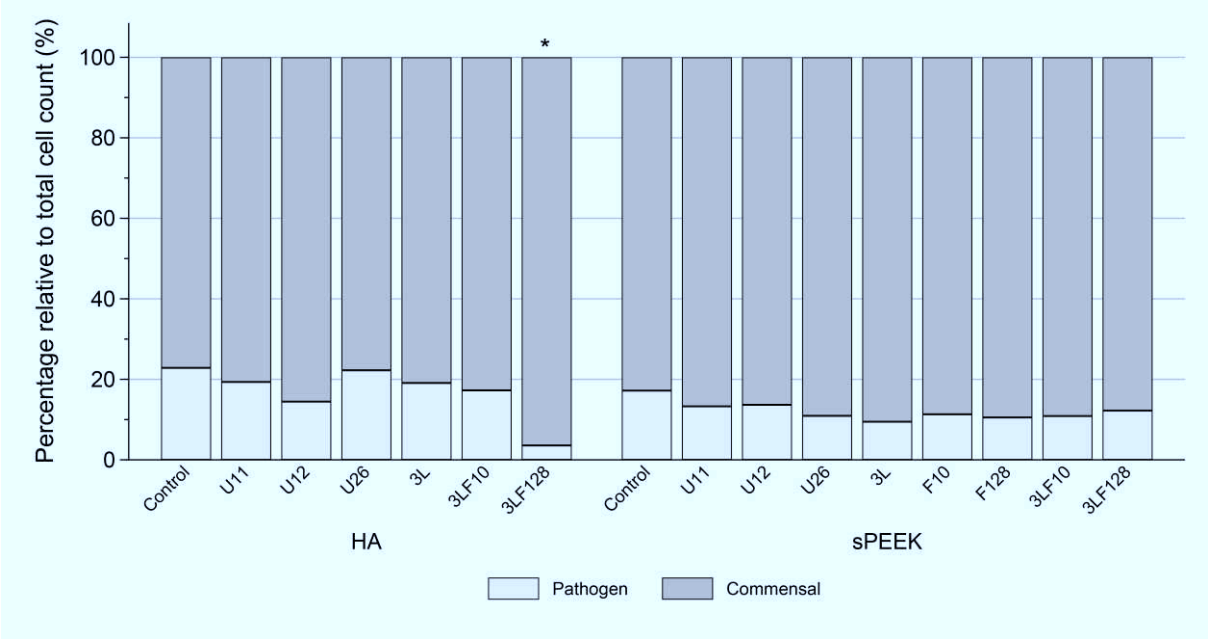

Figure 2. Effect of antibiofilm compounds on multi-species biofilm composition on hydroxyapatite (HA) and sPEEK discs (CFU/mL) in comparison to control group. Note: *Significant statistical difference in comparison to control group [%] ($p<0.05$).

Please insert table 1 here

3.1.2 Multi-species biofilm formation on sPEEK discs - antibiofilm compounds incorporated

The community composition of the control group resulted in 17.2% (±9.9) of pathogens and 82.8% (±9.9) of commensal bacteria, as shown in figure 2. No significant changes in the community composition were observed for all groups when compared to the control. As shown in table 2, only the group sP/3LF128 significantly interfered in the multi-species biofilm formation, increasing the concentration of *S. sobrinus* with 0.65 Log_{10} CFU/mL (±0.43) and of *S. gordonii* with 0.38 Log_{10} CFU/mL (±0.29).

Please insert table 2 here

3.2 Cell Viability assay

The metabolic activity of L929 fibroblasts after 1, 3 and 7 days of cell growth on sPEEK incorporated with the antibiofilm compounds compared to control sPEEK, is summarized in figure 3. An increase in the metabolic activity of the different groups after the 7 days period was observed, except for sP/3LF128 that showed a decrease

at the 7th day with 55% compared to the control ($p<0.05$). All the other tested groups did not show a statistically significant difference compared to the control over this incubation period. More than 70% of viable cells were observed among the evaluated time intervals in all the other groups. The group sP/U11 increased the metabolic activity of L929 after 24h of culture ($p<0.05$), while at the 3rd and 7th day the difference to the control group was not statistically significant.

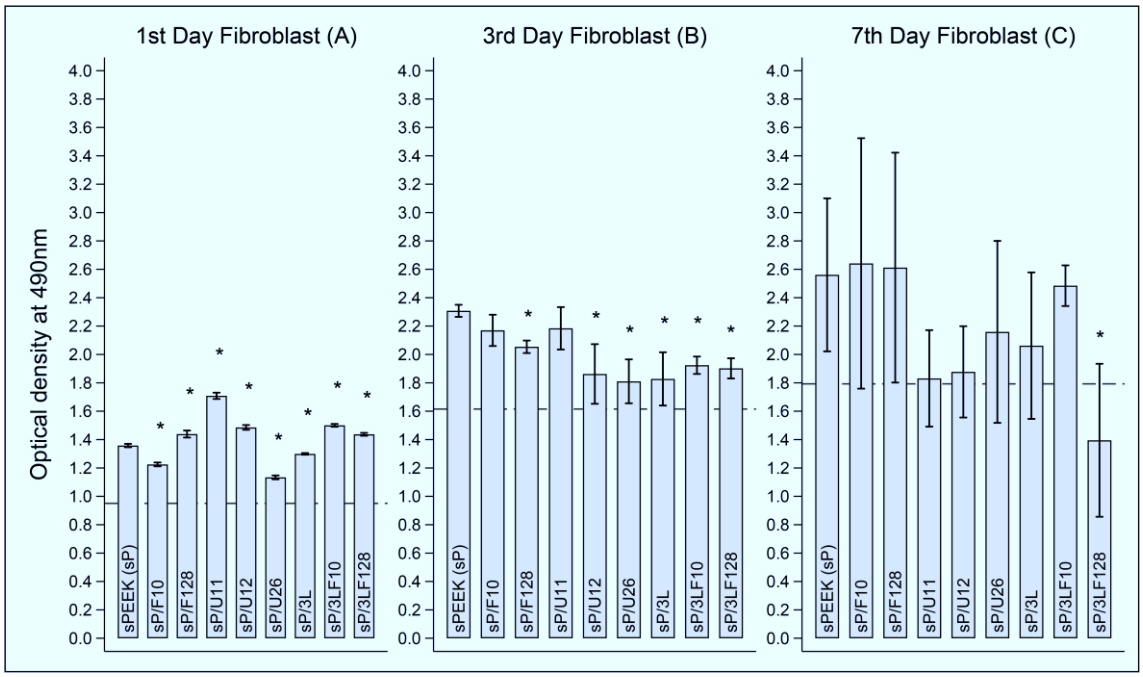

Figure 3. Metabolic activity of L929 fibroblasts after 1 (A), 3 (B) and 7 (C) days.
Note: *Significant statistical difference in comparison to control group [sPEEK] ($p<0.05$); dashed lines denotes cytotoxicity threshold (30% inhibition).

3.3 Scanning Electron Microscopy

The multi-species biofilm inhibition was not sufficient to be observable qualitatively on the SEM images from the hydroxyapatite and sPEEK discs, as shown in Fig. 4 and Fig. 5, respectively.

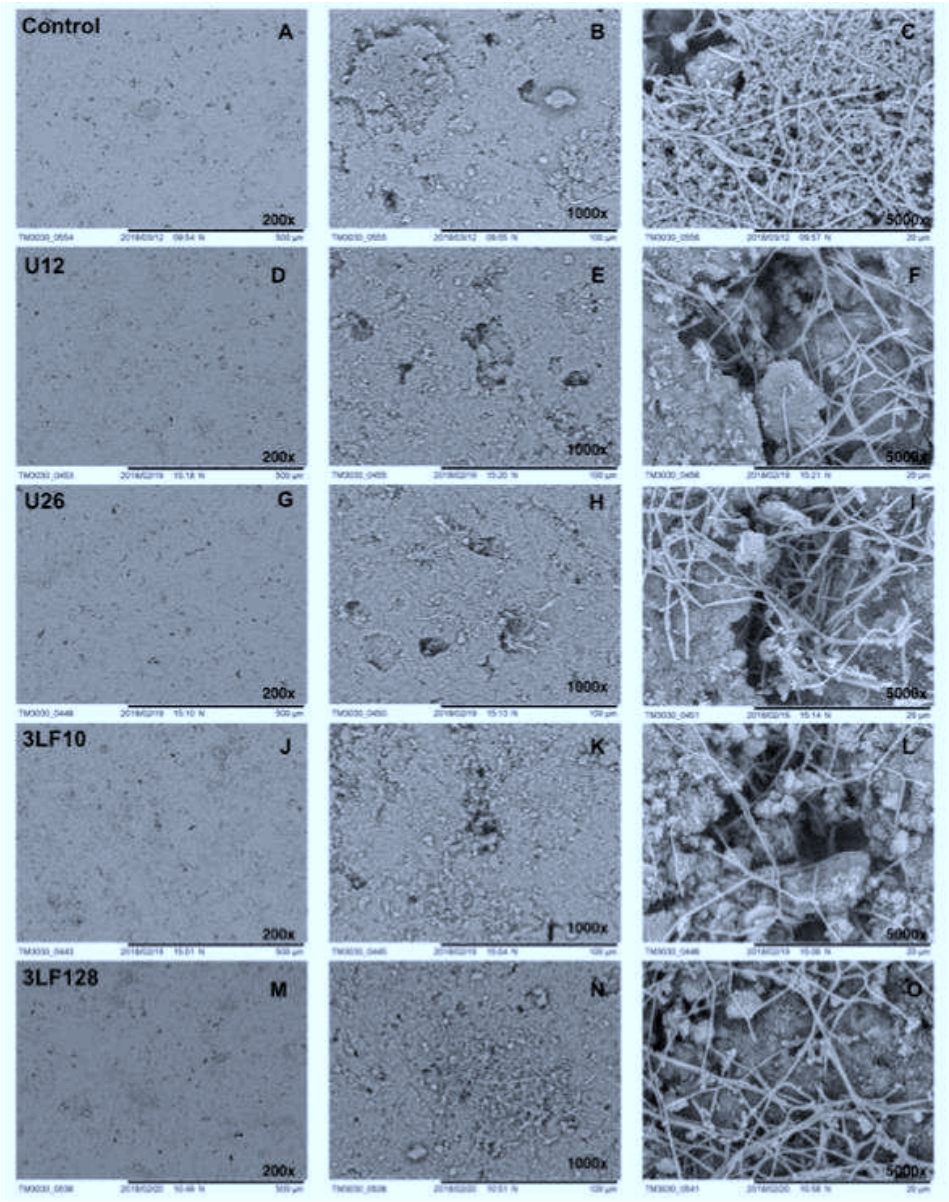

Figure 4. SEM images of multi-species biofilms on hydroxyapatite discs after 24h of growth without antibiofilm compounds (control: A, B, C); with compounds solubilized in liquid medium: U12 (D, E, F); U26 (G, H, I); 3LF10 (J, K, L) and 3LF128 (M, N, O).

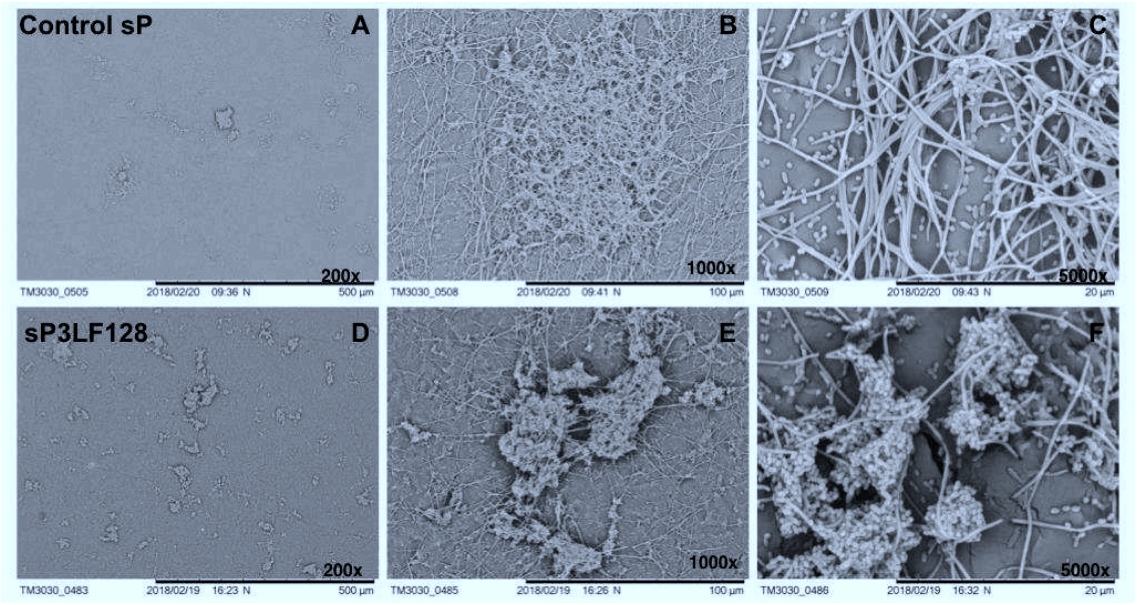

Figure 5. SEM images of multi-species biofilms on sPEEK discs after 24h of growth without antibiofilm compounds (control: A, B, C) and with the 3 lactams and furanone C-30 incorporated at a concentration of 128 µg/mL (sP/3LF128: D, E, F).

4. DISCUSSION

The development of periodontal diseases is multifactorial and biofilms are known as an essential component involved in the process. The dental biofilm community composition is complex and variable. Gram-negative species including *P. gingivalis, Treponema denticola, Tannerella forsythia* and *A. actinomycetemcomitans* are related with these diseases [2]. The present study evaluated the effect of antibiofilm compounds on biofilm formation of 14 oral bacterial species. When solubilized in liquid medium, the groups with the associations of lactams U11, U12 and U26 and furanone C-30 were the most effective, mainly against Gram-negative periodontopathogens. The group 3LF128 significantly reduced the sum of the six pathogenic species witheir the biofilms when compared to the control. By this 3LF128 shifted the biofilm composition to a more beneficial and less pathogenic state. In previous study, furanone C-30 at concentration of 128 µg/mL presented the same effect. [27].

Multi-species biofilm formation of *F. nucleatum,* a bridging specie that link early and late colonizers [28], and of *P. gingivalis*, a keystone bacterial species able to manipulate the overall bacterial population structure [2] was significantly inhibited by 3LF128 in the present study, *as* well as of *P. intermedia*. Similar results were

observed by Passoni (2018), which observed the same effect of lactams on single-species biofilm formation of those species at the same concentration [29] and from Morsch (2019) that showed that furanone C-30 at concentration of 128 µg/mL inhibited single and multi-species biofilm formation of *P. gingivalis* and *P. intermedia*, corroborating results from the present study [27]. In contrast to our data, single and multi-species biofilm formation of *S. mutans* was significantly inhibited by lactams in previous studies [29, 17, 18].

Lactam U12 significantly *S. mitis,* while U26 inhibited *S. sanguinis* in the multi-species biofilm. In contrast, previous study from our group did not observed significant inhibition of single-species biofilm formation of these species [29].

A previous study from Montero et al., 2017 evaluated the effect of the sulfonation process of PEEK on single-species biofilm formation of *S. mutans* and *Enterococcus faecalis*, concluding that the sulfonation process optimized the mechanical properties of the final material and its antibiofilm activity [30]. The same group of authors showed that the incorporation of antibiofilm compounds into the sPEEK structure does not influence the physichochemical characteristics of the modified polymers, and also confirmed the presence of the antibiofilm compounds in the sPEEK structure inhibiting single-species biofilm formation of *S. mutans* without interfering with the planktonic bacterial growth [23, 31]. In contrast to that, the effect of lactams and furanone C-30 when incorporated in sPEEK discs on multi-species biofilms formation decreased considerably in the present study, showing rather then inhibition an increase in the amount of *S. sobrinus* and of *S. gordonii*. The reduction in the antibiofilm activity in a multi-species community is reasonable and related to more complex interspecies interactions.

The effect of antibiofilm compounds incorporated to sPEEK on cell viability of L929 fibroblasts was evaluated by MTS assay. Results indicates that only the group sP/3LF128 showed a cytotoxic effect at day 7 according to the ISO 10993-5:2009(E). Consistent with our findings, the modified sPEEK containing lactam U11 at a concentration of 128 µg/mL and furanone C-30 at concentrations of 10 and 40 µg/mL was not cytotoxic against L929 fibroblasts after a exposure period of 1, 3 and 7 days [31]. Lactams U11, U12 and U26, at the same concentration tested in the present study, showed more than 70% of viable cells of Human Oral Keratinocytes (HOK-18A) after 24h of exposure, which confirms that they are not cytotoxic [29]. The effect of furanone C-30 on HOK-18A and Human Dental Pulp Cells (HDPC's) viability was

also tested in a previous study. Furanone C-30 at concentration of 10 µg/mL maintained 100% of HOK-18A cells viability and 90% of HDPC's, while at 128 µg/mL, furanone showed a cytotoxic effect against both cell types with 36,16% and 7,76% of viable cells, respectively [27]. To be suitable for clinical use antibiofilm agents should not induce a cytotoxic effect on eukaryotic cells. However, chlorhexidine digluconate (CHX), the antimicrobial agent set as gold standard in the treatment of oral diseases, has a cytotoxic effect on human fibroblasts and keratinocytes [32]. In the present study, the effect of group sP/3LF128 after 7 days of exposure resulted in 55% of viable cells. A previous study from de Almeida *et al*., 2018 observed that the metabolic activity of human fibroblasts from the periodontal ligament (PDL) after treatment with lactams was significantly higher when compared to results obtained after treatment with 0.2% CHX, that showed less then 5% of metabolic activity after 24h of incubation [18].

The present data indicate that lactams and furanone C-30 are able to inhibit multi-species biofilm formation of 14 oral species *in vitro* and have potential as adjunctive to classical periodontal therapies for the treatment of periodontal diseases. Further studies should test their inhibitory activity *in vivo*, as the oral environment has even more complex bacterial interactions than the ones simulated in the present study.

5. CONCLUSION

In conclusion, the association of furanone C-30 and lactams solubilized in liquid medium is active mainly against periodontopathogens, while when antibiofilm compounds are incorporated in sPEEK structure their inhibitory activity on multi-species biofilm formation has been lost, also a cytotoxic effect on L929 murine fibroblasts was observed only after 7 days of exposure to the group sP/3LF128.

Acknowledgements

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Variables	Compounds					
	U11	U12	U26	3L	3LF10	3LF128
	mean(±sd)	mean(±sd)	mean(±sd)	mean(±sd)	mean(±sd)	mean(±sd)
Pathogenic						
A. actinomycetemcomitans	-0.06 (0.13)	-0.05 (0.40)	-0.06 (0.35)	-0.15 (0.49)	-0.13 (0.22)	-0.40 (0.08)
F. nucleatum	-0.31 (0.67)	-0.27 (0.33)	-0.02 (0.34)	0.09 (0.30)	-0.32 (0.63)	-0.88 (0.82)*
P. gingivalis	-0.19 (0.39)	-0.37 (0.40)	-0.22 (0.29)	-0.81 (0.66)	-0.41 (0.52)	-0.75 (0.16)*
P. intermedia	-0.42 (0.90)	-0.61 (0.26)	-0.70 (0.64)	-1.70 (1.94)	-2.18 (0.81)*	-2.62 (0.49)*
S. mutans	-0.05 (0.65)	-0.04 (0.13)	-0.12 (0.28)	-0.04 (0.55)	0.37 (0.58)	0.17 (0.12)
S. sobrinus	0.01 (0.64)	-0.20 (0.50)	-0.29 (0.40)	0.06 (0.09)	-0.04 (0.46)	-0.44 (0.07)
Commensal						
A. naeslundii	-0.49 (0.77)	-0.41 (0.31)	-0.61 (0.38)	0.15 (0.20)	-0.10 (0.64)	-0.37 (0.29)
A. viscosus	-0.41 (0.65)	-0.34 (0.43)	-0.23 (0.45)	0.02 (0.45)	0.42 (0.79)	0.05 (0.57)
S. gordonii	-0.29 (0.60)	0.03 (0.75)	0.01 (0.75)	-0.62 (0.82)	-0.10 (0.51)	-0.49 (0.21)
S. mitis	-0.63 (0.35)	-0.86 (0.42)*	-0.64 (0.15)	-0.59 (0.42)	-0.32 (0.15)	-0.52 (0.35)
S. oralis	-0.15 (0.61)	-0.07 (0.25)	-0.43 (0.38)	-0.07 (0.34)	-0.27 (0.56)	-0.74 (0.56)
S. salivarius	-0.02 (0.39)	0.06 (1.00)	-0.46 (0.14)	-0.50 (1.02)	-0.72 (0.94)	-1.39 (0.16)*
S. sanguinis	-0.29 (0.64)	-0.22 (0.26)	-0.52 (0.15)*	-0.69 (0.61)	-0.13 (0.73)	0.13 (0.24)
V. parvula	-0.21 (0.77)	0.07 (0.14)	0.08 (0.70)	0.20 (0.46)	-0.12 (0.97)	0.08 (0.51)
Summary of Species						
Pathogenic	-0.31 (0.68)	-0.28 (0.33)	-0.04 (0.34)	0.07 (0.30)	-0.33 (0.63)	-0.89 (0.81)*
Commensal	-0.21 (0.77)	0.07 (0.14)	0.08 (0.70)	0.20 (0.46)	-0.12 (0.97)	0.08 (0.51)

Table 1. Multi-species biofilm formation (log10 CFU/mL) with antibiofilm compounds solubilized in liquid medium in comparison to control. Experiments on hydroxyapatite discs. Note: *Significant statistical difference in comparison to control group ($p<0.05$).

Variables	Compounds							
	U11	U12	U26	3L	F10	F128	3LF10	3LF128
	mean(±sd)	mean(±sd)	mean(±sd)	mean(±sd)	mean(±sd)	mean(±sd)	mean(±sd)	mean(±sd)
Pathogenic								
A. actinomycetemcomitans	0.19 (0.82)	0.24 (0.59)	-0.05 (0.59)	-0.05 (0.43)	-0.27 (0.40)	-0.24 (0.37)	0.26 (0.71)	0.21 (0.47)
F. nucleatum	-0.33 (1.14)	-0.46 (1.35)	-0.34 (0.69)	-0.34 (0.15)	-0.47 (0.32)	-0.11 (0.18)	-0.25 (0.47)	0.03 (0.27)
P. gingivalis	-0.16 (0.75)	-0.20 (0.80)	-0.31 (0.52)	-0.43 (0.39)	-0.46 (0.40)	-0.23 (0.42)	-0.37 (0.68)	-0.18 (0.65)
P. intermedia	-0.63 (1.22)	0.09 (0.26)	-0.43 (0.48)	-0.25 (0.08)	-0.43 (0.05)	-0.07 (0.14)	-0.09 (0.43)	0.08 (0.21)
S. mutans	0.02 (0.09)	0.13 (0.39)	-0.11 (0.15)	0.05 (0.16)	-0.20 (0.18)	-0.02 (0.13)	0.12 (0.30)	0.13 (0.20)
S. sobrinus	0.28 (0.35)	0.17 (0.61)	0.19 (0.28)	0.21 (0.23)	0.03 (0.08)	0.22 (0.19)	0.37 (0.54)	0.65 (0.43)*
Commensal								
A. naeslundii	0.20 (0.33)	-0.00 (0.73)	-0.14 (0.37)	0.03 (0.37)	-0.18 (0.16)	0.11 (0.25)	0.14 (0.42)	0.46 (0.28)
A. viscosus	0.31 (0.17)	-0.33 (0.68)	-0.24 (0.44)	-0.32 (0.35)	-0.21 (0.36)	-0.19 (0.10)	-0.19 (0.54)	0.24 (0.11)
S. gordonii	0.17 (0.44)	0.09 (0.60)	0.03 (0.42)	-0.04 (0.35)	0.08 (0.31)	-0.01 (0.37)	-0.01 (0.50)	0.38 (0.29)*
S. mitis	0.32 (0.37)	0.41 (0.39)	0.23 (0.06)	0.31 (0.32)	-0.01 (0.18)	0.18 (0.33)	0.38 (0.40)	0.21 (0.14)
S. oralis	0.24 (0.12)	0.03 (0.36)	-0.12 (0.26)	0.01 (0.39)	-0.26 (0.26)	0.04 (0.18)	0.06 (0.27)	0.20 (0.30)
S. salivarius	0.21 (0.48)	0.13 (0.71)	0.10 (0.32)	0.23 (0.38)	0.09 (0.34)	0.07 (0.32)	0.20 (0.42)	0.04 (0.47)
S. sanguinis	0.02 (0.13)	0.22 (0.39)	-0.39 (0.25)	-0.12 (0.07)	-0.22 (0.25)	-0.11 (0.11)	0.04 (0.46)	0.04 (0.10)
V. parvula	0.13 (0.18)	0.07 (0.18)	0.01 (0.06)	-0.01 (0.27)	-0.24 (0.06)	0.08 (0.21)	-0.06 (0.38)	0.19 (0.18)
Summary of Species								
Pathogenic	-0.34 (1.14)	-0.30 (1.06)	-0.34 (0.68)	-0.34 (0.14)	-0.47 (0.31)	-0.11 (0.18)	-0.25 (0.47)	0.03 (0.27)
Commensal	0.13 (0.18)	0.07 (0.18)	0.01 (0.07)	-0.01 (0.27)	-0.24 (0.07)	0.08 (0.21)	-0.06 (0.38)	0.19 (0.18)

Table 2. Multi-species biofilm formation [(og10 CFU/mL) with antibiofilm compounds incorporated in the discs in comparison to control. Experiments on sPEEK discs. Note: *Significant statistical difference in comparison to control group ($p<0.05$).

CAPÍTULO III

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