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

Luciana Solera Sales

Sistemas multiparticulados para liberação controlada de substâncias naturais com potencial biológico e farmacológico

Araraquara

2023

UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

Luciana Solera Sales

Sistemas multiparticulados para liberação controlada de substâncias naturais com potencial biológico e farmacológico

Tese apresentada à Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara para obtenção do título de Doutor em Ciências Odontológicas, área de Odontopediatria.

Orientadora: Profª. Drª. Fernanda Lourenção Brighenti
Coorientadora: Profª. Drª. Andréia Bagliotti Meneguin
Coorientador: Prof. Dr. Hernane da Silva Barud

Araraquara

2023

Sales, Luciana Solera
S163s Sistemas multiparticulados para liberação controlada de substâncias naturais com potencial biológico e farmacológico / Luciana Solera Sales. -- Araraquara, 2023
118 f.: il., tabs., fotos

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araraquara
Orientadora: Fernanda Lourenção Brighenti
Coorientadora: Andréia Bagliotti Meneguin
Coorientador: Hernane da Silva Barud

1. Cárie dentária. 2. Biofilmes. 3. Flavonoides.4. Polímeros. 5. Monoterpenos. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp.
Biblioteca da Faculdade de Odontologia, Araraquara. Dados fornecidos pelo autor(a).

Essa ficha não pode ser modificada.

Luciana Solera Sales

Sistemas multiparticulados para liberação controlada de substâncias naturais com potencial biológico e farmacológico

Comissão julgadora

Defesa para obtenção do grau de doutor em Ciências Odontológicas, área de Odontopediatria

Presidente e orientadora: Profa. Drª Fernanda Lourenção Brighenti

2º Examinador: Profa. Drª Ana Carolina Magalhães

3º Examinador: Profa. Drª Cristiane Yumi Koga Ito

4º Examinador: Profa. Drª Carla Raquel Fontana Mendonça

5º Examinador: Profa. Drª Elisa Maria Aparecida Giro

Araraquara, 08 de dezembro de 2023.

DADOS CURRICULARES

Luciana Solera Sales

NASCIMENTO: 09/08/1991 – Ilha Solteira - SP

FILIAÇÃO: José Roberto da Costa Sales e Marli Solera

2012 – 2016: Graduação em Odontologia pela Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista “Júlio de Mesquita Filho” (Unesp).

2017 – 2019 Mestrado em Odontologia – Área de concentração Odontopediatria pela Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas (Unicamp).

Dedico este trabalho a Deus, fonte de toda sabedoria e inspiração. Agradeço por me guiar ao longo desta jornada acadêmica, por conceder-me força, paciência e discernimento para realizar este trabalho.

Aos meus pais Marli e José Roberto, que me deram a vida e foram meus primeiros mestres e cujo amor incondicional, apoio e incentivo constante fizeram deste momento possível. Este trabalho eu dedico a vocês com todo meu amor e gratidão.

À minha querida vó Rosária, que é sinônimo de amor, alegria e carinho. Seus conselhos e orações me deram forças necessária para seguir meus objetivos. Este trabalho é uma homenagem à sua influência em minha vida e ao amor que compartilhamos.

Amo vocês!

AGRADECIMENTOS

À Faculdade de Odontologia de Araraquara, na pessoa do seu Diretor, Prof. Dr. Edson Alves de Campos e pelo Vice-Diretor Profa. Dra. Patrícia P. Nordi Sasso Garcia.

Ao Departamento de Morfologia e Clínica Infantil e ao Laboratório de Pesquisa em Bioquímica e Microbiológica (LPBM), pela permissão para realização desta pesquisa.

Ao Laboratório de Farmacotécnica da Faculdade de Odontologia de Ciências Farmacêuticas de Araraquara, na pessoa do Prof. Dr. Marlus Chorilli, por permitir a realização de parte desta pesquisa.

Ao Laboratório de Biopolímeros e Biomateriais (BioPolMat) da Faculdade de Araraquara, na pessoa do Prof. Dr. Hernane Barud, por oferecer todo o apoio para a realização deste trabalho

Ao Laboratório de Biomineralização da Faculdade de Odontologia de Araçatuba e ao professor Alberto Carlos Botazzo Delbem, por permitir a realização de análises importantes.

Ao Hospital Odontológico da Universidade de Birmingham, por todo o aprendizado e permissão para a realização de parte deste trabalho.

A todos os professores da Pós – Graduação, em especial aos Professores da Odontopediatria, pelos valiosos ensinamentos sobre pesquisa e docência e pelo apoio.

Aos funcionários da Seção de Pós-graduação da Faculdade de Odontologia de Araraquara, Cristiano Lamounier e José Alexandre Garcia, por toda ajuda.

A todos os funcionários da Faculdade de Odontologia de Araraquara: Reitoria, Diretoria, Biblioteca, Portaria, Limpeza, em especial aos funcionários do departamento de Morfologia e Clínica Infantil.

À CAPES: O presente trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de financiamento 001.

À FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Processo nº 2019/26066-4 e nº 2021/12931-5) pelo apoio financeiro essencial para realização dessa pesquisa.

AGRADECIMENTOS ESPECIAIS

À minha Orientadora, **Fernanda Lourenção Brighenti**, minha sincera admiração e gratidão. Obrigada pela orientação e apoio incansável ao longo deste trabalho. Sua dedicação em me incentivar e me desafiar a ir além de meus limites foi fundamental para meu crescimento acadêmico e pessoal. Muito obrigada por tudo.

À minha coorientadora **Andréia Bagliotti Meneguin**, sua contribuição foi essencial. Obrigada por toda ajuda, paciência e carinho durante a realização deste trabalho. Sempre muito cuidadosa e disposta a ajudar.

Ao meu coorientador **Hernane Barud**, por toda a sua colaboração e disponibilidade. Serei eternamente grata.

Aos meus orientadores em Birmingham **Mike Milward, Mel Grant e Sarah Kuehne**, vocês foram fundamentais durante meu período de Doutorado Sanduíche em Birmingham. Obrigada por todos os ensinamentos, e por terem me recebido tão bem durante esse período. Minha eterna gratidão.

Aos meus pais, **Marli e José Roberto**, que são minha base, sem vocês nada disso seria possível. Amo vocês mais que tudo.

À minha irmã **Ana Clara Lourenço,** por ser sinônimo de amor, carinho e companheirismo. Obrigada por sempre olhar para mim como exemplo e com orgulho. Se eu tento ser melhor a cada dia é por você.

À minha família, em especial, minhas avós **Rosária e Célia**, que sempre acreditaram no meu potencial e contribuíram para essa conquista.

À minha querida amiga **Juliana Rios de Oliveira**, por todo o apoio durante esse período. Em tão pouco tempo nos tornamos mais que amigas, irmãs. Obrigada por tudo, você é especial.

Às minhas amigas Turim, **Mariana e Lilian**, vocês foram essenciais. Muito obrigada por todos os momentos compartilhados, risadas e carinho. Vocês me fizeram amar Araraquara.

Aos meus amigos e colegas de Faculdade durante essa jornada: **Marina, Bianca, Débora, Marcela, Analú, Milena, Karina, Rafael, Elis, Paulo, Molíria**. Obrigada pela amizade, companheirismo e por todos os momentos que passamos juntos. Vocês tornaram o nosso dia a dia no laboratório mais leve e feliz.

Ao **Gabriel Nunes e Amanda Silvestre**, por me ajudarem a desenvolver este trabalho, com muita dedicação e carinho. Muito obrigada por estarem sempre dispostos a me ajudar.

Aos meus amigos do laboratório de Birmingham: **Dario, Lisa, Maria, Ben, Olga, Sandeep, Ona, Peter, Lea, Veksina, Rachel**. Minha eterna gratidão por todos os ensinamentos e por terem me acolhido tão bem. Vocês são especiais.

À minha amiga **Amanda Sardeli,** que nunca me negou uma palavra amiga e me deu todo o suporte e apoio em Birmingham. Você é calmaria nos momentos de turbulência.

Aos meus queridos amigos de infância: **Mirella, Tati, Michele, Carol, Mateus e Arthur,** que compartilharam comigo os primeiros capítulos da minha vida e se fazem presentes até hoje, mesmo com a distância. Obrigada por sempre me apoiarem.

E por fim aos meus dois anjos, meus avôs **José Solera e Roberto**, que embora não estejam mais conosco, sei que estão olhando por mim com alegria e orgulho.

Sales LS. Sistemas multiparticulados para liberação controlada de substâncias naturais com potencial biológico e farmacológico [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2023.

RESUMO

As doenças bucais biofilme-dependentes, como cárie dentária e doença periodontal, são consideradas um problema de saúde pública em todo o mundo. Nosso grupo de estudos tem pesquisado produtos naturais de origem vegetal para o controle mecênico e químico do biofilme bucal. Dessa maneira, o presente estudo teve como objetivo desenvolver, sintetizar e caracterizar in vitro sistemas multiparticulados – micro e/ou nanopartículas- baseados em polímeros naturais para liberação controlada da morina ou carvacrol e avaliá-los quanto à atividade antimicrobiana, antibiofilme e anti-inflamatória. Foram desenvolvidos dois sistemas de liberação controlada, micropartículas contendo morina e nanopartículas contendo morina ou carvacrol. As micropartículas foram obtidas a partir dos polímeros alginato de sódio e goma gelana por meio da técnica secagem por aspersão. As nanopartículas foram obtidas a partir dos polímeros alginato de sódio e quitosana por meio da técnica complexação polieletrolítica. Os sistemas foram então caracterizados quanto aos mecanismos de liberação in vitro, encapsulação das substâncias naturais e morfologia de superfície por meio da microscopia eletrônica de varredura. As nanopartículas foram ainda caracterizadas quanto ao tamanho das partículas e potencial zeta. A atividade antimicrobiana e antibiofilme das micropartículas contendo morina contra biofilme de *Streptococcus mutans* e patógenos periodontais, cultura planctônica de *F. nucletaum* e *P. gingivalis* e biofilme multi-espécies, e das nanopartículas contendo morina ou carvacrol contra biofilmes orais polimicrobianos foi avaliada por meio da viabilidade microbiana e biomassa, respectivamente, após a exposição aos diferentes tratamentos. A acidogenicidade também foi avaliada no biofilme de *S. mutans* e polimicrobiano. Para o biofilme multi-espécies, a viabilidade microbiana no biofilme também foi avaliada por meio da microscopia confocal de varredura a laser e microscopia eletrônica de varredura (MEV). Para obtenção de biofilme polimicrobiano, um inóculo obtido a partir da saliva de um voluntário previamente selecionado foi utilizado. A atividade anti-inflamatória das micropartículas foi avaliada, por meio da modulação de secreção de citocinas pró-inflamatórias. Além disso, um ensaio de citotoxicidade por meio da análise da viabilidade celular foi realizado. A microscopia eletrônica demonstrou que as micropartículas apresentam forma esférica e superfície rugosa e as nanopartículas apresentam tamanho nanométrico, com formato esférico e superfície lisa. As micropartículas controlaram melhor a liberação das substâncias naturais quando comparada com as nanopartículas. As micropartículas contendo morina e as nanopartículas contendo morina ou carvacrol reduziram significativamente a biomassa dos biofilmes, a acidogenicidade e a viabilidade de *S. mutans* e a viabilidade de bactérias totais, acidúricas e estreptococos do grupo mutans, respectivamente. As micropartículas contendo morina reduziram significativamente a biomassa e viabilidade microbiana dos patógenos periodontais (cultura planctônica e biofilme multi-espécies) em comparação com o grupo controle, confirmado pelas imagens do MEV e confocal. As micropartículas contendo morina também atenuaram a secreção de citocinas pró-inflamatórias e não foram tóxicas para as células. Conclui-se que as técnicas utilizadas para obtenção de sistemas multiparticulados são eficazes para o desenvolvimento de sistemas à base de polímeros naturais para liberação de substâncias naturais. Além disso, os resultados

mostraram que a morina e o carvacrol podem ser uma opção promissora para o controle de fatores de virulência das doenças bucais biofilme-dependentes.

Palavras-chave: Cárie dentária. Biofilmes. Flavonoides. Polímeros. Monoterpenos.

Sales LS. Multiparticulate systems for natural substance-controlled release with biological and pharmacological potential [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2023.

ABSTRACT

Biofilm-dependent oral diseases, dental caries and periodontal disease, are considered a public health problem worldwide. Our study group has been researching natural products of plant origin for mechanical and chemical control of oral biofilm. The aim of this study was therefore to develop, synthetize and characterize in vitro multiparticulate systems based on natural polymers for morin and carvacrol controlled release and to assess their antimicrobial, antibiofilm and anti-inflammatory activity. Two controlled release systems were developed: microparticles containing morin and nanoparticles containing morin or carvacrol. The microparticles were obtained from the polymers sodium alginate and gellan gum using the spray drying technique. The nanoparticles were obtained from the polymers sodium alginate and chitosan using the polyelectrolytic complexation technique. The systems were then characterized in terms of in vitro release mechanisms, natural substances encapsulation and surface morphology using scanning electron microscopy. The nanoparticles were also characterized in terms of particle size and zeta potential. The antimicrobial and antibiofilm activity of the microparticles containing morin against *Streptococcus mutans* biofilm and periodontal pathogens, planktonic culture of *F. nucletaum* and *P. gingivalis* and multi-species biofilm, and of the nanoparticles containing morin or carvacrol against polymicrobial oral biofilms was evaluated by means of microbial viability and biomass, respectively, after exposure to the different treatments. Acidogenicity was also assessed in *S. mutans* and polymicrobial biofilms. For the multi-species biofilm, microbial viability in the biofilm was also assessed using confocal laser scanning microscopy and scanning electron microscopy (SEM). To obtain a polymicrobial biofilm, an inoculum obtained from the saliva of a previously selected volunteer was used. The microparticles anti-inflammatory activity was assessed by modulating the secretion of pro-inflammatory cytokines. In addition, a cytotoxicity test was carried out by analyzing cell viability. Electron microscopy showed that the microparticles were spherical in shape and had a rough surface, while the nanoparticles were nanometric in size, spherical in shape and had a smooth surface. The microparticles controlled the release of the natural substances better than the nanoparticles. Microparticles containing morin and nanoparticles containing morin or carvacrol significantly reduced biofilm biomass, acidogenicity and viability of *S. mutans* and viability of total bacteria, aciduric bacteria and mutans streptococci, respectively. Microparticles containing morin significantly reduced the biomass and microbial viability of periodontal pathogens (planktonic culture and multi-species biofilm) compared to the control group, as confirmed by SEM and confocal images. The microparticles containing morin also attenuated the secretion of pro-inflammatory cytokines and were not toxic to the cells. It is concluded that the techniques used to obtain multiparticulate systems are effective for the development of systems based on natural polymers for natural substances release. In addition, the results showed that morin and carvacrol could be a promising option for controlling virulence factors of biofilm-dependent oral diseases.

Keywords: Dental Caries. Biofilms. Flavonoids. Polymers. Monoterpenes.

SUMÁRIO

1 INTRODUÇÃO

As doenças bucais biofilme-dependentes, cárie dentária e doença periodontal, estão entre as doenças mais prevalentes do mundo, sendo consideradas a principal causa de perda dentária[1,2]. Dados do último levantamento epidemiológico demonstram que a prevalência de cárie no Brasil ainda é bastante alta: aos 5 anos de idade, a prevalência da doença é de 49,20%, entre 12 e 19 anos é de 66,7% e na idade adulta atinge 94,8% da população. Para a doença periodontal, a prevalência é de 50,1% para a faixa etária de 15 a 19 anos, 82,2% para os adultos entre 35 e 44 anos e 98,2% para os idosos entre 65 e 74 anos[3]. Apesar dessas doenças estarem associadas a populações que apresentam nível socioeconômico mais baixo e desvantagem social[1,4] países desenvolvidos também sofrem com o problema. A prevalência em crianças de 5 e 6 anos em países que apresentam alta renda é de 49%. Aos 12 anos a prevalência nesses países diminui para 46,6%[5]. Em relação a doença periodontal, a prevalência em todo mundo é de 20 a 50%, sendo mais comum em idosos e na população de países de alta renda[6]. Essa alta prevalência das doenças acarreta na redução da qualidade de vida da população[7,8] e em altos gastos para a sociedade – por ano, bilhões de dólares são gastos para o tratamento da cárie dentária e da doença periodontal[9].

Para que ocorra o desenvolvimento dessas doenças, os microrganismos presentes na cavidade bucal precisam aderir à superfície dentária e formar biofilmes[10]. O biofilme pode ser definido como um grupo de microrganismos envoltos por uma matriz ligada a superfície do dente[11]. Todo biofilme apresenta em comum uma matriz extracelular de polissacarídeos, proteínas e algumas vezes ácidos nucléicos; sua formação se dá por sinais ambientais bacterianos e por fim oferece proteção microbiana contra antibióticos, estresses ambientais, entre eles as respostas imunológicas do hospedeiro[12]. A composição microbiana dos biofilmes é que determina se podem ser considerados não patogênicos ou patogênicos, relacionados com a cárie dentária ou doença periodontal.

Para a doença cárie, as espécies de *Streptococcus sanguinis*, *Streptococcus oralis*, *Streptococcus mitis* e *Actinomyces* spp. são as responsáveis pela colonização inicial da superfície dentária. Essas espécies são capazes de produzir ácidos a partir do açúcar ingerido, diminuindo o pH bucal. Entretanto, esses ácidos são facilmente

neutralizados pelo mecanismo homeostático da cavidade bucal, que incluem o tamponamento por sistemas tampões salivares e a metabolização de arginina ou uréia em amônia[13,14]. Entretanto, se a ingestão do açúcar for frequente, os mecanismos homeostáticos não são capazes de manter o pH próximo à neutralidade, dando início à doença e à desmineralização do esmalte. Se o pH continuar em níveis baixos, ocorrerá a substituição dessas espécies de bactérias por *Streptococcus mutans* e *Lactobacillus* spp., que são espécies acidúricas e toleraram pH menores do que 4, promovendo a progressão da doença e, eventualmente, a formação da lesão[15].

Em condições de saúde periodontal, os microrganismos estimulam o sistema de defesa inato no periodonto, que controla a colonização bacteriana dos tecidos periodontais próximos ao epitélio do sulco gengival. O epitélio pode ser considerado uma barreira entre o ambiente externo e os tecidos do hospedeiro[16]. O desequilíbrio ocorre quando acontece o acúmulo de placa e uma resposta exagerada do hospedeiro, levando a substituição de bactérias Gram positivas, aeróbicas e imóveis por Gram negativas, anaeróbicas e móveis[16-18]. A primeira espécie relacionada com a doença periodontal são as do complexo verde, amarelo e roxo, como *Aggregatibacter actinomycetemcomitans.* O aparecimento dessa espécie permite a colonização de bactérias do complexo laranja, como *Fusobacterium nucleatum*, e do complexo vermelho, como *Porphyromonas gingivalis*, levando a progressão da doença[19].

O controle mecânico associado ao controle químico do biofilme bucal é uma estratégia importante e efetiva para o controle do biofilme oral, consequentemente das doenças biofilme-dependentes, como a cárie dentária e doença periodontal. Entretanto, um controle mecânico satisfatório ainda é muito negligenciado pela população, seja por falta de habilidades motoras ou por falta de conhecimento e motivação. Além disso, a clorexidina, substância mais reconhecida para o controle químico do biofilme, apresenta uma série de efeitos colaterais, como alteração do paladar, manchamento dos dentes, irritação gengival e sensibilidade dentária[20], além de não ser capaz de controlar biofilmes maduros[21]. Como alternativa ao uso da clorexidina e como um complemento da remoção mecânica, as substâncias naturais emergem como uma alternativa promissora. O uso de produtos naturais de origem vegetal tem sido bastante pesquisado e tem apresentado resultados promissores para o controle do biofilme oral [22-24].

A morina (3, 5, 7, 2 ′, 4′-penta-hidroxifavona), um flavonóide de coloração amarela obtida de plantas, frutas e ervas pertencentes a família Moraceae[25,26], é

bastante conhecida por seu efeito antimicrobiano, principalmente por sua capacidade de inibir bactérias Gram positivas, por apresentar atividades farmacológicas, incluindo atividade anti-inflamatória, antioxidante, capacidade de eliminar radicais livres e apresentar efeito anticâncer, antiartrite, gastroprotetor, neuroprotetor, cardioprotetor e hepatoprotetor. Acredita-se que as propriedades farmacológicas da morina estejam relacionadas com a modulação de várias vias de sinalização celular[26]. Em relação às propriedades antimicrobianas, um estudo de Green et al.[27] (2012) mostrou que a morina impediu o desenvolvimento do biofilme *Streptococcus pyogenes*, resultando na redução da biomassa desse biofilme. A morina também se mostrou eficaz em reduzir a formação de biofilme de *S. mutans*, devido a inibição de SrtA, um gene relacionado com a adesão do microrganismo à superfície dentária[28]. Adicionalmente a morina apresenta citotoxicidade muito baixa[26].

O carvacrol (2-metil-5- (1-metiletil) -fenol), um fenol monoterpênico, é um constituinte natural encontrado em muitas plantas aromáticas, incluindo tomilho e orégano[29,30]. Ele apresenta propriedades biológicas e farmacológicas, sendo atividades antioxidantes, anti-inflamatórias, antibacterianas e antifúngicas[31]. Estudos já demostraram que essa substância natural apresenta atividade antimicrobiana contra algumas espécies de bactérias Gram-negativas e Gram-positivas[32,33]. Além dessas características, o carvacrol apresenta baixa toxicidade[34].

Entretanto, ambas as substâncias apresentam estabilidade limitada, sendo degradadas facilmente ou metabolizadas em compostos inativos. Na boca, a presença de forças musculares, como fala, mastigação e deglutição e a presença da saliva acelera não só a degradação das substâncias em sua forma livre como são responsáveis pela sua rápida remoção da cavidade bucal. Além disso, essas substâncias apresentam baixa solubilidade nos fluidos gengivais e saliva, o que pode diminuir a biodisponibilidade oral e consequentemente a distribuição das substâncias no local desejado[35]. Para melhorar essas características, um estudo recente desenvolveu sistemas mucoadesivos de liberação controlada contendo morina Esses sistemas utilizam alginato de sódio e goma gelana, dois polímeros naturais amplamente utilizados para o desenvolvimento de sistemas de liberação controlada. Os resultados desse estudo demonstraram que os sistemas desenvolvidos controlaram as taxas de liberação, apresentaram mucoadesividade e demonstram capacidade em controlar fatores de virulência de biofilmes cariogênicos[37,38].

Entretanto, esses sistemas apresentam diâmetro de 10 a 15 mm e precisam ser aprimorados para aumentar sua aplicabilidade. Uma das alternativas para superar esse desafio seria o desenvolvimento de sistemas multiparticulados utilizando polímeros naturais. Alginato, goma gelana e quitosana são exemplos de polímeros naturais amplamente utilizados na indústria alimentícia e farmacêutica[39].

O uso desses polímeros apresenta diversas propriedades interessantes para o desenvolvimento de um produto para uso bucal, a saber: mucoadesividade, que impede a remoção das substâncias pela saliva[40]; capacidade de liberação controlada de substâncias, mantendo uma distribuição uniforme, aumentando a eficácia do tratamento e diminuindo a toxicidade, baixo custo, baixa toxicidade, biodegradabilidade e capacidade de melhorar a solubilidade de substâncias hidrofóbicas[41]. Dentre as técnicas de obtenção de sistemas multiparticulados, merece destaque a complexação polieletrolítica, uma técnica já bastante conhecida e consolidada na literatura e a secagem por aspersão (“spray drying”), que, apesar de não ser uma técnica muito nova, é considerada uma tecnologia que está em constante evolução; por isso, pesquisas sobre novos materiais que podem ser processados por essa técnica tem sua importância para consolidar essa tecnologia[42].

A complexação polieletrolítica se baseia no uso de polieletrólitos, qualquer material macromolecular que se dissocia em uma molécula polimérica altamente carregada ao ser colocado em um solvente ionizante, formando uma cadeia polimérica carregada positivamente (como, por exemplo, a quitosana) ou negativamente (como, por exemplo, o alginato de sódio)[43]. Quando os polieletrólitos com um ou mais íons de carga oposta se juntam, eles formam o complexo eletrolítico. Essa metodologia tem ganhado interesse porque o processo é simples e facilmente exequível, além de possibilitar o escalonamento do processo, pois requer pouca energia e utiliza solventes seguros para o ambiente e para o ser humano[44]. Em função dos parâmetros utilizados, a complexação polieletrolítica pode resultar na formação tanto de micropartículas quanto de nanopartículas. A possibilidade de obtenção de nanopartículas apresenta importantes diferenciais, dentre os quais se destaca: melhoria da solubilidade, potencialização da ação farmacológica, proteção contra a degradação dos fármacos e diminuição de efeitos colaterais[45].

A secagem por aspersão é um método para produção de produtos secos a partir de líquidos, como soluções, emulsões, óleos, em partículas sólidas com tamanho, forma, densidade e composição química ajustáveis. O líquido é pulverizado

na presença de ar quente, assim o solvente é evaporado enquanto o pó ultrafino é coletado[46,47]. É considerado um processo simples, reprodutível, rápido, contínuo, econômico, de etapa única e que permite condições de temperatura amena adequadas a compostos sensíveis ao calor[42]. Essa técnica tem sido bastante utilizada na indústria farmacêutica para encapsular compostos ativos hidrofílicos e hidrofóbicos, melhorando a biodisponibilidade e a liberação controlada desses componentes ativos. Essas características se devem à geração de partículas menores que os secadores convencionais[46,47].

Desse modo, acredita-se que os sistemas de liberação controlada desenvolvidos previamente têm potencial para combater o acúmulo de biofilme microbiano na cavidade bucal, a inflamação e os efeitos oxidantes resultantes da doença periodontal. Dessa forma, prevenir doenças bucais de origem microbiana como, por exemplo, a cárie dentária e a periodontite. Entretanto, é necessário aperfeiçoar esse sistema, tornando-o mais aplicável.

Sabendo -se dá importância de controlar os fatores de virulência das doenças bucais biofilme-dependentes e objetivando o desenvolvimento de sistemas multiparticulados para liberação controlada de substâncias naturais com potencial biológico e farmacológico, a presente Tese foi dividida em três publicações:

- “Development of multiparticulate systems based on natural polymers for morin controlled release.”
- “Evaluation of chitosan and sodium alginate nanoparticles containing or not morin or carvacrol for polymicrobial oral biofilm control.”
- “Anti-inflammatory and antimicrobial evaluation of multiparticulate system containing morin”.

Na primeira publicação um sistema multiparticulado baseado em alginato de sódio e goma gelana para liberação controlada da morina foi desenvolvido por meio da técnica de secagem por aspersão e avaliado quanto à atividade antimicrobiana e antibiofilme contra *S. mutans.* Na segunda publicação nanopartículas a base de quitosana e alginato de sódio contendo morina ou carvacrol foram desenvolvidas por meio da técnica complexação polieletrolítica e avaliadas quanto à atividade antimicrobiana e antibiofilme contra biofilmes orais polimicrobianos. Na terceira publicação, os sistemas à base de alginato de sódio e goma gelana contendo morina,

previamente desenvolvidos, foram avaliados quanto à atividade anti-inflamatória e antimicrobiana contra patógenos periodontais.

2 PROPOSIÇÃO

O objetivo desse trabalho foi desenvolver, sintetizar e caracterizar sistemas multiparticulados baseados em polímeros naturais para liberação controlada de substâncias naturais e avaliá-los quanto à atividade antimicrobiana, antibiofilme e anti-inflamatória.

2.1 Objetivos específicos

- Padronizar os parâmetros do spray-dryer por um planejamento fatorial experimental (publicação 1);
- Desenvolver um sistema multiparticulado baseado em polímeros naturais, alginato de sódio/goma gelana, para liberação controlada da morina (publicação 1);
- Caracterizar esses sistemas multiparticulados de liberação controlada da morina, quanto à sua morfologia e propriedades físico-químicas (publicação 1);
- Avaliar a atividade antimicrobiana e antibiofilme desses sistemas multiparticulados contendo morina, contra *S. mutans* (publicação 1);
- Caracterizar nanopartículas à base de alginato de sódio e quitosana, com ou sem morina ou carvacrol (publicação 2);
- Avaliar a atividade antimicrobiana e antibiofilme das nanopartículas, contra biofilmes orais polimicrobianos (publicação 2);
- Avaliar o potencial anti-inflamatório, por meio da modulação de secreção de citocinas pró-inflamatórias, dos sistemas multiparticulados à base de alginato de sódio e goma gelana contendo morina (publicação 3);
- Avaliar a atividade antimicrobiana, contra patógenos periodontais, dos sistemas multiparticulados à base de alginato de sódio e goma gelana contendo morina (publicação 3).

3 PUBLICAÇÕES

Este trabalho apresenta as seguintes publicações: “Development of multiparticulate systems based on natural polymers for morin controlled release**”,** “Evaluation of chitosan and sodium alginate nanoparticles containing or not morin or carvacrol for polymicrobial oral biofilm control” e “Anti-inflammatory and antimicrobial evaluation of multiparticulate system containing morin”.

3.1 Publicação 1*

Development of multiparticulate systems based on natural polymers for morin controlled release.

Luciana Solera Sales[a], Milena da Silva Gimenes[b], Andréia Bagliotti Meneguin[c], Hernane da Silva Barud[d], Jorge Alberto Achcar[e], Fernanda Lourenção Brighenti[*].

[a]Department of Morphology, Genetics, Orthodontics and Pediatric Dentistry, São Paulo State University (Unesp), School of Dentistry, R. Humaitá, 1680 – Centro, Araraquara, São Paulo, Brazil – 14801-903. luciana.solera@unesp.br

[b]Department of Morphology, Genetics, Orthodontics and Pediatric Dentistry, São Paulo State University (Unesp), School of Dentistry, R. Humaitá, 1680 – Centro, Araraquara, São Paulo, Brazil – 14801-903. gimenes.milena@yahoo.com.br

[c]Department of Drugs and Pharmaceuticals, São Paulo State University (Unesp), School of Pharmaceutical Sciences, Jaú, Km 1, CP 502, Araraquara, São Paulo, Brazil – 14800-903. abagliottim@hotmail.com

[d]Biopolymers and Biomaterials Laboratory (BioPolMat), University of Araraquara - UNIARA, 14801-340 Araraquara, SP, Brazil. hernane.barud@gmail.com

[e]Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil. achcar@fmrp.usp.br

*Corresponding author: Fernanda Lourenção Brighenti, Department of Morphology, Genetics, Orthodontics and Pediatric Dentistry, São Paulo State University (Unesp), School of Dentistry, R. Humaitá, 1680 – Centro, Araraquara, SP, Brazil - 14801-903. Tel.: +55 16 3301 6551. E-mail address: f.brighenti@unesp.br

* Este artigo foi formatado segundo as normas do periódico International Journal of Biological Macromolecules no qual foi publicado (https://www.sciencedirect.com/science/article/pii/S0141813022030483?via%3Dihub).

Declarations of interest: none

Abstract

This study aimed to develop a multiparticulate system based on sodium alginate/gellan gum polymers for morin controlled release using standardized spray-dryer parameters. A 2^4 experimental factorial design was used to standardize spray-dryer parameters. After standardization, three systems with three different proportions of the natural polymers (50:50, 25:75, 75:25; sodium alginate: gellan gum) with and without morin (control) were developed. The systems were characterized according to its morphology and physicochemical properties. Next, the systems were evaluated regarding antibiofilm and antimicrobial activity against *Streptococcus mutans*. The factorial design indicated the use of the following parameters: i) air flow rate: 1.0 m^3 /min; ii) outlet temperature: 120 °C; iii) natural polymers combination in different proportions; iiii) polymer concentration: 2%. Scanning electron microscopy showed microparticles with spherical shape and rough surface. The samples released 99.86%±9.36; 85.45%±8.31; 86.87%±3.83 of morin after 480 minutes. The systems containing morin significantly reduced *S. mutans* biofilm biomass, microbial viability and acidogenicity when compared to their respective controls. In conclusion, the spray-dryer parameters were standardized to the highest possible yield values and proved to be efficient for morin encapsulation and controlled release. Furthermore, these systems controlled important virulence factors of *S. mutans* biofilms.

Keywords: Biofilm, Morin hydrate, Sodium alginate, Gellan gum

1. Introduction

Dental caries is a chronic and progressive disease of multifactorial etiology, described as a sucrose- and biofilm-dependent disease [1]. Biofilm is formed on the tooth surface, an appropriate location for the development of organized multispecies biofilms. Frequent sugar intake promotes persistent acid production by bacteria. As a result, a pH decrease in the biofilm-tooth surface is observed. Thus, the replacement of non-pathogenic bacteria with aciduric and acidogenic bacteria takes place [2,3]. When natural homeostatic mechanisms are not able to maintain the pH near neutrality, a disruption in the ecological balance is observed and carious lesions might develop [4]. Therefore, biofilm control by tooth brushing with fluoride toothpaste is the main

approach to the therapy of biofilm-dependent oral diseases. However, appropriate biofilm control is still neglected by many individuals [5].

In this context, natural substances with antimicrobial properties have gained attention as an adjuvant to mechanical removal of biofilm and have already shown promising results in the control of oral microorganisms [6–8]. Morin (3, 5, 7, 2′, 4′-pentahydroxyflavone) is a yellow flavonoid isolated from plants, fruits and herbs from the Moraceae family [9]. Evidence suggests that morin is a bioactive compound with biological and pharmacological properties, such as antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, cardioprotective and hepatoprotective effects. In addition, studies have shown that morin inhibits microbial growth, impairs biofilm maturation, destroys cell membrane and downregulates bacterial resistance mechanisms. Furthermore, morin has low cytotoxicity [9,10].

However, like several other natural substances, morin has limited stability in water, is easily degraded and has low solubility in water. Thus, its low availability in the oral environment - which affects substance distribution in the desired location [11,12] - limits morin application. To overcome these limitations, microencapsulation of active compounds with hydrophilic polymers has shown to be an excellent alternative.

Microencapsulation protects active substances from degrading by isolating the core from the external environment. Hence, microencapsulation may increase the stability, the solubility and the availability of morin in oral fluids. Another advantage is the controlled-release capacity of these encapsulated substances, which maintains a uniform distribution in the desired location, increases treatment effectiveness and reduces the toxicity of the active substance by requiring a lower amount of drug to achieve the expected effect [13,14]. In addition, microencapsulation can mask the unpleasant taste and odor of many drugs through the addition of flavoring [15].

Natural polymers, such as sodium alginate and gellan gum, have been widely used for microencapsulation of active compounds due to interesting properties, namely low cost, non-cytotoxicity, biocompatibility, and ability to improve the solubility of hydrophobic substances [16–18]. Besides, they are bio/mucoadhesive, which prevents substances' removal by saliva or muscular forces [19].

Microencapsulation by spray-drying is widely used in pharmaceutical and food industries. Microencapsulation offers many advantages related to compound stability and quick drying, quick evaporation of the solvent without reaching the particles, reproducibility, efficiency and low cost [20,21]. However, to originate powders with

homogeneous size distribution, circularity, low humidity and high yield, spray-drying and formulation parameters needs to be optimized. Factorial design is a powerful tool for the standardization of these parameters, since it allows the evaluation of many variables using a reduced number of experimental trials, in a simple, time saving and low budget method [22].

The microencapsulation process for controlled release of the active compound has been widely explored in pharmaceutical and food industries, but it is still little explored in oral care industry. Thus, the development of a controlled release system containing morin to impair microbial biofilm growth in the oral cavity and to prevent microbial-associated oral diseases - such as dental caries [23]- is a promising approach. These systems might be combined with non-aqueous products, that are not yet available, e.g., mouthwash tablets, toothpaste tablets, chewing gum. The greatest differential of these systems to the already existing products, besides providing the development of a new and different product, is the possibility of the prolonged effect of the antimicrobial agent, with a uniform distribution in the desired location, obtained through controlled release, increasing its antimicrobial effect. Moreover, these controlled release systems have a micrometric particle and are more clinically applicable and accepted by patients than traditional products. The aims of this study were to standardize the spray-dryer parameters by an experimental factorial design [24–26] and to develop a multiparticulate system based on sodium alginate/gellan gum polymers for controlled release of morin. The microparticles were characterized according to its morphology and physicochemical properties and evaluated regarding antimicrobial and antibiofilm activity against the cariogenic bacteria *Streptococcus mutans*.

2. Materials and methods

2.1 Materials

Morin hydrate (2′,3,4′,5,7-Pentahydroxyflavone; Empirical Formula: $C_{15}H_{10}O_7 \cdot xH_2O$; M4008, purity > 85%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium alginate was purchased from Exôdo Científica (Sumaré, SP, Brazil). Gellan gum (type Kelcogel® CGLA) was kindly provided by CP Kelco (Limeira, SP, Brazil). The two anionic polysaccharides were chosen because we aimed to develop microparticles using a physical method (spray-dryer). Moreover, it is known that these

polymers form a hydrogel particle with a structured polymer network, show bio/muco adhesion and can control drugs release.

2.2 Development of microparticles based on sodium alginate and gellan gum

2.2.1 Experimental factorial design

2.2.1.1 Use of a first model in the presence of the four covariates with response yield in %

To obtain high yield multiparticulate systems, controlled drug release and high encapsulation efficiency, a first statistical multiple linear regression model with normal errors was fitted using the data of a 2^4 experimental factorial design (four independent variables with two levels), totalizing 16 experiments [24–26]. The experiments were performed in triplicate and are shown in Table 1. The chosen variable levels were as follows: air flow rate (1.0 and 1.8 m^3/min), outlet air temperature (80 and 120 °C) in the spray drying, polymer concentration (1% or 2% w/v) and polymers used (sodium alginate or gellan gum). The yield in % was chosen as the response variable and calculated by weighing the initial solids (polymers and morin added to the suspension before starting the spray-drying process) and the powder obtained after the spray-drying process. The yield was obtained by Equation 1. These variables were chosen because they are determinants in the drying process.

$$Y\% = (\mathit{Final\ solid\ weight})/(\mathit{Initial\ solid\ weight}) \times 100 \qquad \text{(Eq. 1)}$$

Table 1. Description of the 16 ordered experiments from a 2^4 factorial design used to get the responses of interest and to fit the multiple linear regression model (first model). Polymer A: sodium alginate, Polymer B: gellan gum.

Experiment	Air flow rate (m^3/min)	Temperature (°C)	Concentration (%)	Polymer
01	1.0 (-)	80 (-)	2.0 (+)	A (-)
02	1.8 (+)	80 (-)	2.0 (+)	B (+)
03	1.8 (+)	80 (-)	1.0 (-)	A (-)
04	1.0 (-)	120(+)	1.0 (-)	A (-)
05	1.0 (-)	120 (+)	2.0 (+)	B (+)
06	1.8 (+)	120 (+)	1.0 (-)	B (+)
07	1.0 (-)	120 (+)	1.0 (-)	B (+)
08	1.8 (+)	120 (+)	2.0 (+)	A (-)
09	1.8 (+)	80 (-)	2.0 (+)	A (-)
10	1.0 (-)	80 (-)	1.0 (-)	A (-)
11	1.0 (-)	80 (-)	1.0 (-)	B (+)
12	1.8 (+)	80 (-)	1.0 (-)	B (+)
13	1.0 (-)	120 (+)	2.0 (+)	A (-)
14	1.8 (+)	120 (+)	1.0 (-)	A (-)
15	1.0 (-)	80 (-)	2.0 (+)	B (+)
16	1.8 (+)	120 (+)	2.0 (+)	B (+)

2.2.1.2 Use of a second-order model in the presence of three covariates with response yield in %

Since one of the goals of this study was also to predict maximum responses (yield in %), a second-order model in the presence of three covariates (air flow rate, outlet temperature, polymer) was used. In this section, we fix the polymer gellan gum denoted by +1(B) in Table 1 (results with higher weights and higher responses) considering a central composite design with three factors (23 factorial design with two levels by adding center points). Thus, nine additional experiments were performed, as described in Table 2.

Table 2. Description of nine experiments performed for the second order model for experimental factorial design. Polymer B: gellan gum

Experiment	Air flow rate (m3/min)	Temperature (˚C)	Concentration (%)	Polymer
01	1.4 (0)	100 (0)	1.5	B
02	1.4 (0)	100 (0)	1.5	B
03	1.4 (0)	100 (0)	1.5	B
04	1.2 (-0,5)	100 (0)	1.5	B
05	1.6 (+0,5)	100 (0)	1.5	B
06	1.4 (0)	90 (-0,5)	1.5	B
07	1.4 (0)	110 (+0,5)	1.5	B
08	1.4 (0)	100 (0)	1.25	B
09	1.4 (0)	100 (0)	1.75	B

2.2.2 Preparation of morin-loaded polymeric microparticles by spray drying

After standardization assuming the experimental factorial design, three systems containing sodium alginate (ALG) and gellan gum (GG) were developed in three different proportions (50:50, 25:75, 75:25 ALG:GG) with morin. To prepare 100 mL of suspension, 2% (w/v) sodium alginate and gellan gum, in different proportions (1:1, 1:3 and 3:1), were dispersed in 100 mL of distilled water under magnetic stirring at 60 ˚C. After complete polymer dispersion, 500 mg of morin (20% of the final polymer mass) was added and homogenized. Three systems with the same polymer proportions, without morin, were prepared and used as a control group. This suspension was spray dried in a laboratory-scale spray dryer (MSD 0.5 spray dryer, LabMaq do Brasil, Ribeirão Preto, Brazil), to obtain microparticles. The following spray-drying parameters were used: atomization air pressure (5.0 bar), flow feed pump (0.3 L/h), airflow rate (1.0 m^3/min), outlet temperature (120 °C) and solid content (2%).

2.3 Evaluation of microparticles based on sodium alginate and gellan gum

The three systems were evaluated for i) yield (as described previously); ii) encapsulation efficiency and drug loading; iii) morphological and physicochemical characterization of the microparticles; and iv) antimicrobial and antibiofilm activity.

2.3.1 Encapsulation efficiency (EE, %) and drug loading (mg morin/100 mg powder)

The encapsulation efficiency and drug loading were determined by dispersing 5 mg of the powder obtained after spray drying in 6 mL of phosphate buffer (0.2M; pH

7.4). The set was maintained under magnetic stirring until complete powder solubilization. The samples were then centrifuged at 3000 rpm for 10 min and the supernatant was filtered on 0.45 µm nylon membranes prior to UV–vis (Hewlett Packard-Kayak XA) spectrophotometer quantification at 389 nm wavelengths. To quantify the morin encapsulated in the microparticles and the drug loading, calibration curves were established in the linearity range of 5 to 35 µg/mL (r^2 = 0.9958). The concentration of 35 µg/mL was used to determine the maximum absorption peak of morin, in a UV–vis (Hewlett Packard-Kayak XA) spectrophotometer.

The encapsulation efficiency and drug loading of each sample were determined using Equations 2 and 3, respectively:

EE % = ($Quantified\ morin\ mass$)/($Teorical\ morin\ mass$) X 100 (Eq. 2)

Drug loading = ($Quantified\ morin\ mass$)/($100\ mg\ powder$) X 100 (Eq. 3)

2.3.2 Morphological and physicochemical characterization of the microparticles

2.3.2.1 *In vitro* release of morin

In vitro release of morin from the systems was performed on an SR-8 Plus dissolution station (Hanson Research®, California, USA) using the mini basket apparatus (adapted apparatus I) with stirring at 50 rpm and temperature of 37 °C. Powder containing 10 mg of morin was used. The amount of powder used was determined by morin mass incorporated into the systems, obtained from drug loading. The test was performed in 400 mL phosphate buffer (0.2 M, pH 7.4) for 48 h. Aliquots (2.5 mL) were taken out at a predetermined time and replaced with fresh phosphate buffer at the same temperature. Samples were filtered with a 0.45 µm filter before analysis. The morin released was quantified by measuring the absorbance at 389 nm in a UV–vis (Hewlett Packard-Kayak XA) spectrophotometer. For each system, the experiments were performed in triplicate.

The mechanisms involved in the drug release process were analyzed through the application of several statistical non-linear models with normal errors (Baker-Londsdale, First-Order, Higuchi, Hixson-Crowell, Korsmeyer-Peppas and Weibull) with the aid of the Sigma Plot 10 software. These models were fitted throughout all release profiles, except for the Peppas and Weibull models, which used data referring to 60.0 and 63.2% of drug release, respectively.

2.3.2.2 Field emission gun-scanning electron microscopy (FEG-SEM)

The surface morphology of the systems and the size of the particles obtained were evaluated using a JEOL JSM-7000 F microscope operating at 2 kV. The samples were prepared with a thin layer deposition of carbon, to make them conductive. Photomicrographs were obtained at 500 and 5000 × amplification.

2.3.2.3 Fourier-transform infrared spectroscopy (FTIR)

The FTIR spectra were obtained using a Nicolet Nexus Spectra equipment. The analysis was conducted using 2 cm^{-1} resolution and 32 scans, in the spectral range of 400–4000 cm^{-1}.

2.3.3 Antimicrobial and antibiofilm activity of the developed systems

S. mutans UA159 reference strain was used. This strain was maintained in brain and heart infusion broth ("BHI", Himedia, Mumbai, India) with 20% glycerol at −20 ˚C. Strain reactivation was performed on BHI agar to obtain a saline suspension containing 5 x 10^7 CFU/mL, with the aid of a spectrophotometer (Biotek® Elx800, BioTek Instruments, Inc., Winooski, VT, USA). Incubation was carried out for 48 h at 37 ˚C in 5% CO_2 [27].

Multiparticulate systems containing morin and systems without morin, considered as controls, were used. All these systems were added to the wells at the initial time (t=0). The amount of powder used was determined by morin mass incorporated into the systems, obtained from drug loading. The dose chosen was 2 mg, based on results found by de Farias et al. (2020) [23].

S. mutans biofilms were grown in an active adhesion model [28]. In this model, biofilm formation occurs only with cells with the ability to adhere. Sterile glass coverslips (ø 13 mm; n=3/group) fixed in the active adherence model were attached to 24-well plates containing the microbial inoculum (2.2 mL). The inoculum was prepared in BHI broth to obtain a final concentration of 5 x 10^5 CFU/mL of *S. mutans.* After 6 h, the coverslips were changed to new wells containing BHI broth without sucrose and the systems from time zero. This intermittent sucrose exposure model was proposed by van de Sande et al. (2011) [29] and modified by Albuquerque et al. (2018) [28]. The biofilms were incubated at 37 °C in 5% CO_2 for 24 h.

To analyze the systems' antimicrobial activity, after 24 h, the coverslips were removed from the culture medium and added to glass tubes in 0.9% NaCl. The biofilms were dispersed in an ultrasonic bath (Cristófoli ultrasonic tub, Campo Mourão - PR, Brazil) for 10 s, at 42 kHz. The dispersed biofilms were analyzed for total microbial concentration on BHI agar and incubated at 37 °C in 5% CO_2 for 48 h. The results were expressed in log (1+ CFU/mL). Biofilm acidogenicity was also evaluated during the experiment by reading the pH of the used culture medium, using a specific electrode coupled to an ion analyzer (Quimis pHmeter, Diadema - SP, Brazil). The results were expressed in pH values [28].

For antibiofilm activity analysis of the systems, another biofilm set was grown under the same conditions. The systems' antibiofilm capacity was analyzed by biomass quantification (crystal violet staining) [23]. The results were expressed in absorbance intensity (A570).

2.4 Statistical analysis

The data obtained from the experimental factorial designs were analyzed using the Minitab® software. For yield, encapsulation efficiency and release, the data were not normal (Shapiro-Wilk test) and were expressed as median, minimum and maximum and analyzed by Kruskal-Wallis followed by Dunn tests using Sigma Plot software version 12.0.

For biomass quantification, microbial viability and biofilm acidogenicity, the data distribution was normal, and the data were expressed as mean ± SD. Biomass and microbial viability were analyzed by two-way ANOVA analysis of variance models followed by the Tukey post-test using the SigmaPlot software version 12.0 (SigmaPlot, Systat Software Incorporation, CA, USA). The two independent variables were the systems (50:50, 25:75 and 75:25 ALG:GG) and type of treatment (control and treatment). For biofilm acidogenicity, the data were analyzed with two-way repeated measures ANOVA using Jamovi project (2021), version 1.6. The two independent variables (systems: 50:50, 25:75 and 75:25 ALG:GG; type of treatment: control and treatment) were analyzed at two different times (6 and 24 h). The assumed level of significance was 5% for all cases.

3. Results

3.1 Statistical data analysis obtained from experimental factorial designs

3.1.1 Use of a first model in the presence of the four covariates with response yield in %

The experiments were performed in triplicate using a 2^4 experimental factorial design (four variables and two levels), totalizing 16 experiments [24–26] with a response variable given by yield in %. The factors possibly affecting the response variable were air flow rate (1.0 and 1.8 m^3/min), outlet temperature (80 and 120 ^oC) in spray-drying, polymer concentration (1% or 2%) and polymers used (sodium alginate or gellan gum). Figure 1 shows the scatter plots of the response yield in % (from the average of the three replicates) against each factor (air flow rate, outlet temperature, polymer concentration and polymers). From the plots of Figure 1, we concluded that, apparently, an air flow rate increase (–1 to +1) decreased yield in %; similarly, from –1 to +1 in outlet temperature, polymer concentration and polymer factors, there was an increase in yield in %.

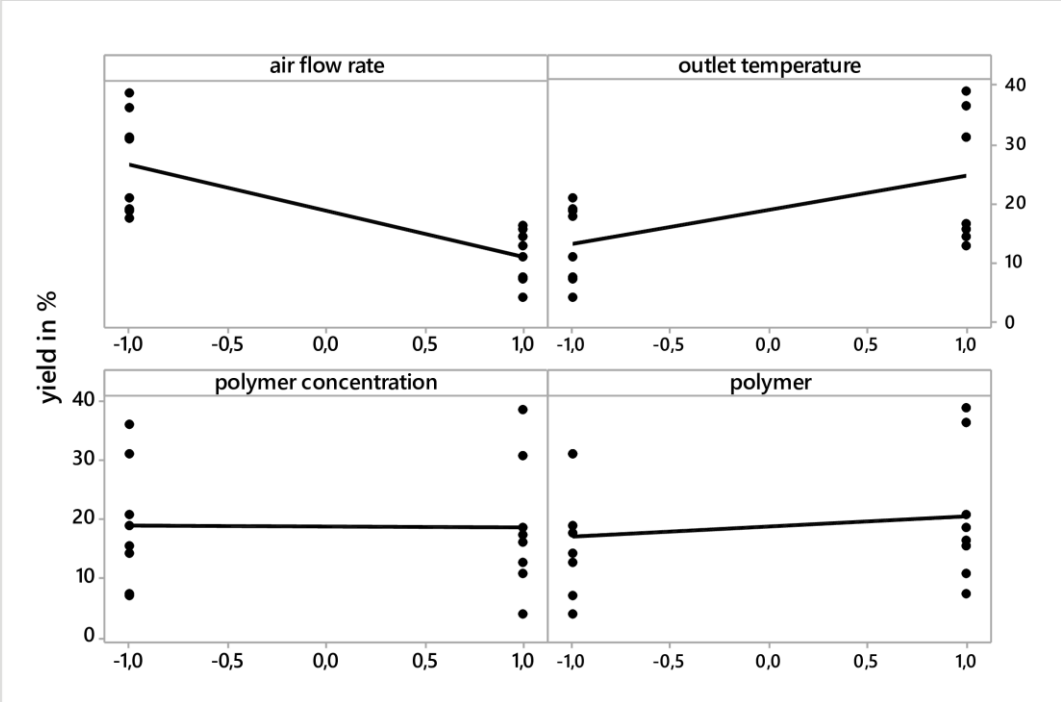

Fig. 1. Scatter plots of the response yield in % average against each factor (air flow rate, outlet temperature, polymer concentration and polymers).

As first statistical data analysis, we assumed only the data of the 24 experimental factorial design (Table 1). With this data set, we fitted a first-order multiple linear regression model [30] in the presence of the four factors and their two-by-two

interactions affecting the response yield in %. Table 3 shows the least-squares estimators (LSE) of the regression parameters, the standard errors (SE) of the estimators, the student-t statistics values and their respective p-values. The needed assumptions for the inferences using the linear regression model (normality and constant variance for the residuals) were verified from residual plots.

Table 3. LSE and p-values (2^4 experimental factorial design)

Factor	LSE	SE	T-Stat	p-value
Constant	18.826	0.505	37.25	0.000
Air flow rate	-7.812	0.505	-15.46	0.000
Outlet temperature	5.687	0.505	11.25	0.000
Polymer concentration	-0.091	0.505	-0.18	0.865
Polymer	1.747	0.505	3.46	0.018
Air flow x temp	-1.981	0.505	-3.92	0.011
Air flow x conc	0.072	0.505	0.14	0.892
Air flow x polymer	-0.253	0.505	-0.50	0.638
Temp X con	0.303	0.505	0.60	0.575
Temp X polymer	0.463	0.505	0.92	0.402
Conc X polymer	0.663	0.505	1.31	0.247

The fitted linear multiple regression model was given by yield in % = 18.826 − 7.812 air flow rate + 5.687 outlet temperature − 0.091 polymer concentration + 1.747 polymer −1.981 air flow x temp + 0.072 air flow X conc − 0.253 air flow X polymer + 0.303 temp X con + 0.463 tempXpolym + 0.663 concXploim (1).

Table 3 shows that three factors (air flow rate, outlet temperature, and polymers) had significant linear effects on the response yield in % (p-values < 0.05). We also observed that the interaction air flow rate x outlet temperature also had a significant effect on the response yield in % (p-value < 0.05). All other two-by-two interactions did not show significant effects on the response yield in % (p-values > 0.05).

Figure 2 shows the plots of the significant interaction (air flow rate x outlet temperature) indicating that the air flow rate effect was negative. The yield in % decreased when the air flow rate went from lower (−1) to higher (+1) levels, but the effect was greater at higher outlet temperature (1).

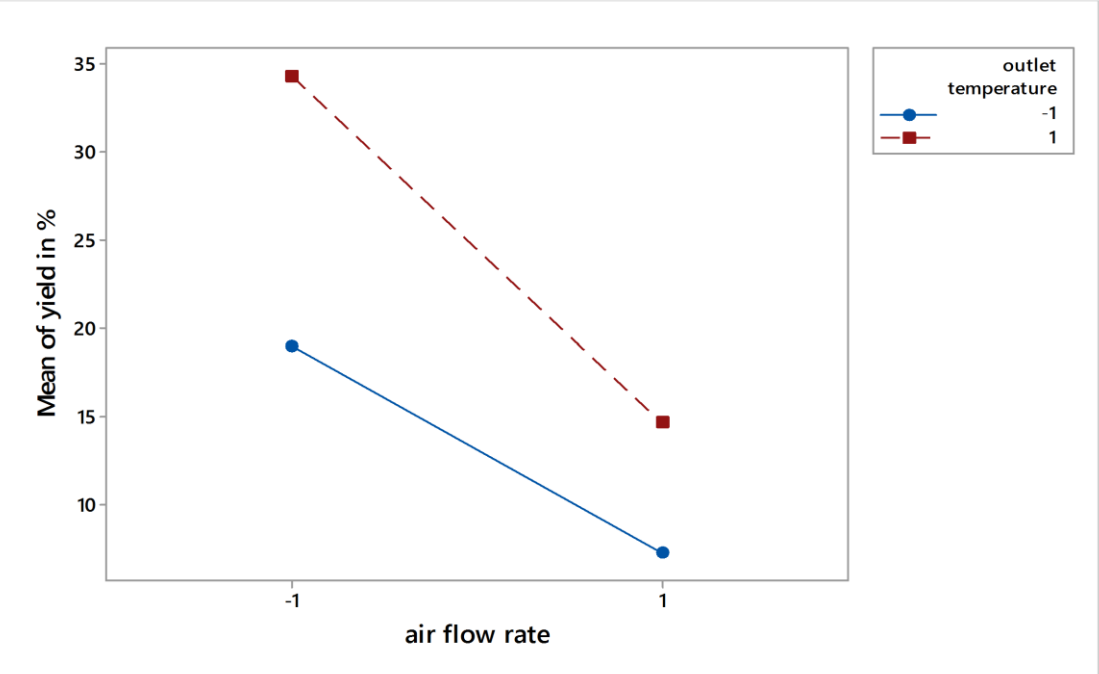

Fig. 2. Interaction plot (air flow rate × outlet temperature).

3.1.2 Use of a second-order model in the presence of three covariates with response yield in %

With this class of experimental designs, it is possible to obtain more accurate inference results including curvature factors in the response surface (quadratic effects of the covariates). In this way, we consider the replicate data obtained from a 23 factorial design (24 experiments) and nine additional experiments in the center of the variability of the factors (a total of 33 observations). The obtained data of this central composite design are presented in Table 4. In other words, we used a central composite design, that is, a factorial or fractional factorial design with center points, augmented with a group of axial points that made it possible to estimate the curvature. With the use of a central composite design, we efficiently estimated the first- and second-order terms and the fitted model of a response variable with curvature by adding center and axial points to a previously created factorial design.

Table 4. Results of a central composite design with three factors (air flow rate, outlet temperature, polymer concentration) with response yield in %

Row	Air flow rate	Outlet temperature	Polymer concentration	Yield in %	Estimated yield %
1	-1.0	-1.0	-1.0	21.70	19.8231
2	1.0	-1.0	-1.0	9.00	7.8485
3	-1.0	1.0	-1.0	34.90	37.4997
4	1.0	1.0	-1.0	12.00	14.5000
5	-1.0	-1.0	1.0	19.10	19.4225
6	1.0	-1.0	1.0	12.50	9.5895
7	-1.0	1.0	1.0	36.50	38.2240
8	1.0	1.0	1.0	15.50	17.3660
9	-1.0	-1.0	-1.0	15.70	19.8231
10	1.0	-1.0	-1.0	7.00	7.8485
11	-1.0	1.0	-1.0	41.20	37.4997
12	1.0	1.0	-1.0	19.00	14.5000
13	-1.0	-1.0	1.0	20.85	19.4225
14	1.0	-1.0	1.0	9.50	9.5895
15	-1.0	1.0	1.0	40.50	38.2240
16	1.0	1.0	1.0	14.25	17.3660
17	-1.0	-1.0	-1.0	25.40	19.8231
18	1.0	-1.0	-1.0	6.00	7.8485
19	-1.0	1.0	-1.0	32.70	37.4997
20	1.0	1.0	-1.0	15.40	14.5000
21	-1.0	-1.0	1.0	15.65	19.4225
22	1.0	-1.0	1.0	10.70	9.5895
23	-1.0	1.0	1.0	39.45	38.2240
24	1.0	1.0	1.0	19.25	17.3660
25	0.0	0.0	0.0	33.10	29.8545
26	0.0	0.0	0.0	34.90	29.8545
27	0.0	0.0	0.0	31.80	29.8545
28	-0.5	0.0	0.0	27.50	25.9152
29	0.5	0.0	0.0	12.40	17.7071
30	0.0	-0.5	0.0	22.50	29.5295
31	0.0	0.5	0.0	39.20	35.8928
32	0.0	0.0	-0.5	29.50	32.4030
33	0.0	0.0	0.5	32.20	33.0193

With this data set, we fitted a second-order multiple linear regression model in the presence of the three factors, their two-by-two interactions and the quadratic effects of the three factors affecting the replicated response yield in %. Using the Minitab® software, Table 5 shows the least-squares estimators (LSE) of the regression

parameters, the standard errors (SE) of the estimators, the student-t statistics values and their respective p-values. The needed assumptions for the inferences using the linear regression model (normality and constant variance for the residuals) were verified from residual plots.

Table 5. LSE and p-values (second order multiple regression model)

Factor	LSE	SE	T-Stat	p-value
Constant	29.85	1.32	22.68	0.000
Air flow rate	-8.208	0.754	-10.89	0.000
Outlet temperature	6.363	0.754	8.44	0.000
Polymer concentration	0.616	0.754	0.82	0.422
Air flow x temp	-2.756	0.762	-3.62	0.001
Air flow x conc	0.535	0.762	0.70	0.489
Temp x conc	0.281	0.762	0.37	0.715
Air flow**2	-32.17	8.63	-3.73	0.001
Temp**2	11.43	8.63	1.32	0.199
Conc**2	11.43	8.63	1.32	0.199

The fitted second-order linear multiple regression model is given by yield in % = 29.85 − 8.208 air flow rate + 6.363 outlet temperature + 0.616 polymer concentration − 2.756 air flow X temp + 0.535 air flow X con + 0.281 temp X conc − 32.17 air flow**2 + 11.43 temp **2 + 11.43 conc**2 (2).

From the results of Table 4, we concluded that

- The linear effects of the air flow rate and outlet temperature were significant on the response yield in % (p-values < 0.05).
- The two-by-two interaction air flow rate x outlet temperature was significant on the response yield in % (p-value < 0.05). All other interactions were not significant (p-values > 0.05).
- The quadratic effect of air flow rate (squared air flow rate) had a significant effect on the response yield in % (p-value < 0.05).

From the estimated second-order model (2), we could obtain the predictions of interest by fixing different values for the three factors (air flow rate, outlet temperature, and polymer concentration). Figure 3 shows the plots of the observed yield in % responses and the estimated means using model (2) versus order, where an excellent fit of the model to the data set was observed.

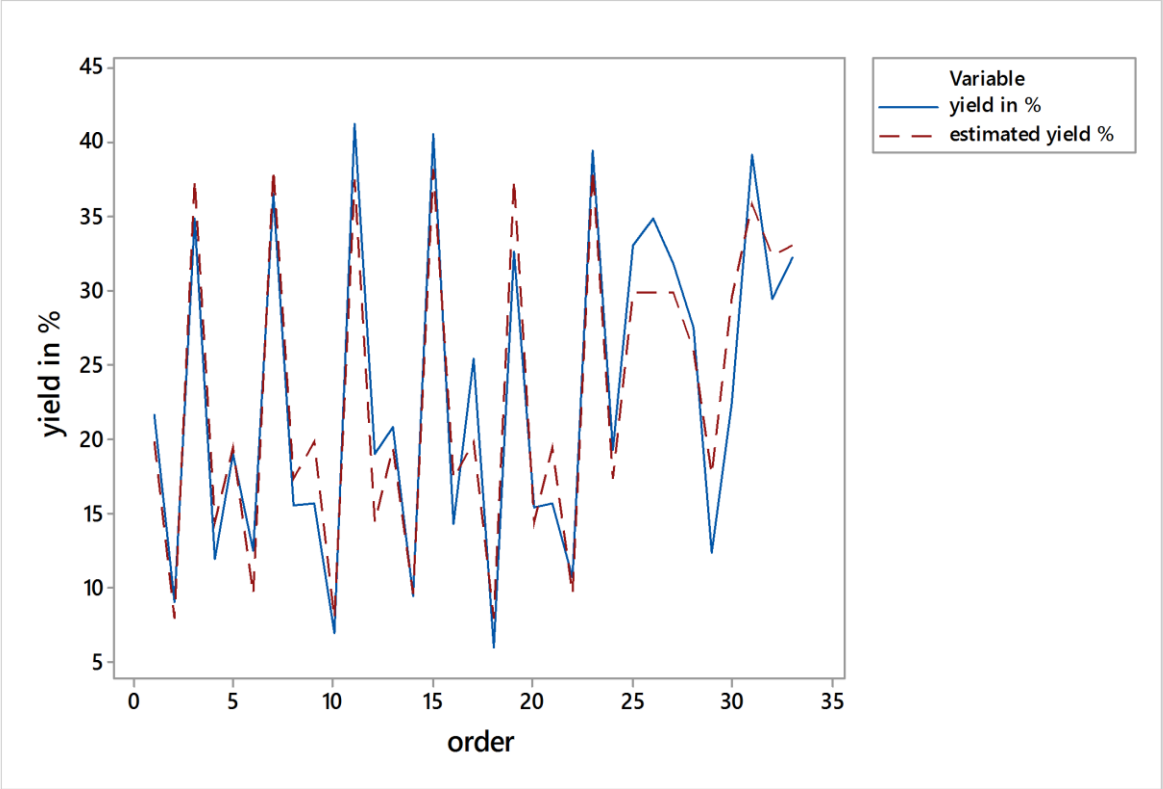

Fig. 3. Yield in % responses and estimated yield in % using the fitted second order model (2).

Table 4 also shows that the combination of coded values (x1, x2, x3) = (−1,1,1) for the factors (air flow rate, outlet temperature, and polymer concentration) shows the maximum estimated yield in % response within the range of the three covariates considered in the experiments. These values corresponded to (air flow rate, outlet temperature, polymer concentration) = (1.0; 120; 2.0) in the original scale.

Thus, the following parameters were used: airflow rate (1.0 m^3/min), outlet temperature (120 °C), and solid content (2%). As there was no difference in the response obtained when using the two different polymers separately, three systems containing sodium alginate (ALG) and gellan gum (GG) were developed in three different proportions (50:50, 25:75, 75:25 ALG:GG) with morin.

3.2 Evaluation of microparticles based on sodium alginate and gellan gum

3.2.1 Yield evaluation, encapsulation efficiency and drug loading

The yield evaluation, encapsulation efficiency and drug loading of the developed systems are described in Table 6. The yield was very similar for the three systems developed, with system 25:75 ALG:GG having the highest value (45.92%) for process yield. These yield values are within the expected range for lab-scale spray-dryer

equipment. For the encapsulation efficiency, the values found were high, compared to the literature, ranging from 46.4% to 66.89%. The highest value (66.89%) was obtained by the 75:25 ALG:GG sample.

Table 6. Median (minimum/maximum) of encapsulation efficiency, drug loading and yield values of the developed systems. ALG: sodium alginate; GG: gellan gum.

Sample	EE (%)	Drug loading (mg morin/100 mg powder)	Y (%)
1. 50:50 ALG:GG – morin	46.40 (44.75/48.04) a	11.60 (11.19/12.01)	43.52 (43.04/44) a
2. 25:75 ALG:GG – morin	61.78 (57.86/63.97) ab	15.44 (14.46/15.99)	44.96 (44/45.92) a
3. 75:25 ALG:GG – morin	66.10 (65.89/68.69) b	16.53 (16.47/17.17)	42.16 (40.96/43.36) a

Different letters indicate statistically significant difference between the groups (Kruskal-Wallis, followed by Dunn test, *p-value* < 0.05), n=3 per group.

3..2.2 Morphological and physicochemical characterization of the microparticles

3.2.2.1 *In vitro* release of morin

In vitro release of morin from the developed systems is shown in Figure 4. After 30 min of testing, about 11%, 13% and 25% of morin were released from 50:50 ALG:GG, 75:25 ALG:GG and 25:75 ALG:GG, respectively. Such behavior shows the influence of ALG proportion on burst release of morin, the intermediate proportion (50:50) being the most suitable for decreasing values. On the other hand, from 240 min of testing, a different behavior was observed, in which the sample containing the highest proportion of GG was able to maintain a constant release rate over time, while the counterparts had an increasing release profile (Table 7).

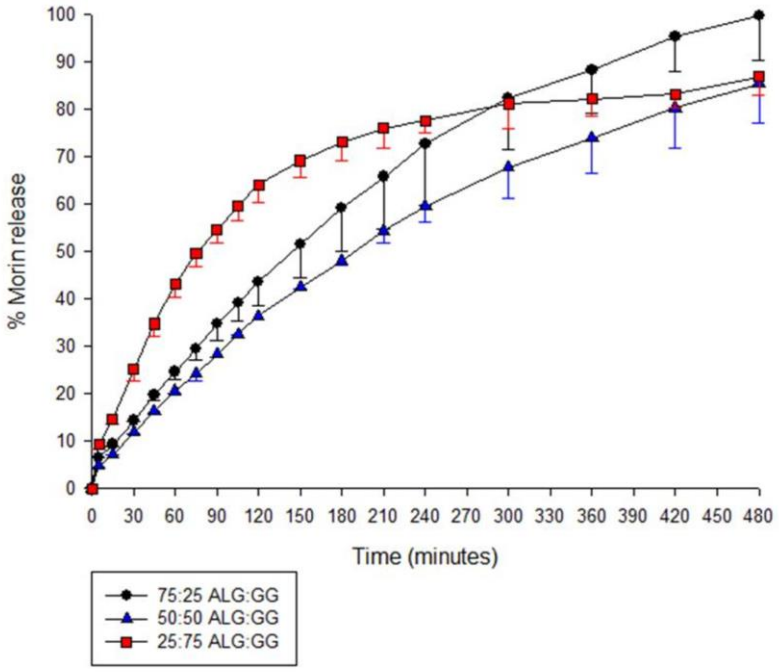

Fig. 4. Mean ± standard deviation of morin controlled release from developed systems. The figure shows the release profile of morin in phosphate buffer (0.2 M, pH 7.4), at 37 °C, for 8 h (n = 3). ALG: sodium alginate; GG: gellan gum.

Table 7. Median (minimum/maximum) of morin release in percentage (%) in 30 and 240 minutes for the developed systems. ALG: sodium alginate; GG: gellan gum.

Sample	Morin release (%) in 30 minutes	Morin release (%) in 240 minutes
1. 50:50 ALG:GG – morin	12.02 (11.43/12.03) a	58.31(57.09/63.45) a
2. 25:75 ALG:GG – morin	24.13 (23.49/27.68) b	77.93 (74.83/79.93) a
3. 75:25 ALG:GG – morin	14.24 (14.2/15.55) ab	75.37 (58.62/84.41) a

Different letters indicate a statistically significant difference between the groups (Kruskal-Wallis, followed by Dunn test, *p-value* < 0.05, non-normal data), n=3 per group.

After 480 min, 75:25, 50:50 and 25:75 ALG:GG samples released 99.86% ± 9.36; 85.45% ± 8.31 and 86.87% ± 3.83 of morin, respectively. Within 30 h, all systems had already released 100% of morin (data not shown).

Assuming six different non-linear statistical models (mathematical models) introduced in the literature applied to the release data for developed systems, Table 8 shows the parameter estimation results and their correlation (observed and fitted) coefficients (r^2).

Table 8. Parameter estimates and correlation coefficients (r^2) for the non-linear statistical models (Baker-Lonsdale, Higuchi, Korsmeyer-Peppas, First order, Hixson-Crowell and Weibull) applied to the release data for developed systems.

RELEASE MODELS		**SAMPLES**		
		25:75 ALG:GG	**75:25 ALG:GG**	**50:50 ALG:GG**
Baker-Lonsdale	*K*	0.0007	0.0005	0.0003
	r^2	0.9699	0.9035	0.9161
Higuchi	*K*	4.7656	4.3916	3.6590
	r^2	0.8981	0.9619	0.9595
Korsmeyer-Peppas	*K*	2.6567	1.1213	0.8416
	r^2	**0.9966**	**0.9942**	**0.9964**
	n	0.6726	0.7617	0.7839
First order	*K*	0.0077	0.0052	0.0038
	r^2	0.9408	0.9883	0.9984
Hixson - Crowell	*K*	0.0022	0.0015	0.0011
	r^2	0.8793	0.9975	0.9927
Weibull	*K*	93.3185	163.4889	128.1622
	r^2	**0.9959**	**0.9940**	**0.9959**
	b	0.9210	0.9274	0.9287

*K = release constant estimate, r^2 = correlation coefficient, n = release exponent for geometric shapes estimates, b = shape parameter estimate.

From the correlation coefficient (r^2) values and according to the systems developed (pristine polymers used), the fitted mathematical models that best correlated with the morin release data for the three systems were the Korsmeyer-Peppas and Weibull models. For the Korsmeyer-Peppas mathematical model, the n obtained estimates for 25:75, 75:25 and 50:50 (ALG:GG) systems were 0.6726, 0.7617 and 0.7839, respectively. Thus, since the particles formed were spherical, the mechanism involved in the release was anomalous transport (Fickian diffusion with polymer chains relaxation). For the Weibull mathematical model, the obtained b estimates for 25:75, 75:25 and 50:50 (ALG:GG) systems were 0.9210, 0.9274 and 0.9287, respectively. This means that the Fickian diffusion was associated with Case II, i.e., controlled by swelling.

3.2.2.2 Field emission gun-scanning electron microscopy (FEG-SEM)

The FEG-SEM image depicted in Figure 5 A-L for the systems developed in the spray dryer shows homogeneous microparticles of 5–9 μm in size and an approximately spherical shape with a rough surface.

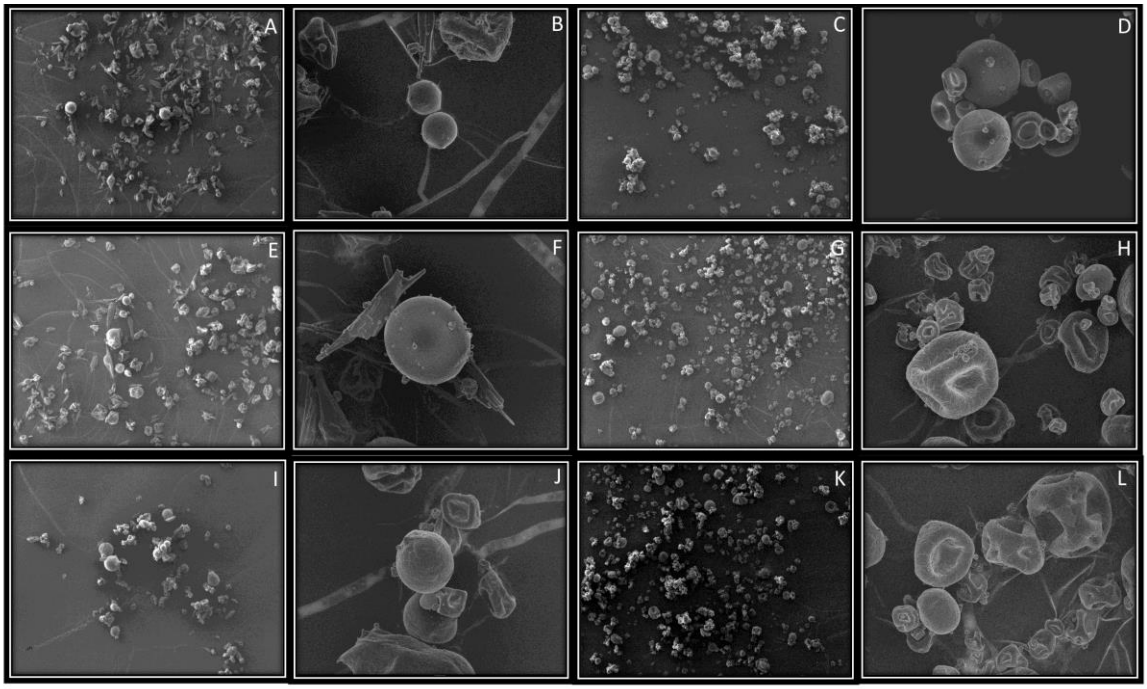

Fig. 5. FEG-SEM photomicrographs of the three developed systems. A: System 75ALG:25GG with morin X500. B: System 75ALG:25GG with morin X5000. C: System 75ALG:25GG control X500. D: System 75ALG:25GG control X5000. E: System 25ALG:75GG with morin X500. F: System 25ALG:75GG with morin X5000. G: System 25ALG:75GG control X500. H: System 25ALG:75GG control X5000. I: System 50ALG:50GG with morin X500. J: System 50ALG:50GG with morin X5000. K: System 50ALG:50GG control X500. L: System 50ALG:50GG control X5000.

3.2.2.3 Fourier-transform infrared spectroscopy (FTIR)

The FTIR analyses were performed to identify the characteristic functional groups of the main compounds present in the developed system composition. The FTIR spectra of the systems are shown in Figure 6.

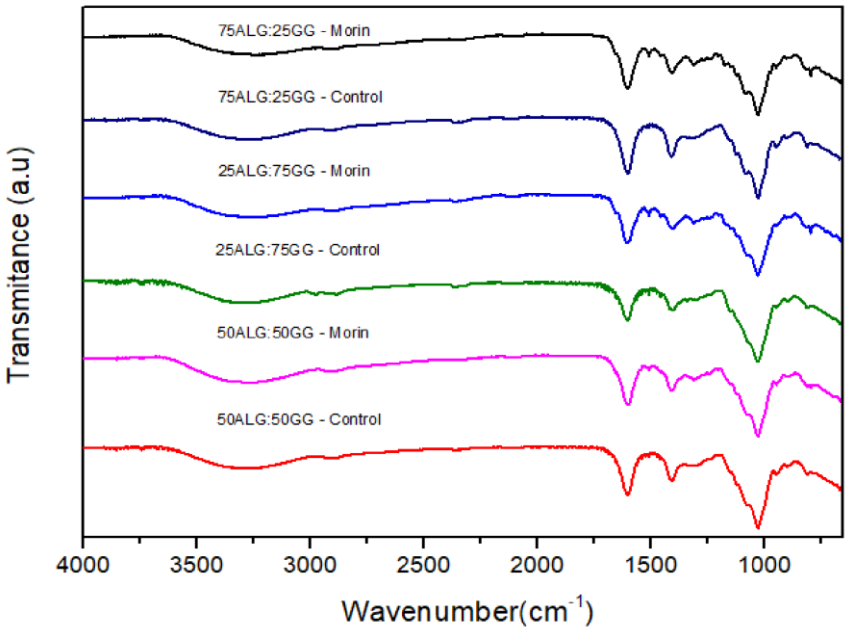

Fig. 6. FTIR of the three developed systems.

The FTIR spectrum of the systems shows bands between 3000 and 3500 cm^{-1} representing the presence of OH groups. The band at 2900 cm^{-1} is related to the C-H bond. The bands between 1400 and 1600 cm^{-1} are related to the carboxyl group. The band at 1000 cm^{-1} corresponds to the C-C and C-O bonds [31]. Overall, the same bands were identified for the microparticles, indicating that the addition of morin did not promote changes in the chemical structure, so the spectra represent the sum of the bands characteristic of each isolated compound.

3.2.3 Antibiofilm and antimicrobial activity of the systems

The biomass (Figure 7A) and viability (Figure 7B) of *S. mutans* were significantly lower in the presence of morin than in their respective controls, regardless of the system (p-value < 0.001). On the other hand, the different proportions of the polymers did not affect the results (p-value = 0.424 and p-value = 0.403, respectively).

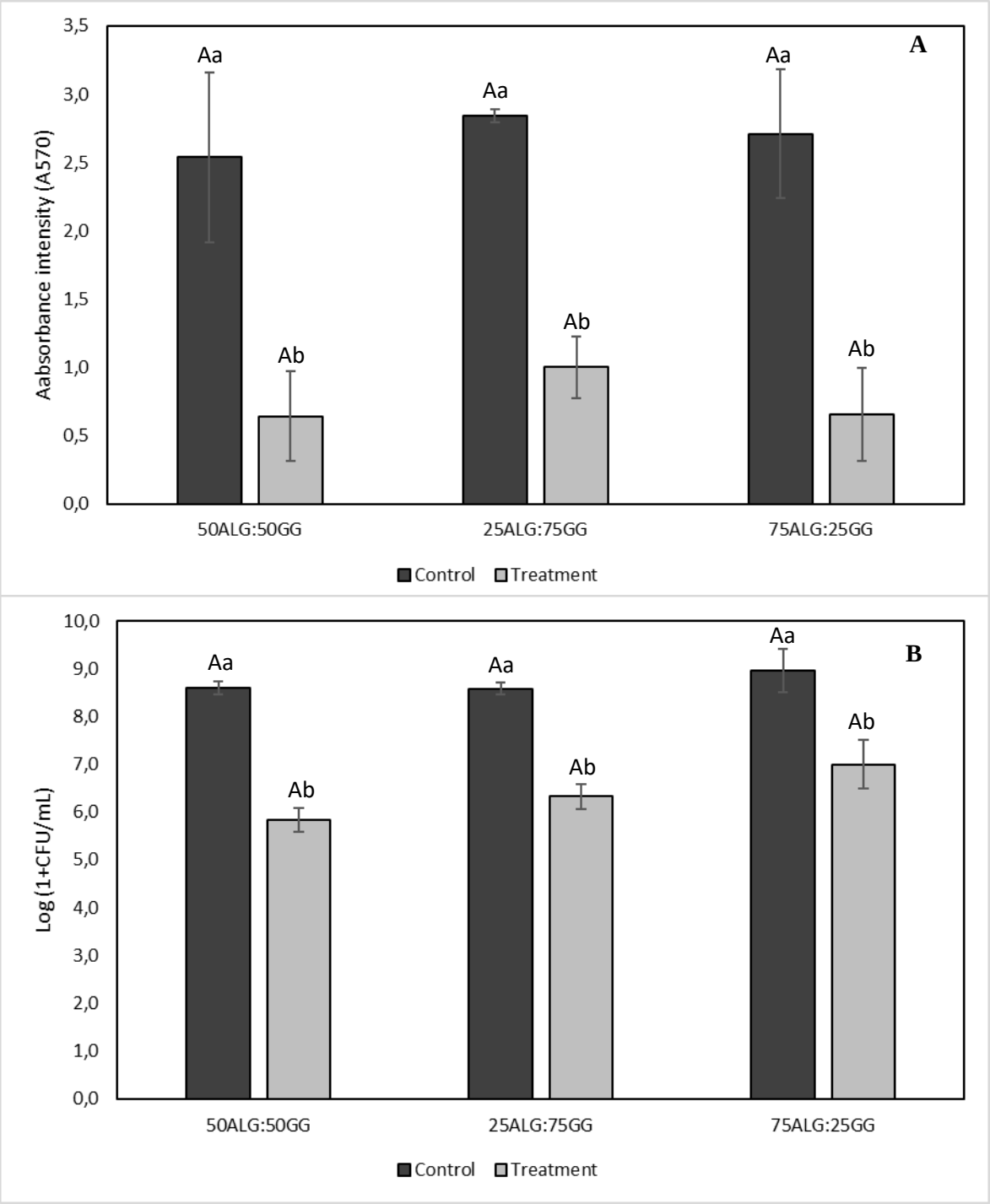

Fig. 7. A: Biomass quantification of S. mutans (mean ± SD - absorbance intensity -A570) after biofilm exposure to the systems with ("treatment") or without ("control") morin. **B:** Viability of S. mutans (mean ± SD - Log 1 + UFC/mL) after biofilm exposure to the systems with ("treatment") or without ("control") morin.

*Different letters indicate a statistically significant difference between the groups.

** Distinct letters indicate statistical differences depicted by 2-Way ANOVA with Tukey's post-hoc test p-value < 0.05 (factors "systems" - capital letter and "type of treatment" - lowercase letter), n = 3 per group. Note that only the factor "type of treatment" was statistically significant.

The acidogenicity data of *S. mutans* biofilm are shown in Figure 8. There was no statistical difference in the time factor (6 h and 24 h) for all groups (p-value = 0.307). No interaction between time and treatment was observed (p-value = 0.518). On the other hand, a statistically significant difference was found between the independent variables (control and treatment) (p-value < 0.001). The 50:50 ALG:GG system was the only one that showed a statistically significantly higher pH value in comparison to its respective control, for either 6 h or 24 h (Figure 8). On the other hand, comparing all groups, the 25:75 and 75:25 ALG:GG treatment groups also showed a statistically significant difference when compared to the 505:50 ALG:GG system (control group). There was no statistical difference between the systems analyzed (independent variables: the systems 50:50, 25:75 and 75:25 ALG:GG).

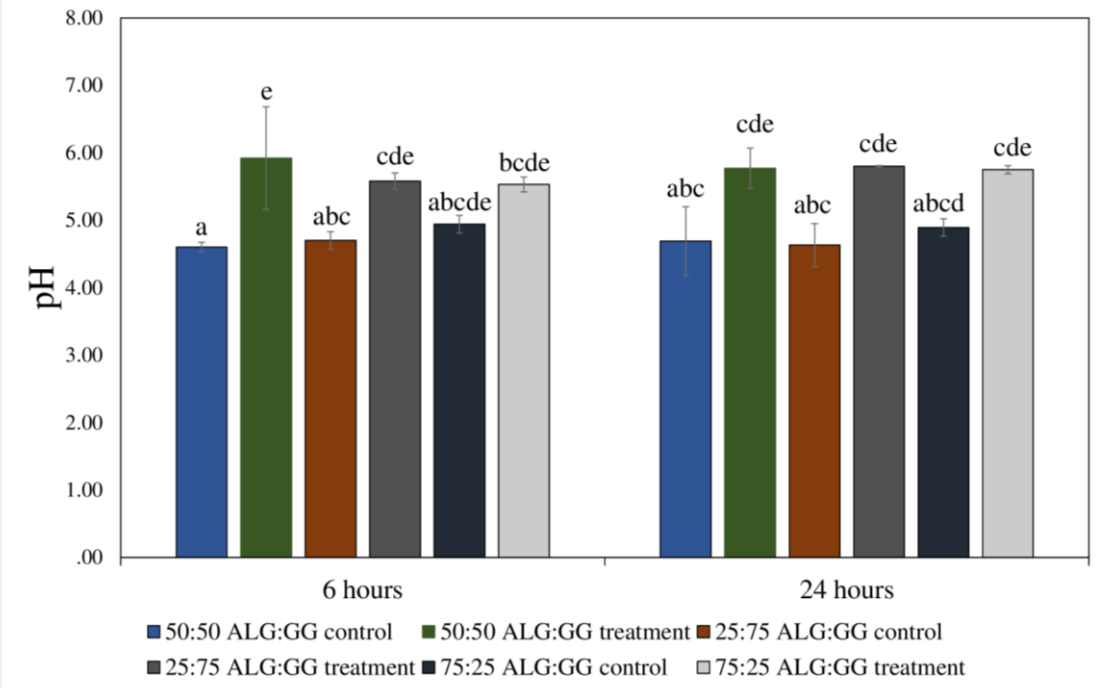

Fig. 8. Acidogenicity in the culture medium (mean ± SD – pH values) after biofilm exposure to the systems with ("treatment") or without ("control") morin. Different letters indicate a statistically significant difference between the groups (Two-Way Repeated Measures ANOVA, followed by the Tukey test, p-value < 0.05), n = 3 per group.

4. Discussion

Microencapsulation by spray-drying is widely used in the pharmaceutical industry but has been scarcely explored in dentistry. This study shows that spray-drying produces systems with high yield and high encapsulation efficiency and with

morin-controlled release. Furthermore, morin was effective in controlling cariogenic bacteria *S. mutans*.

In order to achieve the best yield, we used a 2^4 factorial design because it does not require numerous experiments, saving time and financial resources Four important spray-dryer parameters (air flow rate, outlet temperature, polymer concentration and polymers used) were standardized and resulted in the production of microparticles with high yield, controlled release of morin and systems with relevant characteristics. Polymer concentration determines microparticle morphology, outlet temperature mainly affects microparticle size and airflow rate has the main effect on microparticle yield. Furthermore, outlet temperature is also important to avoid alginate deposit on spray-dryer walls, which decreases the yield and the final product characteristics.

The yields of the systems developed in this study were very similar (~45%), which is suitable for a lab-scale spray-dryer. A yield higher than 50% is hardly obtained with lab-scale spray-dryer because of its reduced dimensions, which might lead to powder accumulation on the walls. Yields close to 100% can be obtained in industrial spray-dryers, since the fraction lost due to adhesion on the walls ends are smaller [32]. The yield values found in this study agree with those of other studies [33,34] or are slightly higher than previously reported [35]. The yield value, the particle size, the particle shape and the microparticle performance observed in the present study are related to the parameters previously established in the spray dryer, such as inlet and outlet temperature, feed flow rate and aspirator.

Parameters standardization is an important step before microparticles are developed, since spray-dryer parameters influence the microparticle characteristics and the subsequent release of the encapsulated active compound [36]. The output temperature is important because low temperatures lead to a viscous deposit of alginate on the equipment walls, which decreases the yield and the final product characteristics. The polymer concentration used in the drying process is important because it determines microparticles morphology [37]. The outlet temperature mainly affects microparticle mean size, and the airflow rate mainly affect microparticle yield. The contribution of this work is precisely that we standardized spray-dryer parameters (air flow rate, outlet temperature, polymer concentration and polymers used) for optimal production of microparticles that shows the desired characteristics (high yield, controlled release of morin and systems with relevant characteristics). The factorial

design was the statistical model used for this purpose, which did not require numerous experiments, saving time and financial resources.

Although different proportions of the polymers did not influence the yield, it influenced morin release profiles. According to the findings of this study, sodium alginate controls the release rates in the first minutes. However, as it presents low molecular weight, it ends up releasing the drug faster with time. This can be explained by the low levels of entanglement between the matrix polymeric chains, hindering the retention of the encapsulated drugs and a faster matrix erosion [38].

While sodium alginate is the most frequently used natural polymer for drug encapsulation by spray-drying, improved microparticle properties were achieved by the addition of gellan gum. The combination of sodium alginate with gellan gum promotes an associative interaction, which provides interesting thermodynamic properties. This interaction is due to the same geometric chain and synergistic gelation of the polymers [39]. Based on the results found in this study, the combination of two polymers proved to be an effective alternative to improve the individual properties of each polymer.

Besides interesting bio/mucoadhesive properties, gellan gum also has the ability to uptake water to increase its volume and mass. Initially, the formation of small clusters of soluble chains occurs. Over time, these chains associate and grow until they form a continuous cross-linked network, when the agglomerate covers the entire solution volume. In this way, the gel formed by gellan gum is a more structured and thermoexpansive gel [39]. This gellan gum property is defined as swelling [40]. The theory of swelling is based on three main causes: polymer-solvent interactions, elasticity and Donnan potential. Elastic pressure holds the gel network together, while Donnan potential and polymer-solvent interactions lead to dissolution [41,42]. Thus, it is suggested that due to the swelling process, a more elastic gel is formed, conferring resistance to drug diffusion and resulting in a more constant and prolonged release profile over time [43]. In the same context, this swelling characteristic makes the sample with gellan gum more viscous for the drying process. This viscosity generates a large drop when the dispersion enters the spray-dryer air chamber, having greater difficulty evaporating. Consequently, the sample has greater retention decreasing encapsulation efficiency. In the present study, the systems with a greater amount of gellan gum showed a constant and delayed morin release profile. On the other hand, the systems with a higher amount of gellan gum showed lower encapsulation

efficiency. Thus, we can state that alginate is responsible for the higher encapsulation efficiency and faster release of morin.

To better understand the release kinetics, mathematical models were applied to release data and correlation coefficients (r^2). A mathematical model describes the relationship between the parameters of the release process using mathematical equations [44]. In this context, four main mechanisms may be involved in the release of bioactive ingredients from the encapsulation matrix: dissolution, diffusion through the matrix, swelling and degradation or erosion. These mechanisms can be detected by mathematical models [45]. The mathematical models Korsmeyer-Peppas and Weibull were the ones that best correlated with morin release data for the three systems developed. The Korsmeyer-Peppas model proposes a simple equation for controlled polymer release. In this model, we found a controlled release by swelling. The n values for this model varied between 0.67 and 0.78 for the systems developed. Thus, because the microparticles were spherical, the release occurred by anomalous transport (non-Fickian). In this model, the swelling or relaxation polymeric chain is the relevant mechanism. This means that the release was not linear with time. The solvent penetration velocity is the most important difference between Case II, Anomalous Case and Super Case II transport non-Fickian mechanisms. In anomalous transport, the polymeric relaxation velocity and solvent diffusion are equal [46]. In the Weibull model, the values of b were between 0.75 and 1 for the three systems, showing a Fickian diffusion release associated with Case II, controlled by swelling. This means that the release occurred in a parabolic curve as a function of time, showing that the release occurred faster at the beginning and then in a more constant manner [47].

The release mechanisms through the mathematical models are in agreement with the predicted outcomes for the developed systems. The polymers used (sodium alginate and gellan gum) are hydrophilic and characterized by absorbing water and increasing dimensions (swelling) when in contact with the aqueous medium. In this way, the polymeric chains loosen, allowing drug diffusion. Although the r^2 value for the first-order and Hixson-Crowell mathematical model was close to 1 for the systems, we cannot say that the release occurred only by drug diffusion through the polymeric matrix (first-order) or only by drug particle dissolution (Hixson-Crowell).

S. mutans biofilm viability and biomass were affected by the presence of three systems containing morin. These findings prove the release capacity of the systems developed, as well as morin antimicrobial and antibiofilm effects. Morin is known to

destroy cell membrane and downregulate the resistance mechanism of microorganisms, inhibiting microbial growth and impairing biofilm maturation [9,10]. Besides, Huang et al. (2014) [48] showed that morin inhibits sortase A, the main gene related to microorganism adhesion to the tooth surface. However, few studies have evaluated the antimicrobial effect of morin incorporated into a controlled release system based on natural polymers. The study by de Farias et al. (2020) [23] was the first to develop this type of system and showed that the systems controlled morin release and virulence factors of cariogenic biofilms. The present study successfully scaled up the systems by spray-dryer and optimized their parameters using experimental factorial design. This was necessary to improve patient acceptability and enhance clinical applicability by developing smaller systems. Moreover, this study demonstrates that different proportions of polymers does not interfere with morin antimicrobial effect, as all morin-containing systems reduced *S. mutans* viability and biomass of the biofilm formed. Substances that also maintain biofilm pH close to neutral have great potential to control dental biofilm and virulence factors of dental caries. The systems containing morin increased the pH when compared to the systems without morin. Even though the difference between the treatment and control systems was small, the findings of this study are promising for the development of oral systems with morin. The different proportions of polymers used also did not interfere with biofilm acidogenicity.

As already mentioned, controlled release systems are still scarcely used in Dentistry. The results of this study showed that the use of natural polymers for the development of controlled release systems represents an interesting strategy. From a clinical point of view, controlling the availability of natural substances in the oral cavity increases the effectiveness of treatment and decreases the toxicity of the substances used [13,14]. Moreover, this study showed that factorial design is an excellent technique for establishing spray-dryer parameters. These systems exhibited high yield and were effective in encapsulation and controlled release of morin. Besides, it was efficient in controlling the virulence factors of *S. mutans* biofilm. Thus, these systems are a promising strategy for use in Dentistry to control dental biofilm and dental caries. Future studies, with other bacterial species, more mature biofilms and polymicrobial biofilms need to be developed to prove the effects achieved in this study.

5. Conclusions

Morin has been extensively studied for oral biofilm control and has shown promising results. However, these studies explored morin biological activity in its free form. The present study showed an efficient alternative for morin use by incorporation in polymeric systems, which improved its aqueous solubility and controlled release to increase its antimicrobial effects. After the parameter's standardization, the chosen technique (spray-dryer) and the polymers (sodium alginate and gellan gum) proved to be efficient for morin encapsulation and controlled release. Furthermore, these systems controlled important virulence factors of S. *mutans* biofilms, such as acidogenicity, microbial viability and biomass, which is useful for the control of cariogenic biofilm. Different from existing products, the controlled release system keeps the morin in the oral cavity longer, increasing its antimicrobial effect and decreases the frequency of product use. Nonetheless, more studies with different bacterial species and more mature biofilm are needed to endorse the findings of the present study.

Acknowledgments

This study was supported in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, by the São Paulo Research Foundation (FAPESP) (grant 2019/08375-0, 2019/26066-4 and 2021/12931-5) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq [grant number 313473/2019-6]. ABM thanks São Paulo Research Foundation (FAPESP) (grant 2019/19817-3). HSB thanks CNPq (grant 407822/2018-6; INCT-INFO), São Paulo Research Foundation (FAPESP) (grants 2018/25512-8, 2013/07276-1 and 2017/50334-3), and TA Instruments Brazil. CAPES, FAPESP, CNPq and TA Instruments Brazil had no involvement in study design, in the collection, analysis and interpretation of data, in the writing of the report, or in the decision to submit the article for publication. This manuscript is based on LSS Doctorate Thesis (Dental Sciences Graduate Program, São Paulo State University (UNESP), School of Dentistry, Araraquara.

References

[1] A. Sheiham, James WPT, Diet and dental caries: the pivotal role of free sugars reemphasized, Journal of Dental Research. 94 (2015), pp. 1341-1347 SAGE Publications Inc.

[2] R.A. Giacaman, Sugars and beyond. The role of sugars and the other nutrients and their potential impact on caries, Oral Diseases, 24 (2018), pp. 1185-1197 Blackwell Publishing Ltd.

[3] Tanner ACR, C.A. Kressirer, S. Rothmiller, I. Johansson, N.I. Chalmers, The caries microbiome: implications for reversing dysbiosis, Adv Dent Res, 29 (2018), pp. 78-85.

[4] N. Takahashi, B. Nyvad, Caries ecology revisited: microbial dynamics and the caries process, Caries Res., 42 (2008), pp. 409-418.

[5] F. Van Der Weijden, D.E. Slot, Oral hygiene in the prevention of periodontal diseases: the evidence, Periodontol, 55 (1) (2000), pp. 104-123

[6] X. Chen, Daliri EBM, N. Kim, J.R. Kim, D. Yoo, D.H. Oh, Microbial etiology and prevention of dental caries: Exploiting natural products to inhibit cariogenic biofilms [Internet], Pathogens, 9 (2020), pp. 1-15.

[7] D.N. Chauhan, P.R. Singh, K. Shah, N.S. Chauhan, Natural oral care in dental therapy, Natural Oral Care in Dental Therapy, Wiley (2020), pp. 1-29.

[8] F.A. Badria, O.A. Zidan, Natural products for dental caries prevention, J. Med. Food, 7 (3) (2004 Sep), pp. 381-384.

[9] A. Caselli, P. Cirri, A. Santi, P. Paoli, Morin: a promising natural drug, Curr. Med. Chem., 23 (8) (2016 Jan 6), pp. 774-791.

[10] S.A. Rajput, Wang X. Qi, H.C. Yan, Morin hydrate: a comprehensive review on novel natural dietary bioactive compound with versatile biological and pharmacological potential, Biomedicine and Pharmacotherapy, 138 (2021).

[11] Y. Shoji, H. Nakashima, Nutraceutics and delivery systems, J. Drug Target., 12 (6) (2004), pp. 385-391.

[12] R. Yazdanshenas, F. Gharib, Solubility and thermodynamic functions measurement of morin hydrate in different alcohols, J. Mol. Liq., 1 (233) (2017 May), pp. 9-14.

[13] D.K. Khajuria, M.C. Konnur, R. Vasireddi, D. Roy Mahapatra, Photonic monitoring of chitosan nanostructured alginate microcapsules for drug release, cited 2021 Mar 29 B. Choi, N. Kollias, H. Zeng, H.W. Kang, Wong BJF, J.F. Ilgner, *et al.* (Eds.), Photonic Therapeutics and Diagnostics XI [Internet], SPIE (2015).

[14] D.K. Khajuria, D. Roy Mahapatra, Photonic hydrogel beads for controlled release of risedronate, [cited 2021 Mar 29]
B. Choi, N. Kollias, H. Zeng, H.W. Kang, Wong BJF, J.F. Ilgner, *et al.* (Eds.), Photonic Therapeutics and Diagnostics X [Internet], SPIE (2014).

[15] I. Siró, E. Kápolna, B. Kápolna, A. Lugasi, Functional food. Product development, marketing and consumer acceptance-A review, Appetite, 51 (2008), pp. 456-467.

[16] A. George, P.A. Shah, P.S. Shrivastav, Natural biodegradable polymers based nano-formulations for drug delivery: A review, International Journal of Pharmaceutics, 561 (2019), pp. 244-264.

[17] J. Singh, K. Kaur, P. Kumar, Optimizing microencapsulation of α-tocopherol with pectin and sodium alginate, J. Food Sci. Technol., 55 (9) (2018 Sep 1), pp. 3625-3631.

[18] K.Y. Lee, D.J. Mooney, Alginate: properties and biomedical applications, Progress in Polymer Science (Oxford), 37, Elsevier Ltd (2012), pp. 106-126.

[19] K. Kesavan, G. Nath, J.K. Pandit, Sodium alginate based mucoadhesive system for gatifloxacin and its in vitro antibacterial activity, Sci. Pharm., 78 (4) (2010), pp. 941-957.

[20] A. Sosnik, K.P. Seremeta, Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers, Advances in Colloid and Interface Science, Vol. 223, Elsevier B.V. (2015), pp. 40-54.

[21] E. Assadpour, S.M. Jafari, Advances in spray-drying encapsulation of food bioactive ingredients: from microcapsules to nanocapsules, Annu. Rev. Food Sci. Technol., 10 (1) (2019 Mar 25), pp. 103-131.

[22] P. Peralta-Zamora, J.L. de Morais, N. Nagata, Por que otimização multivariada? Eng. Sanit. Ambient., 10 (2) (2005 Jun), pp. 106-110.

[23] A.L. de Farias, A.B. Meneguin, Barud H. da Silva, F.L. Brighenti, The role of sodium alginate and gellan gum in the design of new drug delivery systems intended for antibiofilm activity of morin, Int. J. Biol. Macromol., 1 (162) (2020 Nov), pp. 1944-1958.

[24] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistics for Experimenters, John Wiley & Sons. New York, NY, USA (1978).

[25] D.C. Montgomery, Design and Analysis of Experiments, (3rd Editio.), John Wiley & Sons, New York, NY, USA (1991).

[26] G.M. Cox, W.G. Cochran, Experimental Designs, John Wiley & Sons. New York, NY, USA (1957).

[27] F.L. Brighenti, A.C. Medeiros, B.M. Matos, Z.E.A. Ribeiro, C.Y. Koga-Ito, Evaluation of caries-associated virulence of biofilms from Candida albicans isolated from saliva of pediatric patients with sickle-cell anemia, J. Appl. Oral Sci., 22 (6) (2014), pp. 484-489.

[28] Y.E. Albuquerque, M. Danelon, M.J. Salvador, C.Y. Koga-Ito, A.C. Botazzo Delbem, R.Y. Ramirez-Rueda, *et al.*, Mouthwash containing croton doctoris essential oil: in vitro study using a validated model of caries induction, Future Microbiol., 13 (6) (2018 May 1), pp. 631-643.

[29] F.H. van de Sande, M.S. Azevedo, R.G. Lund, M.C. Huysmans, M.S. Cenci, An in vitro biofilm model for enamel demineralization and antimicrobial dose-response studies, Biofouling, 27 (9) (2011 Oct), pp. 1057-1063.

[30] N. Draper, H. Smith, Applied Regression Analysis, John Wiley & Sons. New York, NY, USA (1981).

[31] Fontes M. de Lima, A.B. Meneguin, A. Tercjak, J. Gutierrez, B.S.F. Cury, A.M. dos Santos, *et al.*, Effect of in situ modification of bacterial cellulose with carboxymethylcellulose on its nano/microstructure and methotrexate release properties, Carbohydr. Polym., 1 (179) (2018 Jan), pp. 126-134.

[32] S.H. Lee, D. Heng, W.K. Ng, H.K. Chan, R.B. Tan, Nano spray drying: a novel method for preparing protein nanoparticles for protein therapy, Int. J. Pharm., 403 (1–2) (2011), pp. 192-200.

[33] A.A. Mahmoud, N.A. Elkasabgy, A.A. Abdelkhalek, Design and characterization of emulsified spray dried alginate microparticles as a carrier for the dually acting drug roflumilast, Available from Eur J Pharm Sci [Internet]., 122 (2018), pp. 64-76.

[34] G. Rassu, E. Gavini, G. Spada, P. Giunchedi, S. Marceddu, Ketoprofen spray-dried microspheres based on Eudragit RS and RL: study of the manufacturing parameters, Available from Drug Dev Ind Pharm [Internet]., 34 (11) (2008), pp. 1178-1187.

[35] K. Bowey, B.E. Swift, L.E. Flynn, R.J. Neufeld, Characterization of biologically active insulin-loaded alginate microparticles prepared by spray drying, Available from Drug Dev Ind Pharm [Internet]., 39 (3) (2013), pp. 457-465.

[36] S. Benchabane, M. Subirade, G.W. Vandenberg, Production of BSA-loaded alginate microcapsules: influence of spray dryer parameters on the microcapsule characteristics and BSA release, Available from J Microencapsul [Internet]., 24 (6) (2007), pp. 565-576.

[37] Optimization of spray drying by factorial design for production of hollow microspheres for ultrasound imaging – Narayan, J. Biomed. Mater. Res., 56 (2001), pp. 333-341.

[38] M. George, T.E. Abraham, Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan--a review, Available from J. Control Release, 114 (1) (2006), pp. 1-14.

[39] E.R. Morris, K. Nishinari, M. Rinaudo, Gelation of gellan – a review, Food Hydrocoll., 28 (2) (2012 Aug 1), pp. 373-411.

[40] K.M. Kanyuck, T.B. Mills, I.T. Norton, A.B. Norton-Welch, Swelling of high acyl gellan gum hydrogel: characterization of network strengthening and slower release, Carbohydr. Polym., 1 (259) (2021 May), Article 117758.

[41] M. Annaka, Y. Ogata, N. Takayuki, Swelling behavior of covalently cross-linked gellan gels, Jul 27 [cited 2021 Aug 11], Journal of Physical Chemistry B [Internet]., 104 (29) (2000), pp. 6755-6760.

[42] T. Sakai, Physics of Polymer Gels, John Wiley & Sons (2020), 0–283 p

[43] F.G. Prezotti, I. Siedle, F.I. Boni, M. Chorilli, I. Müller, B.S.F. Cury, Mucoadhesive films based on gellan gum/pectin blends as potential platform for buccal drug delivery, 25 (2) (2019 Feb 7), pp. 159-167.

[44] L. Shargel, B. Andrew, S. Wu-Pong, Applied biopharmaceutics & pharmacokinetics [Internet], [cited 2021 Aug 12]. Available from https://uomustansiriyah.edu.iq/media/lectures/4/4_2016_06_26!10_36_26_AM.pdf (2015).

[45] S. Jafari, Nanoencapsulationof Food Bioactive Ingredients: Principles and Applications, (2017) 2011 Feb.

[46] N. Malekjani, S.M. Jafari, Modeling the release of food bioactive ingredients from carriers/nanocarriers by the empirical, semiempirical, and mechanistic models, Compr. Rev. Food Sci. Food Saf., 20 (1) (2021 Jan 1), pp. 3-47.

[47] P. Costa, J.M. Sousa Lobo, Modeling and comparison of dissolution profiles, Available from, Eur J Pharm Sci [Internet]., 13 (2) (2001), pp. 123-133.

[48] P. Huang, P. Hu, S.Y. Zhou, Q. Li, W.M. Chen, Morin inhibits sortase A and subsequent biofilm formation in *Streptococcus mutans*, Available from Curr Microbiol [Internet]., 68 (1) (2014), pp. 47-52.

3.2 Publicação 2*

Evaluation of chitosan and sodium alginate nanoparticles containing or not morin or carvacrol for oral polymicrobial biofilm control.

Luciana Solera Sales[a], Amanda Letícia Polli Silvestre[b], Marlus Chorilli[c], Andréia Bagliotti Meneguin[d], Hernane da Silva Barud[e], Fernanda Lourenção Brighenti[*].

[a]Department of Morphology, Genetics, Orthodontics and Pediatric Dentistry, São Paulo State University (Unesp), School of Dentistry, R. Humaitá, 1680 – Centro, Araraquara, São Paulo, Brazil – 14801-903. luciana.solera@unesp.br

[b]Department of Drugs and Pharmaceuticals, São Paulo State University (Unesp), School of Pharmaceutical Sciences, Jaú, Km 1, CP 502, Araraquara, São Paulo, Brazil – 14800-903. amanda.silvestre@unesp.br

[c]Department of Drugs and Pharmaceuticals, São Paulo State University (Unesp), School of Pharmaceutical Sciences, Jaú, Km 1, CP 502, Araraquara, São Paulo, Brazil – 14800-903. marlus.chorilli@unesp.br

[d]Department of Drugs and Pharmaceuticals, São Paulo State University (Unesp), School of Pharmaceutical Sciences, Jaú, Km 1, CP 502, Araraquara, São Paulo, Brazil – 14800-903. abagliottim@hotmail.com

[e]Biopolymers and Biomaterials Laboratory (BioPolMat), University of Araraquara - UNIARA, 14801-340 Araraquara, SP, Brazil. hernane.barud@gmail.com

*Corresponding author: Fernanda Lourenção Brighenti, Department of Morphology, Genetics, Orthodontics and Pediatric Dentistry, São Paulo State University (Unesp), School of Dentistry, R. Humaitá, 1680 – Centro, Araraquara, SP, Brazil - 14801-903. Tel.: +55 16 3301 6551. E-mail address: f.brighenti@unesp.br

* Artigo segue a norma do periódico International Journal of Biological Macromolecules para o qual pretende-se submeter.

Abstract

Nanoparticles (NPs) based on chitosan (CS) and sodium alginate (SA) containing morin or carvacrol is an interesting strategy for oral biofilm control. This study aimed to characterize SA/CS-based NPs, with or without morin or carvacrol and to evaluate the antimicrobial and antibiofilm activity against polymicrobial oral biofilms. Polymeric NPs were obtained by polyelectrolytic complexation. Three NPs with three different polymers concentration, with or without morin or carvacrol, were tested (0.15:1; 0.3:1; 0.5:1 CS:SA). The NPs were characterized by particle size, zeta potential and scanning electron microscope (SEM), encapsulation efficiency and drug release. NPs antibiofilm and antimicrobial activity were evaluated using polymicrobial oral biofilms by means of quantifying the biomass (crystal violet staining), assessment of viable microorganisms (CFU/mL) and acidogenicity of the biofilm by pH readings. The NPs presented nanometric size, with spherical shape and smooth surface. Encapsulation efficiency of the samples containing morin was 55.15, 46.87 and 46.17% and for carvacrol was 88.22, 90.15 and 55.30%. Total release of carvacrol and morin occurred within 15 minutes. The NPs with or without morin or carvacrol significantly reduced biofilm biomass and microbial viability of total bacteria, aciduric and mutans streptococci present in the biofilm compared to the control. However, the NPs with or without morin or carvacrol did not significantly increase the biofilm pH when compared to the control. The results suggested the potential of NPs to control polymicrobial oral biofilm. Supported by future studies, this study will provide advances for the use of nanotechnology in dentistry.

Keywords: Oral biofilm; Morin; Carvacrol; Sodium Alginate; Chitosan; Nanoparticles

1. Introduction

Oral biofilm is a complex microorganism organization present on an oral surface, embedded in an extracellular matrix [1,2]. Different microorganism species are present in the oral environment and polymicrobial biofilm are formed naturally [3]. Mechanical control associated with chemical control is still an effective alternative for oral biofilm control [4]. However, the effective mechanical control still depends on the individual's motor skills and chlorhexidine, despite being the most used substance for mechanical control, has side effects when used frequently [5]. In this scenario, natural products

are gaining space by their promising effect in controlling oral biofilm, by not inducing antimicrobial resistance and by having low toxicity, causing little side effects [6,7].

Therefore, two natural substances deserve to be highlighted due to two promising characteristics, morin and carvacrol. Morin (3,5,7,2′ ,4′ -pentahydroxyflavone) is a natural flavonoid, derived from various plant, especially the Moraceae family [8]. Carvacrol (5-Isopropyl-2-methyl-phenol; C10H14O) is an aromatic monoterpenoid isolated from aromatic plants, such as oregano, thyme and sweet majerone [9,10]. Morin and carvacrol have been widely studied for presenting wide biological and pharmacological potential, and for controlling important virulence factors of cariogenic and periodontal biofilms, besides been no-toxic. [11–16]. Moreover, it is known that natural substances are considered a promising alternative for combating antimicrobial resistance, due to the presence of a variety of secondary metabolites, such as flavonoids and monoterpenes, present in morin and carvacrol respectively. These substances are not only related to microorganisms death, but also to the pathogenic process, being able to modulate cellular processes related to the host, such as apoptosis, mitosis and immune response. Therefore, morin and carvacrol have a reduced capacity to develop microbial resistance [17].

Furthermore, the concept of employing controlled release of a substance has sparked attention. Recent studies have developed systems, microparticles, films, tablets, for oral use, based on natural polymers containing morin [18–20]. The aim of these studies was to reduce the undesirable effects of morin, such as low solubility, low biodisponibility and easy degradation in the oral environment and control morin release, increasing its effect and reaching more easily the desired location. The results of these studies were promising, but some points can still be improved. The disadvantages of the systems developed by de Farias et al. (2020) (2022) [19,20] are the large size and thickness of the system, lack of orodispersibility, and swelling in contact with oral fluids. These factors have the potential to cause patient discomfort and reduce treatment compliance. Moreover, many challenges have been faced in the incorporation of carvacrol into these systems mainly due to its high volatility. Films, tablets and microparticles containing carvacrol were only slightly effective against in vitro biofilms [unpublished data]. Moreover, encapsulation efficiency of carvacrol in the systems developed by Sales et al. (2023) [18] were very low (4.0 a 7.7%), impairing its application [unpublished data].

Nanotechnological approaches are a very interesting way to reduce the systems size, the drug dose, increasing safety and consequently potentiating its effect. Moreover, this technology has been widely explored as a drug delivery system in medicine, pharmacy, cosmetic and food industry, but there is still much to be explored in dentistry [21]. In this context, SA and CS have been extensively used for this purpose [22]. CS is a hydrophilic cationic abundant biopolymer consisting of (1→4)-2-amino-2-deoxy-β-D-glucan, derived from chitin [23]. CS is one of the most promising biopolymers to be used as material for delivery and drugs controlled release, due to its biodegradability, biocompatibility, low-cost and antimicrobial and antioxidant properties [24,25]. SA is a hydrophilic biopolymer composed by α-L-guluronate and β-D-mannuronate. Like chitosan, alginate is an inexpensive, biocompatible, and biodegradable [26]. Additionally, CS/SA shows high capacity of electrostatic complexation to produced NPs by polyelectrolyte complexation. Polyelectrolyte complexation is a very easy, cheap, fast technique and does not use dangerous chemicals for NPs development. This reaction involves polymers of different charges, CS, a cationic polymer, needs to be in contact with an anionic polymer, SA [27].

One of the major challenges in eradicating biofilms is the presence of the extracellular matrix, which is known to act as an effective physical barrier to substances penetration [28]. Besides being a physical barrier, the extracellular matrix is also negatively charged, which prevents the deep penetration of positively charged substances, such as chlorhexidine. [29].

It is well documented in the literature that NPs can penetrate more easily into mature biofilms, in the cells and in the biofilm membrane. Moreover, the NPs have greater intracellular retention, improve the agent antimicrobial effect, for it is delivered directly to the bacteria, and decrease the drug toxicity, due to the possibility of using a lower dose compared to the use in its free form. These NPs characteristics are due to their nanometric size, surface charge and contact surface area [30–32].

Given the high prevalence of biofilm-dependent diseases – such as dental caries and periodontal disease – the control of oral biofilm is still a challenge in Dentistry. In this context, this is the first study to explore the unprecedented application of recently developed SA/CS based NPs [33] for morin and carvacrol incorporation and although the NPs development based on alginate and chitosan is already widely explored in other areas, little is said about the evaluation of these NPs for use in combination with toothpastes, mouthwashes or even varnishes to assist in combating oral biofilm. Thus,

the aim of this study is to characterize SA/CS-based NPs, with or without morin or carvacrol and to evaluate the antimicrobial and antibiofilm activity against polymicrobial oral biofilms. With the use of SA/CS-based NPs we expect to improve clinical acceptability of the patients and to improve the penetration of the active substances into the biofilm and to present an alternative to overcome carvacrol evaporation during the preparation of the systems.

2. Materials and methods

2.1 Materials

Morin hydrate (M4008, purity > 85 %), carvacrol (MKCG2266, purity 99%), sodium alginate (SA), chitosan (CS) - low molecular weight (50,000–190,000 Da), 90% deacetylation degree - and glacial acetic acid used were obtained from Sigma-Aldrich (St. Louis, MO, USA). The same batch were used throughout the experiment. The substances were stored at room temperature according to the manufacturer's directions.

2.2 Synthesis of morin or carvacrol nanoparticles (NPs) by polyelectrolyte complexation method

Morin or carvacrol NPs were obtained by polyelectrolyte complexation method as proposed by Silvestre et al. (2023) [33]. Briefly, 0.3 mg/mL CS was dispersed in 0.1 mol/L acetic acid (0.1 mol/L), and 0.5 mg/mL SA was dispersed in purified water. After stirring for 24 h, the dispersions were adjusted to pH 4.0 or 6.0 for, respectively, CS and SA.

For the preparation of NPs, 1 mg/mL morin or 1 mg/mL carvacrol in ethanolic solution were added to the SA dispersion containing 1% Tween 80®. Afterwards chitosan dispersion was dripped onto this dispersion. The dripping occurred uniformly with a peristaltic pump, flow rate 20 ml/h under constant agitation (~100 rpm) for 30 minutes. Three different concentrations (w/w) were tested (0.15:1; 0.3:1; 0.5:1 CS:SA), based on Silvestre et al. (2023) [33] as they presented better conditions according to particle size, polydispersity index and zeta potential. NPs without the drugs and in the same proportions were developed and used as control (blank).

2.3 Characterization of morin or carvacrol nanoparticles

2.3.1 Mean diameter, polydispersity index (PdI) and zeta potential (ZP)

The mean diameter and the polydispersity index (PdI) of the NPs were determined by dynamic light scattering technique (DLS), on Zetasizer Nano-ZS equipment (Malvern Panalytical Instruments, Germany), with a 4.0 mV of He-Ne (λ = 633 nm) laser, detection angle of 173°. The zeta potential (ZP) of the NPs was determined employing the electrophoretic mobility technique with the Smoluchowski equation, using the Zetasizer Nano-ZS particle analyzer, angle of 173°. Measurements were performed in triplicate at 25°C, right after the NPs development. To evaluate the NPs stability, the same measurements were repeated after 7, 15, 30 and 60 days after the NPs development.

2.3.2 Field emission gum Scanning electronic microscopy (FEG-SEM)

NP surface morphology and size were evaluated using a high-resolution field emission gum scanning electron microscopy (FEG-SEM), JEOL JSM-7500 F (Jeol Company, Japan), operating at 2 kV. To avoid particles agglomeration during drying process, the samples were diluted in a dispersion of Tween® 20 (0.5 %, v/v), at 1:30 (v/v). The samples were then dried under vacuum, in a desiccator, for 48 h and covered with a thin layer of conductive material (carbon). Photomicrographs were obtained at ×50,000 magnification.

2.3.3 Encapsulation efficiency (EE%)

Analytical curves for morin or carvacrol were validated by UV-vis (Hewlett Packard-Kayak XA) spectrophotometer. The NPs containing morin or carvacrol were added on Amicon filter (Millipore Corporation, Billerica, MA) with Ultracel-100 (100 kDa) membrane for morin and Ultracel-50 (50 kDa) membrane for carvacrol. The samples were centrifuged for 15 minutes at 6.200 ×g to separate the free drug from the drug incorporated in the NPs [34]. Concentration of free morin or carvacrol was determined using a calibration curve obtained between 5 to 35 μg/mL (r^2 = 0.9958, λ= 389 nm) for morin or between 5 to 75 μg/mL (r^2 = 0.9997, λ= 274 nm) for carvacrol. The highest concentration was used to determine the maximum absorption peak, in a UV–vis (Hewlett Packard-Kayak XA) spectrophotometer. The experiment was performed in triplicate and the EE% was calculated using the following equation:

EE (%) = (Total drug concentration-free drug in the supernatant concentrarion)/(Total drug concentrarion) x100

2.3.4 In vitro release of morin or carvacrol

The release profile of morin or carvacrol from de NPs was performed in vials containing 1.8 mL phosphate buffer (Na2HPO4, 0.2 M, pH 7.4) and 0.2 mL of NPs [containing 16.851 µg (0.15:1), 15.952 µg (0.3:1) and 15.130 (0.5:1) of morin or carvacrol] for 24 h, respecting the sink conditions. The vials were incubated in a shaker (37 ^{o}C, 50 rpm) and removed at predetermined times. The samples were immediately filtered using a 0.22 µm filter and morin or carvacrol concentrations were quantified by measuring the absorbance at 389 nm for morin and at 274 nm for carvacrol in a UV–vis (Hewlett Packard-Kayak XA) spectrophotometer. The results were expressed as drug released (%) x time. The experiments were performed in triplicate for both, morin and carvacrol.

2.4 Effect of NPs with or without morin or carvacrol on cariogenic bacteria

To evaluate NPs effect on cariogenic bacteria a polymicrobial inoculum from saliva of a one pre-selected donor was used. The use of saliva was approved by the Ethics Committee on Human Research from School of Dentistry of Araraquara, UNESP (CAAE: 29584120.8.0000.5416). The volunteer was informed about the study purpose and signed the Informed Consent. The volunteer aged 30 years, had a good general and oral health and has not taken antibiotics, antifungals or mouthwashes in the previous six months and anti-inflammatories and/or immunosuppressants in the last three months [35]. 5 mL of fresh saliva was collected in the morning, stimulated with parafilm and after the volunteer abstained from oral hygiene for 24 h and without having breakfast for 2 h. In addition, the volunteer did not consumed alcohol the night before saliva collection.

Culture media used to grow polymicrobial biofilms was the one originally proposed by McBain et al. (2005) [36]. This medium consists of porcine gastric mucin (2.5 g/L), bacteriological peptone (2.0 g/L), tryptone (2.0 g/L), yeast extract (1.0 g/L) NaCl, (0.35 g/L), KCl (0.2 g/L), CaCl2 (0.2 g/L), cysteine hydrochloride (0.1 g/L), hemin, (0.001 g/L), vitamin K1 (0.0002 g/L), pH 7.0.

Polymicrobial biofilms from 400 µl aliquots of fresh saliva were grown in a sterile glass coverslip (ø 13 mm; n=3/group) using an active adhesion model attached to a 24 well-plate, and intermittent sucrose exposure as described by [18,35,37]. In this model, the glass coverslips are placed vertically by fixing them on 0.7 mm orthodontic wire

with low-viscosity condensation silicone (Zetaplus, Zhermack, Badia Polesine, Italy) and biofilms are exposed to culture media supplemented with 1% sucrose for 6 h/ day. After the first 6 h of biofilm growth, the treatment was performed for 5 minutes with the three different NPs containing carvacrol or morin. The morin or carvacrol dose were 46.5 µg/mL (0.15:1), 44 µg/mL (0.3:1) and 42 µg/mL (0.5:1). NPs without the drugs were used as blank and PBS was used as control. The biofilms were incubated at 37 °C in 5% CO2 for 24 h. NPs antibiofilm activity was evaluated by determining the biomass using a crystal violet assay. The results were expressed in absorbance intensity (A_{570}).

NPs antimicrobial activity was assessed using a separate set of biofilms that were grown under the same conditions for 48 h, with culture media refreshment every 6 or 18 h. The NPs with morin or carvacrol or alone (blank) were added at time 0 and were kept in constant contact with the biofilm throughout its formation (48 h). The control group consisted solely of biofilm growth. After 48 h, the biofilms were removed from the coverslips using an ultrasonic bath (Cristófoli ultrasonic tub, Campo Mourão - PR, Brazil) for 10 s, at 42 kHz. The biofilm dispersions were analyzed for microbial load. Total bacteria on Wilkins-Chalgren agar [36] mutans streptococci on Mitis Salivarius agar supplemented with 15% sucrose and 0.2 IU/mL bacitracin (MSBS) [38] and anaerobic aciduric bacteria on BHI agar pH 4.7 [39]. The plates were incubated at 37 °C in 5% CO_2 for 48 h. The results were expressed in log (1+ CFU/mL). Biofilms acidogenicity were evaluated by assessing the pH of the spent culture medium, using a specific electrode coupled to an ion analyzer (Quimis pHmeter, Diadema - SP, Brazil). The results were expressed in pH values.

2.5 Data analyses

All data present a normal distribution according to the normality test (Shapiro-Wilk) and are therefore expressed as mean ± SD. NPs stability (size, PDI and ZP) were analyzed by one-way ANOVA followed by the Tukey's multiple comparisons test ($p < 0.05$). The effect of the different types of treatment (treatment, blank and control) and NPs (0.15:1; 0.3:1 and 0.5:1 CS:SA) was analyzed by two-way ANOVA followed by the Tukey's multiple comparisons test ($p < 0.05$). All analyses were performed using GraphPad Prism 9.0.

3. Results

3.1 Characterization of morin or carvacrol nanoparticles

The NPs formation occurred spontaneously by the addition of chitosan to the sodium alginate dispersion, visually observed by an opalescent coloration. The addition of morin or carvacrol did not change NPs characteristics as observed by the physicochemical characteristics (size, polydispersity index and zeta potential; table 1).

Table 1. Mean ± standard deviation of the physicochemical characteristics of NPs with and without morin or carvacrol.

Sample	CS:SA	Size (nm)	PDI	ZP (mV)	EE (%)
Blank	0.15:1	275.01 ± 8.08	0.37 ± 0.06	-34.20 ± 0.75	-
	0.3:1	248.4 ± 4.48	0.26 ± 0.01	-34.43 ± 0.64	-
	0.5:1	328.0 ± 9.07	0.40 ± 0.01	-28.73 ± 1.15	-
Morin	0.15:1	201.17 ± 2.67	0.55 ±0.01	-32.27 ± 0.67	55.15 ± 0.22
	0.3:1	272.80 ± 6.85	0.39 ±0.07	-31.23 ±2.07	46.87 ± 0.80
	0.5:1	304.5 ± 10.19	0.37 ±0.05	-27.13 ±1.75	46.17 ±1.10
Carvacrol	0.15:1	229.77 ± 5.26	0.49 ±0.03	-28.40 ±0.44	88.22 ±0.78
	0.3:1	304.13 ± 2.80	0.32 ±0.01	-32.83 ±1.81	90.15 ± 0.08
	0.5:1	315.80 ± 8.36	0.32 ±0.06	-24.33 ±0.38	55.30 ± 1.47

PDI = polydispersity index*; ZP* = zeta potential; EE = Encapsulation efficiency. n=3

According to table 1, the NPs were comparable in particle size, PDI and zeta potential. PDI describes the size distribution of NPs in a sample and the ZP indicates the electrical charge around the NPs dispersed in the aqueous medium. These characterizations are important because they show the stability and physic-chemical properties of the NPs developed. All NPs showed nanometric size (<500 nm [33]), with 0.15:1 NPs showing the smallest size for both morin and carvacrol and 0.3:1 NPs showing the smallest size for blank. On the other hand, the 0.5:1 NPs showed the largest size for both substances and blank. Furthermore, the addition of morin and carvacrol resulted in a reduction of particle size, except for the 0.3:1. PDI of the NPs ranged from 0.26 to 0.55. NPs 0.15:1 with morin or carvacrol showed the highest PDI values, and NPs 0.5:1 with morin or carvacrol showed the lowest PDI values. ZP NPs showed a density of negative charges values around 30 mV, which remained constant after the addition of morin or carvacrol.

The encapsulation efficiency of the NPs containing morin were similar. The sample 0.15:1 showed the highest value (55.15%) when compared with the other samples. On the other hand, the encapsulation efficiency for the NPs containing carvacrol ranged from 55.30 to 90.15%. The sample 0.3:1 showed the highest value (90.15 ± 0.08) and 0.5:1 showed the lowest value (55.30 ± 1.47).

3.1.1 NPs containing morin or carvacrol stability

Table 2 shows the NPs containing morin or carvacrol stability by evaluating their size, PDI and ZP after 60 days. In general, the NPs proved to be stable during the test period for the parameters evaluated.

As shown in Table 2, the average size, PDI and ZP of the NPs containing morin were significantly affected only in the ratio 0.5:1; 0.15:1; 0.3:1 and 0.5:1, respectively, after 60 days of testing. For NPs containing carvacrol, the average size and PDI were affected in the ratio 0.3:1 and 0.5:1; 0.3:1, respectively, after 60 days. ZP was affected in all proportions after the test period for the NPs containing carvacrol.

Table 2. NPs stability (mean ± standard deviation) with morin or carvacrol including size (nm), PDI, ZP (mV) parameters.

Sample	CS:SA	Size (nm)				
		Day 1	**Day 7**	**Day 15**	**Day 30**	**Day 60**
Morin	0.15:1	201.2±2.7^{a}	213.1±1.9^{a}	235.9±10.0^{ab}	252.3 ± 2.8^{b}	210.5±6.9^{a}
	0.3:1	272.8±6.8^{a}	296.6±9.6^{ab}	291.5±19.4^{ab}	302.0 ±9.8^{b}	281.6±8.9^{ab}
	0.5:1	304.5±10.2^{a}	302.1±4.9^{abc}	331.5±6.4^{b}	325.2±6.6^{abc}	336.9±7.5^{c}
Carvacrol	0.15:1	229.8±5.3^{a}	241.5±13.2^{a}	234.2 ± 6.4^{a}	228.1±14.0^{a}	254.5±6.7^{a}
	0.3:1	304.1±2.8^{a}	291.0±6.1^{ab}	278.9 ±1.7^{b}	272.0±43.0^{abc}	235.8±8.9^{c}
	0.5:1	315.8±8.4^{ac}	335.0±8.9^{bc}	350.6±11.4^{ab}	370.1±1.5^{bc}	414.6±15.5^{c}

Sample	CS:SA	PDI				
		Day 1	**Day 7**	**Day 15**	**Day 30**	**Day 60**
Morin	0.15:1	0.55±0.01^{a}	0.56±0.0^{a}	0.56±0.0^{ab}	0.57±0.0^{a}	0.65±0.01^{b}
	0.3:1	0.39±0.07^{a}	0.43±0.08^{a}	0.40±0.05^{a}	0.38±0.07^{a}	0.52±0.03^{a}
	0.5:1	0.37±0.05^{a}	0.36±0.05^{a}	0.45±0.03^{a}	0.42±0.07^{a}	0.47±0.09^{a}
Carvacrol	0.15:1	0.49±0.03^{a}	0.51±0.05^{a}	0.54±0.05^{a}	0.55±0.02^{a}	0.54±0.02^{a}
	0.3:1	0.32±0.01^{a}	0.39±0.05^{a}	0.47±0.05^{a}	0.66±0.01^{b}	0.87±0.20^{ab}
	0.5:1	0.32±0.06^{a}	0.30±0.02^{a}	0.34±0.05^{a}	0.34±0.05^{a}	0.38±0.09^{a}

Sample	CS:SA	ZP (mV)				
		Day 1	**Day 7**	**Day 15**	**Day 30**	**Day 60**
Morin	0.15:1	-32.3±0.7^{a}	-31.5±0.3^{a}	-33.7±1.7^{a}	-29.8±2.3^{a}	-38.5±3.6^{a}
	0.3:1	-31.2±2.1^{a}	-37.2±0.3^{ab}	-38.5±1.2^{b}	-33.8 ± 0.7^{ab}	-37.7±1.4^{b}
	0.5:1	-27.1 ±1.7^{a}	-35.2±2.2^{b}	-33.4±2.5^{b}	-34.8 ± 1.5^{b}	-35.7±1.4^{b}
Carvacrol	0.15:1	-28.4±0.4^{a}	-35.3±0.9^{b}	-31.2±6.1^{ab}	-38.7±0.5^{b}	-36.2±0.9^{b}
	0.3:1	-32.8±1.8^{a}	-41.2±0.5^{b}	-40.2±1.7^{bc}	-37.8±0.9^{cd}	-31.2±2.7^{ad}
	0.5:1	-24.3±0.4^{a}	-33.2±1.4^{b}	-33.5±1.1^{b}	-34.7±0.9^{c}	-36.9±1.9^{bc}

Different letters indicate statistically significant difference between the groups (One - Way ANOVA followed by Tukey's post-hoc test p-value < 0.05), n = 3 per group.

3.1.2 In vitro release of morin and carvacrol

The three NPs formulations containing either morin or carvacrol showed similar behaviors. Within 5 minutes, nearly all morin and carvacrol were released (Figure 1).

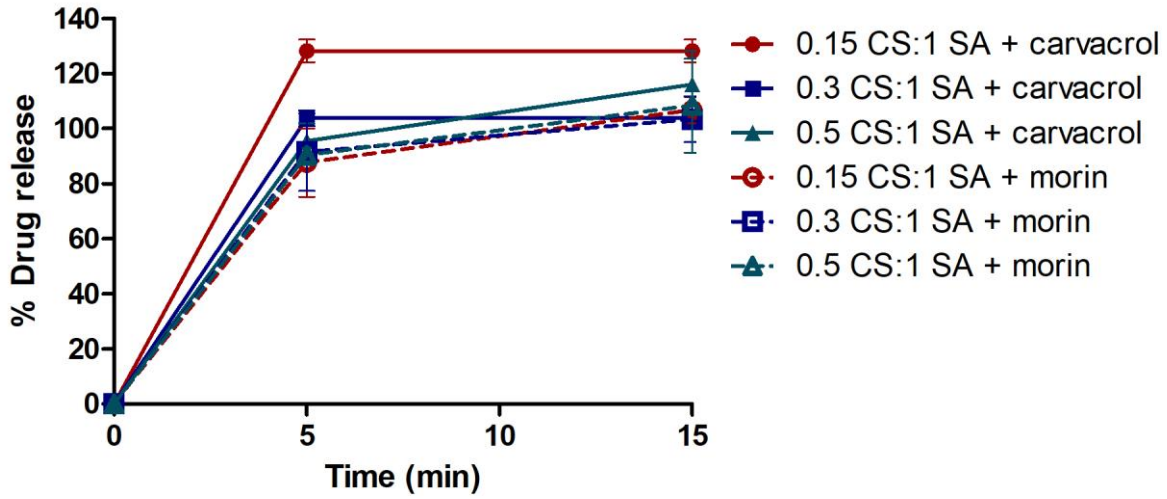

Figure 1. In vitro morin and carvacrol release (Mean ± CI) profile form NPs in phosphate buffer (0.2 M, pH 7.4), n=3. CS: chitosan; SA: sodium alginate.

3.1.3 Scanning electronic microscopy (SEM)

The SEM images (figure 2 A-I) confirmed the NPs nanometric size and homogeneity found in the DLS analyses. It also showed the NPs spherical core/shell shape, without agglomeration and smooth surface. |Morin or carvacrol incorporation did not change NPs structure, which maintained their spherical shape and nanoscale size.

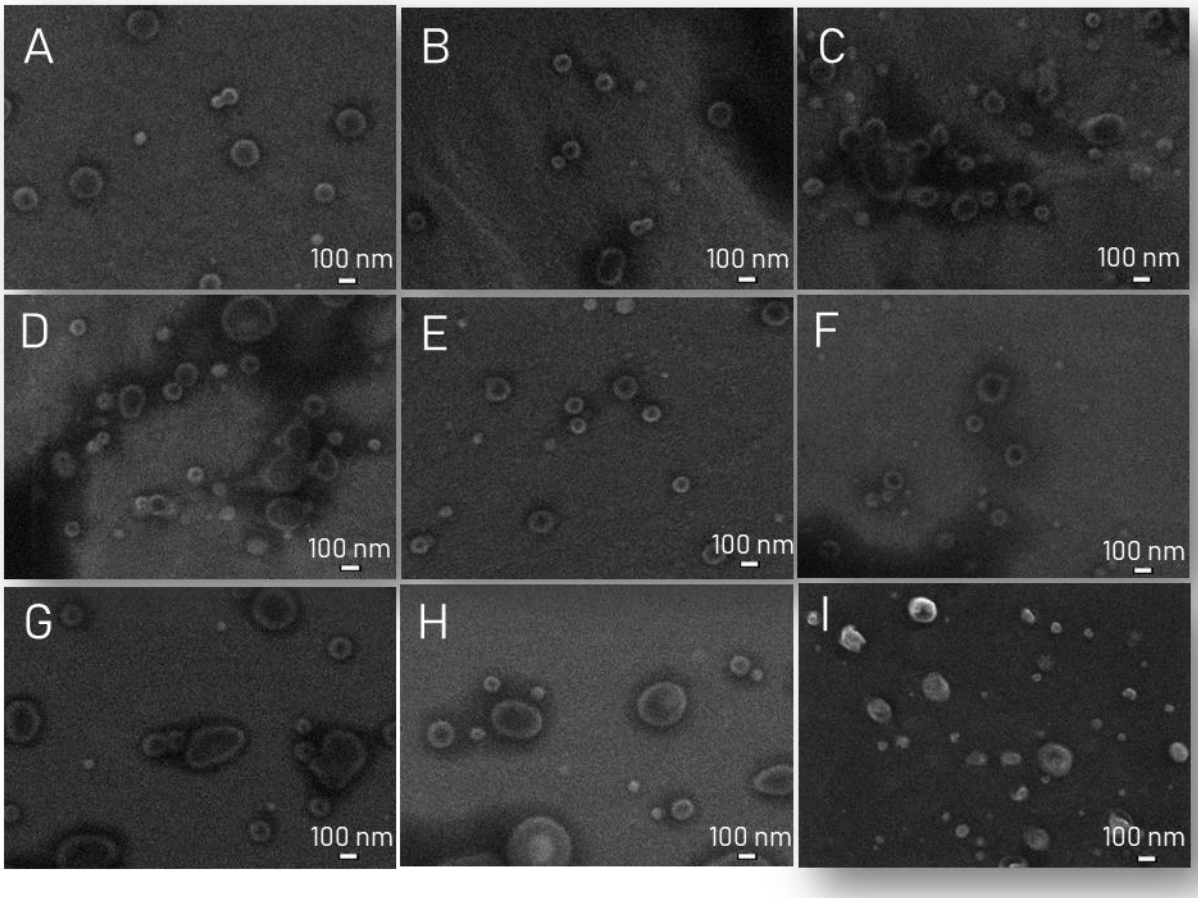

Figure 2. SEM photomicrographs of blank NPs and NPs containing morin or carvacrol. **A:** 0.15CS:1SA blank. **B:** 0.15CS:1SA + carvacrol. **C:** 0.15CS:1SA + morin. **D:** 0.3CS:1SA blank. **E:** 0.3CS:1SA + carvacrol. **F:** 0.3CS:1SA + morin. **G:** 0.5CS:1SA blank. **H:** 0.5CS:1SA + carvacrol. **I:** 0.5CS:1SA + morin. X 50,000 magnification.

3.4 NPs antibiofilm effect

NPs (with or without morin) significantly reduced the polymicrobial biofilm biomass when compared to the control. 0.3:1 CS:AS NPs with morin was the only group that showed a statistically significant difference compared to its corresponding blank control (Figure 3, lower case letters). However, the 0.15:1 ratio (with or without morin) proved to be more effective in reducing biomass when compared to 0.3:1 ratio (with or without morin) but was similar to 0.5:1 ratio (Figure 3, upper case letters).

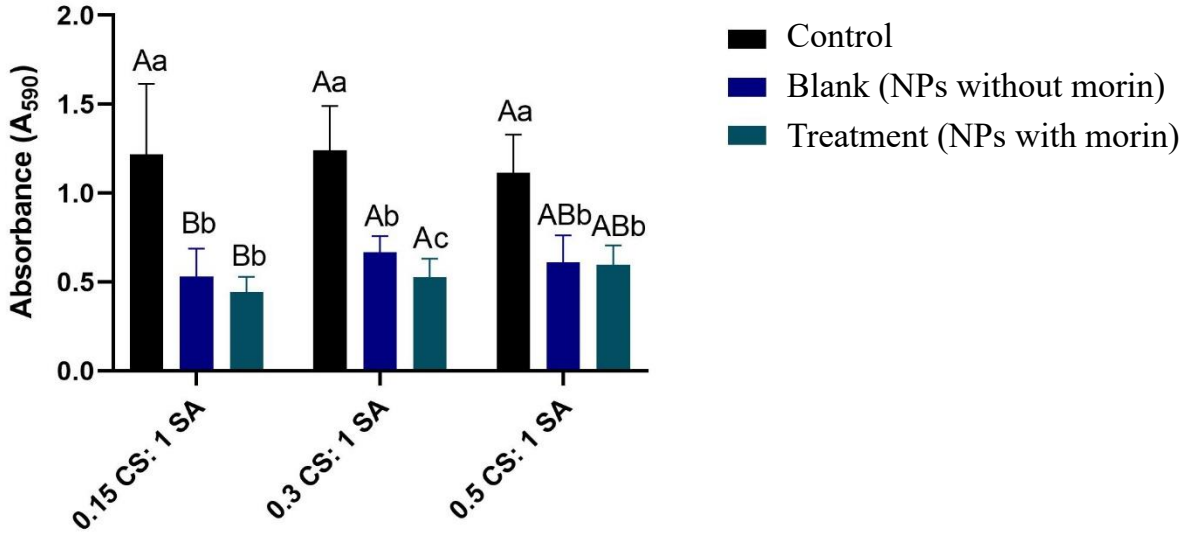

Figure 3. Mean ± SD biomass (A_{590}) after polymicrobial biofilms exposure to NPs with and without morin and control. 2-Way ANOVA with Tukey's post-hoc test p-value<0.05, n=3 per group. *Different letters indicate a statistically significant difference between the groups. Capital letter: differences between the 3 types of NPs within the same type of treatment; lowercase letter: differences between the type of treatment within the same type of NPs.

3.5 NPs antimicrobial effect

The viability of total bacteria, aciduric bacteria and mutans streptococci was significantly reduced in the presence of morin for the three different NPs tested in comparison to the blank and control (Figure 4, lower case letters). The same effect was not observed with carvacrol. The presence of carvacrol led to reduced viability of total bacteria exclusively in the 0.3:1 NP group, aciduric bacteria in the 0.15:1 and 0.5:1 NPs groups and mutans streptococci in the 0.15:1 and 0.3:1 group (Figure 5, lower case letters).

Regarding the different proportions, the 0.15:1 NPs containing morin were more effective against total bacteria and aciduric bacteria and the 0.5:1 NPs were more effective against mutans streptococci. On the other hand, the 0.15:1 NPs with carvacrol were more effective against total bacteria and the 0.3:1 NPs with carvacrol were more effective against aciduric bacteria. For mutans streptococci there were no statistically significant differences between NPs containing carvacrol (Figure 5, upper case letters).

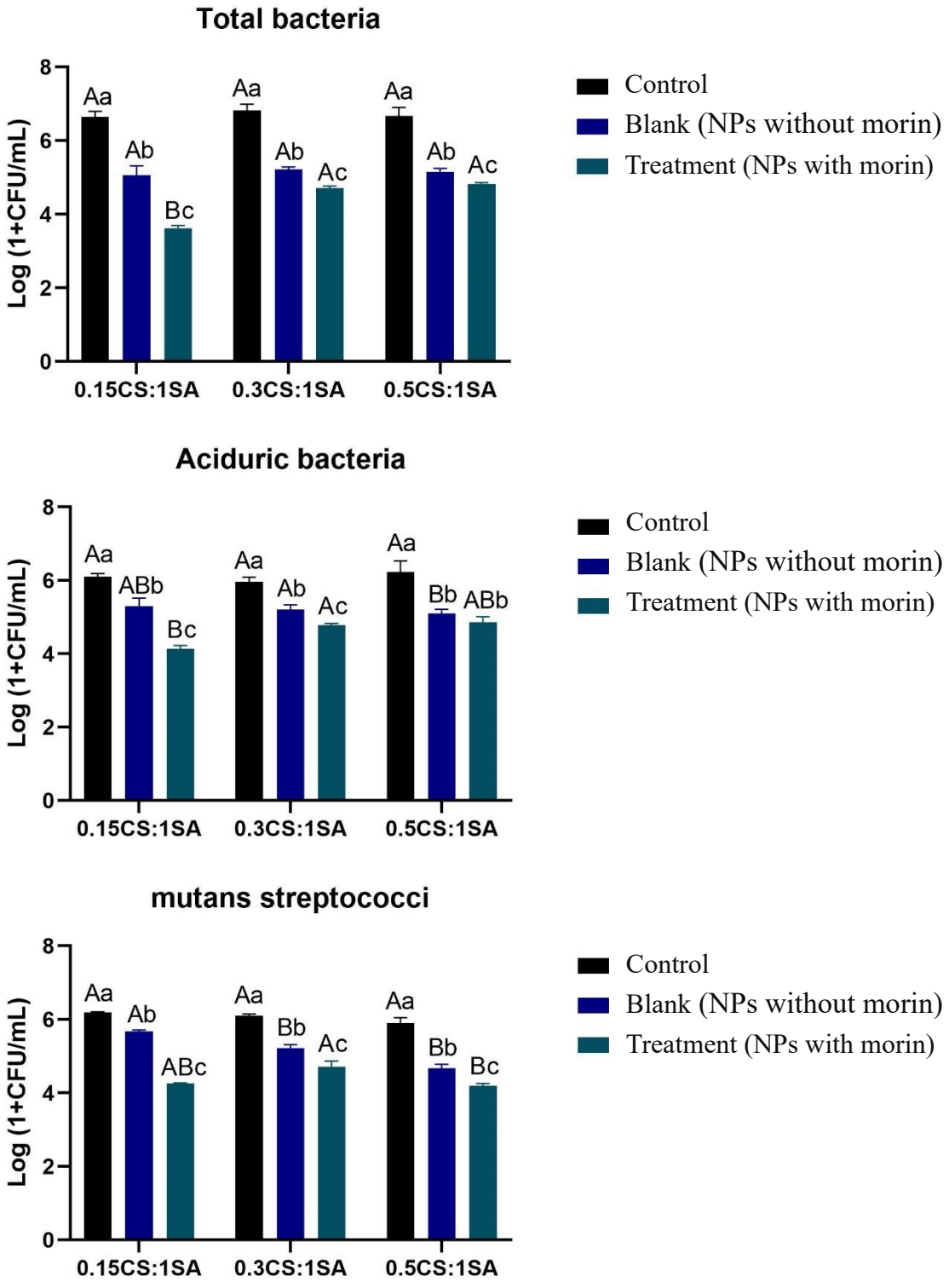

Figure 4. Mean ± SD viability after polymicrobial biofilms exposure to NPs with and without morin and control. 2-Way ANOVA with Tukey's post-hoc test p-value < 0.05, n=3 per group. *Different letters indicate a statistically significant difference between the groups. Capital letter: differences between the 3 types of NPs within the same type of treatment; lowercase letter: differences between the type of treatment within the same type of NPs.

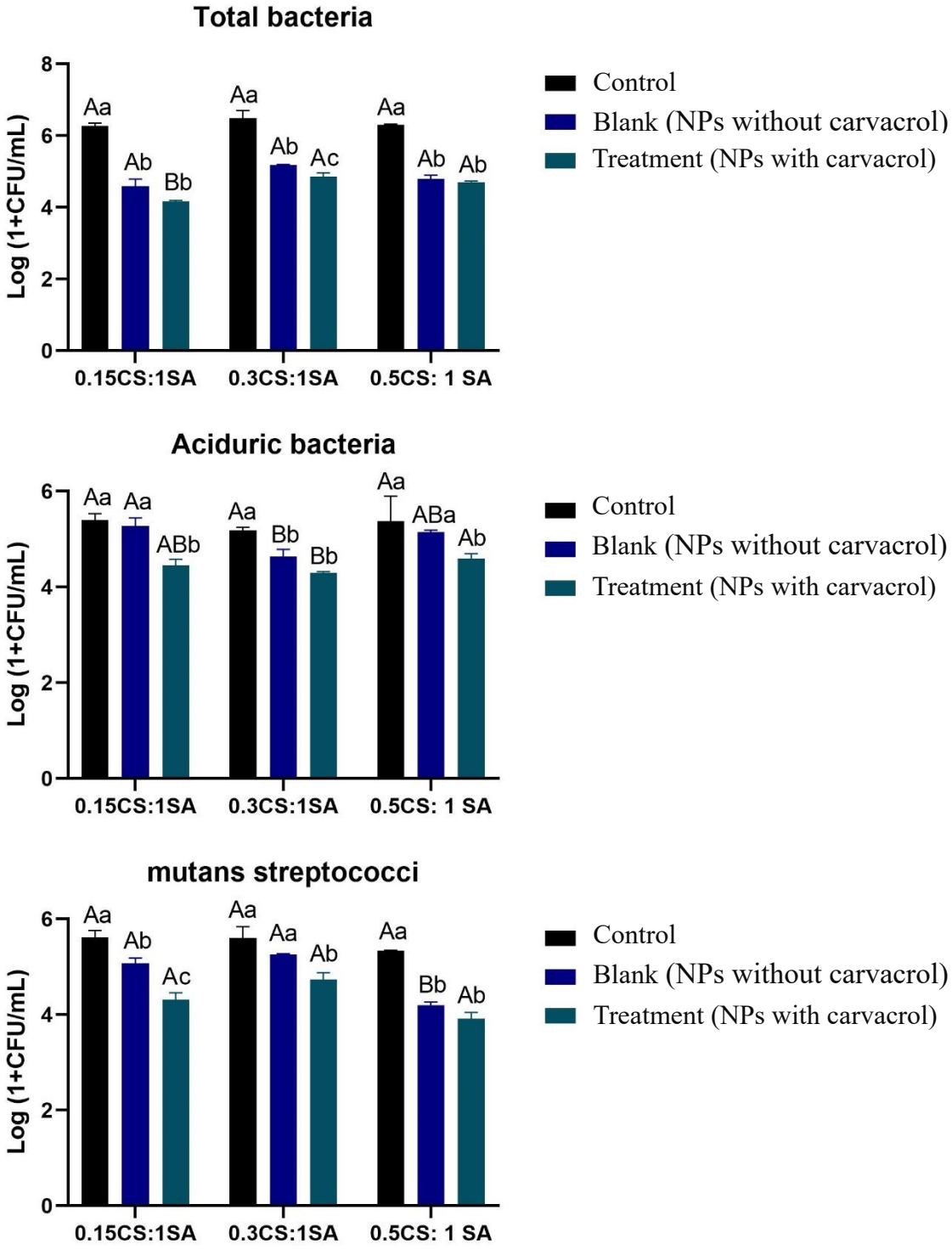

Figure 5. Mean ± SD viability after polymicrobial biofilms exposure to NPs with and without carvacrol and control. 2-Way ANOVA with Tukey's post-hoc test p-value < 0.05, n=3 per group. *Different letters indicate a statistically significant difference between the groups. Capital letter: differences between the 3 types of NPs within the same type of treatment; lowercase letter: differences between the type of treatment within the same type of NPs.

The presence of morin or carvacrol did not decrease the acidogenicity of culture media. Therefore, there was no difference between the control, blank and drug-containing NPs groups (Figure 6).

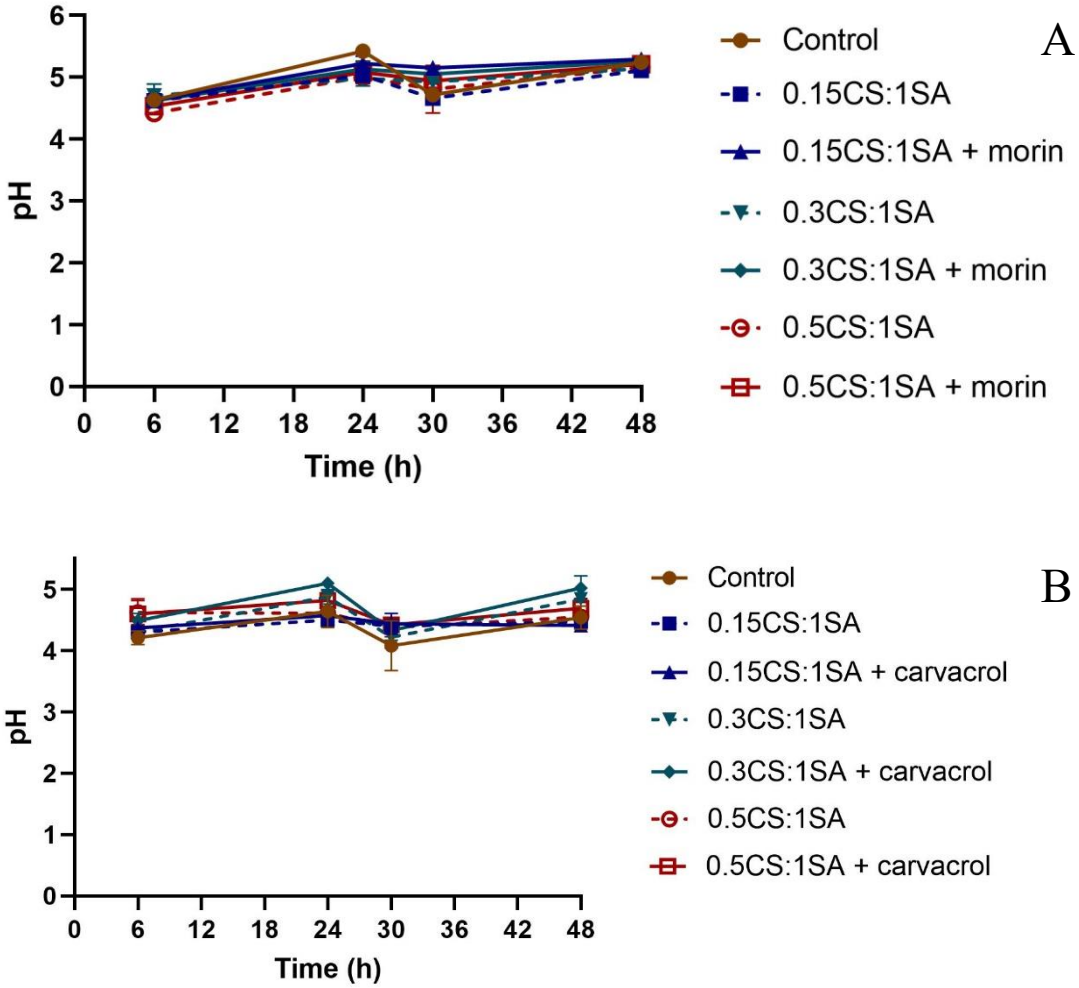

Figure 6. Acidogenicity of the culture medium (mean ± CI – pH) after polymicrobial biofilms exposure to NPs with and without morin **(A)** or carvacrol **(B)** and control. n=3 per group.

4. Discussion

Distinct types of drug delivery have been studied in different fields besides dentistry. In this study, NPs based on CS and SA was employed for the encapsulation and delivery of morin and carvacrol. The selection of CS and SA is attributed to their remarkable attributes, including easy and secure encapsulation, cost-effectiveness and fast preparation techniques. Furthermore, polyelectrolytic complexation offers other advantages such as straightforward process, versatility in its application to a wide

variety of materials, improvement of the physical and mechanical properties of materials and low environmental impact [27]. The results of this study showed that it is possible to prepare NPs with favourable characteristics, including nanometric size, homogeneous size distribution, good stability, high encapsulation efficiency and controlled drug release. Moreover, NPs – especially those loaded with morin - were able to control polymicrobial biofilms.

The parameters used in this study was based on Silvestre et al. (2023) [33], who showed that different pH values, CS and SA dispersion concentrations and different polymers ratios for NPs development can greatly influence NP properties [40,41]. Moreover, as attraction forces between the polysaccharide chains of the polymers and the formation of complexes are influenced by polymers concentration/ratio [42], we chose three different CS:AS concentrations to test with morin and carvacrol.

Physicochemical characterization was done to ensure that NPs were properly formed and stable. The NPs had nanometer sizes [< 500 nm; [33]] and PDI < 0.5 [43,44] as confirmed by DLS and SEM. These parameters are important because NPs larger than 500 nm lose their intrinsic properties, such as efficacy, ability to penetrate biofilms, reduction of the required dosage to achieve the desired effect and, consequently, compromising safety [30,31]. Moreover, high PDI values affect product stability, increasing sedimentation and agglomeration probability [45]. These findings can also be confirmed by SEM images, where no agglomeration of the particles was seen. In addition, core/shell shaped particles were observed, which indicates a greater stability and dispersibility [46]. Future studies are important to confirm these findings and to determine the inner and the outer layer material of the NPs.

However, the addition of morin and carvacrol reduced the particle sizes and increased the PDI. These findings may be explained by strong interactions between the drugs and the polymers, which may have led to NPs contraction by decreasing their size. In addition, PDI may have increased due to the heterogeneity in the incorporation of the drugs. While some particles presented a greater number of incorporated drugs, others presented a lower amount. Despite these observations, the parameters remained within the desired range [47,48].

NP size and distribution was also slightly affected by polymer ratio. The lowest (0.15:1) and the highest (0.5:1) concentration of CS used showed the highest particle size, which agrees to the literature [49,50] and may be explained by two facts. For the 0.5:1 NP, the particle size is increased by protonation of chitosan primary amino

groups, which, in turn is attributed to an increase in repulsion between chitosan chains [51]. On the other hand, the lower the number of molecules available in 0.15:1 NP to react with sodium alginate, results in weak, incomplete, and larger size interactions [33]. Thus, the results of the characterization of the SA/CS NP points out that 0.3:1 ratio is the best CS/SA proportion because it shows the smallest size and the best PDI values, which is also in agreement to the literature [33,52].

Moreover, the NP obtained in the present study presented ideal ZP values, indicating that they are stable and does not have potential to aggregate, as demonstrated by zeta potential and by the physical stability data study [53–56]. These findings are in agreement to [33,52,57] that also developed nanoparticles based on sodium alginate and chitosan.

A crucial feature of drug encapsulation in NP is the encapsulation efficiency, which is directly related a cost-benefit of the biomaterial. For morin, EE% varied from 46.17% to 55.15% which agrees to the values reported in the literature for CS/SA NP [58,59]. On the other hand, the polymer ratio markedly influenced the EE% for NPs containing carvacrol, with EE% ranging from 55.30% (0.5:1) to 90.15% (0.3:1). It is postulated that higher concentration of chitosan in the aqueous phase difficult carvacrol to interact and homogeneously disperse within polymer matrix. Another important consideration is that chitosan may have competed with carvacrol for the hydroxyl ions (OH-) present in the solution [60]. Therefore, ideal concentrations and ratios between the polymers result in optimized interactions between CS's protonated amino groups with SA's carboxylate groups, leading to the formation of more favorable, smaller NPs, which exhibits a stronger drug association [33]. Moreover, this study showed that SA/CS-based NPs showed to be a promising alternative for carvacrol encapsulation, given the difficulty of previous studies to achieve high EE% values due to its high volatility. In this way, the actual antimicrobial potential of carvacrol can be better understood.

On the other hand, morin and carvacrol showed similar release behaviors. Almost 100% of the drugs were released, which differs from results found in the literature (32,33). This difference can be explained by the difference in the methodologies used to assess the release profile. The aforementioned studies [33,34] evaluated release through drug diffusion (Franz diffusion cells), which may have slowed down release process. In the context of our study – aimed at using NPs for oral biofilm control - a faster release is advantageous because it has an immediate effect

upon application, allowing the entire amount of encapsulated drug to directly contact the teeth, which controls oral biofilm.

Besides the physicochemical characterizations, the NPs without any drug showed antimicrobial and antibiofilm effect. This effect can be attributed to the electrostatic interaction between the positively charged amino group of CS and the negatively charged peptidoglycan layer present in Gram-positive biofilm cell membrane, which is in line with previous reports in the literature [24,61], including oral microorganisms [33,62]. This interaction causes changes in permeability and intercellular components, which cause chitosan hydrolysis products to interact with microbial DNA, disturb protein synthesis, preventing mRNA [24].

To enhance the NPs antimicrobial properties, morin or carvacrol were encapsulated by the NPs. These natural substances were chosen due to their known antimicrobial and antibiofilm properties. Overall, the results showed that the incorporation of morin reduced approximately 50% of the biomass with only 5 minutes of treatment and has improved the antimicrobial effect of the NPs. These findings may be explained by several mechanisms of action. Morin in known to promote bacterial aggregation, damage the cell membrane, impair biofilm adhesion and down-regulate resistant mechanisms [12,63]. Furthermore, carvacrol is known to disturb the lipid layer microbial cytoplasmatic membranes and can blend into the biofilm matrix, affecting biofilm formation and growth [64–66]. Moreover, the bioadhesive characteristic of CS may have helped to improve these results, especially in the antibiofilm effect. To the best or our knowledge, this is the first study that developed morin/carvacrol-containing NPs for oral biofilm control and the results obtained provide important insights about the role of NP-containing morin or carvacrol in controlling oral biofilm. In this scenario, the NPs may be a promising alternative to assist biofilm mechanical control, since nowadays chlorhexidine is the most used substance for this purpose, but its prolonged use promotes undesirable side effects [5]. To confirm this effect, future studies should be performed in a more mature biofilm model, using teeth as substrate, and should be accomplished on periodontopathogen biofilms. It is also important to conduct studies to assess NPs cytotoxicity before proceeding to in situ and clinical studies.

It is known that cationic substances are more easily penetrated in biofilms due to the interaction with the anionic bacterial cell membrane. It is known that negatively charged substances are repelled when in contact with anionic biofilms, making contact and consequently penetration difficult [67]. On the other hand, positively charged

substances – such as chlorhexidine – might contract the biofilm, which prevents its penetration into the deeper layers of the biofilm and limits their antimicrobial action to the biofilm's outer surface [57,68,69]. However, the results of the present study showed that, despite the anionic nature of the NPs (ZP negative), they can be considered a good agent for oral biofilm control – with or without active substances. Taken together with the reports in the literature, the findings of the present study demonstrate that the effectiveness of an antimicrobial/antibiofilm agent cannot be solely attributed to their ionic charge. Other factors – such as size and penetration into the deeper layers of the biofilm - should also be taken into account.

A limitation of the present study was the limited amount of morin or carvacrol incorporated into the NPs. This finding can be attributed to their nanometric size, which might explain the low log reduction (less than 2 Log CFU/mL) observed between the control group and the NPs containing morin or carvacrol, which will probably result in non-clinically relevant outcomes [19,20,70]. However, the results obtained for the antibiofilm/antimicrobial assays remain satisfactory if the amount of morin or carvacrol incorporated is considered. The antibiofilm/antimicrobial activity in the present study was reached with a dose up to 47 times smaller in comparison to other release control systems [19,20], which means that a very small amount of morin or carvacrol were sufficient to reduce microbial viability. Moreover, given the high volatility of carvacrol, we can consider that the results of antibiofilm/antimicrobial activity and the EE% was highly successful given the difficulties faced in incorporating it into the release-control systems previously developed [unpublished data].

On the other hand, the amount of morin or carvacrol incorporated into the was not enough maintain the pH at physiological values, which is not in agreement to the literature [18–20]. Besides the low quantity of substances incorporated, this can also be attributed to the acidic nature of the NP, mainly due to the pH of CS dispersion used for NP preparation (pH 4.0). Although biofilm acidogenicity is an important feature to control oral biofilm pathogenicity, CS is also recognized to interfere in the de-remineralization process of dental enamel [71–73]. Future studies to better understand the impact of these findings in enamel demineralization should be carried out in order to evaluate the impact of the low pH of the CS/SA NPs in enamel demineralization. Among the interesting features of CS that might impair undesirable demineralization by CS/SA NPs there are the induction of apatite nucleation, the ability to penetrate into enamel and the ability to bind to dental enamel [72–74].

In a broader context, this study effectively explored the novel application of NPs developed by Silvestre et al. (2023) [33] through morin and carvacrol incorporation and provided a successful alternative for controlling polymicrobial biofilms. It also showed that NPs can be used as a drug delivery platform for oral application of other substances such as fluoride, antifungals, anti-inflammatories, and antivirals. Notably, these NPs offer the advantage due to their reduced size, lower drug dosage and good cost-effectiveness. These findings serve as an impetus for further research aiming to corroborate the potential application of these NPs. It is important that subsequent investigations encompass diverse microorganisms, such as periodontopathogens, and mature biofilms before progressing to in vivo studies for their implementation within the field of dentistry.

5. Conclusion

In this study, the NPs were effectively synthesized, with nanometre size and good size distribution, confirmed also by SEM images. It also showed an excellent zeta potential value and proved an excellent encapsulation efficiency of morin and carvacrol.

Moreover, the NPs developed showed antimicrobial and antibiofilm effect against oral biofilm and the addition of natural substances morin or carvacrol increased this effect. These findings proved the properties combination of chitosan and sodium alginate and addition of morin or carvacrol can be a promising strategy for oral use, fighting biofilm and consequently biofilm dependent diseases.

References

[1] M. Sanz, D. Beighton, M.A. Curtis, J.A. Cury, I. Dige, H. Dommisch, R. Ellwood, R. Giacaman, D. Herrera, M.C. Herzberg, E. Könönen, P.D. Marsh, J. Meyle, A. Mira, A. Molina, A. Mombelli, M. Quirynen, E.C. Reynolds, L. Shapira, E. Zaura, Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases. Consensus report of group 1 of the Joint EFP/ORCA workshop on the boundaries between caries and periodontal disease, J Clin Periodontol. 44 Suppl 18 (2017) S5–S11. https://doi.org/10.1111/JCPE.12682.

[2] P.D. Marsh, Dental plaque as a microbial biofilm, Caries Res. 38 (2004) 204–211. https://doi.org/10.1159/000077756.

[3] W.G. Wade, The oral microbiome in health and disease, Pharmacol Res. 69 (2013) 137–143. https://doi.org/10.1016/J.PHRS.2012.11.006.

[4] C. Valkenburg, D.E. Slot, E.W.P. Bakker, F.A. Van der Weijden, Does dentifrice use help to remove plaque? A systematic review, J Clin Periodontol. 43 (2016) 1050–1058. https://doi.org/10.1111/JCPE.12615.

[5] F. Poppolo Deus, A. Ouanounou, Chlorhexidine in Dentistry: Pharmacology, Uses, and Adverse Effects, Int Dent J. 72 (2022) 269–277. https://doi.org/10.1016/J.IDENTJ.2022.01.005.

[6] D.H. Oh, X. Chen, E.B.M. Daliri, N. Kim, J.R. Kim, D. Yoo, Microbial Etiology and Prevention of Dental Caries: Exploiting Natural Products to Inhibit Cariogenic Biofilms, Pathogens. 9 (2020) 1–15. https://doi.org/10.3390/PATHOGENS9070569.

[7] L. Huang, S. Ahmed, Y. Gu, J. Huang, B. An, C. Wu, Y. Zhou, G. Cheng, The Effects of Natural Products and Environmental Conditions on Antimicrobial Resistance, Molecules. 26 (2021). https://doi.org/10.3390/MOLECULES26144277.

[8] A. Caselli, P. Cirri, A. Santi, P. Paoli, Morin: A Promising Natural Drug, Curr Med Chem. 23 (2016) 774–791. https://doi.org/10.2174/0929867323666160106150821.

[9] C.C. Liolios, K. Graikou, E. Skaltsa, I. Chinou, Dittany of Crete: a botanical and ethnopharmacological review, J Ethnopharmacol. 131 (2010) 229–241. https://doi.org/10.1016/J.JEP.2010.06.005.

[10] M. De Vincenzi, A. Stammati, A. De Vincenzi, M.Silano, Constituents of aromatic plants: carvacrol, Fitoterapia. 75(2004) 801-4. https:// https://doi.org/10.1016/j.fitote.2004.05.002.

[11] A. Caselli, P. Cirri, A. Santi, P. Paoli, Morin: A Promising Natural Drug, Curr Med Chem. 23 (2016) 774–791. https://doi.org/10.2174/0929867323666160106150821.

[12] S.A. Rajput, X. qi Wang, H.C. Yan, Morin hydrate: A comprehensive review on novel natural dietary bioactive compound with versatile biological and pharmacological potential, Biomed Pharmacother. 138 (2021). https://doi.org/10.1016/J.BIOPHA.2021.111511.

[13] P.M. Maquera Huacho, E. Rodriguez Herrero, T. Verspecht, M. Pauwels, E. Marcantonio, D.M. Palomari Spolidorio, W. Teughels, Terpinen-4-ol and carvacrol affect multi-species biofilm composition, Biofouling. 35 (2019) 561–572. https://doi.org/10.1080/08927014.2019.1630386.

[14] L. Marinelli, E. Fornasari, P. Eusepi, M. Ciulla, S. Genovese, F. Epifano, S. Fiorito, H. Turkez, S. Örtücü, M. Mingoia, S. Simoni, A. Pugnaloni, A. Di Stefano, I. Cacciatore, Carvacrol prodrugs as novel antimicrobial agents, Eur J Med Chem. 178 (2019) 515–529. https://doi.org/10.1016/J.EJMECH.2019.05.093.

[15] E.R. Silva, F.O. de Carvalho, L.G.B. Teixeira, N.G.L. Santos, F.A. Felipe, H.S.R. Santana, S. Shanmugam, L.J. Quintans Júnior, A.A. de Souza Araújo, P.S. Nunes, Pharmacological Effects of Carvacrol in In vitro Studies: A Review, Curr Pharm Des. 24 (2018) 3454–3465. https://doi.org/10.2174/1381612824666181003123400.

[16] K. Can Baser, Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils, Curr Pharm Des. 14 (2008) 3106–3119. https://doi.org/10.2174/138161208786404227.

[17] P.D. Gupta, T.J. Birdi, Development of botanicals to combat antibiotic resistance, J Ayurveda Integr Med. 8 (2017) 266-275. https://doi.org/10.1016/j.jaim.2017.05.004.

[18] L.S. Sales, M. da S. Gimenes, A.B. Meneguin, H. da S. Barud, J.A. Achcar, F.L. Brighenti, Development of multiparticulate systems based on natural polymers for morin controlled release, Int J Biol Macromol. 228 (2023) 1–12. https://doi.org/10.1016/J.IJBIOMAC.2022.12.146.

[19] A.L. de Farias, A.B. Meneguin, H. da Silva Barud, F.L. Brighenti, The role of sodium alginate and gellan gum in the design of new drug delivery systems intended for antibiofilm activity of morin, Int J Biol Macromol. 162 (2020) 1944–1958. https://doi.org/10.1016/J.IJBIOMAC.2020.08.078.

[20] A.L. de Farias, M.I.A. Arbeláez, A.B. Meneguin, H. da S. Barud, F.L. Brighenti, Mucoadhesive controlled-release formulations containing morin for the control of oral biofilms, Biofouling. 38 (2022) 71–83. https://doi.org/10.1080/08927014.2021.2015580.

[21] M. Rocha, M.A. Coimbra, C. Nunes, Applications of chitosan and their derivatives in beverages: A critical review, Current Opinion in Food Science, 15 (2017) 61-69. https://doi.org/10.1016/j.cofs.2017.06.008.

[22] H. Dureja, J. Adams, R. Löbenberg, T. Pinto, K. Dua, Natural Polymeric Materials based Drug Delivery Systems in Lung Diseases, (2023).

[23] L.M.B. Ferreira, A.M. dos Santos, F.I. Boni, K.C. dos Santos, L.M.G. Robusti, M.P.C. de Souza, N.N. Ferreira, S.G. Carvalho, V.M.O. Cardoso, M. Chorilli, B.S.F. Cury, D.R.M. de Godoi, M.P.D. Gremião, Design of chitosan-based particle systems: A review of the physicochemical foundations for tailored properties, Carbohydr Polym. 250 (2020). https://doi.org/10.1016/J.CARBPOL.2020.116968.

[24] M.A. Matica, F.L. Aachmann, A. Tøndervik, H. Sletta, V. Ostafe, Chitosan as a Wound Dressing Starting Material: Antimicrobial Properties and Mode of Action, Int J Mol Sci. 20 (2019). https://doi.org/10.3390/IJMS20235889.

[25] Z. Ma, A. Garrido-Maestu, K.C. Jeong, Application, mode of action, and in vivo activity of chitosan and its micro- and nanoparticles as antimicrobial agents: A review, Carbohydr Polym. 176 (2017) 257–265. https://doi.org/10.1016/J.CARBPOL.2017.08.082.

[26] R. Abka-khajouei, L. Tounsi, N. Shahabi, A.K. Patel, S. Abdelkafi, P. Michaud, Structures, Properties and Applications of Alginates, Mar Drugs. 20 (2022). https://doi.org/10.3390/MD20060364.

[27] T. Yilmaz, L. Maldonado, H. Turasan, J.Kokini , Thermodynamic mechanism of particulation of sodium alginate and chitosan polyelectrolyte complexes as a function of charge ratio and order of addition, Journal of Food Engineering, 254 (2019) 42-50. https://doi.org/10.1016/j.jfoodeng.2019.03.002.

[28] Q. Deng, P. Sun, L. Zhang, Z. Liu, H. Wang, J. Ren, X. Qu, Q. Deng, P. Sun, L. Zhang, Z. Liu, H. Wang, J. Ren, X. Qu, Porphyrin MOF dots–based, function-adaptive nanoplatform for enhanced penetration and photodynamic eradication of bacterial biofilms, Wiley Online Library. 29 (2019). https://doi.org/10.1002/adfm.201903018.

[29] M. Fernandez y Mostajo, R.A.M. Exterkate, M.J. Buijs, W. Crielaard, E. Zaura, Effect of mouthwashes on the composition and metabolic activity of oral biofilms grown in vitro, Clin Oral Investig. 21 (2017) 1221–1230. https://doi.org/10.1007/S00784-016-1876-2.

[30] Y. Liu, L. Shi, L. Su, H.C. Van der Mei, P.C. Jutte, Y. Ren, H.J. Busscher, Nanotechnology-based antimicrobials and delivery systems for biofilm-infection control, Chem Soc Rev. 48 (2019) 428–446. https://doi.org/10.1039/C7CS00807D.

[31] M.H. Kim, Nanoparticle-Based Therapies for Wound Biofilm Infection: Opportunities and Challenges, IEEE Trans Nanobioscience. 15 (2016) 294–304. https://doi.org/10.1109/TNB.2016.2527600.

[32] K. Zhou, C. Li, D. Chen, Y. Pan, Y. Tao, W. Qu, Z. Liu, X. Wang, S. Xie, A review on nanosystems as an effective approach against infections of Staphylococcus aureus, Int J Nanomedicine. 13 (2018) 7333–7347. https://doi.org/10.2147/IJN.S169935.

[33] A.L.P. Silvestre, A.M. dos Santos, A.B. de Oliveira, T.M. Ferrisse, F.L. Brighenti, A.B. Meneguin, M. Chorilli, Evaluation of photodynamic therapy on nanoparticles and films loaded-nanoparticles based on chitosan/alginate for curcumin delivery in oral biofilms, Int J Biol Macromol. 240 (2023). https://doi.org/10.1016/J.IJBIOMAC.2023.124489.

[34] L. Mazzarino, R. Borsali, E. Lemos-Senna, Mucoadhesive films containing chitosan-coated nanoparticles: a new strategy for buccal curcumin release, J Pharm Sci. 103 (2014) 3764–3771. https://doi.org/10.1002/JPS.24142.

[35] F.H. van de Sande, M.S. Azevedo, R.G. Lund, M.C.D.N.J.M. Huysmans, M.S. Cenci, An in vitro biofilm model for enamel demineralization and antimicrobial dose-response studies, Biofouling. 27 (2011) 1057–1063. https://doi.org/10.1080/08927014.2011.625473.

[36] A.J. McBain, C. Sissons, R.G. Ledder, P.K. Sreenivasan, W. De Vizio, P. Gilbert, Development and characterization of a simple perfused oral microcosm, J Appl Microbiol. 98 (2005) 624–634. https://doi.org/10.1111/J.1365-2672.2004.02483.X.

[37] Y.E. Albuquerque, M. Danelon, M.J. Salvador, C.Y. Koga-Ito, A.C. Botazzo Delbem, R.Y. Ramirez-Rueda, A.V. Lacerda Gontijo, F.L. Brighenti, Mouthwash containing Croton doctoris essential oil: in vitro study using a validated model of caries induction, Future Microbiol. 13 (2018) 631–643. https://doi.org/10.2217/FMB-2017-0209.

[38] O.G. Gold, H. V. Jordan, J. van Houte, A selective medium for *Streptococcus mutans*, Arch Oral Biol. 18 (1973) 1357–1364. https://doi.org/10.1016/0003-9969(73)90109-X.

[39] M.S. Azevedo, F.H. van de Sande, T.T. Maske, C. Signori, A.R. Romano, M.S. Cenci, Correlation between the cariogenic response in biofilms generated from saliva of mother/child pairs, Biofouling. 30 (2014) 903–909. https://doi.org/10.1080/08927014.2014.948868.

[40] S.G. Carvalho, A.M. dos Santos, A.L.P. Silvestre, A.B. Meneguin, L.M.B. Ferreira, M. Chorilli, M.P.D. Gremião, New insights into physicochemical aspects involved in the formation of polyelectrolyte complexes based on chitosan and dextran sulfate, Carbohydr Polym. 271 (2021). https://doi.org/10.1016/J.CARBPOL.2021.118436.

[41] A. M. dos Santos, S. G. Carvalho, L.M.B. Ferreira, M. Chorilli, M.P.D. Gremião, Understanding the role of electrostatic interactions on the association of 5-fluorouracil to chitosan-TPP nanoparticles, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 640 (2022). https://doi.org/10.1016/j.colsurfa.2022.128417.

[42] A. Makhlof, Y. Tozuka, H. Takeuchi, Design and evaluation of novel pH-sensitive chitosan nanoparticles for oral insulin delivery, Eur J Pharm Sci. 42 (2011) 445–451. https://doi.org/10.1016/J.EJPS.2010.12.007.

[43] H. Cho, J. Bae, V.K. Garripelli, J.M. Anderson, H.W. Jun, S. Jo, Redox-sensitive polymeric nanoparticles for drug delivery, Chem Commun (Camb). 48 (2012) 6043–6045. https://doi.org/10.1039/C2CC31463K.

[44] M. Gaumet, A. Vargas, R. Gurny, F. Delie, Nanoparticles for drug delivery: the need for precision in reporting particle size parameters, Eur J Pharm Biopharm. 69 (2008) 1–9. https://doi.org/10.1016/J.EJPB.2007.08.001.

[45] J. Liu, J. Xiao, F. Li, Y. Shi, D. Li, Q. Huang, Chitosan-sodium alginate nanoparticle as a delivery system for ε-polylysine: Preparation, characterization and antimicrobial activity, Food Control, 91 (2018) 302-310. https://doi.org/10.1016/j.foodcont.2018.04.020.

[46] R. G. Chaudhuri, S. Paria, Core/shell nanoparticles: classes, properties, synthesis mechanisms, characterization, and applications, Chem Rev. 112 (2012) 2373–2433. https://doi.org/10.1021/CR100449N.

[47] A. Zeb, M. Gul, T.T.L. Nguyen, H.J. Maeng, Controlled release and targeted drug delivery with poly(lactic-co-glycolic acid) nanoparticles: reviewing two decades of research, J Pharm Investig. 52 (2022) 683–724. https://doi.org/10.1007/S40005-022-00584-W.

[48] A. Budhian, S.J. Siegel, K.I. Winey, Haloperidol-loaded PLGA nanoparticles: systematic study of particle size and drug content, International Journal of Pharmaceutics, 336 (2007) 367-375. https://doi.org/10.1016/j.ijpharm.2006.11.061.

[49] B. Sarmento, A.J. Ribeiro, F. Veiga, D.C. Ferreira, R.J. Neufeld, Insulin-loaded nanoparticles are prepared by alginate ionotropic pre-gelation followed by chitosan polyelectrolyte complexation, J Nanosci Nanotechnol. 7 (2007) 2833–2841. https://doi.org/10.1166/JNN.2007.609.

[50] S. K. Motwani, S. Chopra, S. Talegaonkar, K. Kohli, F. J. Ahmad, R. K. Khar, Chitosan-sodium alginate nanoparticles as submicroscopic reservoirs for ocular delivery: formulation, optimisation and in vitro characterisation, Eur J Pharm Biopharm. 68 (2008) 513–525. https://doi.org/10.1016/j.ejpb.2007.09.009.

[51] R.S. Tiğli Aydin, M. Pulat, 5-fluorouracil encapsulated chitosan nanoparticles for pH-stimulated drug delivery, J Nanomater. 2012 (2012). https://doi.org/10.1155/2012/313961.

[52] D. Aluani, V. Tzankova, Y. Yordanov, M. Kondeva-Burdina, K. Yoncheva, In vitro protective effects of encapsulated quercetin in neuronal models of oxidative stress injury, Taylor & Francis. 31 (2017) 1055–1063. https://doi.org/10.1080/13102818.2017.1347523.

[53] F.M. Goycoolea, G. Lollo, C. Remuñán-López, F. Quaglia, M.J. Alonso, Chitosan-alginate blended nanoparticles as carriers for the transmucosal delivery of macromolecules, Biomacromolecules. 10 (2009) 1736–1743. https://doi.org/10.1021/BM9001377/SUPPL_FILE/BM9001377_SI_001.PDF.

[54] W.E.G. Müller, S. Wang, E. Tolba, M. Neufurth, M. Ackermann, R. Muñoz-Espí, I. Lieberwirth, G. Glasser, H.C. Schröder, X. Wang, Transformation of Amorphous Polyphosphate Nanoparticles into Coacervate Complexes: An Approach for the Encapsulation of Mesenchymal Stem Cells, Small. 14 (2018). https://doi.org/10.1002/SMLL.201801170.

[55] M.A. Mumuni, F.C. Kenechukwu, K.C. Ofokansi, A.A. Attama, D.D. Díaz, Insulin-loaded mucoadhesive nanoparticles based on mucin-chitosan complexes for oral delivery and diabetes treatment, Carbohydr Polym. 229 (2020). https://doi.org/10.1016/J.CARBPOL.2019.115506.

[56] A. Rafiee, M. H. Alimohammadian, T. Gazori , F. Riazi-rad, S.M.R. Fatemi, A. Parizadeh , I. Haririan , M.Havaskary, Comparison of chitosan, alginate and chitosan/alginate nanoparticles with respect to their size, stability, toxicity and transfection, Asian Pacific Journal of Tropical Disease, 4 (2014) 372-377. https://doi.org/10.1016/S2222-1808(14)60590-9.

[57] Y.L. Liu, M. Nascimento, R.A. Burne, Progress toward understanding the contribution of alkali generation in dental biofilms to inhibition of dental caries, Int J Oral Sci. 4 (2012) 135–140. https://doi.org/10.1038/IJOS.2012.54.

[58] F.N. Sorasitthiyanukarn, P. Ratnatilaka Na Bhuket, C. Muangnoi, P. Rojsitthisak, P. Rojsitthisak, Chitosan/alginate nanoparticles as a promising carrier of novel curcumin diethyl diglutarate, Int J Biol Macromol. 131 (2019) 1125–1136. https://doi.org/10.1016/J.IJBIOMAC.2019.03.120.

[59] S. Bhunchu, P. Rojsitthisak, P. Rojsitthisak, Effects of preparation parameters on the characteristics of chitosan–alginate nanoparticles containing curcumin diethyl disuccinate, Journal of Drug Delivery Science and Technology, 28 (2015) 64-72. https://doi.org/10.1016/j.jddst.2015.05.010.

[60] A. Negi, K.K. Kesari, Chitosan Nanoparticle Encapsulation of Antibacterial Essential Oils, Micromachines (Basel). 13 (2022). https://doi.org/10.3390/MI13081265.

[61] Z. Ma, A. Garrido-Maestu, K.C. Jeong, Application, mode of action, and in vivo activity of chitosan and its micro- and nanoparticles as antimicrobial agents: A review, Carbohydr Polym. 176 (2017) 257–265. https://doi.org/10.1016/J.CARBPOL.2017.08.082.

[62] L. C. de Paz, A. Resin, K. A. Howard, D. S. Sutherland, P. L. Wejse, Antimicrobial effect of chitosan nanoparticles on *Streptococcus mutans* biofilms, Am Soc Microbiol. 77 (2011) 3892–3895. https://doi.org/10.1128/AEM.02941-10.

[63] J. Yang, H. S.Lee, Evaluation of antioxidant and antibacterial activities of morin isolated from mulberry fruits (Morus alba L.), Springer. 55 (2012) 485–489. https://doi.org/10.1007/s13765-012-2110-9.

[64] A. Nostro, A. Marino, A.R. Blanco, L. Cellini, M. Di Giulio, F. Pizzimenti, A.S. Roccaro, G. Bisignano, In vitro activity of carvacrol against staphylococcal preformed biofilm by liquid and vapour contact, Microbiologyresearch.Org. 58 (2009) 791–797. https://doi.org/10.1099/jmm.0.009274-0.

[65] F. Nazzaro, F. Fratianni, L. De Martino, R. Coppola, V. De Feo, Effect of Essential Oils on Pathogenic Bacteria, Pharmaceuticals. 6 (2013) 1451. https://doi.org/10.3390/PH6121451.

[66] L. Marinelli, E. Fornasari, P. Eusepi, M. Ciulla, S. Genovese, F. Epifano, S. Fiorito, H. Turkez, S. Örtücü, M. Mingoia, S. Simoni, A. Pugnaloni, A. Di Stefano, I. Cacciatore, Carvacrol prodrugs as novel antimicrobial agents, Eur J Med Chem. 178 (2019) 515–529. https://doi.org/10.1016/J.EJMECH.2019.05.093.

[67] Y. Jin, B. Zhao, W. Guo, Y. Li, J. Min, W. Miao, Penetration and photodynamic ablation of drug-resistant biofilm by cationic Iron oxide nanoparticles, J Control Release. 348 (2022) 911–923. https://doi.org/10.1016/J.JCONREL.2022.06.038.

[68] C.K. Hope, M. Wilson, Analysis of the effects of chlorhexidine on oral biofilm vitality and structure based on viability profiling and an indicator of membrane integrity, Antimicrob Agents Chemother. 48 (2004) 1461–1468. https://doi.org/10.1128/AAC.48.5.1461-1468.2004.

[69] J. Xiao, M.I. Klein, M.L. Falsetta, B. Lu, C.M. Delahunty, J.R. Yates, A. Heydorn, H. Koo, The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm, PLoS Pathog. 8 (2012). https://doi.org/10.1371/JOURNAL.PPAT.1002623.

[70] F. Cieplik, W. Buchalla, E. Hellwig, A. Al-Ahmad, K.A. Hiller, T. Maisch, L. Karygianni, Antimicrobial photodynamic therapy as an adjunct for treatment of deep carious lesions-A systematic review, Photodiagnosis Photodyn Ther. 18 (2017) 54-62. https://doi.org/10.1016/j.pdpdt.2017.01.005.

[71] Y. Mohamed, R. Ashraf, Remineralization potential of phosphorylated chitosan and silver diamine fluoride in comparison to sodium fluoride varnish: invitro study, Eur Arch Paediatr Dent. 24 (2023). https://doi.org/10.1007/S40368-023-00794-2.

[72] J.L.S. de Souza, T. Alves, L. Camerini, F. Nedel, A.D. Campos, R.G. Lund, Antimicrobial and cytotoxic capacity of pyroligneous extracts films of Eucalyptus grandis and chitosan for oral applications, Sci Rep. 11 (2021). https://doi.org/10.1038/S41598-021-00529-7.

[73] S.A. Yamakami, J.J. Faraoni, N.S.N.D. Lia, F.B. Regula, H. Ohyama, R.G. Palma-Dibb, Effect of an experimental chitosan/casein gel on demineralized enamel under a cariogenic challenge, Dent Med Probl. 59 (2022) 531–538. https://doi.org/10.17219/DMP/146038.

[74] S.B. Nimbeni, B.S. Nimbeni, D.D. Divakar, Role of Chitosan in Remineralization of Enamel and Dentin: A Systematic Review, Int J Clin Pediatr Dent. 14 (2021) 562–568. https://doi.org/10.5005/JP-JOURNALS-10005-1971.

3.3 Publicação 3*

Anti-inflammatory and antimicrobial evaluation of multiparticulate system containing morin.

Luciana Solera Sales[a], Benjamin Hewitt[b], Maria Muchova[c], Fernanda Lourenção Brighenti[d], Sarah A Kuehne[e], Melissa M Grant[f], Michael R Milward*

[a]Department of Morphology, Genetics, Orthodontics and Pediatric Dentistry, São Paulo State University (Unesp), School of Dentistry, R. Humaitá, 1680 – Centro, Araraquara, São Paulo, Brazil – 14801-903. luciana.solera@gmail.com

[b]School of Dentistry, Institute of Clinical Sciences, University of Birmingham, Birmingham, United Kingdom. BXH699@student.bham.ac.uk

[c]School of Dentistry, Institute of Clinical Sciences, University of Birmingham, Birmingham, United Kingdom. MXM1133@student.bham.ac.uk

[d]Department of Morphology, Genetics, Orthodontics and Pediatric Dentistry, São Paulo State University (Unesp), School of Dentistry, R. Humaitá, 1680 – Centro, Araraquara, São Paulo, Brazil – 14801-903. f.brighenti@unesp.br

[e]School of Dentistry, Institute of Clinical Sciences, University of Birmingham, Birmingham, United Kingdom. s.a.kuehne@bham.ac.uk

[f]School of Dentistry, Institute of Clinical Sciences, University of Birmingham, Birmingham, United Kingdom. m.m.grant@bham.ac.uk

*Corresponding author: Michael R Milward, Periodontal Research Group, School of Dentistry, Institute of Clinical Sciences, University of Birmingham, Birmingham, United Kingdom. E-mail address: m.r.milward@bham.ac.uk

* Artigo segue a norma do periódico Journal of Periodontal Research para o qual pretende-se submeter.

Abstract

Periodontitis is the one of the most prevalent diseases in the world. Morin has been widely shown to have anti-inflammatory and antimicrobial properties, and therefore have potential for use in the management of periodontitis. This study aims to evaluate the anti-inflammatory and antimicrobial activity of system containing morin, against periodontal pathogens. These systems improve morin's solubility stability and allow sustained release of morin, thereby enhancing the therapeutic potential. For ani-inflammatory evaluation, H400 oral epithelial cell line was pre-conditioned with system containing morin and exposed to periodontal pathogenic bacteria (*Fusobacterium nucleatum* and *Porphyromonas gingivalis*) for 24 h. The production of cytokines was measured by enzyme-linked immunosorbent assay (ELISA). For antimicrobial evaluation, the minimum inhibitory concentration (MIC) of free morin and system containing morin was determined against *P. gingivalis* and *F. nucleatum*. Planktonic culture (24 h) of *F. nucletatum* and *P. gingivalis* and multi-species biofilms (*Streptococcus oralis*, *F. nucleatum* and *P. gingivalis* – 7 days) were exposed to the treatments. The microbial viability was determined by counting colony forming units (CFU/mL) and confocal laser scanning microscopy. The biofilm biomass was determined using a crystal violet assay. Pre-conditioning of H400 cells with a concentration of free morin (0.125 mg/mL) and system containing morin (0.125 mg/mL) for 8h attenuated secretion of Granulocyte-Macrophage – Colony-Stimulating Factor (GM-CSF). The MIC of free morin was 117.188 µg/mL (*F. nucleatum* and *P. gingivalis*). The MIC of system containing morin was 78.125 µg/mL for *F. nucleatum* and 39.063 µg/mL for *P. gingivalis* respectively. Microbial viability (Log CFU/mL - planktonic culture and mature biofilm) and the biofilm biomass reduced after treatment with free morin and system containing morin compared with the control group. In conclusion, the system containing morin can inhibit two key periodontal pathogens and the excessive inflammation caused by periodontal pathogens and hence offers therapeutic potential.

Keywords: Periodontitis, Biofilm, Morin.

1. Introduction

Periodontitis is the leading cause of tooth loss in adulthood and one of the most prevalent diseases in the world (1,2). Periodontitis is a chronic inflammatory disease,

caused by a dysbiotic shift in the subgingival microbiota of the dental plaque biofilm. These bacteria, particularly *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum* and *Filifactor alocis,* are able to induce an excessive inflammatory response in the periodontium of susceptible patients resulting in progressive destruction in the tooth's supporting tissues (3–6). This is initially characterized by epithelial destruction, with associated attachment loss and ultimately alveolar bone resorption, which, if left untreated, leads to tooth loss.

The acute inflammatory reaction in periodontitis is predominantly mediated by neutrophils, vital component of the innate immune response (7). These cells, stimulated by periodontal bacteria, promote extracellular and intracellular reactive oxygen species (ROS) release (8). This host response is important to combat the disease-causing pathogens. However, excessive ROS release also causes tissue damage and increased osteoclast differentiation in the host to contribute to periodontitis progression (8,9).

Furthermore, there are a number of mechanisms that drive bacterial induced inflammation, one of the key initiators being lipopolysaccharides (LPS), an important cell membrane component. LPS is a key bacterial virulence factor which stimulates sulcular / pocket epithelium resulting in downstream inflammatory responses driven by local cytokine / chemokine production (10,11). These cytokines initiate and propagate the excessive local chronic inflammation that is characteristic of periodontitis ultimately activating osteoclasts responsible for alveolar bone destruction (12). Interleukin-1beta (IL-1β) is one of the first cytokines to be secreted and shows an important role in inflammation processes by inducing the expression of other inflammatory cytokines (6,13). IL-1β release is correlated with two steps. The first step is the activation of the nuclear factor-κB (NF-κB) signaling pathway following periodontal bacteria binding to cell Toll-like receptors, and secondly activation of inflammasomes (12,14). Inflammasomes are innate immune system receptors that control the activation of some cytokines, following detection of bacteria and foreign substances. NLRP3 is one of the inflammasomes that is dysregulated in periodontal disease and is more highly expressed in periodontitis patients when compared to healthy subjects. This inflammasome can be activated by pathogenic microbial components and pathogenic microorganisms, such as *P. gingivalis* and *A. actinomycetemcomitans,* and in response to tissue damage (15–18).

Classically nonsurgical periodontal therapy, aimed at pathogenic biofilm and dental calculus removal from the tooth / root surfaces has been the management strategy of choice producing successful clinical outcomes. However, in certain situations where access to the biofilm is challenging e.g. deep periodontal pockets and furcation defects, treatment outcomes can be compromised (19–21). Therefore, the combined use of mechanical removal with locally administered antibiotics have been used to enhance subgingival biofilm removal and to improve clinical outcomes (20,22). However, concerns with antibiotic use exist due to the challenges of antimicrobial resistance (23–25). In addition, some studies have reported no changes in IL-1β levels after conventional periodontal treatment, with or without the use of antibiotics (26–30). So there exists an unmet clinical need to develop new non-antibiotic based adjunctive therapies to help improve treatment outcomes in patient with periodontitis. Interest has grown in the utilization of natural derived substances that have antimicrobial, anti-inflammatory and antioxidant properties that may have efficacy in chronic inflammatory diseases including periodontitis.

Morin (2′,3,4′,5,7-pentahydroxyflavone), a flavonoid obtained from plants, fruits and herbs, shows antioxidant, anti-inflammatory and antibacterial activity (31–35). Morin presents a well-known ability to inhibit Gram-positive bacteria (36) and ability to inhibit NLRP3 inflammasome expression and NF-κB signaling pathway (37–39) and has low toxicity (40). Recent studies (41–43) developed systems based on sodium alginate and gellan gum for morin controlled release, with the aim of potentiating the effect of free morin, since when it is in its free form it has limited stability and are easily degraded losing functionality which is an issue for clinical use (44,45). These studies not only showed an efficient alternative for incorporating morin into polymeric systems, and a morin controlled release, but also showed an antimicrobial effect by controlling important virulence factors of single and polymicrobial biofilms.

Thus, these modified natural compounds have the key characteristics (antimicrobial and anti-inflammatory) that may offer important clinical benefits when used alongside conventional non-surgical periodontal treatment and might be an alternative not only for controlling cariogenic microorganisms, but also for controlling microorganisms associated with periodontal disease. Therefore, the aim of this study was to evaluate the potential of this controlled release system containing morin, specifically: (i) anti-inflammatory, by modulating the secretion of pro-inflammatory cytokines, and (ii) antimicrobial activity, against periodontal pathogens.

2. Materials and methods

2.1 Materials

The flavonoid morin hydrate (2′,3,4′,5,7-Pentahydroxyflavone, purity > 85 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The same batch (M4008) was used throughout the tests. Dimethyl sulfoxide (DMSO) was used to dissolve morin. When tested as free form, a stock solution (60 mg/mL) was prepared in DMSO DMSO was used as a vehicle control at an equivalent concentration.

The controlled release system based on natural polymers containing morin used was prepared according to (41). Briefly, a suspension containing sodium alginate (ALG) and gellan gum (GG) in a 25:75 ratio (ALG:GG) and 20% of the final polymer mass of morin was prepared and dried in a spray dryer (MSD 0.5 spray dryer, LabMaq do Brasil, Ribeirão Preto, Brazil). The final dose of morin in the system was 15.44 mg morin/100 mg powder. A system without morin was also prepared and used as control.

2.2 Evaluation of anti-inflammatory activity

2.2.1 Cell Culture

Oral epithelial cells (H400) were cultured in flask using Dulbeco's Modified Eagle's Media (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) and 5% glutamine at 37°C in 5% CO_2. The cell line was purchased from the Europe culture collection (ECACC 06092006). Cells were seeded into white-walled 96-well plates (100 µL/ well) or 12-well (1.5 mL/well) plates at a density of 2 x 10^5 mL and allowed to attach overnight before challenge.

2.2.2 Heat-killed bacteria

F. nucleatum (ATCC 23726) and *P. gingivalis* (ATCC 33277) were grown anaerobically (80% N_2, 10% CO_2, and 10% H_2; Don Whitley DG250 Anaerobic Workstation, Don Whitley Scientific, Bingley, UK) at 37 °C on Schaedler anaerobe agar plates (SAA; Sigma-Aldrich/Merck, Darmstadt, Germany) and horse blood agar plates containing, per litre, 15.0 g proteose peptone, 2.5 g liver digest, 5.0 g yeast extract, 5.0 g sodium chloride (Oxoid, Basingstoke, UK), respectively. Schaedler anaerobe broth (SAB; Oxoid, Basingstoke, UK) was used to grow the microorganisms overnight in liquid cultures. After growth, the bacteria were washed two times in sterile phosphate buffered saline (PBS) and heat-killed for 1 hour at 80 ^oC. The optical density at 600 nm

was determined and used to calculate the bacteria concentration for ensuing multiplicity of infection (MOI 100:1) calculations. Heat inactivation was confirmed by plating experiments.

2.2.3 Cytotoxicity

H400 cells were seeded into white-walled 96-well plates (100 µL/ well) at a density of 2 x 10^5 mL overnight before being exposed to different concentrations of free morin (0.001-1 µg/mL), system containing morin (0.001-1 µg/mL) and heat-killed *P. gingivalis* and *F. nucleatum* (MOI 100:1). The toxicity of DMSO (0.1-1000 nL), the solvent used to solubilize morin, was also assessed. Untreated cells were used as a control. Metabolic activity of H400 cells was determined by CellTitre-Glo (Promega) after 24 hours of cells exposure or not (control) to the treatments CellTitre-Glo was added at the end of the experiments, following the manufacturer's protocol and luminescence viability was measured per well, using a Tecan SpectrafluorPlus instrument (Tecan).

2.2.4 Effect of periodontal pathogens on cytokine production in H400 cells

To confirm that bacteria were stimulating cytokine production in H400 cells, the effect of heat-killed periodontal pathogens - *F. nucleatum* and *P. gingivalis*- on IL-8 and GM-CSF secretion was initially evaluated. H400 cells were seeded into 12-well plates (1.5 mL/well), containing culture medium with or without hydrocortisone, at a density of 2 x 10^5 mL overnight before being exposed to heat-killed *F. nucleatum* and *P. gingivalis* (MOI 100:1) for 24 hours. Hydrocortisone was used to control cytokines expression in control group (not infected by periodontal pathogens) and to determine if anti-inflammatory activity would be assessed with or without hydrocortisone supplementation (46).

Cytokine expression in cell culture medium was measured by enzyme-linked immunosorbent assay kit (ELISA; Diaclone; IDS Ltd.) following the manufacturer's protocol. The experiments were carried out in triplicate. Results were presented as pg/mL, by using a calibration curve.

2.2.5 Anti-inflammatory activity

Prior assessing the anti-inflammatory properties, a time- and dose-response study of free morin and system containing morin treatments was conducted to

determine the optimal dose and treatment duration, with the evaluation of IL-8 and GM-CSF expression. H400 cells were seeded into 12-well plates (1.5 mL/well), at a density of 2 x 10^5 mL overnight. Different concentrations of system without morin, DMSO, free morin, system without morin + free morin and system containing morin (encapsulated morin) were added to the cells after overnight growth, for 4, 8 and 24 hours. The experimental groups control (cells without infection) and infection with periodontal pathogens were also evaluated. After treatment time (4, 8 and 24 hours) with the groups mentioned above, the cells were immediately challenged with heat-killed *F. nucleatum* (MOI 100:1) for 24 hours.

For the next experiments, only GM-CSF expression was evaluated, as they presented more consistent values in the preliminary tests. To assess morin stability, free morin and the system containing morin were left in culture medium for 24 hours before being added to the cells. Thus, the experimental groups for this analysis were i) free morin added immediately to culture medium, ii) system containing morin added immediately to culture medium, iii) free morin added after 24 hours in culture medium, and iv) system containing morin added after 24 hours in culture medium. Morin concentration used in all groups was 0.125 mg/mL. The cells were seeded as previously described and treated for 4, 8 and 24 hours. Next, the cells were challenged with heat-killed *F. nucleatum* (MOI 100:1) for 24 hours.

To evaluate the anti-inflammatory effect of the system containing morin, H400 cells were seeded in the same way as described above. The experimental groups were: i) control - cells without infection; ii) *F. nucleatum* or *P. gingivalis* infection (MOI: 100:1) - cells infected with heat-killed periodontal bacteria; iii) system (0.82 mg/mL) - system without morin; iv) DMSO (2 µl/mL); v) free morin (0.125 mg/mL); vi) system without morin (0.82 mg/mL) + free morin (0.125 mg/mL); vii) system with encapsulated morin (final dose of morin was 0.125 mg/mL). After treatment for 8 h, the cells were immediately challenged with heat-killed *F. nucleatum* and *P. gingivalis* (MOI 100:1) for 24 hours.

For all experiments, cytokine expression in cell culture medium was measured by enzyme-linked immunosorbent assay kit (ELISA; Diaclone; IDS Ltd.) following the manufacturer's protocol. The experiments were carried out in triplicate. Results were presented as pg/mL, by using a calibration curve.

2.3 Evaluation of antimicrobial activity

2.3.1 Microorganisms and growth conditions

Periodontal bacteria*, F. nucleatum* (ATCC 23726) and *P. gingivalis* (ATCC 33277) were cultured as described in item 2.2.2. *Streptococcus oralis* (ATCC 35037) was grown at 37 °C in 5% CO_2 on Brain heart infusion agar (BHI). BHI broth was used to grow *S. oralis* overnight in liquid cultures.

2.3.2 Evaluation of minimum inhibitory concentration (MIC)

Broth dilution method was performed to determine the MIC of free morin and the system containing morin, as well as DMSO. Metronidazole was used as a positive control. Free morin and system containing morin were added to 96-well plate for serial dilution in Schaedler anaerobe broth (SAB; Oxoid, Basingstoke, UK) to obtain a range of morin concentrations from 29.297 to 234.375 µg/mL and 9.766 to 156.25 µg/mL, respectively. DMSO was studied under the same conditions within a concentration range of 17.2 to 137.5 mg/mL. *F. nucleatum* and *P. gingivalis* suspensions at 10^6 CFU/mL (A_{600}= 0.001) were prepared and transferred to 96 well-plates. The plates were incubated anaerobically (80% N_2, 10% CO_2, and 10% H_2; Don Whitley DG250 Anaerobic Workstation, Don Whitley Scientific, Bingley, UK) at 37 °C for 24 hours. After incubation, the optical density was read at 600 nm using a microplate reader (Biotek ELX800).

2.3.3 Planktonic culture assay

Overnight *F. nucleatum* or *P. gingivalis* cultures were diluted in fresh Schaedler anaerobe broth to adjust the optical density to OD_{600}= 0.01 (10^7 CFU/mL). The experimental groups: i) system (6.58 mg/mL) -systems developed without morin; ii) DMSO (16 µl/mL); iii) free morin (1 mg/mL); iv) system (6.58 mg/mL) + morin (1 mg/mL) - system developed with encapsulated morin were added to the wells. Next, 1 mL of diluted culture was transferred into a 24-well plate. Untreated wells were used as a control. Plates were incubated at 37 °C for 24 h. To determine bacterial cell viability after the treatment colony forming units (CFU) assays was performed. The results were expressed in log (1+ CFU/mL). The experiments were performed in triplicate.

2.3.4 Multi-species biofilm assay

A multi-species biofilm was formed on 13 mm diameter Thermanox™ coverslips placed in 24-well plates containing artificial saliva (0.25% w/v porcine stomach mucins,

0.35 w/v sodium chloride, 0.02 w/v potassium chloride, 0.02 w/v calcium chloride dihydrate, 0.2 w/v yeast extract, 0.1 w/v lab lemco powder, 0.5 w/v proteose peptone and urea 40%, added after sterilization). Overnight bacterial cultures, prepared on their designated day of use, were washed twice with PBS and standardized to an OD_{600} = 0.5 (*S. oralis*) and OD_{600} = 0.2 (*F. nucleatum* and *P. gingivalis*), to achieve a 10^8 CFU/mL inoculum. Next, the inoculum was diluted in artificial saliva to obtain a working culture containing 10^7 CFU/mL of the microorganisms.

On the first day, *S. oralis* was added and incubated for 24 hours in 5% CO_2. On the second day, the supernatant was removed and *F. nucleatum* was added and incubated anaerobically for a further 24 hours. On the third day, the supernatant was again removed and *P. gingivalis* was added and incubated anaerobically for a further 24 hours. During the next 4 days of biofilm growth, the artificial saliva was changed every day.

The treatments were added on the first day of biofilm growth and replaced with each change of artificial saliva. The concentration of morin used, both in its free form and encapsulated within the system, was 1 mg/mL. To evaluate antibiofilm activity, biomass was assessed using crystal violet assay. The results were expressed in absorbance intensity (A_{590}). To determine bacterial viability after the treatment, colony forming units (CFU) counting and live/dead staining protocol (Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit, ThermoFisher Scientific, USA) were performed. For CFU counting, the biofilm was dispersed in PBS by pipetting with up and down movements. For *S. oralis* counting, biofilm dispersion was seeded on BHI agar and incubated at 37 °C in 5% CO_2 for 24 h. For *F. nucleatum* and *P. gingivalis* counting, biofilm dispersion was seeded on blood agar plate, incubated anaerobically (80% N_2, 10% CO_2, and 10% H_2; Don Whitley DG250 Anaerobic Workstation, Don Whitley Scientific, Bingley, UK) at 37 °C. Differential counting between *F. nucleatum* and *P. gingivalis* was carried out considering colony morphology (*F. nucletaum*: white colour and *P. gingivalis*: black colour). The results were expressed in log (1+ CFU/mL). Live/dead images were obtained using a confocal laser scanning microscopy (CLSM) (LSM 700, Zeiss, Germany) with a ×40 oil immersion objective (Zeiss Objective EC Plan-Neofluar 40×/1.30 Oil DIC M27, FWD = 0.21 mm). The excitation/emission was 488 nm<550 nm for SYTOà9, 555 nm/> 550 nm, for propidium iodide and 490 nm/<525 nm for (WGA) Alexa Fluor$_{T\ M}$ 48. For 3D analysis Z-stacks were taken at 1.2 µm increments from the surface. To examine the biofilm architecture and the treatment

effect, a scanning electron microscope (Zeiss EVO MA10) was used to obtain the images. The images were recorded at 1,000× and 5,000× magnification. The experiments were performed in triplicate.

2.4 Statistical analysis

The data was analyzed using a GraphPad Prism (version 10.0.2 for Windows, GraphPad Software, San Diego, California, USA). As data showed normal distribution (Shapiro-Wilk) and equality of variances (Levene's Test), One way ANOVA, followed by Tukey's post-hoc test was used for statistical analysis. For the effect of periodontal pathogens on cytokine production the data were analyzed by three - way ANOVA, followed by Tukey's post-hoc test. The three independent variables were i) *F. nucleatum* infection or *P. gingivalis* infection x control (without infection), ii) medium with hydrocortisone x medium without hydrocortisone, iii) time (4 x 8 x 24 hours). For time- and dose-response study and morin stability study the data were analyzed by two - way ANOVA, followed by Tukey's post-hoc test. The two independent variables for time- and dose-response were i) time (4 x 8 x 24 hours) or concentration (0.016 x 0.032 x 0.064 x 0.125 x 0.25 x 0.5 mg/mL) and ii) treatment group (control x *F. nucleatum* or *P. gingivalis* infection x system x DMSO x free morin x system + free morin x system + morin). The two independent variables for morin stability were i) time (4 x 8 x 24 hours) and ii) treatment group (free morin added immediately to culture medium x system + morin added immediately to culture medium x free morin added after 24 hours in culture medium x system + morin added after 24 hours in culture medium. A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Evaluation of anti-inflammatory activity

3.1.1 Cytotoxicity

Initially, the cytotoxicity of heat-killed periodontal pathogens, *P. gingivalis* and *F. nucleatum* and of free morin and the system containing morin were evaluated. The microorganisms (MOI 100:1) (Figure 1A), free morin, the system containing morin and DMSO (Figure 1B-D) proved to be not cytotoxic to the H400 cells at the studied concentration.

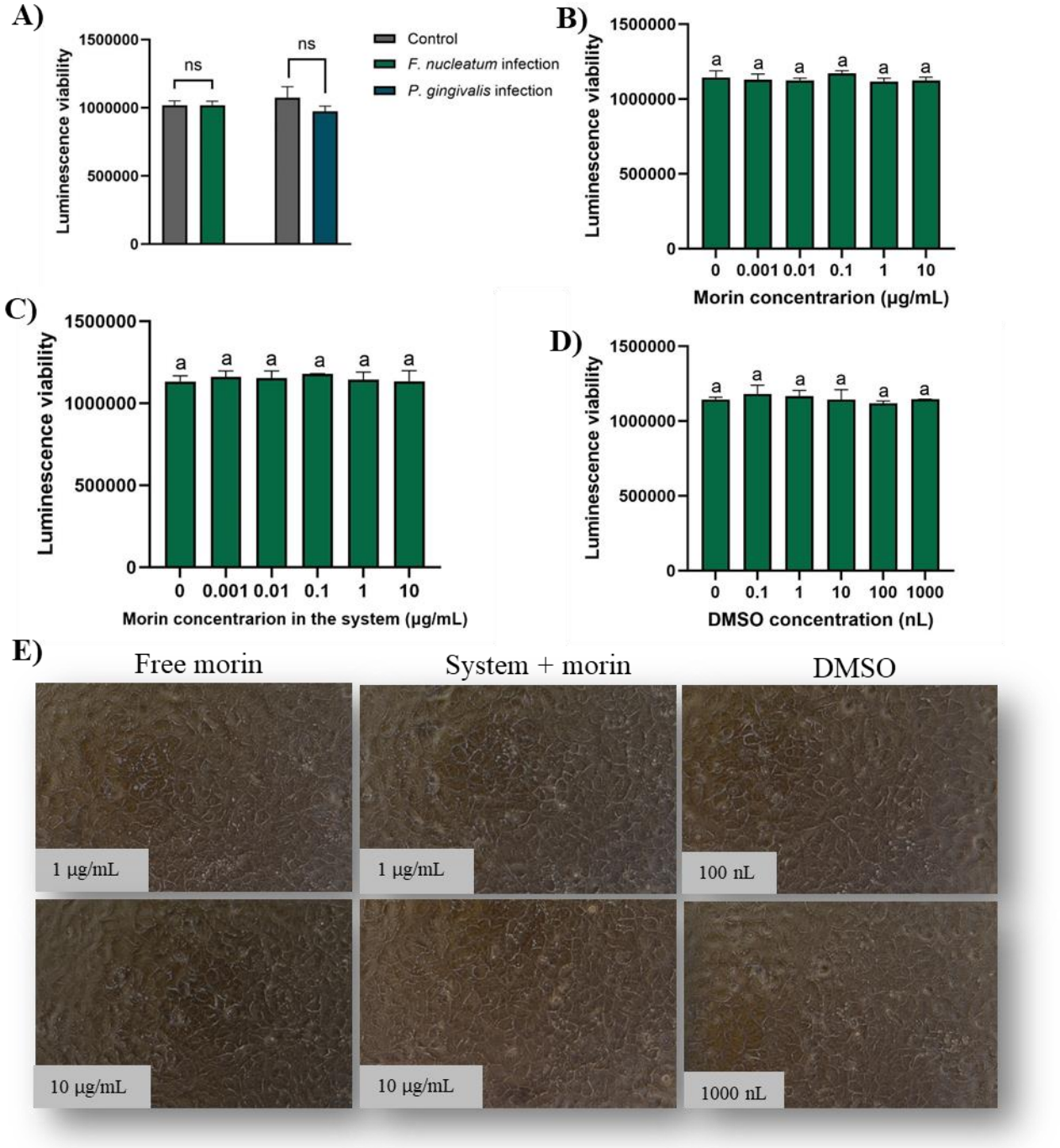

Figure 1. Cytotoxicity of H400 after 24 hours of incubation with **A)** heat-killed *F. nucleatum* and *P. gingivalis*, MOI 100:1. Student t test; **B)** with different concentrations of free morin; **C)** system containing different concentrations of morin, and **D)** different concentrations of DMSO, measured using Cell Titre Glo. One way ANOVA with Tukey's post-hoc test. For all experiments, mean ± SD; n=3. p-value<0.05. **E)** Representative images of H400 after 24 hours of incubation with different concentrations of free morin, system containing morin and DMSO.

3.1.2 Effect of periodontal pathogens on cytokine production in H400 cells

The heat-killed *P. gingivalis* and *F. nucleatum* (MOI 100:1*)* significantly stimulated the secretion of pro-inflammatory cytokines, IL-8 (Figure 2A-B) and GM-CSF (Figure 2C-D) in culture medium with and without hydrocortisone containing H400 cells.

For IL-8, cells stimulated with *F. nucleatum* with and without hydrocortisone showed a significant increase in cytokine expression when compared to control groups, for all times evaluated (4, 8 and 24 hours). However, for cells stimulated with *P. gingivalis*, this difference was only found in the hydrocortisone group, after 4 and 8 hours. There was no statistically significant increase in IL-8 expression over time. Additionally, no statistically significant difference was observed in IL-8 expression when the cells grown in medium with or without hydrocortisone.

For GM-CSF, cells stimulated with *F. nucleatum* and *P. gingivalis* with and without hydrocortisone showed a significant increase in cytokine expression when compared to control groups, for all times evaluated (4, 8 and 24 hours). The addition of hydrocortisone significantly decreased GM-CSF levels in the groups infected with *F. nucleatum* or *P. gingivalis* after 8 and 24 hours when compared to the culture medium without hydrocortisone. After 24 hours, GM-CSF expression increased significantly for the *F. nucleatum* and *P. gingivalis* infection groups, culture medium with and without hydrocortisone, when compared to the other times (4 and 8 hours).

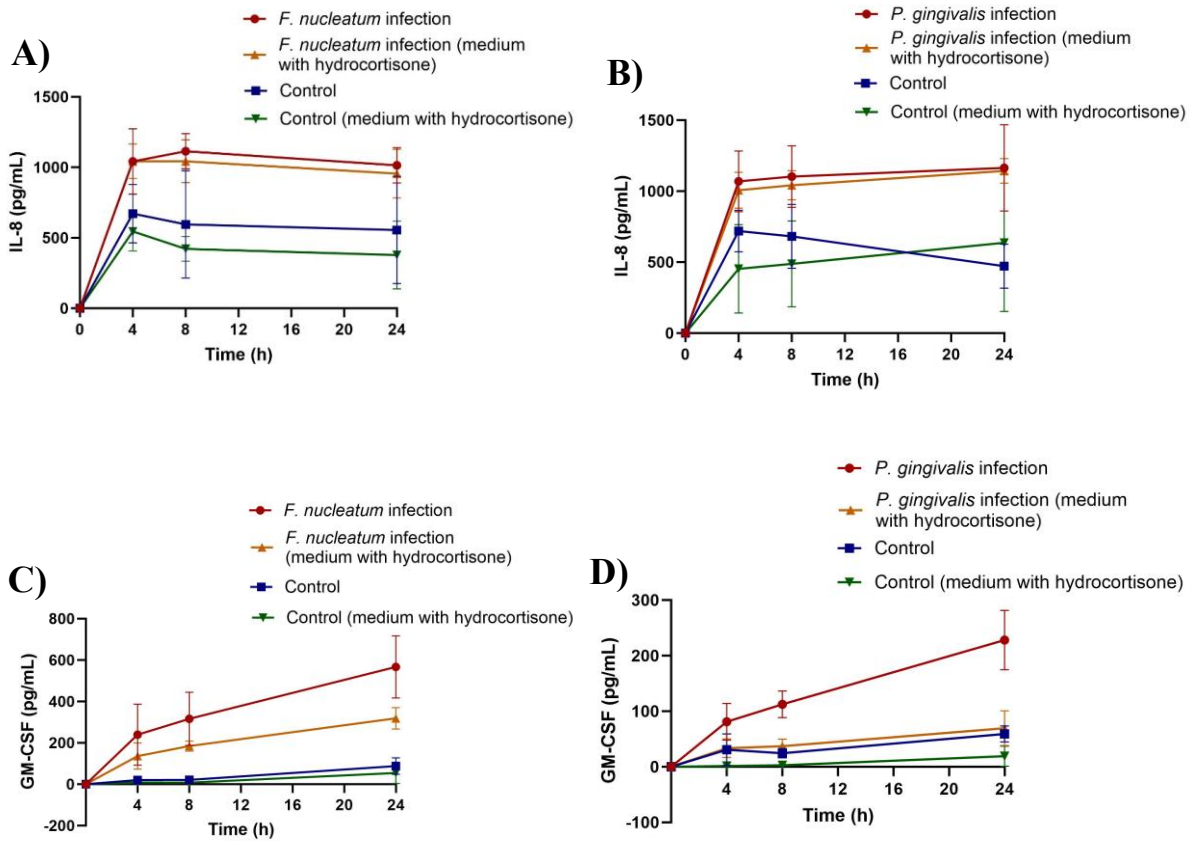

Figure 2. Effect of bacterial stimulation on IL-8 and GM-CSF production by H400 cells after 4, 8 and 24 hours of incubation without (control) and with heat-killed *F. nucleatum* **(A and C)** and *P. gingivalis* **(B and D)** at MOI 100:1. Media without and with hydrocortisone were collected and IL-8 and GM-CSF were measured by ELISA. Three-way ANOVA with Tukey's post-hoc test (mean ± CI n=6).

3.1.3 Anti-inflammatory activity

Preceding the evaluation of the anti-inflammatory properties of the system containing morin, a time- and dose-response study of free morin and the system containing morin treatments was conducted (Figure 3). System + morin (0.25 mg/mL morin) was the only one to significantly decrease IL-8 levels compared to lower concentrations, whereas for free morin and system + free morin IL-8 inhibition was observed at 0.5 mg/mL (Figure 3A). For the other groups, there was no reduction in IL-8 expression. In relation to time-response, IL-8 levels remained constant at 4, 8 and 24 hours for the groups free morin and system containing morin. For the system + free morin group, IL-8 expression remained constant at times 4 and 8 hours. After 24 hours, IL-8 levels decreased significantly when compared to the 8-hour time point (Figure 3C).

For GM-CSF, system containing morin (0.064 mg/mL morin) was enough to start significantly decreasing GM-CSF levels. Total GM-CSF reduction was achieved

at 0.25 mg/mL in the group system + morin. For the group free morin and system + free morin total GM-CSF reduction was achieved at 0.5 mg/mL. GM-CSF levels remained constant for the system and DMSO groups (Figure 3B). Regard to treatment time, 8 hours of treatment was the period in which GM-CSF levels were lowest (Figure 3D).

Another important finding is that the same concentration of morin proved to be more effective at decreasing IL-8 and GM-CSF levels when encapsulated in the system (Figure 3A-D), confirming the findings in figure 3E, in which free morin and the system containing morin added immediately to culture medium showed better results in reducing GM-CSF production when compared to the free morin and the system containing morin that were kept in the culture medium for 24 hours before being used. A significant difference in reducing GM-CSF expression when compared free morin added immediately to culture medium and free morin kept in the culture medium for 24 hours was only found at 24 hours. For the system + morin added immediately and system + morin that were kept in the culture medium for 24 hours, the significant difference in reducing GM-CSF expression was obtained at 4 hours and 24 hours. Afterward, system containing 0.125 mg/mL of morin for 8 hours was used for upcoming experiments.

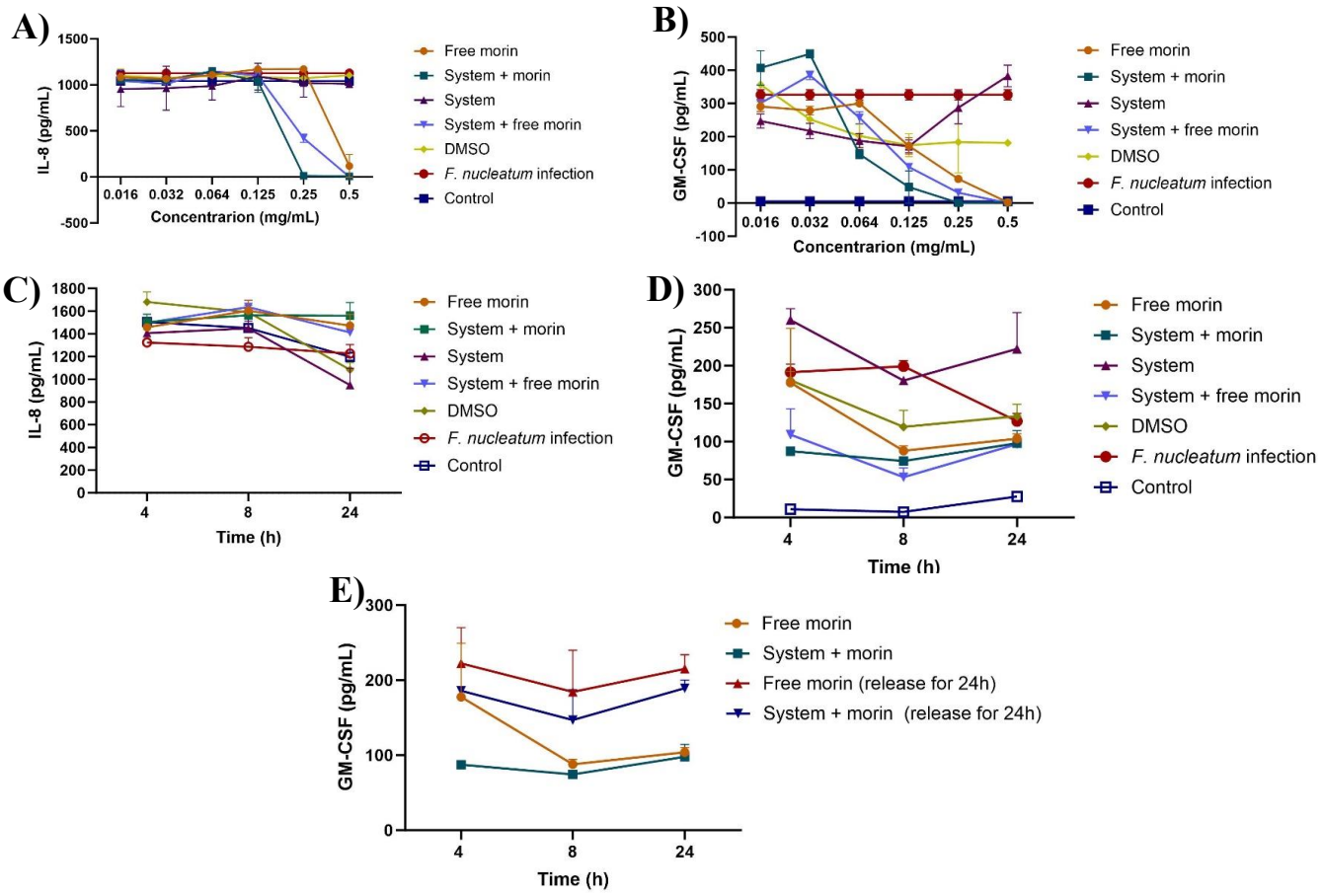

Figure 3. Time and dose response and stability of H400 challenged with heat-killed *F. nucleatum* for 24 h, MOI 100:1. **(A and B):** Dose-response of different treatments and morin concentrations assessed by IL-8 (A) or GM-CSF (B) production; **(C and D):** Time-response of different treatment using 0.125 mg/mL morin assessed by IL-8 (C) or GM-CSF (D) production; **E)** Morin stability assessed by GM-CSF production for 4. 8 and 24 hours, using 0.125 mg/mL free morin and 0.125 mg/mL system + morin added immediately to culture medium and 0.125 mg/mL free morin and 0.125 mg/mL system + morin added after 24 hours in culture medium. Media were collected and IL-8 and GM-CSF measured by ELISA. Two-way ANOVA with Tukey's post-hoc test (mean ± CI; n=3).

To analyse the anti-inflammatory activity of the system containing morin, the potential to reduce GM-CSF in response to *F. nucleatum* and *P. gingivalis* was examined. Free morin and system + morin significantly decreased GM-CSF production by cells stimulated with either *F. nucleatum* or *P. gingivalis* when compared to the control, the system (without morin) and DMSO. The system containing morin proved to be more effective in reducing GM-CSF secretion when compared with free morin and system + free morin (Figure 4).

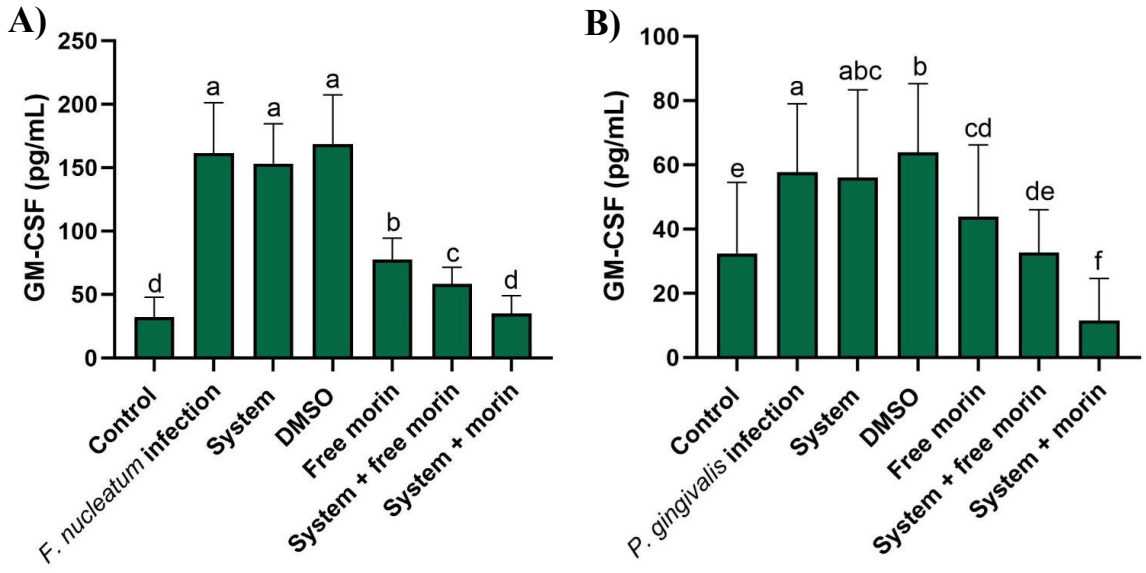

Figure 4. Anti-inflammatory effect of the system without morin (0.82 mg/mL), DMSO (2 µl/mL), free morin (0.125 mg/mL), system without morin (0.82 mg/mL) + free morin (0.125 mg/mL) and system +morin (morin final dose: 0.125 mg/mL) by GM-CSF production by H400 for 8 hours and challenged with heat-killed *F. nucleatum* **(A)** and *P. gingivalis* **(B)** for 24 hours, MOI 100:1. Media were measured by ELISA. One way ANOVA with Tukey's post-hoc test (mean ± SD; n=6). p-value<0.05.

3.2 Evaluation of antimicrobial activity

3.2.1 Minimum inhibitory concentration (MIC)

Prior to evaluating the antimicrobial activity of the system, the minimum inhibitory concentration (MIC) was determined. The MIC of free morin for *F. nucleatum* and *P. gingivalis* was 117.188 µg/mL. For the system containing morin, the MIC was 78.125 µg/mL for *F. nucleatum* and 39.063 µg/mL for *P. gingivalis* (Figure 5).

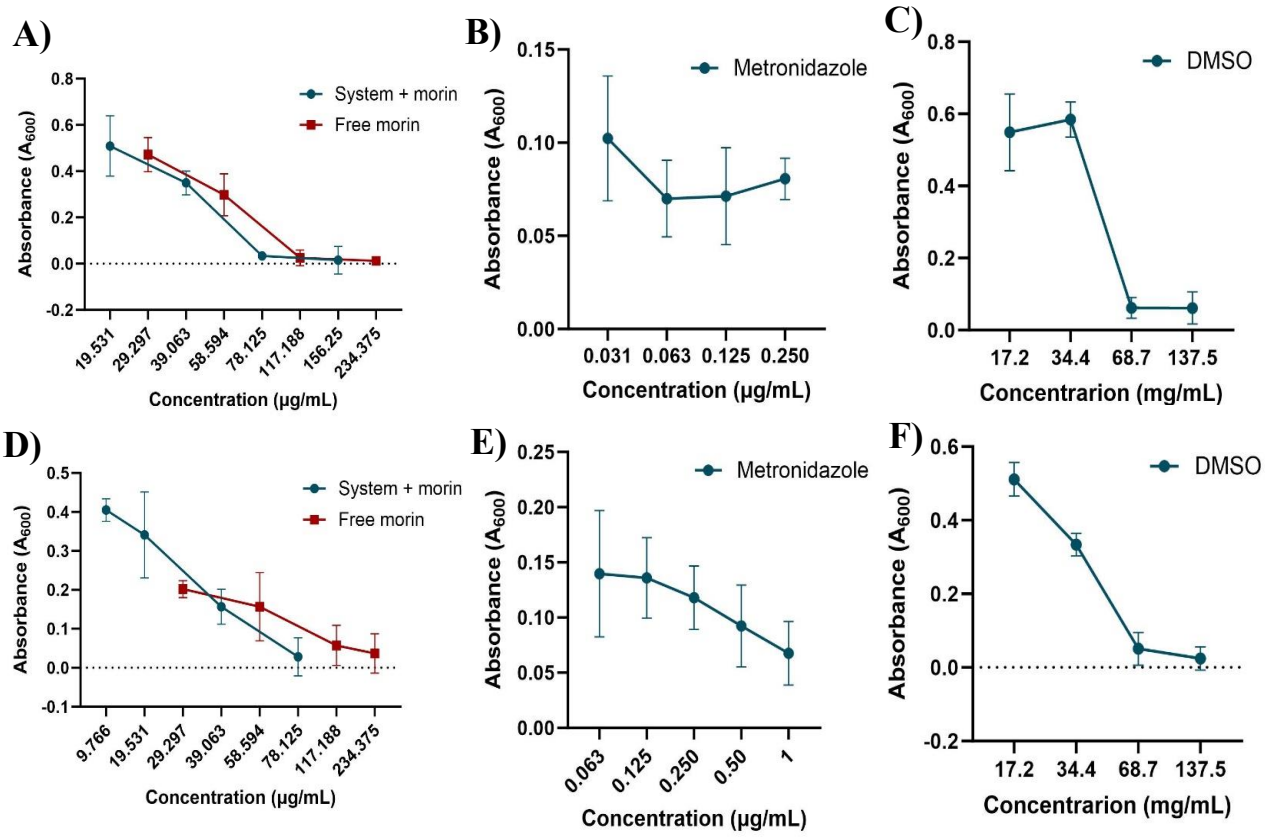

Figure 5. MIC of free morin, morin-containing system **(A and D)**, metronidazole (positive control) **(B and D)** and DMSO **(C and F)** for *F. nucleatum* **(A, B and C)** or *P. gingivalis* **(D, E and F)**, calculated using absorbance values (mean ± SD; n=3).

3.2.2 Planktonic culture assay

The first test to evaluate the antimicrobial effect of the system containing morin was to assess its effect on planktonic cultures of periodontopathogens. According to Figure 6, free morin and the system containing morin (1 mg/mL) significantly inhibited the growth of *F. nucleatum* and *P. gingivalis*.

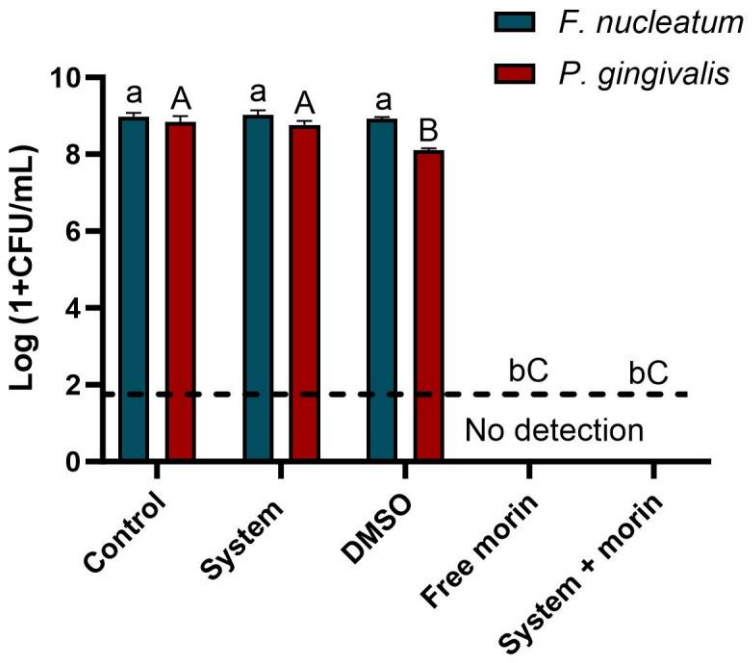

Figure 6. Antimicrobial effect of the system without morin (6.58 mg/mL), DMSO (16 µl/mL), free morin (1 mg/mL) and system (6.58 mg/mL) + morin (1 mg/mL), on planktonic culture, after 24 hours of exposure. One way ANOVA with Tukey's post-hoc test (mean ± SD; n=6). Capital letter: *P. gingivalis*; lowercase letter: *F. nucleatum*. p-value<0.05.

3.2.3 Multi-species biofilm assay

To confirm the findings of previous tests, the effect of the system containing morin was tested on mature multi-species biofilms. Free morin, system + free morin and the system containing morin significantly reduced the viability of *S. oralis* and *F. nucleatum* in multispecies biofilms when compared to the control, system (without morin) and DMSO (Figure 7A). *P. gingivalis* levels were bellow detection limit but was identified *P. gingivalis* in SEM and confocal images (Figures 8 and 9). However, when *P. gingivalis* was isolated, it proved capable of forming biofilm (Figure 8). Free morin, system + free morin and the system containing morin also significantly reduced the biomass of the multispecies biofilm (Figure 7B). For both analyses, the system containing morin proved to be more effective in terms of antimicrobial and antibiofilm effect when compared to morin in its free form. The system without morin also significantly reduced the biomass of the multispecies biofilm when compared to the control (Figure 7B). These findings can also be confirmed by the SEM and confocal images (Figure 8, 9). SEM images show the presence of all three species in the control group biofilm, with *F. nucleatum* identified as the most predominant among them. In the morin-containing system group, it is possible to observe the three species but in a

more isolated way, with no biofilm formation. It is also possible to observe in this group an amorphous mass compatible with polymeric systems. The same was observed in the confocal images. In the control, system (without morin) and DMSO groups it was possible to see the presence of a thick biofilm and live bacteria. In the treatment groups, free morin, system + free morin and system containing morin, there was no biofilm formation, and the bacteria were all dead.

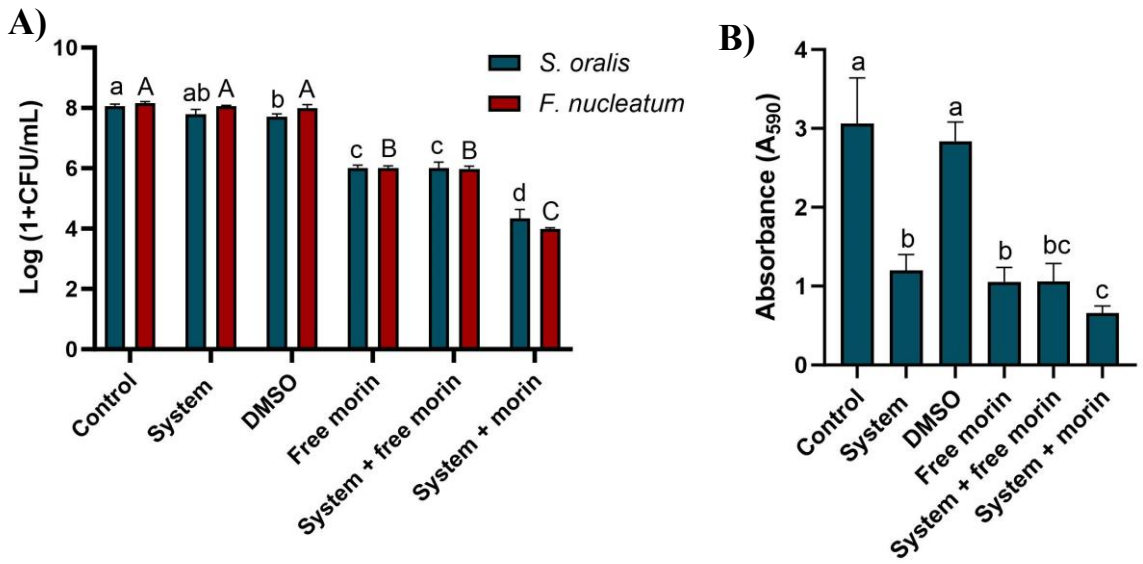

Figure 7. A) Antimicrobial effect, after 7 days, of the system, without morin (6.58 mg/mL), DMSO (16 µl/mL), free morin (1 mg/mL), system (6.58 mg/mL) + free morin (1 mg/mL) and system + morin (1 mg/mL). One way ANOVA with Tukey's post-hoc test (mean ± SD; n=6). Capital letter: *F. nucleatum*; lowercase letter: *S. oralis*. **B)** Biomass after 7 days of exposure to the groups shown. One way ANOVA with Tukey's post-hoc test (mean ± SD; n=6). p-value<0.05.

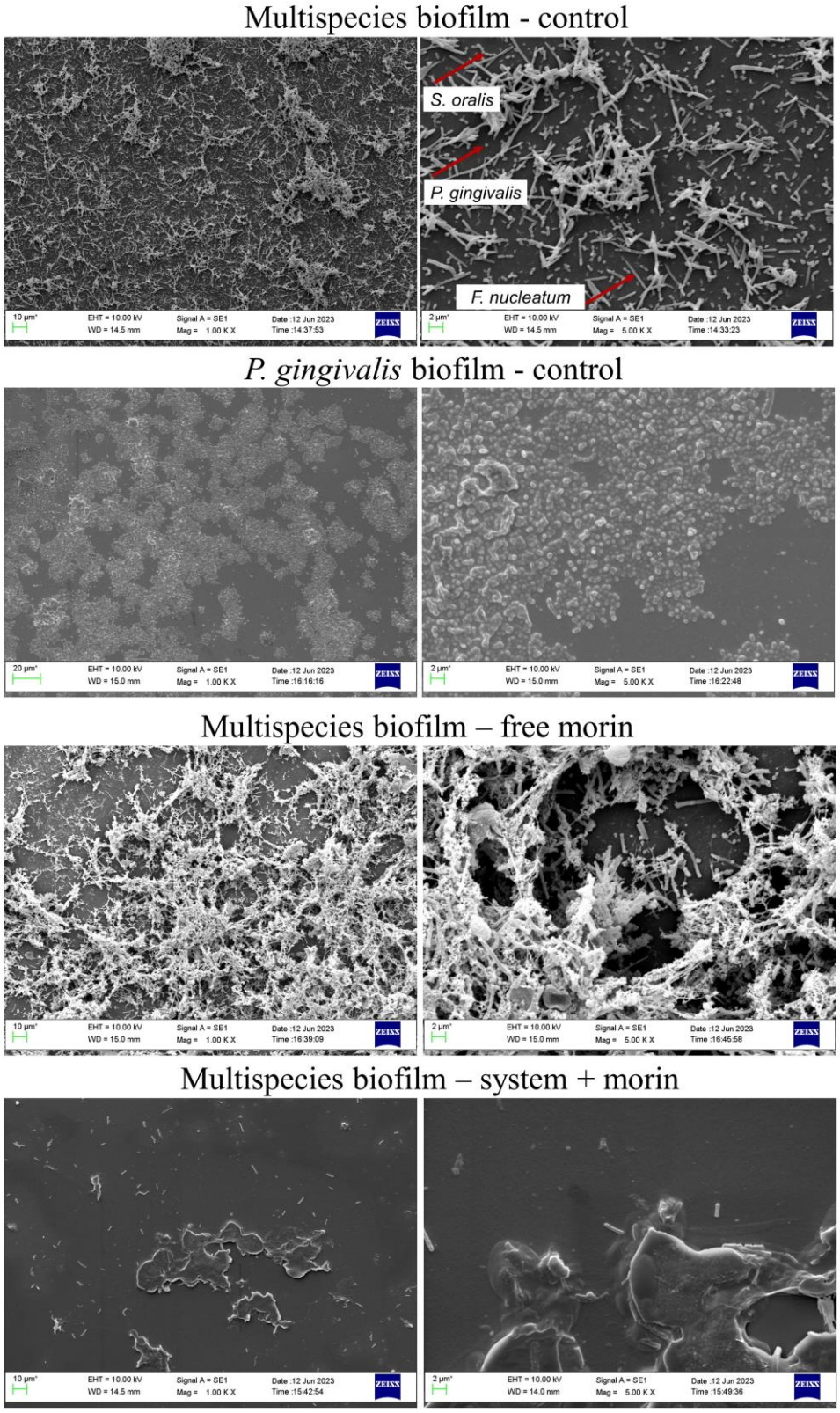

Figure 8. SEM photomicrographs of the the groups shown. X 1,000 and X 5,000 magnification

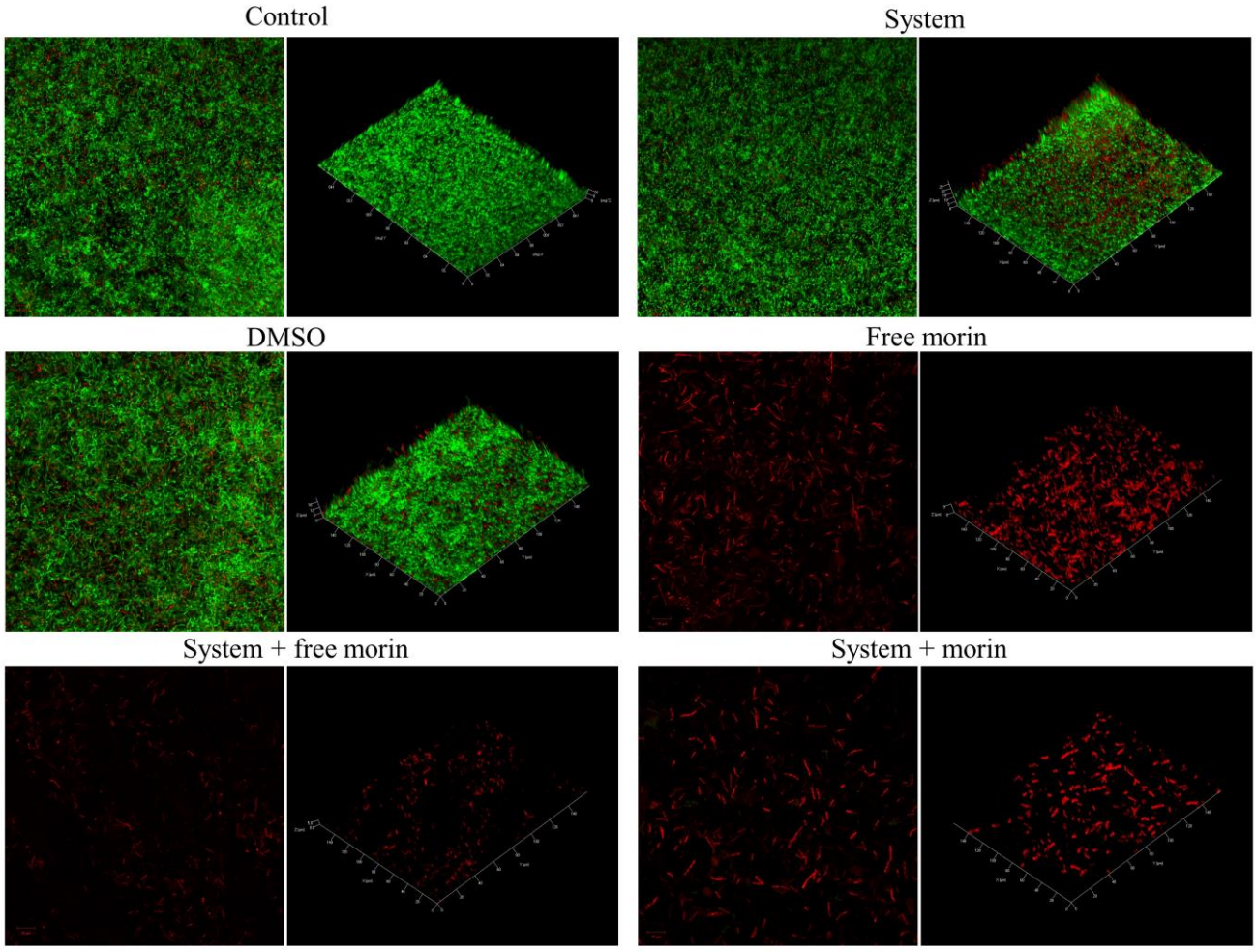

Figure 9. CLSM images of live/dead cells for each group obtained from multispecies biofilm exposed to different experimental treatments. The viable cells emit green fluorescence while dead cells emit red fluorescence.

4. Discussion

Understanding the periodontitis pathogenesis is of paramount importance when trying to combat this condition. Periodontitis is the result of deregulated and exaggerated inflammation, associated to neutrophil infiltration and reactive oxygen species release in periodontal tissues in response to periodontal bacteria, causing tissue damage. The oral epithelium is responsible for initiating the cytokine cascade and neutrophil infiltration when exposed to periodontal bacteria (46,47). Hence, an essential therapeutic approach involves regulating the epithelial responses to inflammatory stimuli. Therefore, we explored the role of free morin and system containing morin as modulators of inflammatory cytokines and of reactive oxygen

species production, as well as their antimicrobial effect against microorganisms associated with periodontitis.

It was initially demonstrated that the periodontal relevant bacterium, *F. nucleatum* and *P. gingivalis* can stimulate the production of important inflammatory cytokines (IL-8 and GM-CSF) by oral epithelia cells (H400) (Figure 2) without causing cytotoxic effects (Figure 1), which is consistent with findings reported in the literature (45–47). This particular cell line was selected due to its extensive use in interaction investigation between oral bacteria and oral epithelial cells (48–51). Grant et al. (2023) (51) have shown that H400 cells activate the same molecular pathways as primary oral epithelial cells. Thus, the results of this study confirm the use of H400 to characterize cellular responses elicited by external inflammatory stimulation (51,52). Furthermore, there was a difference in secretion of inflammatory cytokines when the cells were stimulated with either *F. nucleatum* or *P. gingivalis* (Figure 2), which is in agreement to the literature (48,53,54). This difference may be attributed to more potent virulence factors expressed by *F. nucleatum*. Although *P. gingivalis* is associated with chronic and severe phase of periodontal disease, *F. nucleatum* demonstrates higher adherence to epithelial cells in comparison to *P. gingivalis* (55–57). Consistent with our results, Martinho et al. (2016) (58), showed that various bacteria have distinct lipopolysaccharide (LPS) structures capable of inducing different levels of inflammation. Their work also confirmed that the presence of LPS in the cell membrane of *F. nucleatum* induces a greater release of inflammatory cytokines compared to the LPS present *P. gingivalis* cell membrane. Thus, the results of this study suggest that distinctions found in the abilities of various periodontal pathogens to induce inflammation contribute to a better understanding of periodontal disease. Furthermore, this study validates the use of a robust cell infection model using heat-killed, relevant periodontal relevant bacteria, specifically *F. nucleatum* and *P. gingivalis*. This model allows the use of heat-killed bacteria without affecting the epithelial response. The use of heat-killed bacteria is a safe approach, eliminating the need to expose cells to live bacteria and reducing the risk of contamination. Moreover, it enhances experimental standardization, offering greater control over experimental conditions. This model can be used in future studies to evaluate anti-inflammatory effect of other substances.

Cytokines, such as IL-8 and GM-CSF, are a crucial group of inflammatory mediators that shave a significant impact on inflammation process. According to Grant et al. (2023) (51) IL-8 and GM-CSF were the cytokines secreted in the highest

quantities by H400 cells when stimulated by *F. nucleatum*. However, the results of this study show that the IL-8 secreted by H400 cells were quite high, even in the groups without stimulation and in the groups grown with hydrocortisone-supplemented culture medium (Figure 2). One hypothesis for these findings is that H400 cells are more sensitive to the IL-8 cytokine and respond to it more intensely (59). For this reason, subsequent experiments were carried out without hydrocortisone and only GM-CSF secretion was used as the cytokine marker to evaluate the anti-inflammatory effect of free morin and morin-containing system containing morin. GM-CSF is an important pro-inflammatory cytokine because it is the first line of defence against *F. nucleatum* and *P. gingivalis*. The influence of varying drug doses and treatment durations was assessed by means of IL-8 and GM-CSF expression (Figure 3). The data clearly illustrate the dose-dependent nature in decreasing inflammatory cytokines expression by H400 cells, with higher drug concentrations leading to more significant results in inflammatory cytokines reduction. Notably, the best treatment time to reduce the inflammatory response was 8 hours. As the duration of treatment increased from 8 to 24 hours, there was no improvement in treatment. These findings corroborate those in the literature in which, in order to assess the morin anti-inflammatory effect, in vitro, the treatment time does not exceed 24 hours (60–62). However, the results of this study proved to be more effective as it only took 8 hours of treatment to reduce the effects caused by periodontal bacteria. In this way, we emphasise the importance of treatment time in therapeutic interventions. These findings suggest that an optimal balance between dose and treatment duration is crucial to achieve the desired therapeutic outcomes while minimizing potential adverse effects.

This study showed that pre-treatment for 8 hours with both free morin and systems containing morin (0.125 mg/mL) significantly decreased GM-CSF expression by H400 cells (Figure 4). The findings of this study corroborate with the literature, wherein diverse concentrations of morin, along with varied treatment durations, were experimentally assessed and demonstrated anti-inflammatory effects across multiple cell lines. The precise morin's mechanism of action remains incompletely elucidated. It is postulated that morin may inhibits pro-inflammatory mediators' production, modulates cell signalling pathways and inhibits the immune system cells activation (60–62). Morin has thus emerged as an alternative to help control the exaggerated inflammation caused by periodontitis.

Importantly, the results of this study showed that the systems containing encapsulated morin significantly reduced GM-CSF levels when compared to groups system + free morin and free morin (Figure 4). One of the hypotheses for these results is the limited stability of free morin (44,63). To overcome this challenge and to increase its anti-inflammatory effect, we used polymeric controlled release systems, recently developed by Sales et al. (2023) (41). This system showed to be water-soluble, stable and provided morin controlled release and an antimicrobial effect against *S. mutans*. Therefore, the systems proved to be capable of enhancing the anti-inflammatory effect of free morin, as observed by decreased GM-CSF levels in group system+morin. This is the first study to test morin encapsulated in polymer systems to evaluate its anti-inflammatory effect for use in the periodontitis control.

In addition to its anti-inflammatory effect, morin also showed an antimicrobial effect against periodontopathogens. Morin has been demonstrated to be an effective inhibitor of various microorganisms, such as *S. mutans*, *Staphylococcus aureus* and *Candida albicans* (61,64,65). Its mechanism of action is probably due to facilitating bacterial aggregation, causing cell membrane leakage, interfering with biofilm growth, and down-regulating the PBP2a-mediated resistance mechanism (61,66). In this study, the MIC value found for free morin was 117 µg/mL for *F. nucleatum* and *P. gingivalis* (Figure 5). Although scarce in the literature, this value is in line with values reported in the literature, that varies from 256 to 300 µg/mL *S. aureus* (haemolytic activity of α-hemolysin) (61) and *Streptococcus agalactiae* (67), respectively. While morin antimicrobial effects are well-documented, there is a lack of literature examining the impact of morin on microorganisms responsible for causing periodontitis. In this context, this is the first study to determine MIC values for periodontal relevant bacterial species, providing reference values for future studies. Moreover, future studies to evaluate the effect of morin against periodontal disease-causing species are encouraged.

Apart from assessing the impact of free morin on periodontal pathogens, this study also evaluated the effect of morin encapsulated in polymeric systems. The incorporation of substances into polymeric controlled-release systems is widely explored in the pharmaceutical field, but little has been said about it in dentistry. Recently, studies have developed polymeric systems for incorporating drugs for oral use. De Farias et al. (2020; 2021) (42,43) successfully incorporated morin into films, tablets and microparticles using biopolymers. These systems improved morin

solubility, promoted morin controlled release and demonstrated antimicrobial and antibiofilm activity against *Actinomyces naeslundii* and *S. mutans*, as well as polymicrobial biofilm. These systems presented as a promising alternative to conventional oral biofilm control.

To confirm the antimicrobial effect found, free morin and the system containing morin were evaluated on planktonic culture of *F. nucleatum* and *P. gingivalis* and multispecies biofilms. The treatments proved to be effective in eradicating both *F. nucleatum* and *P. gingivalis* species and reducing biofilm and microbial viability (Figure 7). The antibiofilm effect found is due to both the antimicrobial effect of morin and the bioadhesive characteristics of the polymeric systems, precisely due to the presence of sodium alginate and gellan gum. Both polymers have been shown to have bioadhesive properties (68–71). This is the reason why the systems (without morin) also had an antibiofilm effect, as they adhered to the glass coverslips, preventing biofilm formation. These findings are further supported by SEM images (Figure 8), wherein residual biopolymers are evident, aligning with findings in the literature (42). It is worth noting that the treatments were added at the beginning of biofilm formation and replaced with each change of culture medium. This shows that the antimicrobial effect was evaluated in a preventative rather than therapeutic manner. Studies show that one of the most important antimicrobial effects of morin is its anti-adhesive activity, interfering with biofilm formation and growth (61,66). As this is the first study to evaluate the morin effect on periodontal pathogens, the effect found shows that the systems have potential for controlling periodontal pathogens. Future studies to evaluate the system containing morin effect on established biofilm, as therapeutic effect, are necessary.

One limitation of using morin in Dentistry is its yellow colour, which can potentially cause tooth and restoration discoloration. In this context, systems containing encapsulated morin, besides showing a greater antimicrobial and antibiofilm effect when compared to free morin, demonstrated no observable colour change in biofilms (data not shown). Clinically, this is another advantage of morin-containing systems compared to free morin. Future clinical studies are essential to assess the influence of morin and morin-containing systems on potential tooth and dental restoration colour changes.

In this study, we showed that morin reduces the secretion of inflammatory cytokines generated by periodontal pathogens *F. nucleatum* and *P. gingivalis*. It was also effective in combating microorganisms associated with periodontitis and biofilm

formation. Although the effects achieved with free morin were satisfactory, this study showed that controlled release polymeric systems containing morin previously developed by Sales et al. (2023) (41), play an innovative and important role in reducing the exaggerated inflammatory load present in periodontitis, potentiating the biological effects found with morin in its free form. This finding serves as an incentive for further research to be carried out exploring the microencapsulation of drugs for oral use and also morin effect on microorganisms that cause periodontal disease. Further studies to evaluate the therapeutic effect of morin-containing systems on mature biofilm, and in situ and clinical studies should be carried out to continue exploration of morin's potential in combatting periodontal disease.

5. Conclusion

In conclusion, morin showed an anti-inflammatory effect by modulating pro-inflammatory cytokines produced by H400 cells in response to *F. nucleatum* and *P. gingivalis*. Furthermore, morin showed an antimicrobial effect, as it was effective in combating periodontal pathogens in planktonic culture and in mature biofilm. In addition, the morin controlled release polymeric systems potentiated these effects. These findings could be considered a promising strategy to combat the deregulated and exaggerated inflammation caused by periodontal disease.

References

1. Blanc V, Isabal S, Sánchez MC, Llama-Palacios A, Herrera D, Sanz M, et al. Characterization and application of a flow system for in vitro multispecies oral biofilm formation. J Periodontal Res. 2014;49(3):323–32.

2. Fugazzotto PA. Re: "tooth loss in 776 treated periodontal patients". J Periodontol. 2010;81(8):1105-6; author reply 1106-7.

3. Chapple IL. Time to take periodontitis seriously. BMJ. 2014 Apr 10;348:g2645.

4. Chen WP, Chang SH, Tang CY, Liou ML, Tsai SJ, Lin YL. Composition Analysis and Feature Selection of the Oral Microbiota Associated with Periodontal Disease. Biomed Res Int. 2018;2018:3130607.

5. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. Nat Rev Immunol. 2015;15(1):30-44.

6. Song B, Zhou T, Yang WL, Liu J, Shao LQ. Programmed cell death in periodontitis: recent advances and future perspectives. Oral Dis. 2017;23(5):609-619.

7. Roberts HM, Ling MR, Insall R, Kalna G, Spengler J, Grant MM, et al. Impaired neutrophil directional chemotactic accuracy in chronic periodontitis patients. J Clin Periodontol. 2015;42(1):1–11.

8. Hirschfeld J, White PC, Milward MR, Cooper PR, Chapple ILC. Modulation of neutrophil extracellular trap and reactive oxygen species release by periodontal bacteria. Infect Immun. 2017;85(12).

9. Waddington RJ, Moseley R, Embery G. Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. Oral Dis. 2000;6(3):138-51.

10. Huang N, Li C, Sun W, Wu J, Xiao F. Long non-coding RNA TUG1 participates in LPS-induced periodontitis by regulating miR-498/RORA pathway. Oral Dis. 2020;27(3).

11. Itoh T, Obata H, Murakami S, Hamada K, Kangawa K, Kimura H, et al. Adrenomedullin ameliorates lipopolysaccharide-induced acute lung injury in rats. Am J Physiol Lung Cell Mol Physiol. 2007;293(2).

12. Graves DT, Cochran D. The Contribution of Interleukin-1 and Tumor Necrosis Factor to Periodontal Tissue Destruction. J Periodontol. 2003;74(3):391–401.

13. Kinane DF, Lappin DF. Immune processes in periodontal disease: a review. Ann Periodontol. 2002;7(1):62-71.

14. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. Annu Rev Immunol. 2009;27:229-65.

15. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. Nature. 2012 Jan 18;481(7381):278-86.

16. Aral K, Milward MR, Cooper PR. Inflammasome dysregulation in human gingival fibroblasts in response to periodontal pathogens. Oral Dis. 2022;28(1):216-224.

17. Aral K, Milward MR, Kapila Y, Berdeli A, Cooper PR. Inflammasomes and their regulation in periodontal disease: A review. J Periodontal Res. 2020;55(4):473-487.

18. Lamkanfi M, Dixit VM. In Retrospect: The inflammasome turns 15. Nature. 2017;548(7669):534-535.

19. Iorio-Siciliano V, Ramaglia L, Isola G, Blasi A, Salvi GE, Sculean A. Changes in clinical parameters following adjunctive local sodium hypochlorite gel in minimally invasive nonsurgical therapy (MINST) of periodontal pockets: a 6-month randomized controlled clinical trial. Clin Oral Investig. 2021;25(9):5331-5340.

20. Lang NP, Salvi GE, Sculean A. Nonsurgical therapy for teeth and implants-When and why? Periodontol 2000. 2019;79(1):15-21.

21. Suvan J, Leira Y, Moreno Sancho FM, Graziani F, Derks J, Tomasi C. Subgingival instrumentation for treatment of periodontitis. A systematic review. J Clin Periodontol. 2020;47(22):155-175.

22. Tan OL, Safii SH, Razali M. Commercial Local Pharmacotherapeutics and Adjunctive Agents for Nonsurgical Treatment of Periodontitis: A Contemporary Review of Clinical Efficacies and Challenges. Antibiotics (Basel). 2019;9(1):11.

23. Ansiliero R, Gelinski JMLN, Samistraro QL, Baratto CM, Almeida CA, Locatelli C. Pathogenic Microbial Profile and Antibiotic Resistance Associated with Periodontitis. Indian J Microbiol. 2021;61(1):55–65.

24. Guentsch A. Antibiotics against Periodontal Biofilms. Monogr Oral Sci. 2021;29:119-132.

25. Ferri M, Ranucci E, Romagnoli P, Giaccone V. Antimicrobial resistance: A global emerging threat to public health systems. Crit Rev Food Sci Nutr. 2017;57(13):2857–76.

26. Rosalem W, Rescala B, Teles RP, Fischer RG, Gustafsson A, Figueredo CM. Effect of Non-Surgical Treatment on Chronic and Aggressive Periodontitis: Clinical, Immunologic, and Microbiologic Findings. J Periodontol. 2011;82(7):979–89.

27. Gamonal J, Acevedo A, Bascones A, Jorge O, Silva A. Levels of Interleukin-1β, -8, and -10 and RANTES in Gingival Crevicular Fluid and Cell Populations in Adult Periodontitis Patients and the Effect of Periodontal Treatment. J Periodontol. 2000;71(10):1535–45.

28. Al-Shammari KF, Giannobile W V., Aldredge WA, Iacono VJ, Eber RM, Wang HL, et al. Effect of Non-Surgical Periodontal Therapy on C-Telopeptide Pyridinoline Cross-Links (ICTP) and Interleukin-1 Levels. J Periodontol. 2001;72(8):1045–51.

29. Aral K, Aral CA, Kapila Y. Six-month clinical outcomes of non-surgical periodontal treatment with antibiotics on apoptosis markers in aggressive periodontitis. Oral Dis. 2019;25(3):839–47.

30. Yoshinari N, Kawase H, Mitani A, Ito M, Sugiishi S, Matsuoka M, et al. Effects of scaling and root planing on the amounts of interleukin-1 and interleukin-1 receptor antagonist and the mRNA expression of interleukin-1β in gingival crevicular fluid and gingival tissues. J Periodontal Res. 2004;39(3):158–67.

31. Gopal JV. Morin Hydrate: Botanical origin, pharmacological activity and its applications: A mini-review. Pharmacognosy Journal. 2013;5(3):123–6.

32. Caselli A, Cirri P, Santi A, Paoli P. Morin: A Promising Natural Drug. Curr Med Chem. 2016;23(8):774–91.

33. Ola MS, Aleisa AM, Al-Rejaie SS, Abuohashish HM, Parmar MY, Alhomida AS, et al. Flavonoid, morin inhibits oxidative stress, inflammation and enhances neurotrophic support in the brain of streptozotocin-induced diabetic rats. Neurological Sciences. 2014;35(7):1003–8.

34. Wang J, Guo C, Wei Z, He X, Kou J, Zhou E, et al. Morin suppresses inflammatory cytokine expression by downregulation of nuclear factor-κB and mitogen-activated protein kinase (MAPK) signaling pathways in lipopolysaccharide-stimulated primary bovine mammary epithelial cells. J Dairy Sci. 2016;99(4):3016–22.

35. Can Baser K. Biological and Pharmacological Activities of Carvacrol and Carvacrol Bearing Essential Oils. Curr Pharm Des. 2008;14(29):3106–19.

36. Kang SS, Kim JG, Lee TH, Oh KB. Flavonols inhibit sortases and sortase-mediated Staphylococcus aureus clumping to fibrinogen. Biol Pharm Bull. 2006;29(8):1751–5.

37. Leu WJ, Chen JC, Guh JH. Extract From Plectranthus amboinicus Inhibit Maturation and Release of Interleukin 1β Through Inhibition of NF-κB Nuclear Translocation and NLRP3 Inflammasome Activation. Front Pharmacol. 2019;10:573.

38. Tianzhu Z, Shihai Y, Juan D. The Effects of Morin on Lipopolysaccharide-Induced Acute Lung Injury by Suppressing the Lung NLRP3 Inflammasome. Inflammation. 2014;37(6):1976–83.

39. Yu S, Liu X, Yu D, Changyong E, Yang J. Morin Protects LPS-Induced Mastitis via Inhibiting NLRP3 Inflammasome and NF-κB Signaling Pathways. Inflammation. 2020;43(4):1293–303.

40. Suntres ZE, Coccimiglio J, Alipour M. The Bioactivity and Toxicological Actions of Carvacrol. Crit Rev Food Sci Nutr. 2015;55(3):304–18.

41. Sales LS, Gimenes MDS, Meneguin AB, Barud HDS, Achcar JA, Brighenti FL. Development of multiparticulate systems based on natural polymers for morin controlled release. Int J Biol Macromol. 2023;228:1-12.

42. de Farias AL, Meneguin AB, da Silva Barud H, Brighenti FL. The role of sodium alginate and gellan gum in the design of new drug delivery systems intended for antibiofilm activity of morin. Int J Biol Macromol. 2020;162:1944-1958.

43. de Farias AL, Arbeláez MIA, Meneguin AB, Barud HDS, Brighenti FL. Mucoadhesive controlled-release formulations containing morin for the control of oral biofilms. Biofouling. 2022;38(1):71-83.

44. Yazdanshenas R, Gharib F. Solubility and thermodynamic functions measurement of morin hydrate in different alcohols. J Mol Liq. 2017;233:9–14.

45. Shoji Y, Nakashima H. Nutraceutics and delivery systems. J Drug Target. 2004;12(6):385–91.

46. Milward MR, Chapple IL, Carter K, Matthews JB, Cooper PR. Micronutrient modulation of NF-κB in oral keratinocytes exposed to periodontal bacteria. Innate Immun. 2013;19(2):140-51.

47. Matthews JB, Wright HJ, Roberts A, Ling-Mountford N, Cooper PR, Chapple IL. Neutrophil hyper-responsiveness in periodontitis. J Dent Res. 2007;86(8):718-22.

48. Grant MM, Scott AE, Matthews JB, Griffiths HR, Chapple ILC. Pre-conditioning of gingival epithelial cells with sub-apoptotic concentrations of curcumin prevents pro-inflammatory cytokine release. J Periodontal Res. 2023;58(3):634-645.

49. Milward MR, Chapple IL, Carter K, Matthews JB, Cooper PR. Micronutrient modulation of NF-κB in oral keratinocytes exposed to periodontal bacteria. Innate Immun. 2013;19(2):140-51.

50. Milward MR, Chapple IL, Wright HJ, Millard JL, Matthews JB, Cooper PR. Differential activation of NF-kappaB and gene expression in oral epithelial cells by periodontal pathogens. Clin Exp Immunol. 2007;148(2):307-24.

51. Hewitt B, Batt J, Shelton RM, Cooper PR, Landini G, Lucas RA, Wiench M, Milward MR. A 3D Printed Device for In Vitro Generation of Stratified Epithelia at the Air-Liquid Interface. Tissue Eng Part C Methods. 2022;28(11):599-609.

52. Kim Y, Jo AR, Jang DH, Cho YJ, Chun J, Min BM, Choi Y. Toll-like receptor 9 mediates oral bacteria-induced IL-8 expression in gingival epithelial cells. Immunol Cell Biol. 2012;90(6):655-63.

53. Bierbaumer L, Schwarze UY, Gruber R, Neuhaus W. Cell culture models of oral mucosal barriers: A review with a focus on applications, culture conditions and barrier properties. Tissue Barriers. 2018;6(3):1479568.

54. Abdulkareem AA, Abdulbaqi HR, Milward MR. In Vitro Homeostasis of Rat Oral Epithelial Cell Cultures Following Withdrawal of Periodontal Pathogens. Braz Dent J. 2020;31(2):135-142.

55. Huang GT, Kim D, Lee JK, Kuramitsu HK, Haake SK. Interleukin-8 and intercellular adhesion molecule 1 regulation in oral epithelial cells by selected periodontal bacteria: multiple effects of *Porphyromonas gingivalis* via antagonistic mechanisms. Infect Immun. 2001;69(3):1364-72.

56. Li Y, Guo H, Wang X, Lu Y, Yang C, Yang P. Coinfection with *Fusobacterium nucleatum* can enhance the attachment and invasion of *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans* to human gingival epithelial cells. Arch Oral Biol. 2015;60(9):1387-93.

57. Zhang Z, Liu S, Zhang S, Li Y, Shi X, Liu D, Pan Y. *Porphyromonas gingivalis* outer membrane vesicles inhibit the invasion of *Fusobacterium nucleatum* into oral epithelial cells by downregulating FadA and FomA. J Periodontol. 2022;93(4):515-525.

58. Martinho FC, Leite FR, Nóbrega LM, Endo MS, Nascimento GG, Darveau RP, Gomes BP. Comparison of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* Lipopolysaccharides Clinically Isolated from Root Canal Infection in the Induction of Pro-Inflammatory Cytokines Secretion. Braz Dent J. 2016;27(2):202-7.

59. Grant MM, Kolamunne RT, Lock FE, Matthews JB, Chapple IL, Griffiths HR. Oxygen tension modulates the cytokine response of oral epithelium to periodontal bacteria. J Clin Periodontol. 2010;37(12):1039-48.

60. Wang J, Guo C, Wei Z, He X, Kou J, Zhou E, Yang Z, Fu Y. Morin suppresses inflammatory cytokine expression by downregulation of nuclear factor-κB and mitogen-activated protein kinase (MAPK) signaling pathways in lipopolysaccharide-stimulated primary bovine mammary epithelial cells. J Dairy Sci. 2016;99(4):3016-3022.

61. Rajput SA, Wang XQ, Yan HC. Morin hydrate: A comprehensive review on novel natural dietary bioactive compound with versatile biological and pharmacological potential. Biomed Pharmacother. 2021;138:111511.

62. Singh MP, Chauhan AK, Kang SC. Morin hydrate ameliorates cisplatin-induced ER stress, inflammation and autophagy in HEK-293 cells and mice kidney via PARP-1 regulation. Int Immunopharmacol. 2018;56:156-167.

63. Jangid AK, Pooja D, Kulhari H. Determination of solubility, stability and degradation kinetics of morin hydrate in physiological solutions. RSC Adv. 2018;8(50):28836-28842.

64. Huang P, Hu P, Zhou SY, Li Q, Chen WM. Morin inhibits sortase A and subsequent biofilm formation in *Streptococcus mutans*. Curr Microbiol. 2014;68(1):47-52.

65. Abirami G, Alexpandi R, Durgadevi R, Kannappan A, Veera Ravi A. Inhibitory Effect of Morin Against *Candida albicans* Pathogenicity and Virulence Factor Production: An in vitro and in vivo Approaches. Front Microbiol. 2020;11:561298.

66. Yang JY, Lee HS. Evaluation of antioxidant and antibacterial activities of morin isolated from mulberry fruits (Morus alba L.). The Korean Society for Applied Biological Chemistry and Springer. 2012; 55(4):485–9.

67. Rattanachaikunsopon P, Phumkhachorn P. Bacteriostatic effect of flavonoids isolated from leaves of Psidium guajava on fish pathogens. Fitoterapia. 2007;78(6):434-6.

68. Kesavan K, Nath G, Pandit JK. Sodium alginate based mucoadhesive system for gatifloxacin and its in vitro antibacterial activity. Sci Pharm. 2010;78(4):941-57.

69. Meneguin AB, Beyssac E, Garrait G, Hsein H, Cury BSF. Retrograded starch/pectin coated gellan gum-microparticles for oral administration of insulin: A technological platform for protection against enzymatic degradation and improvement of intestinal permeability. Eur J Pharm Biopharm. 2018;123:84-94.

70. Osmałek T, Froelich A, Tasarek S. Application of gellan gum in pharmacy and medicine. Int J Pharm. 2014;466(1-2):328-40.

71. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog Polym Sci. 2012;37(1):106-126.

4 CONCLUSÃO

Conclui-se que ambas as técnicas utilizadas, secagem por aspersão e complexação polieletrolítica foram eficazes para o desenvolvimento de sistemas multiparticulados a partir de polímeros naturais para a liberação de substâncias naturais, morina ou carvacrol. Os sistemas contendo morina apresentaram efeito antimicrobiano e antibiofilme contra *S. mutans*, biofilme oral polimicrobiano e patógenos periodontais; além de apresentarem efeito anti-inflamatório. Os sistemas contendo carvacrol apresentaram efeito antimicrobiano e antibiofilme contra biofilme oral polimicrobiano. Dessa maneira, os resultados mostraram que os sistemas multiparticulados desenvolvidos podem ser uma opção promissora para o controle de fatores de virulência das doenças bucais biofilme-dependentes.

REFERÊNCIAS*

1. Peres MA, Macpherson LMD, Weyant RJ, Daly B, Venturelli R, Mathur MR et al. Oral diseases: a global public health challenge. Lancet. 2019; 394(10194): 249–60.

2. Kassebaum NJ, Bernabé E, Dahiya M, Bhandari B, Murray CJL, Marcenes W. Global burden of untreated caries: a systematic review and metaregression. J Dent Res. 2015; 94(5): 650–8.

3. Brasil. Ministério da Saúde. Secretaria de Atenção à Saúde. Secretaria de Vigilância em Saúde. SB Brasil 2010: pesquisa nacional de saúde bucal: resultados preliminares. Brasília: Ministério da Saúde, 2012.

4. Watt RG, Mathur MR, Aida J, Bönecker M, Venturelli R, Gansky SA. Oral health disparities in children: a canary in the coalmine? Pediatr Clin North Am. 2018; 65(5): 965–79.

5. Frencken JE, Sharma P, Stenhouse L, Green D, Laverty D, Dietrich T. Global epidemiology of dental caries and severe periodontitis - a comprehensive review. J Clin Periodontol. 2017; 44(18): 94–105.

6. Nazir M, Al-Ansari A, Al-Khalifa K, Alhareky M, Gaffar B, Almas K. Global Prevalence of Periodontal Disease and Lack of Its Surveillance. ScientificWorldJournal.2020: 2146-160.

7. García-Pérez Á, Irigoyen-Camacho ME, Borges-Yáñez SA, Zepeda-Zepeda MA, Bolona-Gallardo I, Maupomé G. Impact of caries and dental fluorosis on oral health-related quality of life: a cross-sectional study in schoolchildren receiving water naturally fluoridated at above-optimal levels. Clin Oral Investig. 2017; 21(9): 2771–80.

8. Fernandes IB, Pereira TS, Souza DS, Ramos-Jorge J, Marques LS, Ramos-Jorge ML. Severity of dental caries and quality of life for toddlers and their families. Pediatr Dent. 2017; 39(2): 118–23.

9. Baelum V, Van Palenstein Helderman W, Hugoson A, Yee R, Fejerskov O. A global perspective on changes in the burden of caries and periodontitis: implications for dentistry. J Oral Rehabil. 2007; 34(12): 872–906.

10. Cvitkovitch DG, Li YH, Ellen RP. Quorum sensing and biofilm formation in *Streptococcal* infections. J Clin Invest. 2003; 112(11): 1626–32.

11. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2010; 8(9): 623–33.

* 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

12. Lemon KP, Earl AM, Vlamakis HC, Aguilar C, Kolter R. Biofilm development with an emphasis on Bacillus subtilis. Curr Top Microbiol Immunol. 2008; 322: 1–16.

13. Nascimento MM, Gordan VV, Garvan CW, Browngardt CM, Burne RA. Correlations of oral bacterial arginine and urea catabolism with caries experience. Oral Microbiol Immunol. 2009; 24(2): 89–95.

14. Morou-Bermudez E, Elias-Boneta A, Billings RJ, Burne RA, Garcia-Rivas V, Brignoni-Nazario V, et al. Urease activity in dental plaque and saliva of children during a three-year study period and its relationship with other caries risk factors. Arch Oral Biol. 2011; 56(11): 1282–9.

15. Takahashi N, Nyvad B. Caries ecology revisited: microbial dynamics and the caries process. Caries Res. 2008; 42(6): 409–18.

16. Herbert BA, Novince CM, Kirkwood KL. *Aggregatibacter actinomycetemcomitans*, a potent immunoregulator of the periodontal host defense system and alveolar bone homeostasis. Mol Oral Microbiol. 2016; 31(3): 207-27.

17. Saliem SS, Bede SY, Cooper PR, Abdulkareem AA, Milward MR, Abdullah BH. Pathogenesis of periodontitis - a potential role for epithelial-mesenchymal transition. Jpn Dent Sci Rev. 2022; 58: 268-278.

18. Socransky SS, Haffajee AD. Periodontal microbial ecology. Periodontol 2000. 2005; 38: 135-87.

19. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998; 25(2): 134-44.

20. Karpiński TM, Szkaradkiewicz AK. Chlorhexidine--pharmaco-biological activity and application. Eur Rev Med Pharmacol Sci. 2015; 19(7): 1321-6.

21. Saleem HGM, Seers CA, Sabri AN, Reynolds EC. Dental plaque bacteria with reduced susceptibility to chlorhexidine are multidrug resistant. BMC Microbiol. 2016; 16(1): 214.

22. Brighenti FL, Salvador MJ, Gontijo AVL, Delbem ACB, Delbem ÁCB, Soares CP, et al. Plant extracts: initial screening, identification of bioactive compounds and effect against *Candida albicans* biofilms. Future Microbiol. 2017; 12: 15-27.

23. Brighenti FL, Salvador MJ, Delbem ACB, Delbem ÁCB, Oliveira MAC, Soares CP, et al. Systematic screening of plant extracts from the Brazilian Pantanal with antimicrobial activity against bacteria with cariogenic relevance. Caries Res. 2014; 48(5): 353–60.

24. Albuquerque YE, Danelon M, Salvador MJ, Koga-Ito CY, Delbem ACB, Ramirez-Rueda RY, et al. Mouthwash containing Croton doctoris essential oil: in vitro study using a validated model of caries induction. Future Microbiol. 2018; 13(6): 631–43.

25. Caselli A, Cirri P, Santi A, Paoli P. Morin: A Promising Natural Drug. Curr Med Chem. 2016; 23(8): 774–91.

26. Rajput SA, Wang XQ, Yan HC. Morin hydrate: A comprehensive review on novel natural dietary bioactive compound with versatile biological and pharmacological potential. Biomed Pharmacother. 2021; 138: 111-511.

27. Green AE, Rowlands RS, Cooper RA, Maddocks SE. The effect of the flavonol morin on adhesion and aggregation of *Streptococcus pyogenes*. FEMS Microbiol Lett. 2012; 333(1): 54–8.

28. Huang P, Hu P, Zhou SY, Li Q, Chen WM. Morin inhibits sortase A and subsequent biofilm formation in *Streptococcus mutans*. Curr Microbiol. 2014; 68(1): 47–52.

29. Monzote L, Stamberg W, Staniek K, Gille L. Toxic effects of carvacrol, caryophyllene oxide, and ascaridole from essential oil of Chenopodium ambrosioides on mitochondria. Toxicol Appl Pharmacol. 2009; 240(3): 337–47.

30. Liolios CC, Graikou K, Skaltsa E, Chinou I. Dittany of Crete: a botanical and ethnopharmacological review. J Ethnopharmacol. 2010; 131(2): 229–41.

31. Baser KH. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. Curr Pharm Des. 2008; 14(29): 3106-19.

32. Rivas L, McDonnell MJ, Burgess CM, O'Brien M, Navarro-Villa A, Fanning S, et al. Inhibition of verocytotoxigenic *Escherichia coli* in model broth and rumen systems by carvacrol and thymol. Int J Food Microbiol. 2010; 139(1–2): 70–8.

33. Rattanachaikunsopon P, Phumkhachorn P. Assessment of factors influencing antimicrobial activity of carvacrol and cymene against *Vibrio cholerae* in food. J Biosci Bioeng. 2010; 110(5): 614–9.

34. Suntres ZE, Coccimiglio J, Alipour M. The bioactivity and toxicological actions of carvacrol. Crit Rev Food Sci Nutr. 2015; 55(3): 304–18.

35. Shoji Y, Nakashima H. Nutraceutics and delivery systems. J Drug Target. 2004; 12(6): 385–91.

36. Meneguin AB, Beyssac E, Garrait G, Hsein H, Cury BSF. Retrograded starch/pectin coated gellan gum-microparticles for oral administration of insulin: A technological platform for protection against enzymatic degradation and improvement of intestinal permeability. Eur J Pharm Biopharm. 2018; 123: 84–94.

37. de Farias AL, Arbeláez MIA, Meneguin AB, Barud HDS, Brighenti FL. Mucoadhesive controlled-release formulations containing morin for the control of oral biofilms. Biofouling. 2022; 38(1): 71-83.

38. de Farias AL, Meneguin AB, da Silva Barud H, Brighenti FL. The role of sodium alginate and gellan gum in the design of new drug delivery systems intended for antibiofilm activity of morin. Int J Biol Macromol. 2020; 162: 1944-1958.

39. George A, Shah PA, Shrivastav PS. Natural biodegradable polymers-based nano-formulations for drug delivery: A review. Int J Pharm. 2019; 561: 244–64.

40. Kesavan K, Nath G, Pandit JK. Sodium alginate based mucoadhesive system for gatifloxacin and its in vitro antibacterial activity. Sci Pharm. 2010; 78(4): 941–57.

41. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog Polym Sci. 2012; 37(1): 106–26.

42. Sosnik A, Seremeta KP. Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers. Adv Colloid Interface Sci. 2015; 223: 40–54.

43. Lankalapalli S, Kolapalli VRM. Polyelectrolyte Complexes: A Review of their Applicability in Drug Delivery Technology. Indian J Pharm Sci. 2009; 71(5): 481–7.

44. Berger J, Reist M, Mayer JM, Felt O, Gurny R. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur J Pharm Biopharm. 2004; 57(1): 35–52.

45. Horev B, Klein MI, Hwang G, Li Y, Kim D, Koo H, et al. pH-activated nanoparticles for controlled topical delivery of farnesol to disrupt oral biofilm virulence. ACS Nano. 2015; 9(3): 2390–404.

46. Arpagaus C, Collenberg A, Rütti D, Assadpour E, Jafari SM. Nano spray drying for encapsulation of pharmaceuticals. Int J Pharm. 2018; 546(1–2): 194–214.

47. Salama AH. Spray drying as an advantageous strategy for enhancing pharmaceuticals bioavailability. Drug Deliv Transl Res. 2020; 10(1): 1-12.

ANEXO A – PARECER DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação da atividade antimicrobiana de substâncias naturais contra biofilme polimicrobiano.

Pesquisador: Fernanda Lourenção Brighenti

Área Temática:

Versão: 2

CAAE: 29584120.8.0000.5416

Instituição Proponente: Faculdade de Odontologia de Araraquara - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 4.107.921

Apresentação do Projeto:

A cárie dentária é considerada um problema de saúde pública em todo o mundo, afetando não apenas crianças, como também adolescentes e adultos. Para que a doença ocorra, os micro-organismos precisam aderir à superfície dentária e formar biofilmes. Nosso grupo de estudos tem pesquisado produtos naturais deorigem vegetal para o controle químico do biofilme bucal. O objetivo desse trabalho será desenvolver in vitrosistemas multiparticulados mucoadesivos para liberação controlada de substâncias naturais e avaliá-los quanto à atividade anticárie, antimicrobiana e antibiofilme. Inicialmente, seis participantes irão realizar a doação de saliva, os quais deverão ter boa saúde geral e bucal, com idade entre 23 e 30 anos e com experiência anterior de cárie. A coleta da saliva será realizada entre 9:00 e 10:00 h com os participantes em jejum de no mínimo 2 h e sem escovar os dentes há 24 h. Será avaliada a concentração salivar microbianae a capacidade em formar biofilme. Para os testes seguintes, será selecionado um participante, o qual deverá apresentar capacidade de formação de biofilme dentro do intervalo de confiança (< 0,05). Será utilizado um inóculo polimicrobiano obtido a partir da saliva do participante selecionado para mimetizar o biofilme formado in vivo com blocos de esmalte bovino. Inicialmente, será realizada uma triagem da atividade

antimicrobiana e antibiofilme dos sistemas. Os sistemas que apresentarem os melhores resultados serão analisados quanto à capacidade em interferir na maturação do biofilme polimicrobiano e citotoxicidade. Os sistemas serão, ainda, caracterizados quanto aos mecanismos de liberação in vitro, mucoadesividade e morfologia de superfície dos sistemas.

Objetivo da Pesquisa:

O objetivo desse trabalho sera desenvolver sistemas multiparticulados mucoadesivos baseados em polimeros naturais para liberacao controlada de substancias naturais e avalia-los quanto a atividade anticarie, antimicrobiana, antibiofilme e citotoxicidade.

Avaliação dos Riscos e Benefícios:

Riscos: Descrição dos riscos inerentes ao pesquisador: Os químicos que apresenta maior risco à saúde são o cloreto de cálcio e o ácido clorídrico portanto, o uso de EPI – obrigatório para manipulação de químicos, será suficiente para controlar os riscos. Os meios de cultura (ágar BHI, Ágar Mitis Salivarius, Peptona bacteriológica, Triptona, Extrato de levedura, Hemina) não são perigosos se as seguintes de controle de exposição e proteção individual forem seguidas: manusear de acordo com as boas práticas de higiene e segurança; lavar as mãos antes de interrupções, e no final do dia de trabalho; utilizar óculos de proteção testado e aprovado de acordo com as normas governamentais adequadas; manusear com luvas; utilizar jaleco de manga longa. Não é necessária proteção respiratória. Todos os participantes já receberam treinamento sobre boas práticas de laboratório. Para mais informações, consultar arquivo: Descrição dos riscos. Descrição dos riscos inerentes à população: O voluntário permanecerá 24 h semhigienização bucal. Esse período não irá trazer prejuízo para a saúde bucal, pois as doenças bucais relacionadas à falta de higiene – cárie e doença periodontal – possuem um aspecto crônico, ou seja, é necessária a abstenção durante um longo período de tempo (21 dias para a cárie dentária) para que os primeiros sinais da doença sejam observados clinicamente. O voluntário poderá sentir desconforto por ficaresse período sem realizar a higienização bucal. Por isso, imediatamente após a coleta da saliva, o voluntário receberá uma profilaxia dental profissional. O voluntário irá mascar um pedaço de uma película resistente à água para estimular a salivação. Esse material é flexível, indolor,incolor, e inerte e não causa alergias. Há o risco de desconforto por excesso de mastigação. Para diminuir o risco desse desconforto, limitaremos o tempo da coleta em 2 minutos.

Benefícios: Esta pesquisa não trará nenhum benefício imediato para o doador de saliva, porém contribuirá do ponto de vista clínico para avaliar a eficácia das substâncias naturais, morina e carvacrol, dois produtos que possuem relatos de serem eficazes para a prevenção da cárie.

Comentários e Considerações sobre a Pesquisa:

A pesquisa está planejada detalhadamente, com as informações para avaliação dos riscos éticos também detalhadamente informados, os riscos cuidadosamente apresentados e dimensionados, e a meu ver, não são expressivos nem para os voluntários, nem para os pesquisadores, nem para a comunidade.

Considerações sobre os Termos de apresentação obrigatória:

Os termos obrigatórios foram apresentados.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências.

Considerações Finais a critério do CEP:

Protocolo APROVADO em reunião de 19 de junho de 2020.

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

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1472315.pdf	20/05/2020 10:57:54		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	20/05/2020 10:56:42	LUCIANA SOLERA SALES	Aceito
Outros	Carta_resposta_pendencias.pdf	20/05/2020 10:54:38	LUCIANA SOLERA SALES	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.pdf	27/01/2020 15:56:23	LUCIANA SOLERA SALES	Aceito
Folha de Rosto	Folhaderosto.pdf	18/12/2019 17:56:21	LUCIANA SOLERA SALES	Aceito
Outros	descricaodosriscos.pdf	17/12/2019 10:11:21	LUCIANA SOLERA SALES	Aceito
Declaração de Instituição e Infraestrutura	declaracao.pdf	16/12/2019 22:22:06	LUCIANA SOLERA SALES	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ARARAQUARA, 24 de
Junho de 2020

_____

Assinado por:
Andréa Gonçalves
(Coordenador(a))

Araraquara, 8 de dezembro de 2023

Luciana Solera Sales