Universidade Estadual Paulista
"Júlio de Mesquita Filho"

Faculdade de Ciências Farmacêuticas

Desenvolvimento de filmes orodispersíveis potencialmente probióticos para promoção da saúde oral

Virgínia Barreto Lordello

Dissertação apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição para obtenção do título de Mestre.

Área de Concentração: Ciência dos Alimentos
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"Pode-se tirar tudo de um homem exceto uma coisa:
a última das liberdades humanas – escolher a própria atitude
em qualquer circunstância, escolher o próprio caminho."

Viktor Frankl

RESUMO

Os microrganismos probióticos associados a produtos alimentícios já são amplamente utilizados para a manutenção da saúde e redução do risco de certas doenças. Nesse contexto, diversos estudos mostram que cepas probióticas específicas têm grande potencial de manutenção e melhora da saúde oral, apresentando atividade anticariogênica, redução da halitose e prevenção de infecções oportunistas, como a candidose, frequentemente presente em indivíduos imunocomprometidos. Essas condições patogênicas apresentadas estão relacionadas à formação e estabelecimento de biofilmes na cavidade oral. As infecções provenientes de biofilmes são duradouras, altamente resistentes à farmacoterapia e de difícil eliminação.

Dessa forma, o objetivo do presente estudo foi obter filmes orodispersíveis (ODF) contendo cepa probiótica *Enterococcus faecium* CRL 183, avaliando sua capacidade de inibir a formação e/ou maturação de biofilme de *Candida albicans in vitro*. Essa cepa foi selecionada devido as suas características de cultivo, informações disponíveis sobre efeito probiótico, segurança e caráter inovador, pois não há na literatura informações sobre a atividade antifúngica dessa cepa na cavidade oral.

A segurança da cepa probiótica foi avaliada através do teste susceptibilidade a antimicrobianos. Para testar a capacidade de inibição de biofilmes de *C. albicans in vitro* foram formados biofilmes polimicrobianos (*C. albicans* + *E. faecium*), em diferentes proporções de fungo: probiótico (1:1; 1:10; 1:100), a serem avaliados durante os períodos de formação (24 horas) e maturação (48 horas) do biofilme. A cepa probiótica foi incorporada em um filme orodispersível (ODF) para avaliar sua resistência durante o processamento e armazenamento e efeito inibitório contra os biofilmes de *C. albicans*. Os resultados evidenciaram que o microrganismo *E. faecium* CRL 183 foi capaz de se estabelecer em um biofilme polimicrobiano com *C. albicans* ATCC 90028 sem perder sua viabilidade celular, ao passo que antagonizou significativamente a formação (até 99,9% de redução em 24 horas) e maturação (até 99% de redução em 48 horas) do biofilme de *C. albicans.* Os filmes orodispersíveis com e sem probióticos (PF e CF, respectivamente) apresentaram aspecto e textura uniformes, pH neutro, baixa atividade de água e não apresentaram contaminação por bolores, leveduras ou coliformes totais durante o armazenamento. O probiótico resistiu à produção do ODF, apresentando viabilidade elevada (> 9 $\log_{10}$ UFC/g), até 90 dias de armazenamento à temperatura ambiente. A incorporação do probiótico na matriz polimérica do ODF não alterou significativamente as propriedades mecânicas, o tempo de desintegração *in vitro*, o intumescimento, a atividade de água e a mucoadesividade em relação a CF. Entretanto o probiótico aumentou o parâmetro de cor amarelo (+b*), o teor residual de umidade, a espessura e a permeabilidade ao vapor d'água dos filmes. O filme probiótico (PF) foi capaz de reduzir em 68,99% (0,52 log) a formação e em 91,24% (1,15 log) a maturação do biofilme de *C. albicans* em comparação aos biofilmes estimulados com o filme orodispersível controle (CF). Dessa forma, conclui-se que os filmes orodispersíveis são uma forma eficiente de veicular o probiótico *E. faecium* CRL 183, com potencial de prevenir o estabelecimento de infecções causadas por *C. albicans* na cavidade oral.

Palavras-chave: Probióticos, atividade anti-*Candida,* Biofilmes, Filmes orodispersíveis, *Candida albicans* ATCC 90028, *Enterococcus faecium* CRL 183.

ABSTRACT

Probiotic microorganisms associated with food are already widely used to maintain health and reduce risk of certain diseases. In this context, several studies show that certain probiotic strains have great potential for maintenance and improvement of oral health, presenting anticariogenic activity, reduction of halitosis and prevention of opportunistic infections, such as candidiasis, often present in immunocompromised individuals. These pathogenic conditions are related to formation and establishment of biofilms in the oral cavity. Infections caused by biofilms are long-lasting, highly resistant to pharmacotherapy and difficult to eliminate.
Thus, the objective of the present study is to obtain orodispersible films (ODF) containing *Enterococcus faecium* CRL 183, evaluating its capacity of inhibiting the formation and / or maturation of *Candida albicans* biofilms in vitro. This strain was selected due to its cultivation characteristics, available information on probiotic effect and safety and innovative character, as there is no information on the antifungal activity of this strain in the oral cavity. The safety of the probiotic strain was evaluated by antimicrobial susceptibility test. In order to test the ability to inhibit the formation of *C. albicans* biofilms in vitro, combined polymicrobial biofilms (*C. albicans* + *E. faecium*) with different proportions of opportunistic pathogens: probiotic were formed (1: 1, 1:10, 1: 100), during the formation (24 hours) and maturation (48 hours) periods of the biofilm. The probiotic strain was incorporated into an orodispersible film (ODF) to evaluate its resistance during processing and storage and inhibitory effect against C. albicans biofilms.
The results showed that *E. faecium* CRL 183 microorganism was able to establish itself in *C. albicans* ATCC 90028 polymicrobial biofilm without losing its cellular viability. It significantly antagonized the formation (up to 99.9% reduction in 24 hours) and maturation (up to 99% reduction in 48 hours) of *C. albicans* biofilm. The orodispersible films with and without probiotics (PF and CF, respectively) presented uniform appearance and texture, neutral pH, low water activity and did not show contamination by molds, yeasts or total coliforms during storage. The probiotic resisted to the ODF production, presenting high viability (> 9 log10 CFU / g), up to 90 days of storage at room temperature. The incorporation of the probiotic in the polymer matrix of ODF did not significantly alter the mechanical properties, in vitro disintegration time, swelling, water activity and mucoadhesiveness in relation to CF. However, the probiotic increased the yellow color parameter (+ b *), the residual moisture content, the thickness and the water vapor permeability of the films. The probiotic film (PF) was able to reduce the formation of C. albicans biofilm in 68.99% (0.52 log) formation and in 91.24% (1.15 log) the biofilm maturation compared to the biofilms stimulated with orodispersible control film (CF). Thus, it is concluded that orodispersible films are an efficient way of transporting the probiotic E. faecium CRL 183, with potential to prevent the establishment of infections caused by C. albicans in the oral cavity.

Keywords: Probiotics, anti-*Candida* activity, Biofilms, Orodispersible films, *Candida albicans* ATCC 90028, *Enterococcus faecium* CRL 183.

SUMÁRIO

INTRODUÇÃO

Probióticos são definidos como microrganismos vivos que, quando administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro (1,2).

Tais microrganismos devem ser preferencialmente de origem humana, dentre os quais destacam-se *Bifidobacterium* spp, as bactérias produtoras de ácido lático como os gêneros *Lactobacillus* spp. e, em menor escala, *Enterococcus* spp. (2–4). Também são representantes dessa classe leveduras como *Saccharomyces* spp. (3,4).

Probióticos devem ainda apresentar resistência às condições adversas do trato gastrintestinal (especialmente se os efeitos esperados forem sistêmicos), modular positivamente os sistemas fisiológicos do paciente, ser inócuos e não devem transmitir genes de resistência antimicrobiana (2,3).

A Agência Nacional de Vigilância Sanitária (ANVISA) recentemente revisou os requisitos para comprovação da segurança e dos benefícios à saúde dos probióticos para uso em alimentos (Resolução da Diretoria Colegiada - RDC Nº 241, de 26 de julho de 2018), estabelecendo que a identificação da linhagem do microrganismo deve ser inequívoca contemplando a nomenclatura binomial, identificação fenotípica e genotípica, origem e depósito em coleção de cultura internacionalmente conhecida (5). Além disso, a comprovação da segurança do probiótico deve ser demonstrada através de estudos científicos e documentos técnicos que assegurem o histórico de uso seguro, ausência de registros de eventos adversos, ausência de fatores de virulência e patogenicidade, ausência de resistência potencialmente transferível a antibióticos relevantes para a saúde humana; e susceptibilidade a, pelo menos, dois antibióticos (5). A resolução ainda determina que os benefícios à saúde devem ser comprovados por meio de estudos que envolvam um grupo representativo da população, considerando a quantidade mínima de cepa probiótica sugerida para obtenção desse benefício (5).

Está bem estabelecido na literatura que probióticos têm um impacto positivo no funcionamento do trato gastrintestinal e auxiliam no tratamento ou redução de risco de diversas doenças como: diarreia decorrente do uso de antibióticos, síndromes metabólicas, vaginites, doenças respiratórias, hipercolesterolemia e

doenças inflamatórias intestinais (6,7).

Em relação à saúde oral, estudos têm evidenciado que o consumo de certas cepas de microrganismos probióticos pode diminuir a halitose, a incidência de cáries em crianças e de doença periodontal, incluindo sangramento causado por gengivite, além de combater infecções orais por *Candida* spp. (8,9).

A maioria das doenças da cavidade oral inicia-se com a formação dos biofilmes microbianos que revestem os dentes, popularmente conhecidos como placa dentária. Os microrganismos presentes nesses biofilmes metabolizam carboidratos da dieta e podem promover a desmineralização e destruição localizada de tecidos dentários, inicialmente na superfície do esmalte, mas podendo atingir a dentina no caso de cáries mais profundas e até comprometer a polpa dental (9,10).

Para que doenças orais ocorram, a interação de três fatores é indispensável: o hospedeiro (dentes, saliva e sistema imune), a constituição da microbiota oral residente e a dieta (tipo e frequência) (10).

Sabe-se que biofilmes são ecossistemas microbianos complexos caracterizados por células ligadas entre si e a um substrato, ou à superfícies bióticas e abióticas envoltas por uma matriz de polímero extracelular (Exopolissacarídeos - EPS) (11). O EPS confere aos microrganismos proteção contra o sistema imune e a agentes antimicrobianos, promove o sequestro de nutrientes do meio, facilita a dispersão para outras regiões não infectadas, entre outras vantagens (12–15).

Na ausência de higiene oral um biofilme é capaz de estar completamente formado em um período de apenas quatro horas após as refeições e, mesmo com a prática da escovação dental diária e o uso do fio dental, não ocorre a eliminação completa desses biofilmes que, além de estarem envolvidos na gênese da cárie dentária, podem também atuar na periodontite e candidíase oral (8)

Diferentes microrganismos estão envolvidos no desenvolvimento de doenças que afetam a saúde bucal, entre eles merecem destaque: *Streptococcus mutans, Candida albicans e Lactobacillus* spp. (16–20).

Diversas cepas probióticas apresentam capacidade de se estabelecer em biofilmes e de reduzir a população de patógenos altamente prevalentes na cavidade oral como *Actinomyces viscosus*, *Staphylococcus aureus*, *Candida albicans* e *Streptococcus mutans*. (4,7). Apesar de algumas espécies de *Lactobacillus* spp. estarem presentes em lesões de cárie (17), o consumo de produtos contendo cepas

específicas, como *Lactobacillus rhamnosus GG,* foi relacionado à diminuição na incidência de cáries, bem como, redução na população de *Streptococcus mutans* em crianças, comparado a um grupo controle que não ingeriu o produto contendo essa cepa probiótica (21). Samot e Badet (2013) investigaram a ação probiótica de 66 espécies de lactobacilos, e observaram que todas essas espécies foram capazes de inibir o crescimento de *S. mutans e A. viscosus*, e a maioria desses lactobacilos foram capazes de antagonizar *Fusobacterium nucleatum* e *Porphyromonas gingivalis,* duas cepas presentes em doenças periodontais (22).

Além do gênero *Lactobacillus* spp., as cepas probióticas *Enterococcus faecium* WB2000, *Bifidobacterium adolescentes* SPM1005 e *Bifidobacterium* BB12 foram efetivas na inibição da formação e crescimento de biofilmes (23).

Os mecanismos pelos quais os microrganismos probióticos atuam na melhora ou manutenção da saúde oral incluem redução de microrganismos patogênicos formadores de biofilmes, em função da produção de substâncias com propriedade antimicrobiana (peróxido de hidrogênio e bacteriocinas), proteases, e por competição direta pelos nutrientes disponíveis e sítios de adesão na cavidade oral (3). Pujia e colaboradores (2017) sugeriram que os probióticos também afetam os patógenos por serem capazes de modular as ações locais e sistêmicas do sistema imune, neutralizar fatores de virulência e interferir nos sistemas de sinalização bacteriana (*quórum sensing*) (9).

O fungo oportunista da pele e mucosa humana, *Candida albicans,* está presente na cavidade oral de mais de 75% da população (19). Altamente associado a infecções hospitalares provenientes de sondas e cateteres, é o quarto microrganismo mais isolado da corrente sanguínea nesses casos (24).

Devido a mudanças ambientais, alterações na microbiota e competência do sistema imunológico do hospedeiro, a espécie *C. albicans* pode se tornar patogênica, especialmente em pacientes em quimioterapia, transplantados, soropositivos, idosos, pacientes com debilidades motoras, usuários de prótese totais ou parciais e recém-nascidos de baixo peso (19, 25–27).

As principais doenças causadas por *C. albicans* em seres humanos são as infecções superficiais, como a candidíase oral (muito prevalente em imuno-comprometidos) ou vaginal, que acomete 75% das mulheres ao menos uma vez na vida (19). Os pacientes imunossuprimidos correm o risco dessas infecções

superficiais tornarem-se invasivas, podendo atingir a corrente sanguínea (candidemia) ou órgãos vitais como rins, fígado e coração (27).

C. albicans é considerada a espécie mais prevalente e virulenta do gênero, pois entre os seus principais fatores de virulência, pode-se destacar o polimorfismo entre leveduras e hifas, que é um mecanismo de evasão do sistema imune inato por dificultar a fagocitose, bem como a formação de biofilmes e produção de toxinas e enzimas hidrolíticas (25, 28, 29).

Como a maioria das infecções causadas por biofilmes, as infecções por *C. albicans* são duradouras, de difícil eliminação e com elevada resistência à farmacoterapia. Os biofilmes de *C. albicans* geralmente apresentam resistência aos principais antifúngicos existentes como: anfotericina B, fluconazol, voriconazol e nistatina (15).

Tanto os fungos quanto os humanos são eucariontes e, no nível molecular, suas células são semelhantes. Isso torna mais difícil encontrar ou projetar drogas que atinjam fungos sem afetar as células humanas (30). Dessa forma, é urgente a busca por alternativas para o tratamento e prevenção de doenças, em especial fúngicas, causadas por biofilmes.

Como uma dessas alternativas, a literatura demonstra que muitas cepas potencialmente ou comprovadamente probióticas apresentam atividade anti-*Candida*. Em uma revisão de literatura conduzida em 2016 demonstrou-se através de estudos *in vivo* e *in vitro,* que cepas probióticas como *Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium bifidum, Saccharomyces boulardii, Streptococcus thermophilus, Streptococcus salivarius, Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus fermentum, Lactobacillus plantarum,* bem como vários isolados clínicos, teriam atividades inibitórias contra *Candida* spp. (4).

Um estudo duplo-cego randomizado avaliou o efeito de uma mistura de probióticos - *Lactobacillus rhamnosus* HS111, *Lactobacillus acidophillus* HS101 e *Bifidobacterium bifidum* – na forma de cápsulas liofilizadas, no desenvolvimento de candidíase oral assintomática em pacientes com prótese total (dentadura). Após cinco semanas de tratamento, a taxa de detecção de *Candida* spp. foi de 92% no grupo placebo e 16,7% no grupo tratado com probióticos, evidenciando a sua capacidade de reduzir a colonização da cavidade oral por esse microrganismo (31).

Matsubara e colaboradores (2016) observaram que suspensões de *L. rhamnosus, L. casei e L. acidophilus* reduziram significativamente a formação de biofilmes de *C. albicans* SC5314 em 32, 59 e 56,3%, respectivamente. Quando biofilmes maduros (48 horas) foram estimulados com as mesmas suspensões probióticas, a redução dos biofilmes foi de 61,8% (*L. rhamnosus),* 54,7% (*L. casei*) e de 34,2% (*L. acidophilus*) (27). Ao estimular os biofilmes de *C. albicans* com o sobrenadante filtrado dessas culturas de lactobacilos*,* a redução foi menos efetiva na formação, e não houve redução na maturação dos biofilmes. Esse resultado demonstra que a ação inibitória dessas cepas sobre *C. albicans* não é mediada unicamente pela produção de metabólitos (27).

Mailänder-Sánchez e colaboradores (2017) observaram que a cepa probiótica *Lactobacillus rhamnosus* GG impediu a adesão, invasão, formação de hifas (prevenindo danos epiteliais), depleção de glicose e da síntese de ergosterol de *C. albicans* (32).

Vilela e colaboradores (2015) relataram redução de até 57,5% da contagem de *C. albicans* em biofilmes estimulados com culturas de *L. acidophilus* ATCC 4356 e redução de 45,10% em biofilmes estimulados com sobrenadante filtrados dessa cultura probiótica (33).

Ambos os estímulos (suspensão de células probióticas ou filtrado) foram eficientes na supressão da formação de hifas. Nesse estudo, *Galleria mellonella* foi utilizada como modelo de candidíase experimental e o tratamento e a profilaxia com células de *L. acidophilus* ou com seus metabólitos aumentou consideravelmente a sobrevivência desse inseto (33).

Ribeiro e colaboradores (2017) observaram efeito semelhante na interação de *C. albicans* com células de *L. rhamnosus:* diminuição de 52,2 % na quantificação de biofilme em UFC/ mL e redução de 48% na atividade metabólica. O sobrenadante filtrado de *L. rhamnosus* diminuiu o crescimento de *C. albicans* em 39,4% e atividade metabólica em 61% (34). *L. rhamnosus* também promoveu a regulação negativa de diversos genes associados a fatores de virulência de *C. albicans* como ALS3 e HWP1 (relacionados à adesão e filamentação) e de BCR1 e CPH1 (genes reguladores do processo de transcrição celular) (34). *L. rhamnosus* foi capaz de proteger *G. mellonella* de candidíase experimental (34).

Sabe-se também que não apenas os microrganismos probióticos podem competir com *C. albicans* influenciando sua viabilidade e patogenicidade. Há na literatura diversos estudos que avaliam a interação entre esse fungo e bactérias patogênicas como *Staphylococcus aureus, Streptococcus mutans*, *Streptococcus gordonii, Actinomyces viscosus*, espécies de *Fusobacterium* spp., *Enterococcus* spp., *Escherichia* spp. e até mesmo *Pseudomonas aeroginosa* em casos de fibrose cística (29, 35).

Dentre esses microrganismos, a relação entre os gêneros *Enterococcus* spp. e *Candida* spp. mostrou-se particularmente interessante, pois a literatura mostra que o fungo favorece a bactéria, porém essa relação não é mutuamente benéfica. Dois estudos conduzidos por Mason e colaboradores (2012) demonstraram que *C. albicans* SC5314 promoveu aumento e persistência da colonização gástrica e entérica de *Enterococcus* spp. ao passo que antagonizou outras espécies de bactérias láticas como, por exemplo, *Lactobacillus* spp., durante a recuperação da microbiota após tratamento com antibiótico cefoperazona (36,37).

Apesar do mecanismo envolvido não ter sido elucidado, os autores observaram efeito promotor semelhante em relação a populações de *Bacteroidetes* e sugerem que *C. albicans* poderia auxiliar a sua adesão e fixação ao ceco; que glucanos da levedura poderiam ser fonte potencial de alimento para essas bactérias, ou ainda, *C. albicans* poderia suprimir outras bactérias que antagonizam *Bacteroidetes* (37). Por outro lado, *Enterococcus* spp. diminui a patogenicidade de *C. albicans* através da ação de proteases, peptídeos e bacteriocinas. Pesquisadores identificaram a ação de duas proteases (peptídeos de ativação da biossíntese de gelatinase - GBAP) ativadoras do sistema regulador da virulência de *Enterococcus* spp., que ao serem secretadas por essa bactéria, impediram o polimorfismo de *C. albicans* (38). O polimorfismo é um dos principais fatores de virulência associados a esse fungo e a formação de hifas está intrinsecamente ligada à formação de biofilmes e à invasão das células hospedeiras (19,25,38).

Cruz e colaboradores (2013) demonstraram ainda que *Caenorhabditis elegans*, modelo de estudo de infecções polimicrobianas, apresentou uma mortalidade ocasionada por *C. albicans* menor quando era co-inoculado por

Enterococcus spp. Sendo a taxa de sobrevivência do nematódeo dose-dependente, quanto maior o número de colônias de *Enterococcus* spp. inoculadas, maior a proteção contra a patogenicidade de *C. albicans* (38). Este estudo determinou ainda que a patogenicidade de ambos os microrganismos era atenuada durante a infecção polimicrobiana em *C. elegans*. O mesmo efeito foi observado ao tratar *C. elegans,* já contaminado por *C. albicans,* com o sobrenadante filtrado e esterilizado de um inóculo de *Enterococcus faecalis*, constatando-se redução considerável da formação de hifas e, consequentemente, da patogênese do fungo (38). Shekh e Roy (2012) caracterizaram e purificaram parcialmente uma proteína anti-candida (ACP) produzida por *Enterococcus faecalis* que apresentou atividade de amplo espectro contra oito cepas de *C. albicans* multirresistentes (30).

Em um estudo de 2017 foi descrita a ação da bacteriocina EntV, codificada por um gene que está presente em todas as cepas de *E. faecalis* sequenciadas até o momento, o gene Ef1097 (39). Essa enterocina sozinha, bem como seu análogo sintético, foi suficiente para impedir o polimorfismo de *C. albicans* SC5314 e a formação de biofilmes em diferentes substratos e condições experimentais. Biofilmes de *C. albicans* pré-formados (24 horas/ ∼30 μm de espessura) foram tratados com EntV e sofreram grande perturbação, com redução de sua espessura para ∼15 μm. Os biofilmes que não foram tratados com EntV aumentaram sua espessura para > 50 μm (39). O peptídeo também protegeu macrófagos aumentando sua atividade antifúngica, reduziu a invasão epitelial, a inflamação e a carga fúngica em um modelo murino de candidíase orofaríngea (39).

Bachtiar e colaboradores (2016) observaram que *E. faecalis cps*2 (um isolado clínico não encapsulado) e *E. faecalis* ATCC 29212, bem como os metabólitos produzidos por essas cepas, não inibiram a formação de biofilmes de *C. albicans* ATCC 10231 e promoveram a expressão de genes associados à formação de biofilme. No entanto, *E. faecalis cps*2 e *E. faecalis* ATCC 29212 reduziram significativamente (>50%) a maturação desses biofilmes (40). Esses resultados foram confirmados pela regulação negativa promovida pelas cepas de *E. faecalis* e seus metabólitos sobre genes de adesão ALS1 e ALS3 e sobre EFB1, gene utilizado para medir quantitativamente os efeitos prejudiciais de agentes antifúngicos contra biofilmes de candida maduros (40).

É importante salientar que em nenhum dos estudos citados (36-40) que avaliaram o potencial anti-*Candida* de *Enterococcus* spp.*,* a cepa bacteriana utilizada foi descrita como sendo inócua, muito menos tendo alegação probiótica. Sabe-se, também que existe muita preocupação em relação a *Enterococcus* spp. devido à sua alta incidência em infeções nosocomiais, e alta capacidade de tornar-se resistente a antibióticos e transferir genes de resistência, em especial à vancomicina, para outros patógenos (41). Provavelmente por essa razão, nesses estudos foram explorados majoritariamente a ação de metabólitos, peptídeos e bacteriocinas ao passo que a interação célula – célula não foi considerada viável.

Como visto anteriormente, a ação probiótica também pode ser mediada pela secreção de metabólitos, porém a competição célula-célula por sítios de adesão e nutrientes pode ser um potencializador do efeito anti-*Candida* (9,27,31,33,34). Dessa forma, seria interessante avaliar se o efeito dos metabólitos produzidos *Enterococcus* spp. seria também potencializado por essa competição direta, uma vez que algumas cepas desse gênero são consideradas seguras e probióticas (2, 41).

A cepa probiótica *Enterococcus faecium* CRL 183 foi isolada do queijo Tafi, tradicional no Vale do Tafí, noroeste da Argentina e tem documentada a sua nomenclatura binomial, identificação genotípica, histórico de uso seguro, ausência de registros de eventos adversos, ausência de fatores de virulência, patogenicidade e resistência antimicrobiana (42).

Vários benefícios sistêmicos promovidos por essa cepa probiótica estão documentados, como modulação do sistema imunológico e da microbiota intestinal, redução dos sintomas relacionados à colite ulcerativa (43), regulação do perfil lipídico e inibição do desenvolvimento da lesão aterosclerótica (44-45) sendo uma alternativa ou adjuvante para terapia medicamentosa. *E. faecium* CRL183 também preveniu o adenocarcinoma de mama (46), câncer de cólon quimicamente induzido (47) e ganho de peso corporal (48) em diferentes modelos murinos. É importante ressaltar que, em alguns desses estudos, o *E. faecium* CRL183 foi utilizado como cultura inicial de um produto à base de soja fermentado, combinado ou não com outras cepas probióticas, avaliando seus efeitos sistêmicos (43,45,47,48).

Objetivando ampliar a utilização da referida cepa probiótica, Witzler e colaboradores (2017) demonstraram que a cepa probiótica *E. faecium* CRL 183

liofilizada após microencapsulação pelo método de coacervação complexa, manteve-se viável à temperatura ambiente por um período de 280 dias e foi capaz de inibir o crescimento de *S. mutans,* constatado pela formação de um halo de inibição 1,7 ± 0,3 mm de diâmetro (43). Esse mesmo estudo evidenciou que *E. faecium* CRL183 foi resistente à saliva humana. Sendo capaz de se liberar da matriz alimentícia, sobreviver às enzimas salivares e se multiplicar (aumento de até 3,64 log_{10} UFC/mL) na saliva após 24h, indicando uma possível capacidade de colonização da cavidade oral por esse probiótico (49). A cepa probiótica encapsulada e liofilizada foi posteriormente incorporada em uma pastilha alimentícia *diet* com potencial funcional. Entretanto, a viabilidade da cepa durante 28 dias de armazenamento à temperatura ambiente foi reduzida a valores considerados baixos para produtos probióticos (1,86±0,07 UFC/g) (49).

Outros estudos veicularam cepas probióticas como *L. reuteri* ou *L. brevis* em gomas de mascar e pastilhas de manitol obtendo efeitos benéficos como redução significativa de placa, população de *S. mutans* e de sangramentos gengivais (50, 51). Porém esses estudos não avaliaram aspectos tecnológicos da formulação como a sobrevivência do probiótico ao longo do tempo de armazenamento.

Tais resultados apontam para a necessidade do aprimoramento do processo de obtenção de formulações probióticas orais, que permitam a liberação controlada de doses conhecidas de organismos viáveis, resistam ao processo de fabricação, mantenham a estabilidade durante o armazenamento a longo prazo, de preferência sem necessidade de refrigeração, e cujos custos de produção sejam economicamente favoráveis (52).

Uma opção de veículo para microrganismos probióticos são os filmes orodispersíveis (ODF), que foram desenvolvidos na década de 1970, para populações com dificuldades de deglutição, ou disfagia, tornando-se uma alternativa a comprimidos, cápsulas e xaropes (53).

Porém, a primeira forma comercializada deste produto surgiu apenas em 2001, como refrescante bucal “Listerine” da Pfizer (53). Esta forma farmacêutica pode ser definida como uma fina película de fácil dissolução e elevada superfície de contato, que permite a liberação de todo componente ativo na cavidade oral. Dentre as vantagens atribuídas ao ODF pode-se citar: facilidade de manuseio e administração, não há necessidade de ingestão de água, evita o metabolismo de primeira

passagem e maior adesão do paciente ao tratamento (54).

Tanto a nomenclatura oficial, quanto a popular tem ampla variação. O FDA utiliza o termo “filmes solúveis”, enquanto que a agência europeia de medicamentos (EMA) adotou “filmes orodispersíveis”. Popularmente, esses filmes são conhecidos como “strip oral”, “filmes orais”, “tira oral”, “filme solúvel oral”, “dissofilme”, “filme solúvel bucal”, “filme mucoadesivo” e “filme transmucoso”, porém, apesar de serem usados de forma intercambiável, podem designar diferentes produtos (53).

A permanência do filme solúvel na cavidade oral, durante o tempo de desintegração, pode promover uma liberação mais eficiente de princípios ativos se comparado a outros produtos como géis orais que são facilmente retirados pelo fluxo salivar (55).

Os filmes orodispersíveis são compostos por macromoléculas, naturais ou sintéticas, formadoras de matriz polimérica, plastificantes - que aumentam as propriedades filmogênicas dos polímeros conferindo maior flexibilidade e menor fragilidade – e o ingrediente farmacêutico ativo (API) ou composto bioativo de interesse (53,54). A composição e proporção desses excipientes pode ser alterada de acordo com as características desejadas para o produto e a adição de edulcorantes, estimulante salivar, aromatizantes, flavorizantes, pigmentos, tensoativos e estabilizantes pode ser considerada (53).

De acordo com as características dos filmes, estes constituem veículos de substâncias bioativas ideais para pacientes com condições que favoreçam a disfagia como idosos, pacientes com debilidades motoras, imunocomprometidos, portadores de Alzheimer ou Parkinson. Coincidentemente, nesta população se observa alta prevalência de candidose (19, 25-27,53-55).

Considerando o exposto, o objetivo do presente estudo é avaliar se a cepa probiótica *E. faecium* CRL 183 é capaz de desestabilizar a formação e/ou maturação de biofilme de *C. albicans* ATCC 90028 *in vitro.* E se os filmes orodispersíveis seriam uma forma eficiente de veicular essa cepa na cavidade oral, promovendo a redução de risco de doenças orais ocasionadas por este fungo oportunista.

Objetivo Geral

Desenvolvimento de filmes orodispersíveis contendo *Enterococcus faecium* CRL 183, capaz de inibir a formação e/ou maturação de biofilme de *Candida albicans in vitro.*

Objetivos específicos

- Avaliar se a cepa probiótica *Enterococcus faecium* CRL 183 é capaz de se estabelecer e sobreviver em biofilmes polimicrobianos com *Candida albicans* ATCC 90028.

- Avaliar se essa cepa probiótica é capaz de desestabilizar a formação e/ou maturação de biofilmes de *Candida albicans in vitro*.

-Incorporar o microrganismo probiótico em filmes orodispersíveis e avaliar a viabilidade da cepa, bem como a estabilidade físico-química do produto durante o período de armazenamento.

- Avaliar se o filme orodispersível probiótico é capaz de desestabilizar a formação e/ou maturação de biofilmes de *Candida albicans in vitro*.

Capítulo 1.

Probiotic *Enterococcus faecium* CRL 183 inhibits *Candida albicans in vitro.*

(Artigo submetido para a revista Applied Microbiology and Biotechnology)

Title: Probiotic *Enterococcus faecium* CRL 183 inhibits *Candida albicans in vitro.*

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Abstract: *Candida albicans* is the most prevalent fungal microorganism of human microbiota presenting a commensal or pathogenic phenotype depending on the host's immune defense capacity. Similarity between fungal and host cells promotes several adverse effects during antifungal pharmacotherapy and antimicrobial resistance increase is a major concern. Therefore, the search for alternative treatments and prevention strategies is urgent. In this context, probiotic bacteria seem to be a viable alternative with their benefits documented in the literature, through *in vitro*, *in vivo* and even clinical studies. The probiotic strain *Enterococcus faecium* CRL 183 has a well-documented safety use and history of benefits to the immune system, and activity against pathogens. Thus, the aim of this study was to evaluate if this probiotic strain was able to prevent *C. albicans* biofilm colonization *in vitro*. In order to test the anti-*Candida* activity of the probiotic strain *E. faecium* CRL 183 against *Candida albicans* ATCC 90028, combined polymicrobial biofilms (*C. albicans* + *E. faecium*) with different proportions of funghi: probiotic were formed (1:1, 1:10, 1: 100), during the formation (24 hours) and maturation (48 hours) periods of the biofilm. The results showed that *E. faecium* was able to establish itself with *C. albicans* in polymicrobial biofilms without losing its cellular viability. The probiotic strain significantly antagonized ($p<0,0001$) *C. albicans* biofilm formation (up to 99.9% reduction in 24 hours) and maturation (up to 99.43% reduction in 48 hours). According to these results *E. faecium* CRL183 may be a promising resource to prevent the formation of those biofilms.

Key words: Probiotics, Biofilms, Anti-*Candida* activity, Polymicrobial biofilms, *Enterococcus faecium* CRL 183, *Candida albicans* ATCC 90028

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Introduction

Candida albicans is the most prevalent fungal species of human microbiota that innocuously colonizes the human skin and mucosa, as well as the gastrointestinal and reproductive tract (Nobile and Johnson 2015). It is also one of the few fungi capable of causing diseases in humans, which is commonly characterized as superficial infections such as oral or vaginal candidiasis, which affects 75% of women at least once in their lifetime (Mayer et al. 2013; Nobile and Johnson 2015). In certain groups such as: transplant patients, under chemotherapy, seropositive, elderly, patients with motor weakness, users of prostheses, catheters or pacemakers, and low birth weight infants, there is a greater risk that superficial infections may become invasive reaching the bloodstream (candidemia) or vital organs such as kidneys, liver and the heart (Han et al. 2013; Mattei et al. 2013; Mayer et al. 2013; Matsubara et al. 2016b).

This can occur when *C. albicans* overgrows because an imbalance in the host microbiota, due to changes in local pH and nutritional availability, or impairment of the host immune defenses (Nobile and Johnson 2015). Highly associated with nosocomial infections, it is the fourth most isolated microorganism in the bloodstream with mortality rates up to 40% (Andes et al. 2004; Nobile and Johnson 2015).
Among its main virulence factors, we can highlight the polymorphism, production of toxins and hydrolytic enzymes as well as the formation of biofilms (Niewerth and Korting 2001; Han et al. 2013; Zago et al. 2015).

Like most infections caused by biofilms, *C. albicans* infections are long-lasting, difficult to eliminate and with high resistance to pharmacotherapy such as: amphotericin B, fluconazole, voriconazole and nystatin (Fanning and Mitchell 2012). Furthermore, both fungi and humans are eukaryotes and, at the molecular level, their cells are similar. This makes it more difficult to find or design drugs that target fungi without affecting human cells (Shekh and Roy 2012). In this way, the search for alternatives for the treatment and prevention of diseases, especially the ones caused by fungal biofilms is urgent. Among the current alternatives, researchers have been investigating the action of probiotics, which are defined as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill et al. 2014). Such microorganisms should preferably be of human origin, among

which stand out the genera *Lactobacillus*, *Bifidobacterium* and, to a lesser extent, *Enterococcus*. *Streptococcus*, and *Saccharomyces* (Matsubara et al. 2016a)

It is well established that a great variety of probiotic strains were able to impair *C. albicans* biofilm formation and maturation (Vilela et al. 2015; Matsubara et al. 2016b; Ribeiro et al. 2017), inhibit the fungal polymorphism, adhesion and invasion of host tissues (Ribeiro et al. 2017; Mailänder-Sánchez et al. 2017). Probiotics were also able to downregulate several genes associated with virulence factors of *C. albicans* such as ALS3, HWP1, BCR1 and CPH1 (Ribeiro et al. 2017).

In vitro, *in vivo* and even clinical studies have demonstrated the ability of probiotics to prevent and treat mucosal candidiasis (Ishikawa et al. 2015; Matsubara et al. 2016a). But not only the interaction between probiotic-fungus has been investigated, there are several studies in the literature which evaluate the effect caused on *Candida* spp., by pathogenic bacteria such as: *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus gordonii*, *Actinomyces viscosus*, species of *Fusobacterium*, *Enterococcus*, *Escherichia* and *Pseudomonas aeroginosa* in cases of cystic fibrosis (Nobile and Johnson 2015; Zago et al. 2015).

Among these microorganisms, the interaction between the genera *Enterococcus* spp. and *Candida* spp. has been shown to be particularly interesting. Both are commonly co-isolated from the same niches, either as commensal members of the microbiota, colonizing the gut, or in polymicrobial infections losing in prevalence in those cases only to *S. aureus* (Garsin and Lorenz 2013). The literature also shows that the fungus favors this bacterium (Mason et al. 2012a; Mason et al. 2012b), whereas *Enterococcus* spp. significantly attenuates the fungus virulence in polymicrobial infections through the secretion of proteases, bacteriocins and lactic acid.(Shekh and Roy 2012; Garsin and Lorenz 2013; Cruz et al. 2013; Bachtiar et al. 2016; Graham et al. 2017). These findings show that several *Enterococcus* spp. metabolites where able to considerably reduce polymorphism and biofilm total biomass (Cruz et al. 2013; Bachtiar et al. 2016; Graham et al. 2017), increase the survival of *Caenorhabditis elegans* infected with *Candida albicans* (Cruz et al. 2013) and reduce epithelial invasion, inflammation, and fungal load in a murine model of oropharyngeal candidiasis through the increase of macrophages antifungal activity (Graham et al. 2017).

Another anti-*Candida* protein (ACP) characterized by Shekh and Roy (2012) showed a broad-spectrum activity against eight strains of multidrug resistant *C. albicans.* It is important to highlight that in none of those studies, the bacterial strain used against *Candida* was described as being innocuous, much less having probiotic claim (Shekh and Roy 2012; Garsin and Lorenz 2013; Cruz et al. 2013; Bachtiar et al. 2016; Graham et al. 2017). There is a lot of concern about *Enterococcus* spp. due to its high ability to become resistant to antibiotics and to transfer resistance genes, especially to vancomycin, to other pathogens (Hanchi et al. 2018). Probably because of that, the anti-*Candida* activity of *Enterococcus* spp. metabolites was evaluated whereas the cell - cell interaction was not considered suitable. However, the direct competition for adhesion sites and nutrients may be a potentiator of the anti-*Candida* effect promoted by bacteria metabolites (Vilela et al. 2015; Ishikawa et al. 2015; Matsubara et al. 2016b; Pujia et al. 2017; Ribeiro et al. 2017).

Witzler *et al* (2017) shown that the probiotic strain *E. faecium* CRL 183, isolated from an Argentinian traditional cheese, was able to inhibit *S. mutans* growth verified by the formation of an inhibition halo of 1.7 ± 0.3 mm diameter. This same study pointed out that *E. faecium* CRL183 was resistant to human saliva enduring the salivary enzymes and being able to grow (up to 3.64 $\log_{10}$ CFU / mL) in this environment after 24 hours, indicating a possible ability of oral cavity colonization by this probiotic strain (Witzler et al. 2017).

There is no documentation of adverse effects caused by *E. faecium* CRL 183, this strain has known genotypic identification, absence of virulence factors, pathogenicity and antimicrobial resistance(Saavedra et al. 2003). Nevertheless, several beneficial systemic effects promote by this probiotic strain are documented, i.e. modulation of the immune system and intestinal microbiota, reduction of symptoms related to ulcerative colitis (Celiberto et al. 2017), regulation of lipid profile and inhibition of atherosclerotic lesion development (Cavallini et al. 2009; Cavallini et al. 2011), being an alternative or adjuvant for drug therapy. *E. faecium* CRL183 also has prevented breast adenocarcinoma (Kinouchi et al. 2012), chemically induced colon cancer (Sivieri et al. 2008) and body weight gain (Marchesin et al. 2018) in different murine models. It is important to highlight that in some of those studies *E. faecium* CRL183 was used as a starter culture of a fermented soy-based product,

combined or not with other probiotic strains (Cavallini et al. 2011; Kinouchi et al. 2012; Celiberto et al. 2017; Marchesin et al. 2018).

Therefore, the aim of this study was to evaluate if *E. faecium* CRL 183 would be able to prevent *C. albicans* biofilm formation and maturation through direct competition, once the antifungal probiotic strain has never been tested.

Material and methods

Material

The probiotic strain *E. faecium* CRL 183 was obtained from Reference Center for Lactobacilli – CERELA/CONICET (San Miguel de Tucumán, Argentina) and *C. albicans* ATCC 90028 were kindly provided by the Laboratory of Clinical Microbiology of the School of Pharmaceutical Sciences, UNESP (Araraquara, Brazil).

The microorganisms were kept frozen at -80ºC in a cryogenic tube containing proper culture media with addition of 20% glycerol, up to the time of its use.

Methods

Strains and growth conditions

Prior to their use, the strains were thawed and subcultured in specific culture media: *E. faecium* in Bile Esculin Agar (Acumedia, Michigan, EUA) and *C. albicans* in Sabouraud Dextrose Agar supplemented with chloramphenicol (0.05 g L^{-1}) *(*SDA - Acumedia, Michigan, EUA) and incubated at 37°C for 48h.

Assurance of probiotic strain safety

As stated before, *E. faecium* CRL 183 has its safety consumption attested through many *in vivo* and clinical studies. It does not show any antibiotic resistance genes nor virulence factors (Saavedra et al. 2003). The *E. faecium* probiotic strain was submitted to the antimicrobial susceptibility test by disc diffusion performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2019).

A standard inoculum (turbidity equivalent to a standard solution of McFarland 0.5) was seeded with sterile swab in 140 x 15 mm Petri dishes (Corning, New York, USA) containing Mueller Hinton Agar (Difco, Detroit, USA). Disks containing antimicrobials: Ciprofloxacin 5 mg; Chloramphenicol 30 mg; Erythromycin 15 mg; Nitrofurantoin 300

mg; Norfloxacin 10 mg; Tetracycline 30 mg; and Vancomycin 30 mg (SENSIFAR - Cefar Diagnostica, São Paulo, Brazil), were placed on the inoculated agar with the aid of sterile forceps. The plates were incubated at 35 ± 2ºC, with incubation time of 16-18 h for most antimicrobials, except for vancomycin, which requires 24 hours of incubation. After this period, the presence and diameter of the growth inhibition halos caused by the antimicrobial discs were assessed. The results were interpreted according to the criteria recommended by the CLSI (2019).

Biofilm formation

The biofilm assays were carried out following the methods described by Fontana et al. (2009) and Zago et al., (2016), with modifications. The microorganisms freshly cultivated as described in growth conditions were inoculated with a sterile loop in 2mL of enriched broth containing: 26 g/L of brain–heart infusion (BHI- Kasvi, Curitiba, Brazil), 10 g/L of yeast extract (YE- Acumedia, Michigan, USA), 20 g/L of trypticase soy broth (TSB - Acumedia, Michigan, USA) and supplemented with 20% sucrose (Synth, Diadema, Brazil) to obtain yeast and probiotic bacteria inoculum. The suspensions obtained were standardized by spectrophotometry reading on a Synergy H1M microplate reader (Biotek, Winooski, USA). The two standardized microbial suspensions were then diluted to obtain different inoculum of yeast (10^6 or 10^8 CFU/mL) and probiotic (10^7 or 10^8 CFU/mL).

Monospecies biofilms assays were conducted on 96-well microplates with each well containing 75 µL of yeast or probiotic suspension plus 75 µL of enriched broth, i.e. 150 µl per well.

Polymicrobial biofilm assay were also performed in 96-well microplates with each well containing the suspension of each microorganism in the following proportions: 75 µL *C. albicans* 10^8 CFU/mL+ 75 µL *E. faecium* 10^8 CFU/mL (1:1), 75 µL *C. albicans* 10^6 CFU/mL 75+ µL *E. faecium* 10^7 CFU/mL (1:10) and 75 µL *C. albicans* 10^6 CFU/mL 75+ µL *E. faecium* 10^8 CFU/mL (1:100). The microplates were incubated at 35 ± 2ºC for 24h to evaluate the interaction of microorganisms in the biofilm adhesion and formation and for 48h to evaluate it in the biofilm maturation.

In the case of the 48 hours biofilms, they were split in two groups (48A and 48B) to evaluate if the metabolites produced by *E. faecium* within the first 24 hours play or not a role in the destabilization of *C. albicans* biofilms. In the group 48A after

the first 24 hours, the supernatant from each well was carefully replaced with 150 µL of fresh enriched broth and the plates returned to the incubator. Therefore, the metabolites produced were removed and new nutrients were added to the microenvironment. In the group 48B no intervention has been made during the 48 hours of incubation.

Biofilm pH evaluation

After the formation (24h) and maturation (48h) times of the biofilms, all the supernatant from the wells were aspirated carefully to avoid removal of the adhered biofilms and the pH of those supernatants were measured with pH-fix (Neumann-Neander, Düren, Germany). After this procedure the supernatant was discarded.

Biofilm quantification of viable cells

The adhered biofilms were scraped off the wells and resuspended vigorously in 150 µL of enriched broth with a sterile pipette tip for 30s. Serial decimal dilutions of those suspensions were made using as diluent the culture medium itself. Monospecies biofilms were quantified by plating the obtained dilutions in SDA supplemented with chloramphenicol (for *C. albicans)* and Bile Esculin Agar (for *E. faecium).* The polymicrobial biofilms were plated onto both culture media. The plates were incubated for 24h at 35 ± 2ºC and the number of fungal and probiotic cells present in the biofilms was determined by counting colony-forming units (CFUs). The experiments were performed in four replicates and repeated in three independent assays.

Determination of the anti-*Candida* activity of *E. faecium* CRL 183.

The decimal reduction (DR) of *Candida albicans* cell viability in the presence of probiotic strain was determined by equation 1:

$$DR = \text{Log}_{10}(Ca_m) - \text{Log}_{10}(Ca_p) \quad (1)$$

Where Ca_m is the colony-forming units (CFU/ mL) values of *C. albicans* present in the monospecies biofilms (positive control) and Ca_p is the colony-forming units (CFU/ mL) values of *C. albicans* present in the polymicrobial biofilms.

Then, the percentual reduction (PR) of fungal population in the polymicrobial biofilms after 24 or 48 hours were calculated with the equation 2:

$$PR = (1\text{-}10^{-DR}) \times 100 \quad (2)$$

Where DR is the decimal reduction obtained by equation 1.

Scanning electron microscopy (SEM)

This analysis was performed through FEG- SEM (JEOL JSM-7500F) to visualize the interactions between probiotic and yeasts within the biofilms. The experimental conditions were: polymicrobial biofilm at 1:100 pathogen: probiotic ratio and monospecies biofilms: *C. albicans* (initial inoculum of yeast: 10^6 CFU/mL) and *E. faecium* (initial inoculum of bacteria 10^8 CFU/mL). Biofilms were formed as described above in a sterile coverslip placed at the bottom of 6 -well microtiter plates.

After the periods of incubation (24h or 48h), the biofilms supernatants were gently removed and the coverslip were washed with PBS. The biofilms were fixed with a 2.5% glutaraldehyde solution (Merck, Darmstadt, Germany) then dehydrated with an ethanol series (30%, 50%, 70%, 85% and 95% ethanol solution for 15 min each; two washes with 100% ethanol for 15 min). The samples were dried at 37ºC and kept in a vacuum desiccator until the analysis day.

The coverslips containing adhered biofilms were then attached to the stub surfaces with double sided adhesive tape, coated with a layer of carbon and observed through FEG- SEM (JEOL JSM-7500F). The photomicrographs were taken at 1000x; 3000× and 5000x magnifications.

Statistical analysis

To verify the statistical significance, the results were submitted to One-way ANOVA followed by Tukey's multiple comparisons test (parametric data). It was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California USA) with a minimum significance level of 5% ($p \leq 0.05$).

Results

Assurance of probiotic strain safety.

The antimicrobial susceptibility test results presented in table 1 were interpreted according to the standard table for interpretation of inhibition halos present in the diagnostic test package insert following CLSI recommendations (CLSI 2019). The

probiotic strain was sensitive to all classes of antimicrobials tested, including vancomycin.

Table 1: *E. faecium* CRL 183 antimicrobial susceptibility test by disc diffusion

Antimicrobials	Inhibition halos (millimeters)	Interpretation
Ciprofloxacin 5 mg	30	Sensitive
Chloramphenicol 30 mg	27	Sensitive
Erythromycin 15 mg	22	Sensitive
Nitrofurantoin 300 mg	30	Sensitive
Norfloxacin 10 mg	25	Sensitive
Tetracycline 30 mg	24	Sensitive
Vancomycin 30 mg	19	Sensitive

Biofilm pH evaluation

For *C. albicans* monospecies biofilms in formation, the supernatant pH was 7.0 at all concentrations tested. After 48 hours, with or without culture medium renewal, the pH was reduced to 5.0. There was no difference between the pH of *E. faecium* monospecies biofilms supernatants and the polymicrobial biofilms supernatants in any experimental conditions (initial concentration, incubation time, culture medium exchange), which remained in the order of 5.0.

Quantification of viable cells and determination of the anti-*Candida* activity of *E. faecium* CRL 183

The interaction between fungus and probiotic was analyzed in polymicrobial biofilms. The survival of *E. faecium* in an environment co-colonized by *C. albicans* was evaluated by quantifying its colonies and comparing it with the results obtained in the *E. faecium* monospecies biofilm (control). It was observed that this probiotic is able to establish itself and survive in the microenvironment, competing for space and nutrients with *C. albicans.* There was significant statistical difference in relation to the control group only in the 1:1 ratio of fungus/ probiotic (Figure 1). Renewal of the culture medium in the 48 hours biofilms (48A) did not result in a statistically significant difference in relation to the non-renewal group (48B) in the ratios 1:10 and 1:100 (Figure 1B). In the ratio 1:1 the renewal of culture medium (48A) impaired *E.*

faecium survival in the presence of *C. albicans* if compared with the group 48B. Presumably, metabolites produced within the first 24 hours helped *E. faecium* to survive in the microenvironment where there was a high concentration of *C. albicans.*

Despite this positive effect that *C. albicans* exerts in relation to *Enterococcus* spp. the relation between these microorganisms cannot be considered as mutually beneficial. Especially in the context of the present study, since the presence of the fungus impaired the viability of *E. faecium* only in the 1:1 ratio (Figure 1), while the probiotic negatively affected the cellular viability of *C. albicans* in all experimental conditions (Figure 2). The reduction of *C. albicans* within the 24 hours biofilms range from 1.57 to 3.08 Log_{10} CFU/mL, these results indicate that, during the formation of biofilms, the fungal reduction depends on probiotic concentration. However, in terms of percentual reduction, which ranged from 97.12% to 99.92% for 24 hours biofilms, there was no statistical difference between 1:1; 1:10 and 1:100.

In mature polymicrobial biofilms (48 hours) *C. albicans* reduction ranged from 2.00 to 2.30 Log_{10} CFU/mL (Figure 2) corresponding to PR of 99 to 99.43% for the group where was the removal of metabolites and renewal of nutrients (48A). Which was not significantly different from 48B group where it was observed reduction from 1.46 to 2.15 Log_{10} CFU/mL (Figure 2) or 96.54 to 99.30%. The 48 hours results indicate that, for mature biofilms, the fungal reduction was not dependent on the initial probiotic dose, since there was no significant statistical difference between the groups (1:1, 1:10 and 1: 100).

Scanning electron microscopy (SEM)

The SEM analysis showed in Figure 3 allowed to illustrate the results obtained by counting colony-forming units. In monospecies biofilms, or controls, it was possible to observe cellular aggregates, surrounded by EPS, scattered throughout the coverslips. A lower number of fungal cells (3A,3B,3C and 3D) was observed in comparison to bacteria (3E,3F,3G,3H), which corroborates the cell counting results obtained for monospecies biofilms. In polymicrobial biofilms (3I,3J,3K and 3L), it is possible to observe that there was a considerable reduction in the number of *C. albicans* cells compared to the control biofilms (3A,3B,3C and 3D) of this species. This does not occur with the number of *E. faecium* seen in the polymicrobial biofilm which is apparently equal to that found in the monospecies biofilm. Furthermore, the

images of the polymicrobial biofilms (3I,3J,3K and 3L) allow to evaluate that there is a physical interaction between the fungus and the probiotic cells.

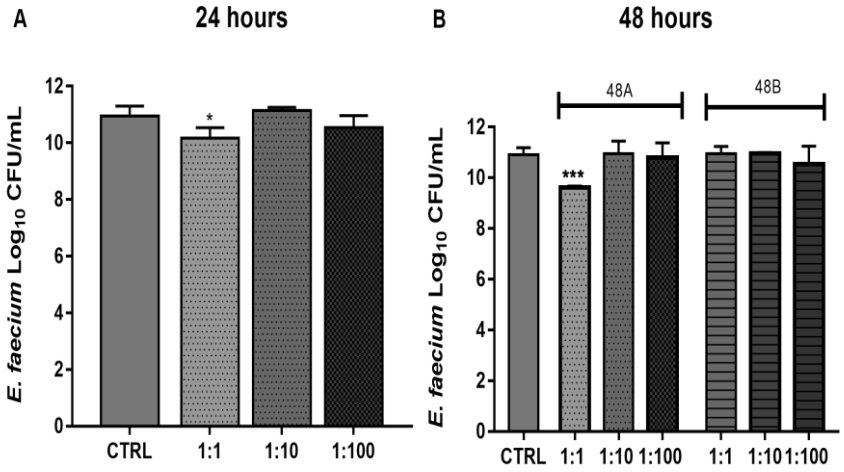

Fig. 1 - Cell viability of *E. faecium* in the presence of *C. albicans*. A. 24 hours – biofilm formation. B. 48 hours – biofilm maturation; 48A: group with metabolites withdrawal and nutrients renewal. 48B: group without interventions. CTRL - monospecies biofilms of *E. faecium* (control); 1: 1; 1:10; 1: 100 - polymicrobial biofilms in different ratios of *C. albicans / E. faecium*. Each column represents the mean of three independent experiments, each performed in quadruplicate (n = 12), the bars represent the standard deviation. The asterisks indicate when there was a statistically significant difference in relation to the control group (*p = 0.03; ***p = 0.0003). ANOVA, followed by Tukey's post-test.

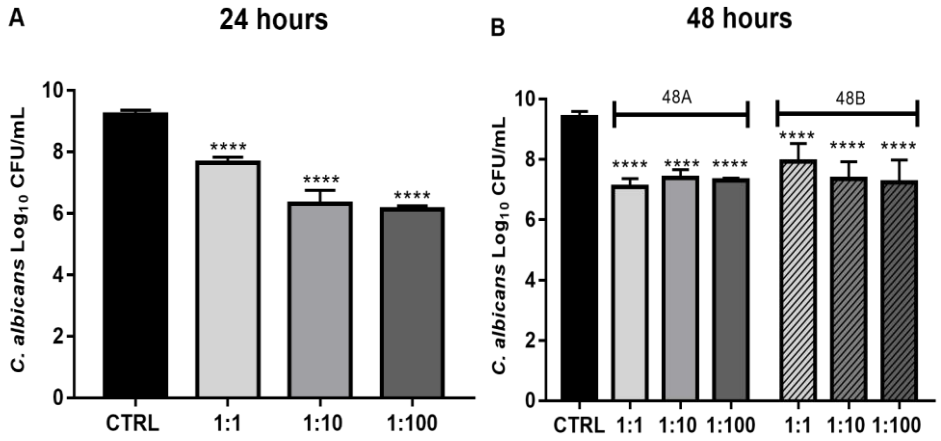

Fig. 2 - Cell viability of *C. albicans* in the presence of *E. faecium*. A. 24 hours – biofilm formation. B. 48 hours – biofilm maturation; 48A: group with metabolites withdrawal and nutrients renewal. 48B: group without interventions. CTRL - monospecies biofilms of *C. albicans* (control); 1:1; 1:10; 1:100 - polymicrobial biofilms in different ratios of *C. albicans / E. faecium*. Each column represents the mean of three independent experiments, each performed in quadruplicate (n = 12), the bars represent the standard deviation. The asterisks indicate when there was a statistically significant difference in relation to the control group (****p < 0.0001). ANOVA, followed by Tukey's post-test.

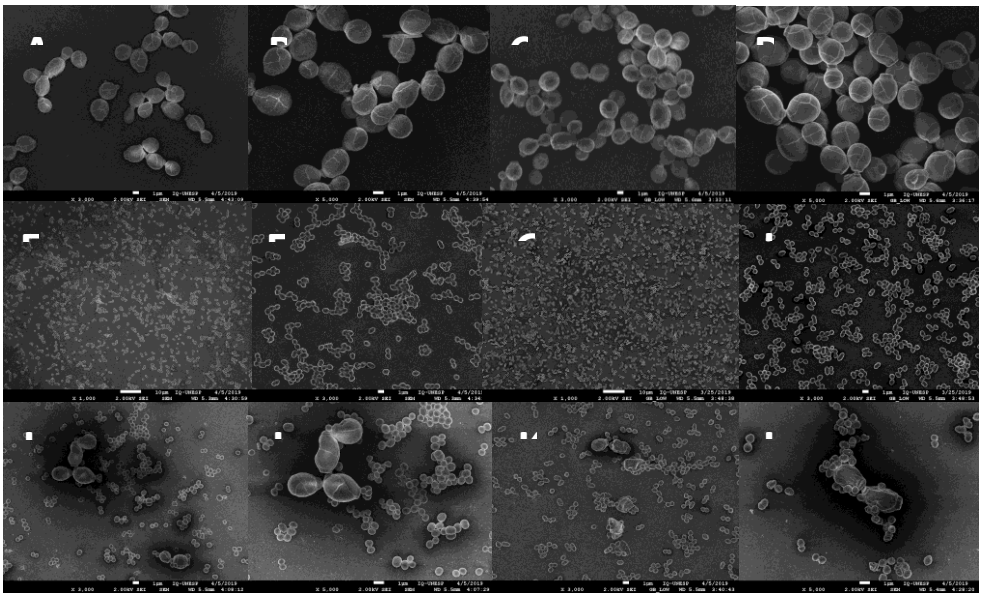

Fig. 3. Scanning electron micrographs (SEM) of biofilms recorded at 1000x (E, G); 3000x (A, C, F, H, I, K) or 5000x (B, D, J, L) magnifications. A-B: *C. albicans* monospecies biofilm at 24 hours. C-D: *C. albicans* monospecies biofilm at 48 hours. E-F: *E. faecium* monospecies biofilm at 24 hours. G-H: *E. faecium* monospecies biofilm at 48 hours. I-J: Polymicrobial biofilms at 24 hours. K-L: Polymicrobial biofilms at 48 hours.

Discussion

Assurance of probiotic strain safety

Whilst adverse effects due to administration of probiotic bacteria are uncommon, it is mandatory to assure its susceptibility to antimicrobials. It is known that antibiotic resistance can be intrinsic or acquired as a result of a chromosomal mutation or by horizontal gene transfer (Celiberto et al. 2018). There is a special concern about the spread of Vancomycin-Resistant Enterococci (VRE) since some species of the genus may become a nosocomial pathogen and a reservoir for resistance genes, leading to clinical isolates resistant to all antibiotics (Hanchi et al. 2018). The strain *E. faecium* CRL 183 has documented absence of antimicrobial resistance genes (Saavedra et al. 2003) and was susceptible to all classes of antimicrobials tested and, therefore, it can be considered safe.

Biofilm pH evaluation

Factors such as nutrients availability, temperature and pH are determinants for the survival and behavior of microorganisms, especially in polymicrobial environments. It is a consensus that probiotic strains can produce lactic acid and

other organic acids. Which, in your turn, can cross the plasma membrane of yeast cells, increasing the activity of H^+-ATPase. This mechanism promotes energy exhaustion of yeast cells inhibiting their growth and promoting cell death (Jørgensen et al. 2017). Furthermore, neutral to alkaline is the optimal extracellular pH for *Candida* spp. because it aids hyphal morphogenesis, among other fungal virulence strategies (Davis 2003). *E. faecium* is a lactic acid bacteria (LAB) and the acid production could be one of the key mechanisms by which this probiotic impairs *C. albicans* viability (Hanchi et al. 2018). A study which evaluated the inhibition on several *Candida* species promoted by *L. reuteri* has shown that, the almost complete inhibition was only achieved in pH 3.6, which is a very acidic level. However, extensive acidification may be a concern regarding the application in oral cavity due to the role of acid in caries lesions (Jørgensen et al. 2017). In our study the biofilm supernatants pH remained within the range of healthy human saliva pH which is 5.3 to 7.8, depending on the stimulation state (Gittings et al. 2015).

Quantification of viable cells and determination of the anti-*Candida* activity of *E. faecium* CRL 183

Similar to the results shown in Figure 1; Mason et al. (2012a, b) demonstrated that *C. albicans* SC5314 promoted gastric and enteric colonization of *Enterococcus* spp. while antagonizing other lactic bacteria such as *Lactobacillus* spp., during recovery of gut microbiota following treatment with the antibiotic cefoperazone. Although the mechanism involved was not elucidated, the authors suggest that it was similar to the one *C. albicans* exerts on bacteria from phylum *Bacteroidetes.* The fungus could aid its adhesion and fixation to the cecum; that yeast glucans could be a potential source of food for these bacteria, or else, *C. albicans* could suppress other bacteria that antagonize *Enterococcus* spp. (Mason et al. 2012a).

The reduction of *C. albicans* viable cells during biofilm formation was affected by the fungal: probiotic ratio. Cruz et al. (2013) demonstrated that *Caenorhabditis elegans*, a study model of polymicrobial infections, presented less mortality caused by *C. albicans* when it was co-inoculated by *Enterococcus* spp. The survival rate of the nematode was dose-dependent: the higher the number of colonies of *Enterococcus* spp. inoculated, the greater the protection against *C. albicans* pathogenicity. This study further determined that the pathogenicity of both microorganisms was attenuated during polymicrobial infection in *C. elegans* (Cruz et

al. 2013).

For biofilm maturation, the microorganism ratio did not influence in the result. Matsubara et al. (2016b) also did not observe a significant relationship between inoculated probiotic dose and biofilm destabilization.

Among the *Enterococcus* spp. mechanism of action to downregulate *C. albicans* spp. pathogenicity the literature majorly suggests the action of proteases, peptides and bacteriocins secreted by the bacteria(Shekh and Roy 2012; Cruz et al. 2013; Bachtiar et al. 2016; Graham et al. 2017). For example, researchers identified the action of two proteases (gelatinase biosynthesis activating cluster peptide - GBAP) that activate the virulence regulating system of *Enterococcus* spp., which when secreted by this bacterium prevented *C. albicans* polymorphism and, consequently, fungal pathogenesis (Cruz et al. 2013). The same effect was observed when *C. elegans*, previously infected with *C. albicans*, was treated with the supernatant (filtered and sterilized) of an *Enterococcus faecalis* inoculum. Thus, Cruz et al. (2013) determined that in their study, the inhibitory activity was secreted instead of a direct cellular competition between the bacterium and the fungus.

In a more recent research, the action of the EntV, an enterocin encoded by Ef1097 gene, which is present in all strains of *E. faecalis* sequenced to date was described (Graham et al. 2017). This enterocin alone, as well as its synthetic version, was able to prevent *C. albicans* polymorphism and biofilm formation on different substrates and experimental conditions. Preformed *C. albicans* biofilms (24 hours / ~ 30 μm thick) were treated with EntV and underwent a large perturbation with a reduction of their thickness to ~15 μm. Biofilms that were not treated with EntV increased their thickness to >50 μm. The peptide also protected macrophage by enhancing its antifungal activity, reduced epithelial invasion, inflammation, and fungal load in a murine model of oropharyngeal candidiasis (Graham et al. 2017).

Bachtiar et al. (2016) observed that *E. faecalis* cps2 (a non-encapsulated clinical isolate) and *E. faecalis* ATCC 29212, as well as the metabolites produced by them, did not inhibit *C. albicans* biofilm formation. However, both strains and their secreted metabolites significantly reduced (>50%) the maturation of these biofilms. These results were confirmed by the downregulation promoted on ALS1 and ALS3 (adhesion related genes) and on EFB1, a gene used to quantify the harmful effects of antifungal agents against mature candida biofilms (Bachtiar et al. 2016).

In our study, it was not possible to determine if the anti-*Candida* activity of *E. faecium* CRL 183 was modulated by its metabolites as suggested in literature cited above. However, as can be seen in Figure 2B, the renewal of the culture medium (48A) did not significantly influence *C. albicans* survival compared to the group in which there was no intervention (48B). Therefore, the removal of *E. faecium* metabolites secreted during the first 24 hours and the new nutrient supply did not favor the cellular viability of *C. albicans*. In addition, the *E. faecium* population was approximately 11 $\log_{10}$ CFU/mL in the polymicrobial biofilms and *C. albicans* population was approximately 7 $\log_{10}$ CFU/mL, under all experimental conditions. It means that the probiotic growth was much greater than the fungal growth within polymicrobial biofilms. This may suggest that in our study model, the inhibitory action on *C. albicans* may not be only mediated by metabolites produced by *E. faecium*, but most likely due to direct cell competition for nutrients and space. This mechanism of direct competition and limitation of environmental nutrients can provoke a metabolic reprogramming on *C. albicans*, reducing its virulence. Mailänder-Sánchez et al. (2017) suggest that this is the mechanism by which the probiotic strain *Lactobacillus rhamnosus* GG prevented adhesion, invasion, formation of hyphae (preventing epithelial damage), glucose depletion and ergosterol synthesis of *C. albicans*. Another inhibitory mechanism may have been the culture medium acidification in polymicrobial biofilms promoted by *E. faecium* which is a lactic acid bacteria (LAB). This pH reduction promotes an energy exhaustion of yeast cells, inhibiting their growth and disfavor *C. albicans* filamentation (Davis 2003; Jørgensen et al. 2017). Hypha formation is one of the main virulence factors of *C. albicans* and is directly related to its pathogenicity and to biofilm stability (Mayer et al. 2013; Nobile and Johnson 2015).

Vilela et al. (2015) suggest this same mechanism in the suppression of *C. albicans* polymorphism when it was stimulated with cultures of *L. acidophilus* ATCC 4356 or filtered supernatants from this probiotic culture. They also reported a reduction of up to 57.5% in CFU-counting of *C. albicans* biofilms stimulated with the probiotic cell culture and 45.10% reduction in biofilms stimulated with probiotic culture filtered supernatants. *Galleria mellonella* was used as an experimental candidiasis model and either treatment or prophylaxis with *L. acidophilus* cells, or their metabolites, greatly increased the survival of this insect (Vilela et al. 2015).

Ribeiro et al. (2017) observed a similar effect on the interaction of *C. albicans* with *L. rhamnosus* cells: a 52.2% reduction in biofilm quantification in CFU / mL and a 48% reduction in metabolic activity. The stimulation with only the filtered supernatant of *L. rhamnosus* decreased, in a lesser extent, *C. albicans* growth (39.4%) and metabolic activity (61%).

Matsubara et al. (2016b) observed that *L. rhamnosus*, *L. casei* and *L. acidophilus* cell suspensions significantly reduced *C. albicans* SC5314 biofilm formation in 32, 59 and 56.3%, respectively. When 48 hours biofilms were stimulated with the same probiotic suspensions, the *C. albicans* reduction was 61.8% (*L. rhamnosus*), 54.7% (*L. casei*) and 34.2% (*L. acidophilus*). By stimulating *C. albicans* biofilms with the filtered supernatant of these *lactobacillus* cultures, the reduction was less effective in the formation, and there was no reduction in the biofilm maturation. This result demonstrates that the inhibitory action on *C. albicans* was also not mediated only by probiotic secreted metabolites(Matsubara et al. 2016b).

Therefore, our results seem to be very promising once *E. faecium* CRL183 presented an ability to impair *C. albicans* biofilm formation in up to 99.92% and its biofilm maturation in up to 99.43%, which are much higher rates than those presented by other *Enterococcus* spp. (Shekh and Roy 2012; Cruz et al. 2013; Bachtiar et al. 2016; Graham et al. 2017) or probiotic strains (Vilela et al. 2015; Matsubara et al. 2016b; Jørgensen et al. 2017; Ribeiro et al. 2017).

Scanning electron microscopy (SEM)

The images obtained through SEM permitted to visually confirm the hypothesis that in the present study direct competition and limitation of environmental nutrients, play an important role. It is very interesting to observe that *C. albicans* not only appears in a lesser amount but also with its morphology very damaged in 48 hours polymicrobial biofilms (3K and 3L) in comparison with *C. albicans* control biofilms at the same period (3C and 3D), which could be caused by the lactic acid crossing yeasts membrane (Jørgensen et al. 2017). It was expected to see hyphal formation in *C. albicans* biofilms in our SEM images, which did not occur. It may be explained by the lack of molecular factors favoring *C. albicans* polymorphism in the culture medium used in the experimental protocol, since it is known that the culture medium plays a fundamental role in the growth, morphology and biofilm formation of

C. albicans (Weerasekera et al.; Davis 2003). Another reason could be the several washes with ethanol removed hyphae and pseudohyphae, since these structures develop in the outermost layers of biofilms, while yeasts initially inoculated, are found in the basal layers adhered to the biotic or abiotic substrate (Uppuluri et al. 2010). Yet, in the context of prevention, our results remain promising as yeasts are the infecting forms which initiates biofilms and are responsible for spread to no infected sites (Uppuluri et al. 2010; Lohse et al. 2017).

Both the counting colony-forming units technique and SEM analysis showed that *E. faecium* CRL 183 promotes a great reduction in *C. albicans* viability.

In conclusion, according to the methodologies applied in this *in vitro* study, *E. faecium* CRL 183 was able to significantly inhibit the formation and maturation of fungal biofilms in all evaluated pathogen / probiotic relationships. These promising results demonstrate the need for in-depth studies to investigate the mechanisms by which *E. faecium* CRL183 acts against *C. albicans*. In addition, local release forms of probiotics in regions susceptible to candidiasis also need to be evaluated.

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Capítulo 2.

Development of orodispersible films loaded with probiotic *E. faecium CRL* 183 for oral candidiasis prevention.

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Development of orodispersible films loaded with probiotic *E. faecium CRL* 183 for oral candidiasis prevention.

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Abstract:
The aim of this study was to develop and characterize an orodispersible film (ODF) capable of deliver the probiotic *Enterococcus faecium* CRL 183 in the oral cavity evaluating its *in vitro* antifungal activity. The ODF was composed by cheap and natural polymers and physical-chemically characterized; the probiotic resistance and viability during processing and storage was evaluated as well as the *in vitro* anti-*Candida* activity. The obtained films were thin, resistant and flexible, with neutral pH, low Aw values and microbiologically safe. Furthermore, they presented good barrier properties. The probiotic resisted the obtaining process of ODF, demonstrating high viability (> 9 $\log_{10}$ CFU / g), up to 90 days of storage at room temperature. The Probiotic Film (PF) promoted 68.9% of reduction in fungal biofilm formation (24h) and 91.24% in its maturation (48h) compared to the group stimulated with the control film (CF). Those promising results were confirmed through SEM images.

Keywords: Probiotics, anti-Candida activity, Biofilms, Orodispersible films, *Candida albicans* ATCC 90028, *Enterococcus faecium* CRL 183.

1. Introduction

It is well established that probiotics, which are defined as living microorganisms that, when administered in suitable amounts, confer benefits to the host health, are able to improve dysbiotic microbiota, regulate intestinal transit, neutralize carcinogens, promote vitamin synthesis and bile salt metabolism, reinforce gut barrier and the immune system and directly antagonize and compete with pathogens (Hill et al., 2014).

In this context, several studies have shown that certain probiotic strains have great potential for maintenance and improvement of oral health, presenting anticariogenic activity, reduction of halitosis and prevention of opportunistic infections, such as candidiasis, often present in immunocompromised individuals (Ishikawa et al., 2015; Jørgensen, Kragelund, Jensen, Keller, & Twetman, 2017; Mailänder-Sánchez et al., 2017; Matsubara, Bandara, Mayer, & Samaranayake, 2016; Pujia et al., 2017).

These pathogenic conditions are related to formation and establishment of biofilms in the oral cavity. Infections caused by biofilms, especially fungal biofilms, are long-lasting and difficult to eliminate. Therefore, high doses of antifungals are often required, which promote serious adverse effects due to the similarity between host and fungal cells, and leads to pharmacotherapy resistance (Fanning & Mitchell, 2012; Pujia et al., 2017; Shekh & Roy, 2012).

Thus, alternatives for fungal infections must be searched and prevention seems to be the best strategy. Our research group has demonstrated that the probiotic strain *Enterococcus faecium* CRL 183 was able to inhibit up to 99.9% *in vitro* formation of *Candida albicans* biofilms and 99% biofilm maturation, without losing its own viability. This study also has shown that probiotic initial inoculum, previously considered low (10^7 CFU / mL), was as capable of impairing the fungal biofilms as higher initial inoculums (data not-published).

Witzler et al. (2017) has shown that this same probiotic strain was able to inhibit the growth of *Streptococcus mutans* ATCC 25175, another important pathogen of oral cavity. Furthermore, *E. faecium* CRL 183 resisted to salivary enzymes, being able to significantly grow in this environment. The probiotic strain was incorporated in a lozenge in an attempt to develop an alternative way to deliver it into the oral cavity,

but the probiotic viability was quickly reduced in that vehicle (Witzler, Pinto, Font de Valdez, de Castro, & Cavallini, 2017).

One of the greatest challenges concerning probiotic products is to develop non-dairy alternatives focusing on local, instead of systemic actions, such as: capsules, gels or incorporation in food. These novel products should grant probiotic viability during processing and storage, should not alter product flavor or consistency and, on the oral cavity concern, should stay in the mouth long enough to release the probiotic (Heinemann, Carvalho, & Favaro-Trindade, 2013; Saha, Tomaro-Duchesneau, Daoud, Tabrizian, & Prakash, 2013; Witzler et al., 2017)

An option that meets all these requirements is the ODF, also known as soluble films, oral disintegrating films, oral thin films or mucoadhesive films – the nomenclature changes depending on their characteristics and functionalities (Lee, Kim, Kim, Choi, & Jeong, 2017). This pharmaceutical form can be defined as a thin film of easy dissolution and high contact surface, which allows the release of all active component into the oral cavity. Among the advantages attributed to those films are: ease of obtention and handling, storage at room temperature, and easy administration, since it does not need water intake, facilitating patient compliance to treatment. Lastly, as the oral mucosa is highly vascularized, ODF can also avoid the first-pass metabolism (Dixit & Puthli, 2009).

Another advantage is that the permanence of the ODF in the oral cavity, during the disintegration time, can promote a more efficient release of active components compared to other products such as oral gels that are easily removed by the salivary flow (Borges, Silva, Cervi-Bitencourt, Vanin, & Carvalho, 2016). Researchers have shown that this dosage form may be the best strategy to immobilize probiotics and deliver them into specific areas such as the oral cavity. Probiotic ODF presented high microbial viability after processing allowing a proper shelf-life, suitable disintegration time allowing the local probiotic release, and good appearance and mechanical properties (de Barros, Scherer, Charalampopoulos, Khutoryanskiy, & Edwards, 2014; Heinemann et al., 2013; Saha et al., 2013).

Those films are usually composed of macromolecules, natural or synthetic, forming polymer matrix, plasticizers - which increase the filmogenic properties of the polymers giving greater flexibility and less fragility - and the active pharmaceutical ingredient (API) or bioactive compound of interest. The composition and proportion of

such excipients can be altered according to the characteristics desired for the product and the addition of sweeteners, salivary stimulants, flavorings, pigments, surfactants and stabilizers can be considered (Lee et al., 2017). The chosen polymers should have a good shelf life, should not be toxic to the probiotic, guaranteeing its viability and metabolic activity, as well as a quick and efficient release. In addition, they should not cause damage to the patient (Heinemann, Vanin, De Carvalho, Trindade, & Fávaro-Trindade, 2017; Saha et al., 2013). Carboxymethylcellulose (CMC) and hydroxypropyl methylcellulose (HPMC) appears in several studies as a good choice for films that carry probiotics (De Barros et al., 2014; Heinemann et al., 2013; Saha et al., 2013). However, Heinemann et al (2013) demonstrated that films composed of CMC and gelatin blend were more efficient at protecting probiotic cells during the drying process of the films. And that films composed of CMC, gelatin and starch allowed the survival of probiotics in a high concentration (> 9 CFU / g) for a longer period (Heinemann et al., 2013).

Borges et al. (2016) also suggests that the gelatin, nor only confers advantageous properties to the product as viscoelasticity, transparency and excellent solubility at room temperature, but it also can be an important source of peptides promoting a healthier nutritional status (Borges et al., 2016).

According to the characteristics of the films, these constitute carriers of bioactive substances ideal for patients with conditions that favor dysphagia such as: the elderly, patients with motor debilities and immunocompromised. Coincidentally, in this population there is a high prevalence of candidiasis (Borges et al., 2016; Dixit & Puthli, 2009; Lee et al., 2017; Nobile & Johnson, 2015). Considering the above, this study aimed to develope and characterize a probiotic orodispersible film with the addition of *E. faecium* CRL 183, evaluating its antifungal potential against *C. albicans* ATCC 90028, *in vitro*.

2. Material and methods

2.1. Material

The probiotic strain *E. faecium* CRL 183 was obtained from Reference Center for Lactobacilli – CERELA/CONICET (San Miguel de Tucumán, Argentina) and *C. albicans* ATCC 90028 were kindly provided by the Laboratory of Clinical Microbiology of the School of Pharmaceutical Sciences, UNESP (Araraquara, São Paulo,

Brazil).The microorganisms were kept frozen at -80ºC in a cryogenic tube containing proper culture media with addition of 20% glycerol, up to the time of its use.

Carboxymethylcellulose (CMC) was donated by Biovital (São Carlos, São Paulo, Brazil), gelatine (260 bloom / 30 mesh) was provided by Gelita (Cotia, São Paulo, Brazil), Sorbitol (Neosorb P60W) was supplied by Labonathus (São Paulo, São Paulo, Brazil), alcohol-free mint flavoring was provided by Firmenich, (Cotia, São Paulo, Brazil) and potato starch was purchased from Yoki (Pouso Alegre, Minas Gerais, Brazil).

2.2. Methods

2.2.1. Strains and growth conditions

Prior to their use, the strains were thawed and subcultured in specific culture media: *C. albicans* in Sabouraud Dextrose Agar supplemented with chloramphenicol (0.05 g L^{-1}) (SDA - Acumedia, Michigan, EUA) and *E. faecium* in Bile Esculin Agar or M17 agar (Acumedia, Michigan, EUA) and incubated at 37°C for 48h. *C. albicans* colonies, freshly cultivated as described above, were inoculated with a sterile loop in enriched broth (26 g/L of BHI- Kasvi, Curitiba, Brazil; 10 g/L of YE - Acumedia, Michigan, USA; 20 g/L of TSB - Acumedia, Michigan, USA; 20% of sucrose Synth, Diadema, Brazil). This yeast suspension was standardized (10^6 CFU/mL) by spectrophotometry reading on a Synergy H1M microplate reader (Biotek, Winooski, USA).

To prepare the probiotic inoculum, *E. faecium* freshly cultivated colonies were inoculated in 5 mL of M17 Broth (Himedia, Mumbai, India) followed by incubation at 35 ± 2 ° C for 14-16 hours. After this period, the tubes containing the inoculum were pelleted by centrifuging at 4000 rpm (80-2B Centribio-Equipar, Curitiba, PR, Brazil) for 20 min, washed twice with 0.9% (w/v) NaCl solution (Synth, Diadema, SP, Brazil) and resuspended in lower volume of 0.9% (w/v) NaCl. The bacterial suspension obtained was standardized at ~10^{10} CFU/mL.

2.2.2. *Orodispersible films preparation*

Two formulations of ODFs - Probiotic Film (PF) and Control Film (CF) - were prepared by solvent casting as described by Heinemann et al. (2013), with modifications. The procedure is outlined in Figure 1.

Probiotic Film (PF)

Three dispersions of macromolecules (polymers) and plasticizer were prepared using a magnetic stirrer (Fisatom, São Paulo, Brazil): G - 2.00 g gelatin + 0.4 g sorbitol in 100 mL distilled water; PS - 2.00 g potato starch + 0.4 g sorbitol in 100 ml distilled water; CMC- 1.00 g carboxymethylcellulose + 0.2 g sorbitol in 100 ml distilled water. These dispersions were mixed in the proportion 1CMC: 2PS: 2G (v / v) to obtain the film-forming dispersion (FD), to which 0.50% (w/w) mint powder flavoring was added. The film-forming dispersion was sterilized at 121 ° C / 15 minutes in a vertical autoclave (Phoenix, Araraquara, Brazil) and then cooled to 45 °C. After that, all the steps were conducted within the laminar flow under sterile conditions. To the sterile film-forming dispersion 10% v / v. of *E. faecium* CRL 183 inoculum freshly cultured as described in strains and growth conditions was added, under constant magnetic stirring, at 45ºC. Two milliliters of FD were poured into 2x2 cm holes of silicone molds to obtain films with standardized size and weight. The silicone molds were arranged in laminar flows with constant air circulation at room temperature (28 ± 2 ° C) for 24 hours. The obtained films were carefully removed from the silicone molds and conditioned in petri dishes at room temperature within a vacuum desiccator. The films were produced in three batches.

Control Film (CF)

The same procedure was used to obtain control films, except the addition of *E. faecium* CRL 183 inoculum.

2.2.3. Macroscopic observations, color parameters, thickness analysis, pH and moisture content of ODFs

The films were inspected in relation to appearance, texture, flexibility and occurrence of bubbles and fissures. A colorimeter (Konica Minolta® Spectrophotometer CM 600d) was used for the assessment of color parameters, in triplicate. The thickness was evaluated with a digital micrometer MDC-25SX (Mitutoyo®, Kawasaki, Japan) in three random positions of each film, in sextuplicate. The pH of the films was determined using a digital potentiometer (Qualxtron®, Model 8010), through the dissolution of a 0.1 g-sample of the films in 20.0 mL of 0.9% (w/v) NaCl, in triplicate. The moisture content was measured in an infrared analyzer (Sartorius®, MA35), right after the end of the drying period of the films (T0).

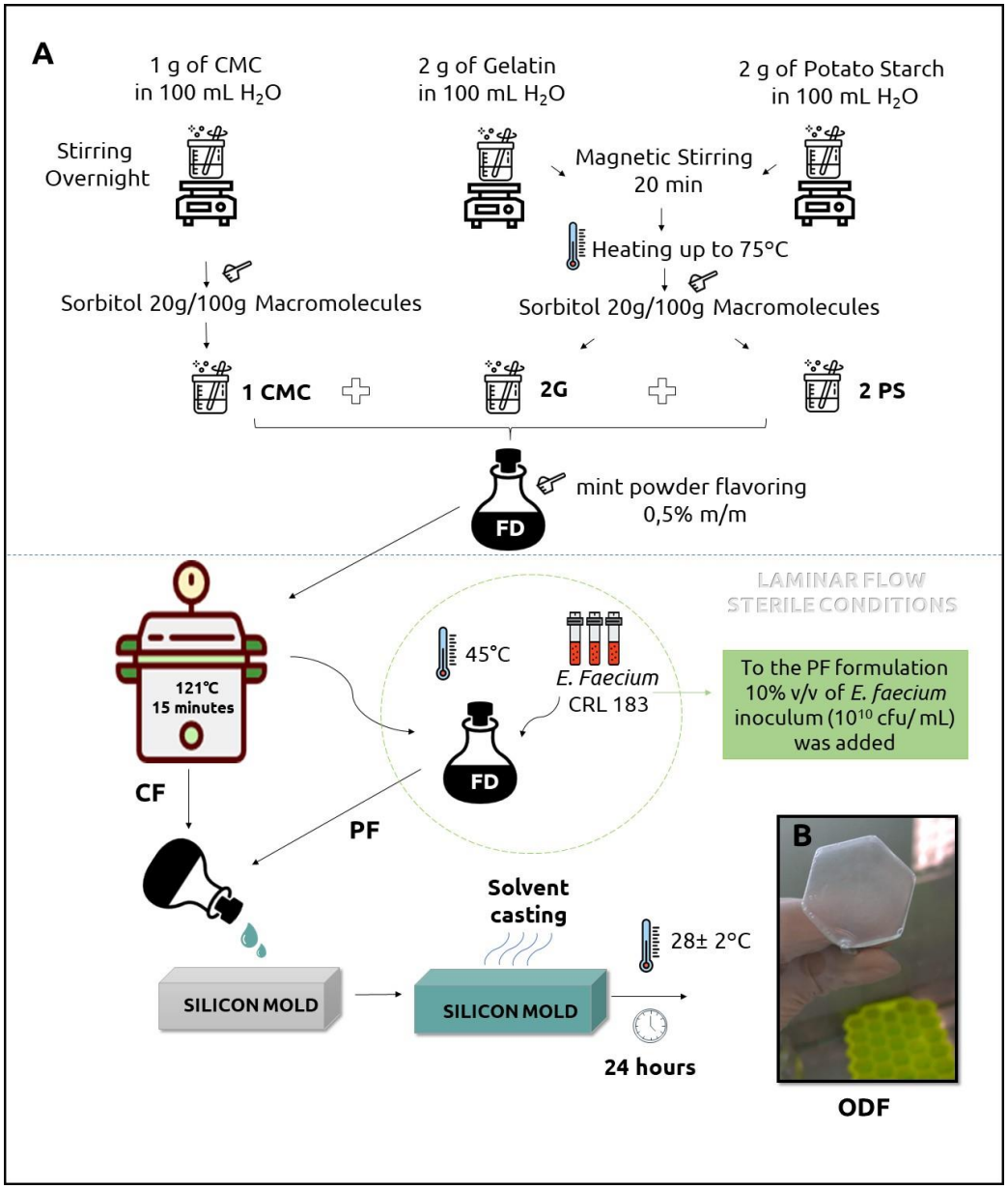

Fig 1. Orodispersible films preparation protocol (A). Orodispersible film (B)

2.2.4. Probiotic survival in PF after drying process and storage.

To evaluate the cell viability of *E. faecium* in the orodispersible films, PF samples (0.1 g) were solubilized in 20 mL of 0.9% (w/v) NaCl (Synth, Diadema, SP, Brazil), sterile solution, at 37° C. From this solution, serial decimal dilutions were made which were plated through spread-plate technique in M17 agar (Himedia, Mumbai, India). The results were expressed as log CFU/g after incubation performed in aerobic conditions at 37°C / 48 hours. The analyses were performed immediately after the drying process of ODF (T0) and compared to the probiotic amount added to

the filmogenic solution, to evaluate the probiotic resistance to processing. To evaluate the probiotic viability overtime, the analysis was performed fortnightly, during the storage period of 90 days.

2.2.5. Microbiological safety of ODFs and Water activity (Aw)

The films water activity (Aw) was measured fortnightly using an Aqualab® CX2 analyzer (Decagon devices, USA) after stabilization of 0.1g samples at 25ºC for 30 minutes. Microbiological safety of ODFs were determined monthly through the dissolution of a 0.1 g-sample in 20.0 mL of sterile NaCl 0.9% (w/v). This solution was then serial diluted and plate on specific culture media to evaluate contamination by yeasts and molds (PDA - Potato Dextrose Agar, Himedia, India) and incubated at 30.0ºC, for 120h. Total coliforms contamination was assessed with Petrifilm® (3M®, USA), incubated at 37.0 ºC, for 48h.

2.2.6. In vitro disintegration time

The disintegration test was performed as described by Garsuch and Breitkreutz (2015), with modifications. PF or CF samples were placed at the top of 5 mL Erlenmeyer Flask, into which was placed a filter paper. Two hundred microliters of artificial saliva (at 37ºC) was placed on the films surface. Artificial saliva, without enzymes, was prepared as follows: 0.844g of sodium chloride; 1.2g of Potassium Chloride; 0.193g of calcium chloride dihydrate; 0.111g of magnesium chloride hexahydrate and 0.342g of potassium phosphate dibasic were accurately weighed and added to 1000ml of distilled water. The pH was adjusted with 0.1N sodium hydroxide to 7.0 (Nalluri, Sravani, Anusha, Sribramhini, & Maheswari, 2013). The time taken for the drop to pass through the film (T1) or to dissolve the film and form a hole in it (T2) was recorded. The tests were performed at least in triplicate.

2.2.7. Liquid Uptake

An adapted Enslin device (Prezotti et al., 2012; Voigt, 2000) was used to measure the liquid uptaking ability of the ODFs. Samples of the films were weighted and placed at bottle of the sintered glass filter of funnel. The changes on artificial saliva volume on the graduated pipette of the device due to sample absorption were measured at 1, 2, 5, 10, 15, 20, and 30 min.

The tests were performed in triplicate and the results were expressed as medium absorbed (%) in relation to initial mass of sample as a function of time.

2.2.8. Water vapor permeability (WVP)

PF and CF samples were cut in circular sections and fixed at the top of 1.1 cm opening glass cups. The cups were previously filled with 10 mL of water (100% relative humidity gradient at 25ºC*).* The set was accurately weighed and stored in a vacuum desiccator containing silica gel. Therefore, the relative humidity inside the cell was always higher than in the outside, and the water vapor transport was determined from the set mass loss*.*

The set weight was measured after 0, 24, 48, 72 and 96h and the mass changes were plotted as a time function. The slope of each line was calculated by linear regression ($r^2 > 0.99$), and the water vapor transmission rate (WVTR) was measured from the slope of the straight line ($g.h^{-1}$) by the test area (m^2). All values correlated to WVTR were corrected for the effect of gradient of concentration established in the stagnant air gap inside the cup, as seen on Eq. (1) (Gennadios, Weller, & Gooding, 1994):

$$(1) \qquad \mathrm{WVTR_C} = \mathrm{WVTR}\left[\frac{(\mathrm{Pw0} - \mathrm{Pw2})}{(\mathrm{Pw1} - \mathrm{Pw2})}\right]$$

In which:
WVTR: measured water vapor transmission rate ($g\ m^{-2}\ h^{-1}$);
Pw_0: partial pressure of water vapor in air at the surface of distilled water (Pa);
Pw_1: partial water vapor pressure on the underside of film (Pa);
Pw_2: partial water vapor pressure at the film surface outside the cup (Pa).

Subsequently to the permeation tests, WVP ($g\ mm\ m^{-2}\ h^{-1}\ Pa^{-1}$) was calculated using Eq. (2):

$$(2) \qquad \mathrm{WVP} = \frac{\mathrm{WVTR}_c \times \mathrm{L}}{\Delta \mathrm{P}}$$

Where:
WVTRc: corrected water vapor transmission rate ($g\ m^{-2}\ h^{-1}$);
L: average film thickness (mm);
ΔP: water vapor pressure difference between dry atmosphere and pure water.

2.2.9. Mechanical properties of ODFs

The mechanical properties of CF and PF were evaluated on a texture analyzer TA-XT2 (Stable Micro Systems), using a spherical-ended puncture probe (5 mm). The films sections were fixed on a metallic holder with a circular hole (D = 10 mm) and the probe was moved down at 1 mm s^{-1} with constant velocity (0.1 mm s^{-1}). The trigger force was 0.005 kg and force versus displacement curves were recorded until the film rupture and used to determine the puncture strength (Ps) and elongation at break (Eb %) parameters, according to the following equations (Eqs. (3) to (4)) (Meneguin et al., 2017).

(3) $$Ps = \frac{F}{A}$$

Where:
F (N): force required to rupture the film;
A (m^2): sectional area of the film (A = 2rh, where r is the hole radius and h is the film thickness).

(4) $$E_b\,\% = \frac{\sqrt{r^2+d^2}-r}{r} \times 100$$

Where:
r (mm): radius of the exposed film on the orifice plate and d is the displacement.

2.2.10. Mucoadhesive force

The mucoadhesive strength of PF and CF was evaluated by measuring the force required to detach the ODFs from a mucin disc using a TA-XT2 Texture Analyzer (Stable Micro Systems) (Bruschi et al., 2007). Mucin discs were hydrated with artificial saliva (25µl) and placed on a support for mucoadhesion test. ODFs were attached with double-sided tape to the analytical probe (10 mm) which was then lowered (10 mm.min^{-1}) until the mucin disc was in contact with the surface of the films. The complex mucin disc-films were kept in contact for 60s and then the probe was moved up at a constant speed (20 mm min^{-1}). The maximum force needed to detach the films from mucin discs, mucoadhesive force (FMA), was recorded.

2.2.11. Evaluation of the Probiotic film (PF) anti-Candida activity

To evaluate whether *C. albicans* biofilms would be impaired by PF, biofilm assays were carried out following the methods described by Fontana et al., (2009) and Zago et al., (2016), with modifications. The assays were conducted on 6-well microplates with each well containing 2 mL of standard yeast inoculum (prepared as described in strains and growth conditions) plus 2 mL of enriched broth, i.e. 4mL per well. In the test group, to each well was added a sample of 0.1g of PF (containing 10^9 CFU/g of *E. faecium*). To the control group 0.1g of CF was added to the wells. The microplates were incubated at 35 ± 2ºC for 24h to evaluate the action of the probiotic film in *C. albicans* biofilm adhesion and formation and for 48h to evaluate it in the biofilm maturation. After that, the pH of the biofilms supernatants were measured with pH-fix (Neumann-Neander, Düren, Germany) and the adhered biofilms were scraped off the wells and resuspended vigorously in 4 mL of enriched broth with a sterile pipette tip for 30s. Serial decimal dilutions of those suspensions were made using as diluent the culture medium itself. Microorganisms were quantified by plating the obtained dilutions in SDA supplemented with chloramphenicol. The plates were incubated for 24h at 35 ± 2ºC and the number of fungal cells present in the biofilms was expressed in CFU/ml. The experiments were performed in triplicate and repeated in three independent assays.

The decimal reduction (DR) of *Candida albicans* cell viability in the presence of PF was determined by equation 5:

$$(5) \qquad DR = Log_{10} (Ca_{CF}) - Log_{10} (Ca_{PF})$$

Where:
Ca_{CF}: CFU/ mL values of *C. albicans* present in the biofilms stimulated with control films (CF);
Ca_{PF}: CFU/ mL values of *C. albicans* present in the biofilms stimulated with probiotic films (PF).

Then, the percentual reduction (PR) of fungal population after 24 or 48 hours were calculated with the equation 7:

$$(6) \qquad PR = (1-10^{-DR}) \times 100$$

Where: DR is the decimal reduction obtained by equation 5.

2.2.12. Field emission gun scanning electron microscopy (FEG-SEM)

PF and CF surface and transversal section morphology as well as the interactions between *C. albicans* and PF or CF in the biofilms of 24 and 48 hours were assessed by FEG-SEM (JEOL JSM-7500F).

Biofilms were formed as described above in a sterile coverslip placed at the bottom of 6-well microtiter plates. After the periods of incubation (24h or 48h), the biofilms supernatants were gently removed and the coverslip were washed with PBS. The biofilms were fixed with a 2.5% glutaraldehyde solution (Merck, Darmstadt, Germany) then dehydrated with an ethanol series (30%, 50%, 70%, 85% and 95% ethanol solution for 15 min each; two washes with 100% ethanol for 15 min). The samples were dried at 37ºC and kept in a vacuum desiccator until the analysis day. For cross-section observations PF and CF samples were cryofractured by immersion in liquid nitrogen. PF, CF or the coverslips containing adhered biofilms were attached to the slab surfaces with double sided adhesive tape and then coated with a layer of carbon and observed through FEG- SEM (JEOL JSM-7500F).

2.2.13. *Statistical Analysis*

To verify the statistical significance, the results were submitted to One-way ANOVA followed by Tukey's multiple comparisons test or T-student test (for parametric data) and Mann Whitney test (for non-parametric data). It was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, California USA) with a minimum significance level of 5% ($p \leq 0.05$).

3. Results and discussion

3.1. Macroscopic observations, color parameters, thickness analysis, pH and moisture content of ODFs:

Both CF and PF were macroscopically homogeneous, a little brittle and without bubbles or fissures (Figure 1B). There was significant difference ($p < 0.0001$) in thickness between CF and PF (Table 1), which was expected, once there was an incorporation of a great amount (10^{10}CFU/mL) of probiotic, which dimensions are in the order of micrometers, into the PF polymeric matrix.

The probiotic incorporation into the ODF did not alter the color parameters lightness (from black to white; L*) and chroma a (from green to red; a*). But it did

promote a significative increase in the b* coordinate (from blue to yellow). This was also expected once the free cells of probiotics incorporated in PF present a cream color, promoting this increase ($p<0.0001$) in the yellow parameter compared to CF. The difference between the pH ($p<0.003$) between formulations can be explained by the production of lactic acid by *E. faecium* (Celiberto, Bedani, Rossi, & Cavallini, 2015), which decrease the ODF pH.

There was a higher moisture content in PF than in CF ($p<0.0001$) at T0, possibly corresponding to water present in bacterial cells. One of the main objectives of deploying probiotic strains in dehydrated matrices is the possibility of increasing the stability of the microorganism in the product, once dehydrated cells can maintain their cellular viability for longer than in liquid cultures (De Barros et al., 2014). However the optimum residual moisture content for greater stability of the microorganism in a given product varies with the composition of the medium in which the organisms were dehydrated, with the storage atmosphere, species, strain and physiological state of the microorganisms and therefore should be determined for each strain (De Barros et al., 2014).
The residual moisture content in CF was very similar to the results obtained by Heinemann et al. (2017), in a formulation also composed by CMC, gelatin and starch.

Table 1. Physicochemical characterization of orodispersible films (means±SD)

	Thickness (mm)	Color (L*, a*, b*)	Moisture (%)	pH
CF	0.080±0.001^{A}	L*=33.53±1.67 a*=-0.24±0.13 b*=-0.49±0.26^{a}	12.57±0.14^{A}	6.94±0.003^{A}
PF	0.100±0.001^{B}	L*=34.28±1.12 a*=-0.22±0.04 b*=+0.80±0.16^{b}	15.41±0.26^{B}	6.91±0.005^{B}

Means in the same column followed by different capital letters are significantly different by T- test ($p < 0.05$). Means in the same column followed by different lowercase letters are significantly different by MannWhitney test ($p < 0.05$).

3.2. Probiotic survival in ODF after drying process and storage.

Maintaining the cell viability of probiotic microorganisms in oral formulations or in food matrices throughout shelf-life is a major challenge. During products

processing and storage, variations in pH, temperature, humidity, mechanical stress, enzymatic activity, exposure to oxygen, or other chemical components promote the reduction of probiotic viability with impact in health benefits to the consumer (de Barros et al., 2014; Witzler et al., 2017). To assure the integrity of probiotic microorganisms in these products, strategies such as microencapsulation (involving probiotic cells in a polymer membrane) or freeze-drying (which sublimes water under reduced pressure resulting in the formation of dry and porous microcapsules) are taken (Cozentino, 2013). The micro-encapsulation (M), microencapsulation followed by freeze-drying (MF), and freeze-drying (F) on *E. faecium* CRL 183 free cells were evaluated by Cozentino (2013). It was noticed that all three techniques were able to maintain high cell viability of the probiotic (M ≥ 8 $\log_{10}$ CFU / g for up to 56 days; MF ≥ 8 $\log_{10}$ CFU / g for up to 232 days; F ≥ 10 $\log_{10}$ CFU / g for up to 119 days). However, these techniques present limitations such as high complexity, lyophilization can be very expensive, and there is a great loss of cell viability in relation to the free probiotic cells in the microencapsulation process. Cozentino (2013), for example, lost approximately 3 $\log_{10}$ CFU / g after the microencapsulation of *E. faecium* CRL 183. This reduction in cell viability of microorganisms can be influenced by factors such as species, strain, growth phase, use of cytoprotectant, and composition of the excipients used in the cell protection process (de Barros et al., 2014).

The loss in *E. faecium* cell viability, during the processing of the films developed in the present study was 1.24 ± 0.15 $\log_{10}$ CFU /g (Figure 2A). Heinemann et al. (2013) obtained a similar loss of *Lactobacillus acidophilus* (LA) and *Bifidobacterium animalis* subsp. *lactis* (BL) loaded in films also composed of CMC, gelatin and starch. Soon after the ODF drying process, the cellular viability obtained was 9.0 log CFU / g for LA and 9.6 log CFU/g for BL, and the probiotic inoculum added to filmogenic solution was between 10^8 and 10^{10} CFU/ml (Heinemann et al., 2013). De Barros et al. (2014) obtained a loss of only 0.5 $\log_{10}$ CFU / mL when incorporating *Bifidobacterium breve* NCIMB 8807 into a polymeric matrix composed of hydroxypropyl methylcellulose (HPMC). In a 2013 study, which entrapped *Lactobacillus fermentum* NCIMB 5221 on Carboxymethylcellulose (CMC) films, the loss was 1.96x 10^{10} ± 8.97x10^8 after film formation (Saha et al., 2013).

Therefore, soluble films can be a simple and inexpensive way of protection and delivery of probiotic microorganisms, since they do not use complex equipment

and can be composed by simple and cheap polymers. In addition, loss of microorganisms viability during processing, is not as drastic because the drying process can be performed at room temperature (de Barros et al., 2014; Lee et al., 2017).

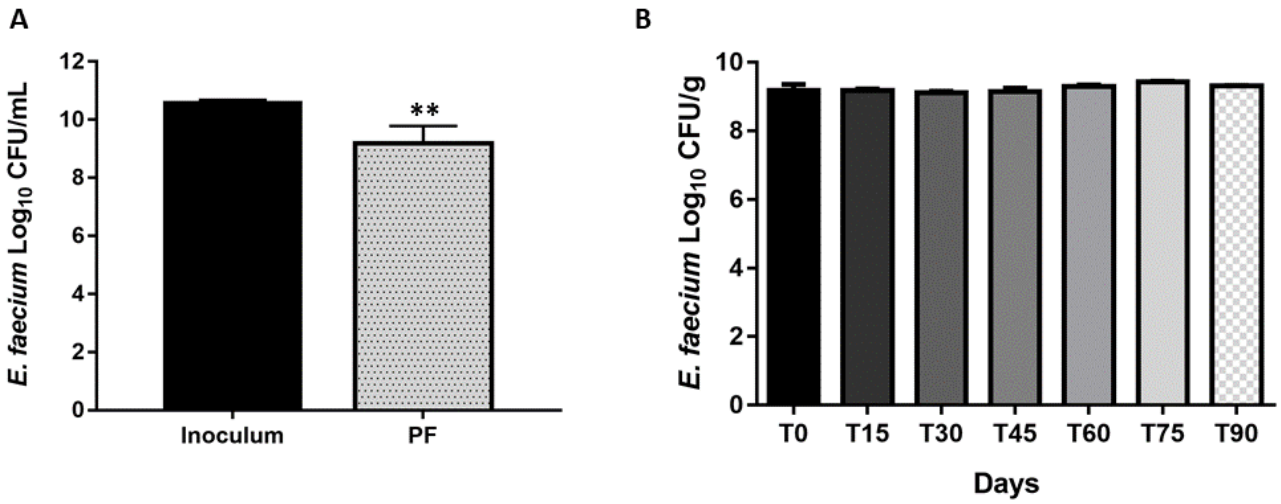

Fig 2. Probiotic resistance. (A) Probiotic viability in the inoculum and in PF (Probiotic Film) after drying, according to T-test (**p= 0.002). (B) Probiotic viability during storage, according to ANOVA followed by Tukey's test.

Concerning the viability during storage, the population of *E. faecium* in PF was high and constant. Probiotic cell viability was 9.19 ± 0.58 log10 CFU / g right after the drying process and remained stable (> 9 log10 CFU / g) up to 90 days after the films were formulated (Fig 2B), evidencing the efficiency of the film composed of CMC, gelatin and starch used in this study as a vehicle for this probiotic strain. Witzler et al. (2017) incorporated microcapsules of the same probiotic into lozenges in order to develop a formulation for delivering that strain into the oral cavity. However, the probiotic cell viability decreased from 8.73 $\log_{10}$ CFU/g to 5.53 $\log_{10}$ CFU/g shortly after its incorporation, reaching only 1.86 $\log_{10}$ CFU/g after 28 days of storage. On the other hand, probiotics loaded in ODF after one month of formulation presented cell viability close to 9 log CFU / g and only after 90 days of storage there was a statistically significant decrease in the viability of the probiotics (Heinemann et al., 2013). Saha et al., (2013) obtained significantly constant viability ($8.60x10^8$ ±

5.86x10^7 CFU / g) of *Lactobacillus fermentum* NCIMB 5221 in a CMC orodispersible film for up to 142 days of storage (Saha et al., 2013).

3.3. Microbiological safety of ODFs and Water activity (Aw)

The water activity is the main intrinsic factor of food related to its deterioration. It is a qualitative measure of the amount of free water of the product, i.e. water that is not bound to any other molecule and is therefore available to undergo physico-chemical reactions (Scott, 1957). The Aw analysis is also related to the multiplication of microorganisms in food. Beauchat (1981) listed the minimum Aw values required for the development of various microorganisms and production of toxins. According to this author, Aw values lower than 0.6 are unfavorable to the growth of contaminating and pathogenic microorganisms. Thus, the Aw value allows to evaluate susceptibility to food spoilage and is related to its shelf life. The Aw values (mean+SD) for the ODFs were: CF (0.378±0.04) and PF (0.404±0.05), and it was less than 0.5 at all analyzed times (0, 15, 30, 45, 60, 75 and 90 days), for both formulations. This is in accordance with the microbiological safety results obtained, as there was no contamination by yeasts and molds or total coliforms in 0, 30, 60 and 90 days neither in PF nor in CF. On the other hand, the maintenance of viability throughout the storage period of microorganisms intentionally added to products, as in the case of PF developed in this study, is increased the lower the water activity of the product (Meng, Stanton, Fitzgerald, Daly, & Ross, 2008). Thus, this analysis was fundamental in the context of this study, since low Aw values favor the cellular viability of microorganisms purposely incorporated into food matrices while disfavoring the multiplication of contaminating microorganisms.

3.4. In vitro disintegration time

When ODFs obtained with natural water-soluble polymers come into contact with biological fluids; first there is a wetting of the surface of the films, in which pores are created where the water molecules fade from the outer side to the inner side, creating channels throughout the system. The absorption of water weakens the polymer chains, leading to volume expansion, known as swelling, until the point of contact is broken, leading to disintegration. The speed of this process is closely

related to the concentration of polymers (the higher, the slower the disintegration rate) and the interaction of the bioactive compound with the polymer.

In this test it was possible to observe that the films in touch with artificial saliva rapidly swell and form a hydrogel, allowing that part of the saliva volume cross the film (T1) and later there is the appearance of a hole (T2) in the films, characterizing the complete disintegration, as described by (Garsuch & Breitkreutz, 2010). There was no difference between the disintegration time (T1 and T2) for PF and CF (Figure 3A). The literature and pharmacopoeias do not establish an official threshold for the endpoint of *in vitro* disintegration tests for products such as ODFs. The European Pharmacopeia states that ODF should 'disperse rapidly' but it does not provide information about how long it should take, neither *in vivo* nor *in vitro* (Dixit & Puthli, 2009; Lee et al., 2017; Preis, 2015). The T1 observed (PF: 1.35 min and CF 1.28 min) was in accordance with the European Pharmacopeia (2013) orientation for the disintegration threshold for orodispersible tablets (ODTs) which should be within three minutes (180s). Furthermore, there was an instant swell of the films resulting in a hydrogel which, with the mouth movements, would have been disintegrated (Preis, Gronkowsky, Grytzan, & Breitkreutz, 2014).

Considering the complete disintegration and appearance of a hole (T2), Heinemann et al. (2017) suggested the average time of four minutes as the ideal for the in vitro disintegration of ODF, since this time allows the gradual release of bioactive compounds incorporated into the polymer matrix, without causing fatigue to the consumer. In this same study, the mean *in vitro* disintegration time was (6.50 ± 0.67 minutes), very similar to the T2 obtained for us (PF: 6.43 min and CF 5.5 min), for an ODF also composed of CMC, gelatin and starch (Heinemann, Vanin, De Carvalho, Trindade, & Fávaro-Trindade, 2017).

The results presented in Figure 3A are also in the same time interval of other formulations composed by CMC (Saha et al., 2013) or Gelatin and hydrolyzed collagen (Borges, Tagliamento, Silva, Sobral, & De Carvalho, 2013).

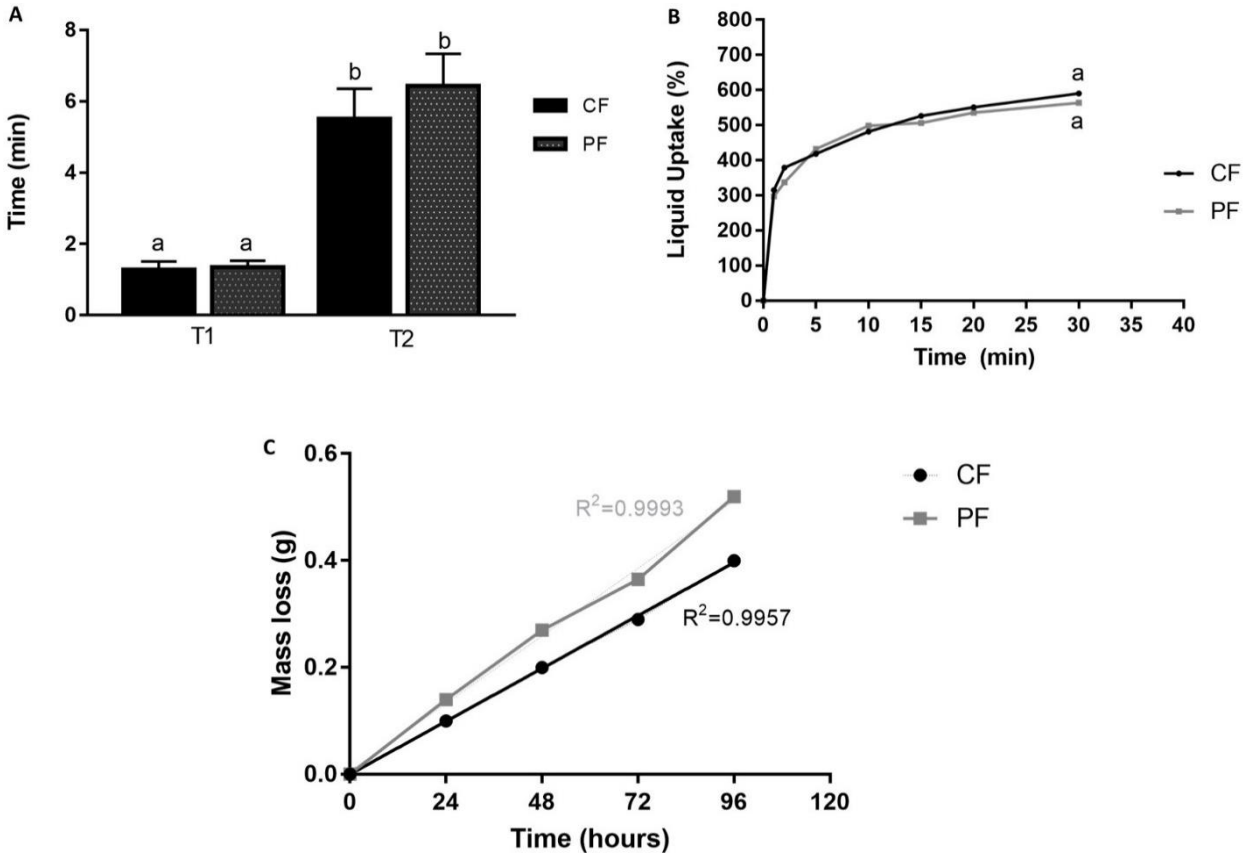

Fig 3. Disintegration time of ODFs; (A) T1: time in which artificial saliva cross the films; T2: time to complete disintegration (appearance of a hole). (B) CF and PF uptake of artificial saliva over time. (C) Mass loss in water vapor permeability test over time. Same lowercase letters show statistical equality according to T-test

3.5. Liquid Uptake

There is a correlation between liquid uptake and effective release of API or bioactive compounds from drug delivery systems thus this information can be valuable during the development of new pharmaceutical delivery systems. This is also a critical parameter in administration routes in which there is a shortage of liquid, such as buccal administration (Welch & Strømme, 2003). The liquid uptake can also predict the enzymatic degradation of the polymeric matrix and consequently the release of API (Prezotti, Meneguin, Evangelista, & Ferreira Cury, 2012). Either PF and CF presented a high and fast ability of artificial saliva uptake, i.e. (PF: 297%; CF: 315%) after only 1 minute, reaching approximately 600% after 30 minutes of analysis. This was expected once the films were composed of high hydrophilic compounds such as CMC and starch (Heinemann et al., 2017; Prezotti et al., 2012). CMC contains hydroxy and carboxyl groups, for which pH values around 7.0

promotes the ionization and consequent repulsion of chains with network dilation, favoring the water entrance. Furthermore, the entanglement level of the structures can also play a role in the high liquid uptake presented by CF and PF. The low concentration of polymers in PF and CF composition promotes a looser network which favors water molecule diffusion, compared to stronger structures (Meneguin, Stringhetti, Cury, & Evangelista, 2014).

The incorporation of the probiotic did not alter significantly the liquid uptake ability of the films (Figure 3B).

The probiotic incorporation into the matrix enhanced significantly (p=0.01) WVP values, which can be explained by the greater thickness in these films. The higher polymeric mass promotes more water absorption (Meneguin et al., 2014; Prezotti et al., 2012). Furthermore, the probiotic incorporation probably added diffusional pathways in the film structure, enhancing its permeability (Nielsen, 1967).

Table 2. Mechanical properties, WVP and mucoadhesive force of PF and CF

Films	WVP ($\times 10^{-5}$ g.mm. m−2.h^{-1}. Pa^{-1})	Ps (MPa)	Eb (%)	FMA (N)
CF	1.27±0.1^{A}	25.61± 2.2^{A}	4.13±0.87^{A}	0.060 ± 0.01^{A}
PF	1.93±0.2^{B}	25.92± 2.9^{A}	9.40±0.95^{B}	0.064± 0.01^{A}

Results presented as mean ± SD. (n=3). Means in the same column followed by different capital letters are significantly different by T- test ($p < 0.05$). WVP: Water vapor permeability; Ps: Puncture strength; Eb: Elongation at break; Ep; FMA: Mucoadhesive Force.

3.7. Mechanical properties

The resistance to cracking during processing, transport and storage is an important characteristic of polymeric films and its determined by its mechanical properties (Prezotti et al., 2012). The films CF and PF were considerably resistant and the probiotic incorporation did not alter the puncture strength (Ps) of the films (Table 2). Elongation at break (Eb %) determines the maximum deformation that a material can undergo before it cracks and for oral applications films must be flexible enough to comfortably follow the movement of the mouth (Santos et al., 2017). PF were twice more flexible than CF (Table 2) which can be explained by the residual moisture difference between the two polymeric films; water can act as plasticizer

intercalating with the polymer chains and therefore enhancing flexibility (Dixit & Puthli, 2009). Furthermore, loaded films can promote a rearrange in the polymeric microstructure in a more flexible way (Meneguin et al., 2017).

3.8. Mucoadhesive force

Mucoadhesive systems can promote a higher residence time in the biological target site, enhancing contact with the epithelial barrier and, therefore, increase bioavailability of the active compound (Carvalho, Bruschi, Evangelista, & Gremião, 2010). The mucoadhesive force presented by PF and CF were considerably low and it did not differ statistically between one another (Table 2). This was a surprising result once the films were composed by CMC and gelatin which are mucoadhesive polymers (Dekina, Romanovska, Ovsepyan, Tkach, & Muratov, 2016). CMC is considered as first generation mucoadhesive polymer - there are formation of a hydrogen bond between its carboxylic acid group and glycoproteins of mucin (Chatterjee, Amalina, Sengupta, & Kumar Mandal, 2017). However, mucoadhesive properties can vary with environment-related factors such as pH, contact time, swelling and physiological characteristics (Carvalho et al., 2010). Although the mucoadhesive force for PF and CF obtained in the present study was lower than other films composed by similar polymers, this is not necessarily an issue. Once the aim was to release efficiently the probiotic into the mouth, the mucoadhesive force obtained is enough to make the films resist the salivary flow during their disintegration time.

3.11. Evaluation of the Probiotic film (PF) anti-Candida activity

The probiotic was efficiently released from the polymeric film and impaired in 68.9% (DR=0,52 $\log_{10}$) *C. albicans* biofilm formation (24 hours) and in 91,24% (DR =1,15 $\log_{10}$) biofilm maturation, compared to the group stimulated with CF (Figure 4). It was determined by our research group that *E. faecium* CRL 183 free cells were able to impair *C. albicans* biofilm formation and maturation in 99,9% and 99,43%, respectively (data not published).

Among the inhibitory mechanisms exerted by the probiotic on fungal biofilms, it was suggested the, competition for nutrients and space, culture media acidification and secretion of anti-*Candida* metabolites (data not published). the pH of biofilms

stimulated with PF were around 5.0 and with CF around 7.0. The lactic acid can cross the cell yeast membrane causing energy exhaustion and inhibiting its multiplication and leading to cell death (Jørgensen et al., 2017).

The lesser percentual reduction in the stimuli with PF compared to the stimuli with free cells of the previous study was expected, once the probiotic must be firstly released from the polymeric matrix in which it is entrapped in a latent phase without metabolic activity (de Barros, Scherer, Charalampopoulos, Khutoryanskiy, & Edwards, 2014).

In the context of candidiasis prevention, the results presented in this study are very promising since the *C. albicans* viability reductions was similar to results obtained by other studies with different probiotic strains as free cells (Matsubara, Wang, Bandara, Mayer, & Samaranayake, 2016; Ribeiro, de Barros, Rossoni, Junqueira, & Jorge, 2017; Vilela et al., 2015). The application of probiotic free cells into the oral cavity could be easily removed by the salivary flow and present unpleasant sensory properties. Therefore, the present study demonstrates that the ODF containing *E. faecium* CRL 183 may be a more advantageous way to prevent oral candidiasis since it permits local probiotic release in an adequate amount of time with good ability to impair *C. albicans* biofilm formation and maturation.

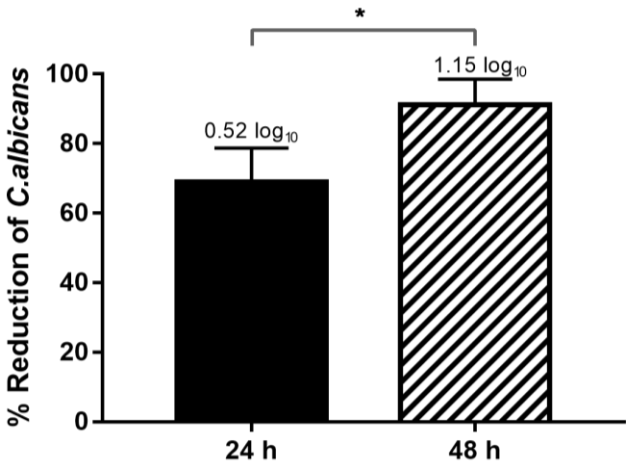

Fig 4. Percentual reduction of *C. albicans* biofilm formation (24 hours) and maturation(48h) in the presence of PF (Probiotic Film). Each column represents the mean of three independent experiments, each performed in triplicate (n = 9), the bars represent the standard deviation. Numbers above columns represent the decimal reduction (DR) The asterisk indicates statistically significant difference(*p=0,03) between 24h and 48h, according to T-test.

3.12. Field emission gun scanning electron microscopy (FEG-SEM)

The photomicrographs shown in Figure 5 reveal that both CF and PF present whole surface without fissures in them structure. Both films surface was very rough, and there were some pores in PF films (5E) which was not observed for CF (5B). This finding confirms the results obtained in WVP tests, which shows that PF was more permeable to water vapor than CF. It was not possible to visually identify the microorganisms in the polymeric film through SEM-technique, which was already reported in other study (Heinemann et al., 2013).

Cross-section micrographs (5C-5F) confirms the greater thickness of PF films and shows that the probiotic incorporation promoted the appearance of fissures in the bottom side of the film. Nevertheless, the probiotic did not change the structure of the polymeric film.

The photomicrographs of *C. albicans* biofilms stimulated with CF (5G and 5I) or PF (5H and 5J) confirm the results obtained through CFU counting, showing reduction in *C. albicans* cells in the PF group. Furthermore, the biofilms stimulated with CF (5G and 5I) presented *C. albicans* cells surrounded by a very thick matrix which occur in a lesser amount in the biofilms stimulated with PF (5H and 5I). This matrix can be a blend of the polymers that compound the ODFs and exopolysaccharides (EPS) produced by *C. albicans*. In the 48 hours biofilm stimulated with PF it is possible to notice that beyond the reduction yeast cells presented a wrinkled morphology (indicated by the arrow), which may be a sign of apoptosis.

4. Conclusion

The Probiotic Film developed is a promising strategy to prevent oral candidiasis, since it permits the probiotic local delivery which, in its turn, was able to reduce *C. albicans* biofilm formation (68%) and maturation (91%). Furthermore, the ODF made of simple and cheap polymers was able to maintain a high and long-lasting probiotic viability (9 $\log_{10}$ CFU/g up to 90 days), being a suitable way to protect this microorganism during storage, showed by its good mechanical properties and WWP. Yet, the film was flexible and present great liquid uptake ability, being capable of efficiently release the probiotic from polymeric matrix within 6 minutes.

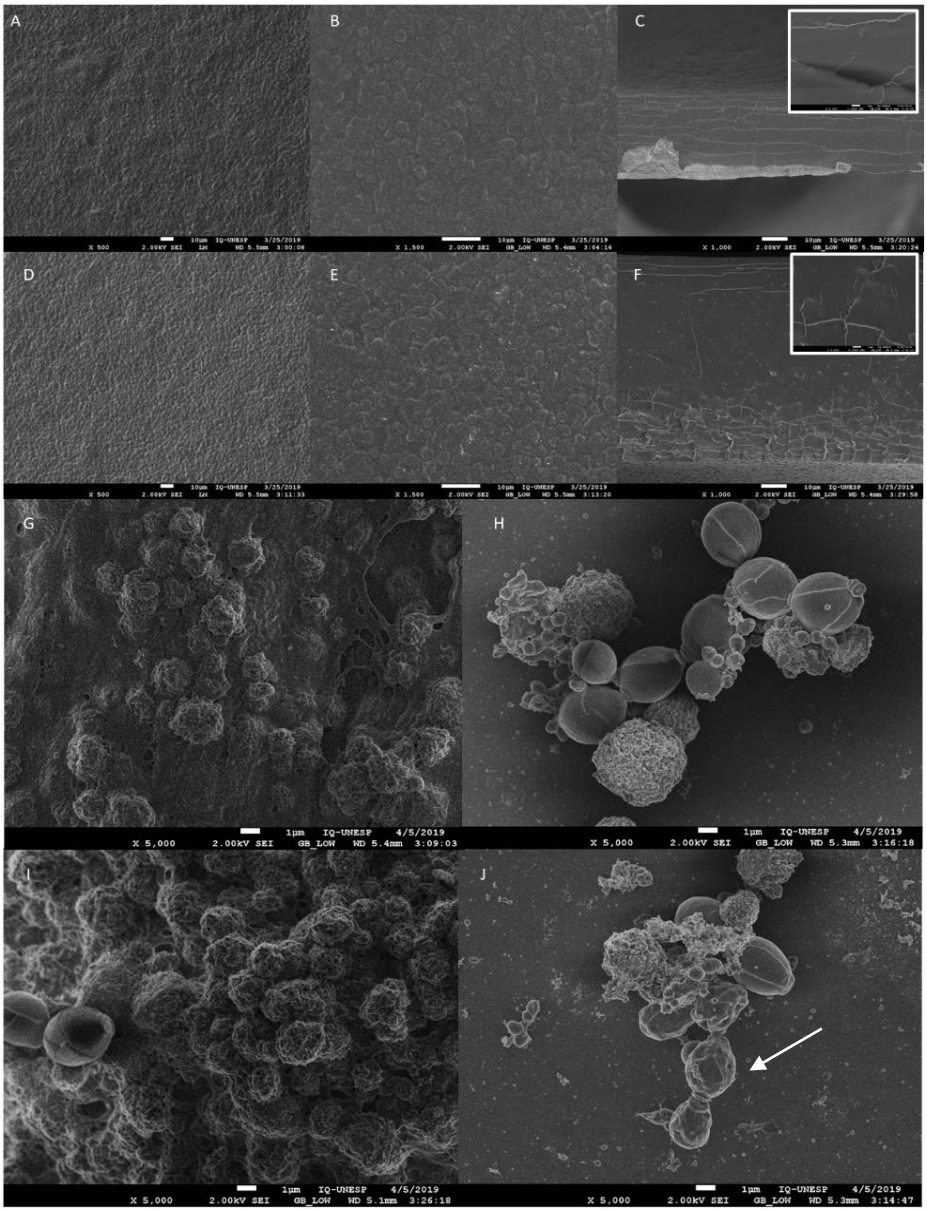

Fig. 5. FEG-SEM images. Surface of the ODFs: (A) CF 500x, (B) CF 1000x, (D) PF 500x, (E) PF 1000x. Cross-sections were taken at 1000× and those recorded at 8000 x magnification are embedded into the right-hand corner: (C) CF, (F) PF. Biofilms at 5000x magnification: *C. albicans* + CF 24H (G); *C. albicans* + PF 24H (H); *C. albicans* + CF 48H (I); *C. albicans* + PF 48H (J).

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Andreia Bagliotti Meneguin declares she has no conflict of interest. Sarah Raquel de Annunzio declares she has no conflict of interest. Maria Pía Taranto declares she has no conflict of interest. Carla Raquel Fontana declares she has no conflict of interest. Marlus Chorilli declares he has no conflict of interest. Daniela Cardoso Umbelino Cavallini declares she has no conflict of interest.

Ehical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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CONSIDERAÇÕES FINAIS

O microrganismo probiótico *E. faecium* CRL 183 é capaz de se estabelecer em um biofilme polimicrobiano com *C. albicans* ATCC 90028, sem ter sua viabilidade celular comprometida. O probiótico foi capaz de inibir significativamente a formação e a maturação do biofilme fúngico *in vitro,* em todas as proporções de patógeno/probiótico testadas. A redução da carga de *C. albicans* foi superior às obtidas por abordagens semelhantes descritas na literatura utilizando outras cepas probióticas (99 a 99,9%, dependendo da condição experimental). Apesar da acidificação do meio, pela produção de ácido lático, ser um dos mecanismos que explicam o antagonismo entre *E. faecium* e *C. albicans*, o pH do meio na presença de *E. faecium* CRL 183 manteve-se dentro da faixa de pH da saliva humana saudável.

A matriz polimérica composta por carboximetilcelulose, gelatina e fécula de batata produz um filme orodispersível de fácil obtenção e de baixo custo, capaz de envolver o microrganismo probiótico em sua estrutura sem lhe causar danos. Os filmes obtidos apresentaram boas propriedades mecânicas e de barreira, o que pode proteger o microrganismo probiótico das ações do ambiente explicando a alta viabilidade durante 90 dias de armazenamento à temperatura ambiente. A flexibilidade, alta capacidade de absorção de líquidos e o tempo de desintegração obtidos indicam que o ODF pode ser eficiente na liberação local de *E. faecium*, sendo mais resistente ao fluxo salivar do que enxaguantes ou géis orais, por exemplo. Por fim, filmes contendo *E. faecium* CRL 183 foram capazes de desestabilizar a formação (68 %) e a maturação (91%) de biofilmes de *C. albicans,* indicando que o consumo regular do ODF probiótico pode prevenir a colonização local por esse fungo. Entretanto, estudos mais aprofundados que comprovem os mecanismos pelos quais *E. faecium* CRL 183 antagoniza *C. albicans* ATCC 90028, bem como, a ação dos filmes probióticos em modelos de candidíase *in vivo* devem ser considerados.

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