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KÁTIA REAL ROCHA

AÇÃO ANTIBACTERIANA DE ENTEROCINAS PRODUZIDAS POR ESPÉCIES DE *ENTEROCOCCUS* SOBRE CÉLULAS PLANCTÔNICAS E BIOFILMES

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina, como requisito final à obtenção do título de Doutora em Microbiologia.

Orientadora: Profa. Dra. Márcia Cristina Furlaneto
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“The greater our knowledge increases the more our ignorance unfolds”

John F. Kennedy

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RESUMO

Enterocococcus spp. pertencem ao grupo bactérias ácido láticas produtoras de bacteriocinas (enterocinas) que apresentam atividade contra várias bactérias patogênicas de origem alimentar. Este estudo foi realizado primeiramente para selecionar enterococos produtores de enterocinas contra *Listeria innocua* 12612, bem como seu espectro de ação contra outras bactérias oportunistas e em seguida avaliar a influência de suas enterocinas em biofilme bacteriano. Para tal, os isolados potencialmente produtores de enterocinas (Ent+) contra *L. innocua* 12612 foram caracterizados quanto a presença de genes estruturais para enterocinas e produção de componente hemolítico. Em seguida foi obtido o sobrenadante de cultivo livre de células (CFS) dos Ent+, para avaliar sua ação contra *L. innocua* 12612 por método de disco-difusão, após tratamento com calor (80°C e 100°C), enzimas proteolíticas (α- quimiotripsina, protease e tripsina), resistência a catalase, quantificação da atividade antibacteriana do CFS e do CFS parcialmente purificado por unidade arbitrária (UA), bem como determinar seu respectivo modo de ação e espectro antibacteriano contra outras bactérias. Dos 76 enterococos de diferentes fontes, 11 foram considerados Ent+ (5 isolados provenientes de alimentos e 6 de ambiente), além disso 9 destes apresentavam ao menos um gene codificador de enterocina, *ent*A, *ent*B, *ent*P, *ent*1071 e/ou *ent*X. Não houve isolados com atividade hemolítica. Após a obtenção dos CFS 7/11 dos isolados Ent+ (5 de alimentos e 2 de ambientes) apresentaram halo de inibição contra *L. innocua*. Esses 7 CFS foram termoestáveis, de natureza proteica e a ação antibacteriana por peróxido de hidrogênio foi descartada pela utilização de catalase, indicando a ação antagônica pela presença de enterocina. Ao concentrar a enterocina (CFS parcialmente purificado) os valores de UA tenderam a aumentar. O modo de ação observado foi bactericida. A atividade antagônica dos 7 CFS contendo enterocina contra outras 33 bactérias indicadoras ocorreu principalmente em *L. innocua*, *Listeria monocytogenes* e *Listeria ivanovii*. Os CFS que apresentaram maiores valores de UA e espectro de ação foram os provenientes de alimentos, provavelmente isso possa estar relacionada com a maior quantidade de genes codificadores de enterocinas por parte desses isolados. Além disso, foram selecionados 4 CFS provenientes de alimentos para avaliar sua atividade anti-biofilme de *Listeria* spp., no desenvolvimento e após a pré- formação do biofilme por 24h a 30°C e 37°C. O biofilme foi formado em superfícies abiótica e avaliado por biomassa total (cristal violeta), atividade metabólica (redução de XTT) e microscopio de fluorescência (com marcador de célula morta utilizando iodeto de propidio). Para tal, 8 *Listeria* spp. foram avaliadas quanto a formação de biofime por biomassa total. Dessas foram selecionados 3 *Listeria* spp. (*Listeria monocytogenes* 2032*,* *Listeria ivanovii* 2056 e *Listeria innocua* 2050) para serem submetidas ao tratamento com os CFS. Os resultados mostraram que houve atividade anti-biofilme das *Listeria* spp., por parte de todos os CFS avaliados (desenvolvimento e no biofilme pré-formado), em ambas temperaturas avaliadas. De uma maneira geral, a atividade anti-biofilme listerial provocadas pelos CFS, foi acompanhada por uma menor atividade metabólica, bem como redução da estrutura do biofilme e maior quantidade de células mortas nas imagens obtidas, quando comparado com o controle. Concluindo, com base nos esforços consideráveis para a busca por novas bacteriocinas para serem aplicadas na área alimentícia principalmente contra *Listeria*, um importante patógeno alimentar, tem que os enterococos aqui avaliados

apresentam-se como potenciais candidatos no controle de bactérias patogênicas sejam em células planctônicas ou sésseis.

Palavras-chave: Bacteriocina; *Enterococcus faecalis; Enterococcus faecium; Listeria monocytogenes; Listeria ivanovii; Listeria innocua*.

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ABSTRACT

Enterococcus spp. belong to the group lactic acid bacteria, which produce bacteriocins (enterocins) that presents activity against several foodborne pathogens. This study was firstly carried out to select enterococci producing enterocin against *Listeria innocua* 12612, as well as its spectrum of action against other opportunistic bacteria and then to evaluate the influence of its enterocins on bacterial biofilm. For this, isolates potentially producing enterocin (Ent+) against *L. innocua* 12612 were characterized for the presence of structural genes for enterocins and production of hemolytic component. Then, the cell-free supernatant (CFS) of the Ent+ was obtained to evaluate its action against *L. innocua* 12612 by disc-diffusion method, after heat treatment (80°C and 100°C), proteolytic enzymes (α-chymotrypsin, protease and trypsin), catalase resistance, quantification of the antibacterial activity of CFS and partially purified CFS by arbitrary unit (AU), as well as determine their respective mode of action and antibacterial spectrum against other bacteria. Of the 76 enterococci from different sources, 11 were considered Ent+ (5 isolates from food and 6 from environment), in addition 9 of these had at least one enterocin encoding gene, *ent*A, *ent*B, *ent*P, *ent*1071 and /or *ent*X. There were no Ent+ isolates with hemolytic activity. After obtaining the CFS 7/11 of the Ent+ isolates (5 from food and 2 from environments) presented halo of inhibition against *L. innocua*. These 7 CFS were thermostable, of a protein nature and the antibacterial action by hydrogen peroxide was discarded by the use of catalase, indicating the antagonistic action by the presence of enterocin. By concentrating the enterocin (partially purified CFS) the AU values tended to increase. The mode of action observed was bactericidal. The inhibitory activity of the 7 CFS containing enterocin against other 33 indicator bacteria occurred mainly in *L. innocua, Listeria monocytogenes* and *Listeria ivanovii*. The CFS that presented higher values of AU and action spectrum were those from food, probably this may be related to the greater amount of genes coding for enterocins by these isolates. In addition, 4 CFS from food were selected to evaluate their anti-biofilm activity of *Listeria* spp., in the development and after pre-formation of the biofilm for 24 h at 30 °C and 37 °C. The biofilm was formed on abiotic surfaces and evaluated by total biomass (violet crystal), metabolic activity (XTT reduction) and fluorescence microscopy (with a dead cell marker using propidium iodide). For this, 8 *Listeria* spp. were evaluated for biofilm formation by total biomass. Of these, 3 *Listeria* spp. (*Listeria monocytogenes* 2032, *Listeria ivanovii* 2056 and *Listeria innocua* 2050) were selected to be submitted to treatment with CFS. The results showed that there were anti-biofilm activity, by all evaluated CFS (development and preformed biofilm), in both evaluated temperatures. In general, the listerial anti-biofilm activity by CFS was accompanied by a lower metabolic activity, as well as a reduction of the biofilm structure and a greater number of dead cells in the obtained images, when compared to the control. In conclusion, based on the considerable efforts to search for new bacteriocins to be applied in the food area mainly against *Listeria*, an important food pathogen, the enterococci present here are potential candidates for the control of pathogenic bacteria in planktonic cells or sessile.

Key words: Bacteriocin; *Enterococcus faecalis; Enterococcus faecium; Listeria monocytogenes; Listeria ivanovii; Listeria innocua.*

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1. INTRODUÇÃO

O gênero *Enterococcus* compreende bactérias Gram-positivas, pertencente ao grupo das bactérias ácido lática (BAL). Por serem desse grupo os enterococos sobrevivem em condições ambientais diversas, o que permite serem encontrados nos mais variados lugares, bem como alimentos, principalmente os fermentados.

Quando em alimentos, os enterococos podem conferir benefícios por diversas razões, como auxiliar na fermentação ou fornecer sabores aos alimentos. Além disso, sua capacidade de produzir produtos antimicrobianos como as bacteriocinas, permite que atuem também como agentes protetores contra agentes patogênicos de alimentos.

Bacteriocinas produzidas por enterococos são denominadas enterocinas. As bacteriocinas são peptídeos antimicrobianos sintetizados nos ribossomos, as quais atuam na sua grande maioria na membrana da bactéria alvo. A produção de bacteriocinas desperta interesses em várias áreas, principalmente a de alimentos, uma vez que por serem consideradas seguras à saúde humana, podem ser utilizadas como bioconservadores de alimentos. A aplicação de bacteriocina pode ajudar a reduzir o uso de aditivos químicos que por sua vez podem ser tóxicos a saúde humana.

Vários estudos demonstram a atividade antibacteriana de enterocinas contra diversas espécies patogênicas ou deteriorantes de alimento como *Salmonella* spp., *Staphylococcus aureus, Bacillus* spp., *Escherichia coli, Clostridium* spp., principalmente, *Listeria monocytogenes.*

Muitas enterocinas já foram caracterizadas e a grande maioria é ativa contra *Listeria* spp., importante patógeno alimentar. Seu interesse na área industrial se deve tanto à aplicação das enterocinas nos alimentos, funcionando como bioconservador, bem como em equipamentos de processamento de alimento para evitar ou erradicar biofilmes bacterianos indesejáveis.

O biofilme bacteriano é um mecanismo de defesa, pois protege a bactéria de condições adversas ao ambiente e agentes antimicrobianos, (ex. os biocidas comumente utilizados na indústria alimentar). A presença de biofilmes de patógenos e deteriorantes de alimento nos equipamentos relacionados a produção de alimento representa um grande problema, uma vez que é uma fonte de contaminação alimentar. Assim, tem-se despertado o interesse das bacteriocinas também no controle desses biofilmes.

Sendo assim, é de grande interesse o conhecimento de novos antimicrobianos para o desenvolvimento de novas ferramentas biotecnológicas na conservação de alimentos,

uma vez que os benefícios fornecidos pelas bacteriocinas em relação a outros antimicrobianos são muito desejados principalmente na indústria de alimento. Para tal, esta tese teve por objetivo verificar a produção de enterocinas por isolados de enterococos de diversas fontes, bem como caracterizar estas bacteriocinas em termos de espectro de atividade, modo de ação e sua influência na formação e inibição de biofilmes bacterianos.

2 REVISÃO BIBLIOGRÁFICA

2.1 CARACTERÍSTICAS GERAIS DOS *Enterococcus*

O gênero *Enterococcus* compreende bactérias Gram-positivas, anaeróbicas facultativas, catalase negativa, pertencente ao grupo das bactérias ácido láticas (BAL), com desenvolvimento em condições de alta salinidade e temperatura, permitindo sua sobrevivência em diversos ambientes como solo, água, microbiota de seres humanos e animais. Ainda, podem ser encontrados em alimentos crus, como leite e carne, multiplicando-se durante o processo de fermentação ou processamento desses alimentos (GIRAFFA, 2002; FOULQUIÉR-MORENO et al., 2006; FISHER; PHILIPS, 2009).

Atualmente foram descritas mais de 58 espécies pertencentes ao gênero *Enterococcus* (EUZÉBY, 2018) sendo as mais importantes são *Enterococcus faecium* e *Enterococcus faecalis*, comumente encontradas no trato intestinal de animais e humanos. Ambas espécies são importantes na área clínica como na de alimento. Por outro lado, alguns enterococos se tornaram reconhecidos também como patógeno humanos emergentes nos últimos anos (FOULQUIÉ-MORENO et al 2006, FRANZ et al 2011; FISHER; PHILIPS, 2009; KHAN et al., 2010). Portanto, pode-se concluir que os enterococos que não apresentam atividade hemolítica e não possuem genes de resistência à citolisina e à vancomicina podem ser consideradas seguros e podem ser utilizados como probióticos (KHAN et al., 2010).

Em diversos países europeus, os enterococos são utilizados como cultura *stater* em produtos fermentados, fornecendo propriedades organolépticas desejáveis, relacionadas à consistência e aroma característicos (GIRAFFA, 2002; FOULQUIÉR-MORENO et al., 2006). As culturas *starter* também servem para prolongar o prazo de validade do produto, pelo aumento da ácidez no alimento, principalmente através da conversão de lactose em ácido láctico (KHAN et al., 2010).

Alguns enterococos ocorrem em maior número em certos tipos de alimentos tradicionais, como queijos, legumes e embutidos (ex. salsichas). Eles podem ser usados como probióticos e ingeridos em alto número, geralmente em preparação farmacêutica, para tratamento de diarreia, síndrome do intestino irritável, na diminuição de níveis de colesterol e melhora na imunidade do hospedeiro. O uso de enterococos em ração animais podem estimular o sistema imunológico destes, além de implicar na redução e/ou eliminação de patógenos zoonóticos, fatores importantes do ponto de vista da segurança alimentar (FRANZ et al 2011).

Além disso, por sua capacidade de produzir compostos antimicrobianos como as bacteriocinas, fazem dos enterococos um microrganismo extensivamente estudado, visando sua capacidade de agirem como agentes protetores contra agentes patogênicos de alimentos.

2.2 Bacteriocinas

2.2.1 Características gerais

Bacteriocinas são pequenos peptídeos antimicrobianos sintetizado nos ribossomos que são ativos contra outras bactérias, sendo que a bactéria produtora protege-se de sua própria bacteriocina por mecanismos de imunidade específicos. As bacteriocinas são liberadas no meio extracelular e comumente tem ação contra bactérias relacionadas que compartilham nichos ecológicos semelhantes, geralmente são catiônicas e hidrofóbicas (COTTER et al. 2005, COTTER et al. 2013). As bacteriocinas apresentam espectro antibacteriano restrito e amplo, as quais podem atuar contra bactérias patogênicas Gram-positivas, e algumas Gram negativas (COTTER et al. 2013).

A primeira bacteriocina, designada posteriormente como colicina, foi descoberta em 1925 por Gratia, no qual envolvia o antagonismo bacteriano mediado por uma substância produzida por *Escherichia coli*.

As bacteriocinas têm distintos mecanismos de ação que podem ser amplamente divididos naqueles que funcionam principalmente no envelope celular e os que são ativos principalmente dentro da célula, afetando a expressão gênica e a produção de proteínas (COTTER et al. 2013).

Sua produção ocorre em todas as fases de crescimento da bactéria produtora e cessa no fim da fase exponencial, podendo ocorrer por falta de nutrientes, decorrente do

aumento populacional ou e em algumas espécies por indução por peptídeos ferormônios (COOTER et al 2005; OGAKI et al. 2015).

Dentre as bacteriocinas de BAL amplamente utilizadas na indústria de alimentos e certificadas tem-se a nisina e a pediocina PA-1. A nisina foi considerada segura pela Food and Agriculture Organization/Word Health Organizagion (FAO/WHO) desde 1969, e já encontrou aplicação como bioconservador em uma ampla gama de alimentos em todo o mundo e na prevenção da mastite bovina. A pediocina Pa1/AcH é uma bacteriocina produzidas por *Pediococcus* spp. (COTTER et al., 2005). Porém, outras bacteriocinas também estão presentes em alimentos através da sua produção por culturas iniciadoras ou como resultado de sua presença em fermentos bacterianos que são adicionados como ingredientes alimentares (FRANZ et al., 2007).

2.2.2 Classificação

Desde a primeira classificação das bacteriocinas BAL, proposta por Klaenhammer (1993), diferentes esquemas têm sido propostos. As classificações das bacteriocinas concordam que as características básicas, tais como sua massa molecular, espectro de atividade, modo de ação, estabilidade, especificidade na via de biossíntese, composição química, modificações pós-traducionais e maquinaria de processamento de secreção, todos precisam ser incluídos (COTER et al. 2005, 2013; FRANZ et al., 2007; YANG et a, 2014; ALVAREZ-SIEIRO et al 2016). De uma maneira geral as bacteriocinas de Gram-positiva foram divididas em três classes. Classe I (peptídeos modificados pós tradução, lantibióticos), classe II (peptideo sem modificação pós tradução, não lantibióticos) e classe III (grandes proteínas, não termoestáveis) (COTER et al. 2005, 2013; FRANZ et al., 2007; YANG et a, 2014).

A classe I corresponde aos lantibióticos, nos quais são modificados pós tradução (com adição de aminoácidos incomuns e modificados, como a lantionina e a β-metil-lantionina), tendo massa molecular menor que 5 kDa podendo se apresentar em duas estruturas linear (nisina) ou estrutural (mersacidina). Esse classe é produzida principalmente pelo *Lactococcus* spp.

Na classe II tem-se as bacteriocinas com menos de 10 kDa, termoestáves, toleram ampla faixa de pH e apresenta carga positiva. Os gêneros produtores dessa classe são os *Pediococcus* spp., *Lactococcus* spp., *Lactobacillus* spp., e *Enterococcus* spp. Essa classe é subdividida em 5 grupos por COTER et al. (2013). A sub-classe IIa, são as bacteriocina

antilisterial ou do tipo pediocinas. Essa sub-classe apresenta uma região conservada tipo dupla glicina (YGNGV) em sua extremidade N-terminal, que tem papel essencial na atividade antilisterial. Por outro lado, a classe IIb apresenta uma estrutura complexa que contêm ao menos dois peptidos para atividade antimicrobiana. Enquanto a classe IIc são as bacteriocinas circulares, como a enterocina AS-48. A sub-classe IId corresponde as bacteriocinas não modificadas, lineares, do tipo não pediocina, de peptideo único, incluindo lactococcinaA e epidermicina NI01. As bacteriocinas que contêm uma região carboxi-terminal rica em serina com uma modificação pós-traducional do tipo sideróforo não ribossômico pertencem a classe IIe, como as microcinas E492. Essa subclasse deve ser consideradas nas classificações de bacteriocinas de Gram-negativas, que são divididos em peptídeos pequenos, como as microcinas, e as grandes proteínas, como as colicinas. Quando da presença ou ausência de modificações significativas as microcinas são classificadas em classe I e II, respectivamente.

A classe III consiste nas bacteriocinas maiores que 30 kDa, não termoestáveis e principalmente produzidas pelos gêneros *Lactobacillus* spp. e *Enterococcus* spp. Elas podem ser divididas em dois grupos distintos. A pertencentes ao grupo A são bacteriolíticas, como a enterolisinaA e as do grupo B são as não líticas como caseicin 80 (YANG et al., 2014).

No entanto, essa classificação está sendo continuamente revisada e está evoluindo com o acúmulo de conhecimento e o surgimento de novas bacteriocinas (ALVAREZ-SIEIRO et al., 2016). Esses autores subdividiram a classe I em 6 subclasses, com base nas modificações que sofrem após a tradução, porém essa nova classificação ainda não foi adotada nos artigos mais recentes.

2.2.3 Importância na indústria de alimento

As bacteriocinas de BAL foram usadas pela primera vez para melhorar a segurança e qualidade dos alimentos, por ser considerados como “friendly” isto é, não apresentarem risco associado à saúde, devido a seu status Generally Recognized as Safe - GRAS (OGAKI et al. 2015; ALVAREZ-SIEIRO et al. 2016; CAMARGO et al 2018). Outros críterios que são adotados para aplicação da bacteriocina em alimento são que a bactéria bacteriocinogênica apresente status como seguro, forneça qualidade/ sabor aos alimentos, bem como sua bacteriocina tenha um amplo espectro de inibição, incluindo

patógenos, tolerantes a pH e calor; e prontamente digeridas por enzimas proteolíticas no estômago devido a sua natureza proteica (COTTER et al. 2005; 2013).

Tendo em vista o aumento da distribuição global de alimentos que provocou um aumento na disseminação de doenças transmitidas por alimentos, soluções são necessárias para combater essa tendência. Além disso, o maior interesse dos consumidores por alimentos seguros que são minimamente processados despertou o interesse no desenvolvimento de antimicrobianos naturais eficazes ou combinações antimicrobianas para controlar esses patógenos de origem alimentar (MARTHUR et al., 2017).

Diferentemente dos conservantes químicos, que por sua vez podem afetar a saúde humana, as bacteriocinas, prometem uso seguro como conservante de alimentos em vegetais, laticínios, queijos, carnes e outros produtos alimentícios, pois inibem a contaminação de microrganismos durante o processo de produção (KHAN et al., 2010; YANG et al 2014). Isto acontece por seu espectro de ação abranger boa parte dos microrganismos que deterioram alimentos e causam intoxicações alimentares.

Dentre os vários patógenos alimentares, um dos mais estudados para sua eliminação com a utilização de bacteriocinas é a *Listeria monocytogenes*. A *L. monocytogenes* é o agente causado da listeriose e pode causar infecções oportunistas que podem levar a meningite e sepse em casos graves (MARTHUR et al., 2017; CAMARGO et al., 2018). A incidência desta doença é muito maior em populações suscetíveis, incluindo indivíduos imunocomprometidos, grávidas e idosos. *Listeria* é uma bactéria amplamente distribuída no meio ambiente e o seu controle em instalações de produção de alimentos requer atenção devido ao risco a sáude pública associado a essa bactéria (BUCHANAN et al., 2017). *L. monocytogenes* já foi isolada de uma diversidade de produtos alimentícios crus e processados, incluindo leite e produtos lácteos, carnes, ovos, frutos do mar, vegetais e outros alimentos prontos para consumo (FERREIRA et al., 2014).

Além disso, as bacteriocinas têm muitas propriedades que sugerem que são alternativas viáveis aos antibióticos. Tendo em vista o aumento da resistência aos antibióticos na área clínica, as bacteriocinas têm despertado interesse para substituir ou potencializar a ação dos antibióticos comumente utilizados contra os patógenos humanos (COTTER et al. 2013; YANG et al 2014; DRIDER et al. 2016). Bem como, já foi observado que as bacteriocinas apresentam outras aplicações como: atividade antivirais; reguladores da microbiota intestinal, podendo eliminar/ controlar a colonização intestinal

por parte das bactérias multiressistentes, sem afetar a microbiota normal; atividade antitumoral devido a interação das bacteriocinas (cargas postivias) com a membrana das células cancerígenas (cargas negativas), permitindo a sua desestabilização; e promotora de crescimento de plantas (DRIDER et al. 2016).

Em decorrência da utilização de bacteriocina nas diversas áreas, já há estudos que destacam a importância de se compreender os possíveis mecanismos de resistências uma vez que aplicação de bacteriocinas específicas podem ser reduzidas pela seleção de microrganismos resistentes (COTTER et al 2013; BASTOS et al 2015).

Dentro do grupo complexo de BAL, as cepas mais estudadas pertencem aos gêneros *Lactobacillus, Lactococcus* spp., *Streptococcus* spp., *Pediococcus* spp., *Leuconostoc* e *Enterococcus* spp. (KHAN et al., 2010; CAMARGO et al., 2018).

2.2.4 Enterocinas

2.2.4.1 Características gerais

Enterocinas são bacteriocinas produzidas por enterococos, sendo que a primeira foi descrita por Kjems (1955), enquanto estudava bacteriófago, desde então um grande número de enterocinas tem sido estudada.

Assim como as outras bacteriocinas, as enterocinas podem ser aplicadas nos alimento de várias maneiras, utilizadas purificadas, parcialmente purificadas, ou mesmo com a utilização da cepa bacteriocinogênica como cultura *starter* (FOULQUIÉR-MORENO et al., 2006; FISHER; PHILIPS, 2009).

Muitas enterocinas são ativas contra *L. monocytogenes*, um importante patógeno alimentar, como mencionado no tópico anterior, e algumas também foram descritas como sendo ativas mesmo contra bactérias Gram-negativas, uma propriedade incomum para as bacteriocinas produzidas pela BAL. Estas propriedades resultaram em muitos estudos descrevendo o uso de enterocinas como conservantes em alimentos de origem animal e vegetal (KHAN et al., 2010).

E. faecium e *E. faecalis* são conhecidamente produtores de enterocinas, Franz et al. (1999) Foulquié-Moreno et al. (2006), porém Jaouani et al (2014) mostrou atividade antibacteriana de sobrenandante de cultivo contendo enterocinas de diferentes espécies de enterococos (*E. faecium, E. faecalis, Enterococcus hirae, Enterococcus mundtii* e

Enterococcus durans) contra *L. monocytogenes* pelo método de difusão em ágar sendo que *Listeria ivanovii* e *Listeria innocua* também foram inibidos pela maioria desses enterococos. De Kwaadsteniet et al (2005) observou que a enterocina produzida por *E. mundtii* isolado de feijão foi ativa contra Gram-positiva e Gram negativa.

2.2.4.2 Biossíntese, classificação e transporte

A biossíntese de enterocinas requer a ação de quatro genes: um gene responsável pela produção do pré-peptídeo; um relacionado a imunidade específica que confere a produção de uma proteína de imunidade à célula produtora; outro que codifica proteínas do transportador ABC, responsável por exteriorizar a bacteriocina, e o gene que codifica uma proteína essencial para a exteriorização da bacteriocina. Esses genes estão organizados em um ou dois *operons.* Por exemplo, as enterocinas A e B possuem genes organizados em dois *operons*, referentes aos *loci ent*A e *ent*B (FISHER; PHILIPS, 2009; OGAKI et al. 2015).

A classificação das enterocinas são encontradas nas classes de bacteriocinas I (citolisina), IIa (A, CRL35, bacteriocin 31, P, Munditin e citolisina), IIc (AS-48, B, L50A, L50B, EJ97, Q, 1071A, 1071B) e III (Enterolysin A) (FOULQUIÉR-MORENO et al., 2006). O espectro restrito da classe IIa fornece uma solução interessante na atividade antilisterial em alimento fermentados, uma vez que sua atividade não afeta o processo de fermentação do alimento por não inibir a atividade lactococcos (COTTER et al 2005).

Como observado, a grande maioria das enterocinas pertence à classe II, que são sintetizadas como pré-peptídeo sinal tipo dupla glicina, porém há algumas do tipo Sec-dependente, o que acarreta dois tipos diferentes de transporte intracelular para o extracelular (OGAKI et al. 2015).

No tipo pré-peptídeo tipo dupla glicina, tem se que na região N-terminal dos pré-peptídeos, há dois resíduos de glicina conservados, que têm por funções impedir a atividade da bacteriocina intracelularmente e fornecer o sinal de reconhecimento para o transportador ABC. Inicialmente, ocorre a síntese do pré-peptídeo e também de um fator de indução (IF), em seguida, os pré-peptídeos tipo dupla-glicina são exportados através da membrana citoplasmática por um cassete transportador ABC ligado a ATP. O transportador ABC é uma protease, cujo domínio proteolítico reside na região N-terminal da proteína; portanto, tem por função a remoção da sequência-líder (que mantém as bacteriocinas inativas) e a translocação do peptídeo maduro através da membrana

citoplasmática. Após a liberação da bacteriocina, a proteína quinase histidina detecta a presença do fator de indução e se autofosforila, permitindo que o grupo fosfato (P) seja transferido para o regulador, que ativa a transcrição de genes regulados e a produção da proteína de imunidade (OGAKI et al. 2015). Exemplos dessas bacteriocinas são elas as enterocinas A,B, 1071, Munditcina KS e CRL35 (FRANZ et al., 1999, 2007).

Nas enterocinas que apresentam o pré-peptídeo do tipo Sec-dependente, o movimento deste através da membrana plasmática é mediado por um complexo de proteínas multiméricas citosólicas (SecB e SecA) e presentes na membrana (SecYEG), chamado de translocase (Figura 1). No citoplasma, a proteínas SecB captura o pré-peptídeo sintetizado e o direciona à proteína SecA. Essa proteína por sua vez direciona o pré-peptideo para a proteína SecYEG contida na membrana celular. O pré-peptídeo se insere para manter o canal aberto e a proteína SecA também é inserida pela dupla hélice IRA1 (regulador intramolecular de hidrólise de ATP). Após a translocação, a sequência Sec é removida do pré-peptídeo por peptidases periplásmicas de sinal (SP) e, consequentemente, há a formação da bacteriocina madura (OGAKI et al. 2015). Exemplo de enterocinas com esse tipo de transporte são as bacteriocinas P e 31 (FRANZ et al., 1999, 2007).

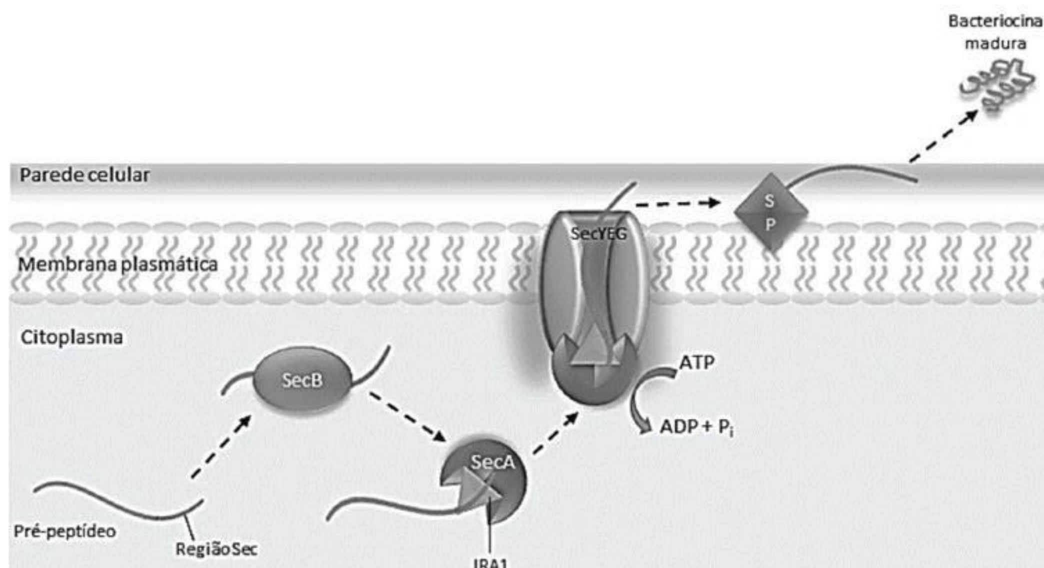

Figura 1. O esquema de transporte de bacteriocinas pela via Sec translocase. Fonte: Ogaki et al, (2015).

2.2.4.3 Modo de ação

As enterocinas, como a maioria das bacteriocinas de BAL, têm a membrana citoplasmática como seu alvo primário. Elas formam poros na membrana celular,

esgotando o potencial transmembrana e/ou o gradiente de pH, resultando no vazamento de moléculas intracelulares indispensáveis (CLEVELAND et al., 2001).

Há dois modelos de formação de poros na membrana citoplasmática, que diferem quanto a inserção do peptídeo na membrana - alvo: Modelo *Barrel stave* e *Wedge-like* (Figura 2). No modelo *Barrel stave* o início da ligação ocorre com a presença de um resíduo de aminoácido no meio da sequência de peptídeo da classe II que facilita sua inserção na membrana citoplasmática. A porção hidrofóbica dessa bacteriocina se liga às cadeias de ácidos graxos dos lipídios da membrana, enquanto a porção hidrofílica em alfa-hélice fica voltado para a parede celular. No modelo *Wedge-like*, o peptídeo de classe I se liga a superfície da membrana provocando sua desestabilização, com consequente formação de poros. A inserção da porção C-terminal na bicamada lipídica é promovida pela força próton motriz, o que possibilita o não contato da bacteriocina com a porção hidrofóbica da membrana (OGAKI et al., 2015).

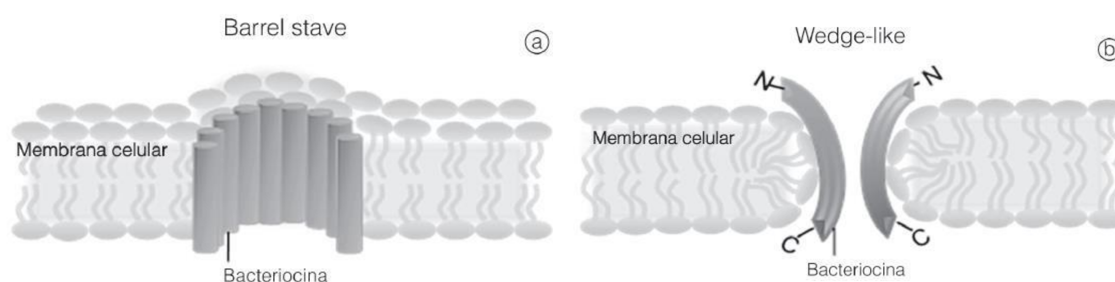

Figura 2. Modelos para a formação de poros pelas bacteriocinas. (a) modelo *Barrel stave* e (b) modelo *Wedge-like*. Fonte: Ogaki et al., (2015).

2.2.4.4 Tipos de enterocinas e seus espectros de ação

Muitos das enterocinas são isoladas de produtos de origem alimentar, animais e vegetais (Tabela 1), sendo que muitas delas como as enterocinas A, B, P, bacteriocina 31, AS-48, dentre outras apresentam características em comum, uma vez que todas têm ação contra *L. monocytogenes* (FOULQUIÉR-MORENO et al., 2006; FRANZ et a., 2007; KHAN et al. 2010).

Tabela 1. Bacteriocinas produzidas por *Enterococcus* spp.

Bacteriocina	**Produzido por**	**Isolado de**
Enterocina A e B	*E. faecium* P21	Salsicha fermentado a seco
Enterocina EJ97	*E. faecalis* S-47	
Sem nome	*E. faecium* A2000	Queijo
Enterocina CRL35	*E. faecium* CRL35	Queijo

Bacteriocin N15	*E. faecium* N15	Nuka
Enterocina A e B	*E. faecium* WHE81	Queijo
Sem nome	*E. faecium* RZS C5; *E. faecium* DPC 1146	
AS-48	*E. faecalis* S-48	Trato intestinal de suíno
Enterocina 012	*E. galinarrum*	Duodeno de avestruz
Sem nome	*E. faecium* CRL 1385	Frango
Enterocina P	*E. faecium* P13	Salsicha fermentada a seco
Enterocina 1071 A e 1071 B	*E. faecalis* BFE 1071	Fezes de suínos
Mundticina AT06	*E. mundtii* AT06	Vegetais
Mundticina KS	*E. mundtii* NFRI 7393	Ensilagem de capim

Fonte: De Kwaadsteniet et al. (2005)

A enterocina A foi descrita em 1996 por Aymerich, que a isolou de *E. faecium*. Esse peptídeo é codificado por cromossomo, contendo 47 aminoácidos, com peso molecular de 4829 Da, formando quatro resíduos de cisteína contendo pontes dissufeto intramoleculares (FRANZ, et al., 1999).

A enterocina B foi isolada por *E. faecium* e descrita por Casaus et al. (1997). Essa enterocina é codificada por cromossomo, contendo 53 amonoácido, com peso molecular de 5463 Da (FRANZ, et al., 1999).

A enterocina A e enterocina B atuam sinergicamente apresentando forte atividade anti-listerial, bem como potencial como conservantes em alimentos. Ambas já foram aplicadas em suco de maçã contra *Salmonella enterica*, em frutas e legumes enlatados contra *Bacillus coagulans*, em alimentos mal cozido tendo como alvo *L. monocytogenes* dentre outros alimentos como revisado por Khan et al., (2010). Sonsa-Ard et al (2015), isolou uma enterocina de *E. faecium* CN-25, de ovas de peixe fermentados com atividade antilisterial. A bactéria produtora apresentava o gene para enterocina A e B. Foi observado que o peptídeo CN-25 aumentou a permeabilização da membrana ocasionando a depleção do potencial eletrônico transmembranar de *Listeria* sp.

Enterocina P é um peptídeo cromossomal isolado de *E. faecium* por Cintas et al (1997) contendo 43 aminoácidos com peso molecular 4443 Da (FRANZ et al., 1999). Apresenta um amplo espectro de ação contra *Enterococcus, Lactobacillus* e *Pediococcus spp., B. cereus, C. botulinum, C. perfringens, L. monocytogenes* e *S. aureu* (CINTAS et al., 1997; FRANZ et a., 2007).

Foulquié-Moreno et al. (2003) observou a atividade antimicrobiana de vários *E. faecium* em sobrenadante livre de célula (CFS) contra *L. innocua,* bactérias ácido láticas e até de *Clostridium* sp sendo esse somente inibido após a purificação parcial do CFS.

Um dos isolados *E. faecium* RZS C5 apresentou gene codificadores de enterocinas A, B e P, porém apesar de saber que a enterocina B depende da expressão da enterocina A, só foi possivel isolar a enterocina B. Estes dados demonstram que as enterocinas nem sempre sao expressas ao mesmo tempo ou que as suas concentrações nos sobrenadantes de cultivo não foram suficientes para serem detectadas. Javed et al (2011) também observaram a atividade antimicrobiana das enterocinas em CFS e em CFS parcialmente purificados de *E. faecalis* e *E. faecium* isolado de produtos lácteos fermentados, que abrigavam o gene para enterocinas A, B e P. Ao contrário dos isolados de *E. faecium*, o *E. faecalis* só exibiu atividade antimicrobiana após a purificação parcial. Esses CFS foram ativos contra *L. monocytogenes, Bacillus subtilis* e *Bacillus cereus.*

A bacteriocina 31, isolada de *E. faecalis*, tem seus genes localizados em plasmídeo pYI17 que codificam um peptídeo de 43 aminoácido após sua maturação. Apresenta atividade contra *L. monocytogenes* e *E. faecium* e *E. hirae* (TOMITA et al., 1996, FRANZ, et al., 1999).

Edalation et al., (2012) observaram a produção de enterocinas em sobrenadante de cultivo por *E. faecium, E. faecalis* e *E. casseliflavus* que apresentaram alta atividade contra *L. innocua*. Os genes encontrados nos enterococos produtores de bacteriocinas foram enterocinas A, B, P, X e em alguns a bacteriocina 31.

Enterocina X foi isolada de *E. faecium*, pertencente a classe IIb das bacteriocinas composta por dois peptídeos antibacterianos, Xα e Xβ contendo 4420 Da e 4068 Da, respectivamente, que quando juntos apresentam um espectro antibacteriano totalmente diferente do que aqueles obtidos em peptídeos isolados. Vale salientar que o peptídeo Xα apresenta uma forte atividade antilisterial (HU et al., 2010).

As enterocinas L50, isoladas de *E. faecium* são codificadadas por plasmídeo em dois peptídeos L50A e L50B, apresentando peso molecular de 5190 e 5178 Da respectivamente, não apresentando modificação pós tradução (CINTAS et al., 1998; FRANZ, et al., 1999). Estas enterocinas já foram utilizadas em cerveja com e sem álcool contra *Lactobacillus brevis* e *Pediococcus damnosus* (KHAN et al. 2010).

A enterocina 1071 produzida por *E. faecalis* é composta por dois peptídeos 1071 A e 1071 B, sintetizado por plasmídeo. Apresenta um amplo espectro de ação contra Gram-positiva, como *Clostridium*, *Enterococcus, Lactobacillus, Propionibacterium, Streptococcus, Micrococcus* e *Listeria* spp (FRANZ et al., 2007).

A enterocina Mundticina KS, produzida por *E. mundtii,* isolada de vegetais processados, contem 43 aminoácidos contendo peso molecular 4287 Da, apresenta

peptídeo líder do tipo dupla glicina e apresenta espectro de atividade contra *Enterococcus, Lactobacillus, Leuconostoc e Pediococcus spp.,* além de *L. monocytogenes e Clostridium botulinum* (FRANZ et al., 2007).

A enterocina AS-48 isolada de *E. faecalis* é codificada por genes localizados em plasmideo pMB2, tendo na sua exportação celular a clivagem do pré-peptídeo formando um peptídeo maduro com 70 aminoácido, pela clivagem do peptídeo líder (MARTÍNEZ-BUENO et al 1994). A AS-48 foi a primeira enterocina purificada e caracterizada. Apresenta um amplo espectro inibitório sendo capaz de inibir muitas bactérias não relacionadas com Gram positivas tais como *Bacillus spp., Corynebacterium glutamicum, Corynebacterium bovis, Mycobacterium phlei, Micrococcus luteus, S. aureus* e também algumas espécies Gram-negativas tais como *Salmonella typhimuriam, Shigella sonnei, Escherichia coli, Pseudomonas spp.*e *Klebsiella pneumoniae* (KHAN et al., 2010). Lucas et al., (2006) observaram a ação da AS-48 contra o crescimento vegetativo de *Bacillus coagulans*, em refrigeração e altas temperaturas, em alimentos enlatados. Enquanto Grande et al., (2005) observaram o aumento da sensibilidade dos endosporos de *Bacillus* spp. com aplicação da AS-48. O que demonstra a importância dessa enterocina na redução de células vegetativas, bem como formas de resistências. Ananou et al., (2005) avaliou a atividade contra o crescimento de *S. aureus* utilizando bactéria produra de AS-48 ou adição do AS-48 semi-purificada em salsicha. A enterocina nas concentrações de 30 ou 40 µg/g inibiu a contagem de unidade formadora de colônia (UFC) de *S. aureus* quando comparado ao controle. Enquanto a utilização da bacteriocina produtora (10^7 UFC/g) na salsicha produziu enterocina AS-48 suficiente para controlar o crescimento de estafilococos. Ananou et al., (2010) relataram que a enterocina AS-48 pode ser produzida em subprodutos da indústria de laticínios (leite desnatado), reduzindo os custos da produção industrial. A enterocina apresentou atividade contra *L. monocytogenes* e inibição parcial de *S. aureus*.

Outra bacteriocina que apresenta uma amplo espectro de atividade foi relatada por De Kwaadsteniet et al. (2005). Esses autores isolaram uma enterocina ST15 de *E. mundtii*, com peso molecular de 3944 Da no qual apresentou ação contra bactérias Gram-positivas e Gram-negativas: *Acinetobacter baumanii, Bacillus cereus, Clostridium tyrobutyricum, Enterococcus faecalis, Klebsiella pneumoniae, Lactobacillus sakei, Propionibacterium sp., Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus caprinus* e *Streptococcus pneumoniae.*

A enterocina CRL35, contem 43 aminoácido e peso molecular 4900 Da que apresenta ação antilisterial (FRANZ et al., 2007) e a primeira estudada com propriedades antiviral (DRIED et al., 2016).

Existe uma enterocina que não apresenta interesse para aplicação na industria alimentícia, a citolisina. Essa bacteriocina pertence a classe I, que apresenta atividade bacteriocina e também hemolitica, no qual é sintetizada na forma de um pré-peptídeo inativo que sofre clivagem na porção N-terminal durante sua exportação da célula (FRANZ et al 1999; 2007).

Muitas dessas enterocinas, algumas já mencionadas, foram testadas em diversos produtos alimentícios como na indústria de laticínios e conservação de vegetais, frutas, sucos de frutas (MOLINOS et al., 2005; GRANDE et al., 2005; LUCAS et al., 2006; ANANOU et al., 2010) e carnes (ANANOU et al., 2005) tendo como alvos espécies patogênicas de Gram-positivas e Gram-negativas.

Como visto há vários estudos utilizando enterocinas contra vários patógenos que persistem nos sistemas alimentares em seus estados planctônicos, porém apesar de pouco há crescentes estudos em relação a essa bacteriocina contra os microrganismos indesejáveis em seus estados sésseis (biofilmes).

2.2.4.5 Bacteriocinas/ enterocinas na inibição de biofilme na indústria de alimento

Biofilme têm sido definido como agregados de microrganismos ligados a um substrato ou entre si, capaz de persistir em diferentes superfícies de materiais, embutidos em uma matriz de substância polimérica extracelular (EPS), que comumente compreende lipídios, proteínas e exopolissacarídeos (HOBLEY et al., 2015).

O biofilme de bactérias patogênicas, em ambientes de processamento de alimento, apresenta um grande risco para a saúde pública, uma vez que essas bactérias podem levar a doenças humanas graves. Essas contaminações podem ocorrer em qualquer estágio do processamento de alimentos por meio de manipuladores de alimentos, equipamentos contaminados e superfícies de preparação de alimentos (ABDALLAH et al. 2014).

O biofilme tem quatro estágios comuns. O primeiro sendo a adesão superficial da bactéria a uma superfície, seguido pela formação de micro-colônias e sua maturação. Após esse estágio, começa a fase de dispersão, no qual algumas células se desprendem da estrutura e se instalam em outras superfícies (ABDALLAH et al., 2014). Vários fatores influenciam na adesão da bactéria, incluindo superfícies com propriedades de

hidrofobicidade, cargas elétricas, composição da comunidade microbiana, temperatura, disponibilidade de alimentos, comunicação célula-célula, bem como fatores de virulência. Após a adesão celular na superfície, começa o desenvolvimento de micro colônias e produção de EPS, que protegem o biofilme de agentes químicos, o que dificulta a sua eliminação. O que faz dos biofilmes um grande problema na indústria de alimento, pois permite que os patógenos e/ou deteriorantes de alimento sobrevivam e persistam nas instalações de processamento, resultando na contaminação cruzada dos produtos finais (CAMARGO et al., 2018).

No entanto, os regimes convencionais de limpeza e desinfecção também podem contribuir para o controle ineficiente do biofilme e para a disseminação da resistência. Consequentemente, novas estratégias de controle estão emergindo constantemente, com grande incidência no uso de bio-soluções, como por exemplo as bacteriocinas (SIMÕES et al., 2010).

Bacteriocinas, comumente usadas como bioconservadores, como nisina, lauricidina, reuterina e pediocina, foram bem documentadas para o seu potencial de controle de biofilme contra microrganismos comumente encontrados em instalações de processamento de laticínios. Surtos de patógenos associados a biofilmes têm sido relacionados à presença de *Listeria monocytogenes, Yersinia enterocolitica, Campylobacter jejuni, Salmonella* spp. *Staphylococcus* spp. e *Escherichia* coli O157: H7 (SIMÕES et al 2010). Porém são poucos os estudos similares utilizando enterocinas.

Zhao et al., (2004) observaram a redução de biofilme de *L. monocytogenes* por CFS de *Enterococcus durans* e *Lacotoccus lactis.* Camargo et al (2016) observaram que CFS de diversas BAL, incluindo *Enterococcus* spp. foram capazes de prevenir biofilme de *L. monocytogenes* isoladas de ambientes de processamento de carne.

Al-Seraih, et al. (2017) relataram uma enterocina produzida por *E. faecalis* B3A-B3B com atividade contra *L. monocytogenes, S. aureus* resistente à meticilina e *Clostridium perfringens* e *Salmonella* Newport. Essas enterocinas foram capazes de impedir a formação de biofilme de *L. monocytogenes*. Além disso, sua utilização com nisina reduziu concentração inibitória mínima necessária para inibir células planctônica ou de biofilme de *L.monocytogenes.*

É reconhecida que a nisina tem um importante papel na redução e eliminação da formação de biofilme de *S. aureus, Staphylococcus epidermidi e L. monocytogenes* (NOSTRO et al. 2010). Porém Davison et al., (2010) observou o efeito da nisina sob a

integridade de membrana celular de biofilme de *Staphylococcus epidermidis,* porém sem qualquer remoção do biofilme.

Com o intuito de melhorar a eficiência sobre a eliminação de biofilme na industria alimentícia, há um crescente número de estudos que visem a utilização de bacteriocina associada a biocidas comumente (cloro, peróxido de hidrogênio, iodo, ozônio e ácido peracético) utilizados na indústria de alimentos.

A enterocina AS-48 reduziu a aderência e formação de biofilme de *Listeria* sp (CABALLERO-GÓMEZ et al 2012). Esses autores observaram o efeito satisfatório da combinação de enterocina e biocida em células planctônicas e sésseis de *L. monocytogenes*. Aliado a isso, esses autores demonstraram que essa enterocina ao ser adsovida em placas de microtitulação de poliestireno antes da adição de *L. monocytogenes* evitou a adesão e formação do biofilme por parte dessa bactéria. Dado esse que demonstra o potencial de proteção à adesão bacteriana da enterocina. Já em 2013 esses mesmos autores obtiveram resultados parecidos de ação de AS-48 e biocidas, porém contra biofilme de *S. aureus*. Esses autores acreditam que o acúmulo de danos celulares causados por biocidas, enterocina AS-48 e o esgotamento do suprimento de energia necessários para o reparo de danos celulares poderiam explicar o aumento observado nas atividades antimicrobianas nas combinações bacteriocina-biocida.

Os dados expostos acima sugerem que as bacteriocinas podem ser aplicadas em ambientes de processamento de alimentos para controlar o crescimento, formação e/ou eliminação de biofilme de bactérias importantes que representam um sério problema para o ambiente industrial. Assim, busca-se a aplicação de tratamentos com bacteriocinas associados ou não a biocidas que reduzam ou impeçam a adesão e/ou promovam a desestabilização da matriz extracelular, permitindo assim que os protocolos atuais de limpeza sejam otimizados, aumentando a eficácia na redução de biofilmes.

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3. OBJETIVOS

3.1 OBJETIVO GERAL

Selecionar *Enterococcus* spp. produtores de enterocinas e caracterizá-las quanto ao modo de ação, espectro de atividade e influência na formação de biofilme.

3.2 OBJETIVOS ESPECÍFICOS

- Caracterizar isolados de enterococos produtores de bacteriocinas quanto a presença de genes estruturais de enterocinas e atividade contra o crescimento de *Listeria innocua*; bem como quantificar essa atividade antibacteriana, determinar seu modo de ação e seu espectro de atividade contra outras bactérias oportunistas.

- Avaliar a atividade antibacteriana de enterocinas contra biofilme de *Listeria* spp., tanto no desenvolvimento, quanto no biofilme pré-formado, por biomassa total e atividade metabólica.

ARTIGOS

ARTIGO 1

Antimicrobial activity of enterocins against *Listeria* spp. and other food opportunistic bacteria

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Abstract

Many of the *Enterococcus* species are important producers of antimicrobial peptides against several bacteria, mainly pathogenic foodborne bacteria such as *Listeria* sp. The objective of this study was to determine enterocin producers among enterococci isolates, the prevalence of enterocin structural genes and to detect the spectrum of activity against various bacteria. Seventy-six isolates of enterococci were evaluated of which 11 showed as potential enterocin producers against *Listeria.innocua* CLIP 12612. By PCR analysis, the isolates were determined to harbor at least one enterocin-encoding gene, which included *ent*A, *ent*B, *ent*P, *ent*1071 and *ent*X, in proportions of 45.4, 54.5, 45.4, 18.2 and 54.5%, respectively. None of these isolates produced hemolysin. Cell-free supernatants (CFSs) from 7/11 isolates possessed activity against *L. innocua* CLIP 12612 in an agar well diffusion assay. CFSs were thermostable, protein nature, resistant to catalase and did not present bacteriophage activity. Partial precipitation of CFSs increased the arbitrary units during a 6 h period. Mode of action was determined by OD to be a bactericidal effect against *L. innocua* 12612. Regarding the spectrum of activity of the 7 CFSs against other 33 indicator bacteria, the elimination of mainly *L. innocua, L. monocytogenes* and *L. ivanovii,* as well as clinical enterococci, was observed. Some isolates evaluated here may be candidates for the production of enterocins for use as food biopreservatives, since they have high anti-listerial activity even after 24 h of experimentation, and for use in the pharmaceutical area since they inhibit clinical microorganisms.

Keywords: bacteriocins, *Listeria monocytogenes, Listeria ivanovii, Listeria innocua*, CFS.

Introduction

Food safety has been an important global issue due to increasing foodborne diseases and changes in food consumption habits. Therefore, the need to avoid economic losses due to microbial-induced spoilage and the preservation of foods by natural methods may be a satisfactory approach to solve many of the current food-related issues.

The development of biopreservation technologies with lactic acid bacteria (LAB) and/or their metabolites represents an additional hurdle in the protection of food against microbial contamination as these bacteria produce several antimicrobial substances, including organic acids, hydrogen peroxide and bacteriocins (Perin *et al*., 2013). Many bacteriocin-producing LAB strains have been isolated from milk, plants and fermented products of dairy, cereals or meats, many of which have been identified and characterized (Ivanova *et al*., 2000), and studies have demonstrated that bacteriocin from BAL has considerable inhibitory activity against pathogenic and spoilage microorganisms in foods (Liu *et al*., 2008), mainly by the production of bacteriocins (Rehaiem *et al*., 2012; Leroy and De Vuyst, 2004).

Bacteriocins are ribosomally synthesized antimicrobial proteins/peptides produced by many bacteria that kill or inhibit the growth of other bacteria, either in the same species (narrow spectrum of activity) or across genera (broad spectrum of activity) (Cotter *et al*. 2013). Bacteriocins produced by the genus *Enterococcus* are called enterocins (Foulquié-Moreno et al, 2006; Castellano *et al*., 2008) and have been extensively studied and isolated from a variety of sources (Bayoub *et al*., 2011; Hadji-Sfaxi *et al*., 2011).

Enterocins are found within class I, IIa, and IIc bacteriocins (Cotter *et al*., 2013). The cytoplasmic membrane is their primary target, and similar to most bacteriocins, they form pores, thereby depleting transmembrane potential and/or a pH gradient, resulting in cell death (Cleveland et al., 2001). Enterocins show high activity, particularly against *Listeria* species at nanomolar concentrations (Herranz, et al., 2001). Enterocins has proven to effectively inhibit *L. monocytogenes* in fermented foods (Liu *et al*., 2008; Rehaiem *et al*., 2012). However, low levels of bacteriocins secreted from natural strains do not meet the requirements of industrial-scale production and have limited applications to study stages.

Listeria genus can be found as a contaminant in meat, milk and other food processing facilities, and the most important species is *L. monocytogenes*, which causes

septicemia, meningitis, encephalitis or death/stillbirth of neonates, especially in high-risk groups in humans (including immunocompromised persons and the elderly) (Vázquez-Boland *et al*., 2001). These bacteria are exceptional because they can tolerate high salt concentrations, remain viable at temperatures and are also difficult to eliminate by ordinary disinfectants (Pan *et al*., 2006).

Relevant information that must be investigated includes possible bacteriocins that the strains are able to produce, which can be assessed by the identification of specific genes related to known bacteriocins and thermal and protease resistance, followed by the inhibitory spectrum (Perin *et al*., 2012). These data can justify further studies with purified bacteriocins to investigate the diversity of characteristics that allow their use in the food industry as biopreservatives.

In this study, we focused on the isolation and inhibitory effects of enterocins from a collection of *Enterococcus* sp. isolates obtained from soft cheese and environments (rivers and soil), where common foodborne pathogens or standard strains originate. The inhibitory effect of cell-free culture supernatants (CFSs) was tested against several food-borne microorganisms.

Materials and methods

Bacterial strains and culture conditions

A collection of 76 isolates of enterococci was isolated from foods, clinical samples and the environment (Table 2), and 34 indicator bacteria (Table 3). All bacterial isolates were stored in brain heart infusion (BHI) broth (Neogen Culture Media, USA) with 20% (v/v) glycerol (Merck, Germany) at -20 °C. *Listeria innocua* 12612 from the American Type Culture Collection (ATCC) was used.

Screening for antagonism against *Listeria innocua*

The strains of enterococci were inoculated in trace forms on the surface of BHI and incubated at 37 °C for 24 h. Next, 1 ml of chloroform (Baker Analyzed, USA) was deposited in each of the lids of the plates, and plates were inverted and closed for 20 min. Then, 10 ml of BHI soft agar, which was prepared by adding 0.8% bacteriological agar (Neogen Culture Media, USA) to BHI broth and sterilizing the solution, containing 1 x 10^8 CFU ml^{-1} of *L. innocua* 12612 was deposited on the surface of the inoculated medium. The plates were incubated at 37 °C for 24 h, and the appearance of clear zones around

colonies was indicative of potential enterocin producers by enterococci. Each condition was tested in duplicate.

Genotyping of genes encoding enterocins

The strains selected by previous experiments were submitted to molecular identification of enterocin-producing genes, which included enterocin A (*ent*A), enterocin B (*ent*B), enterocin P (*ent*P), enterocin L50A/B (*ent*L50A/B), enterocin 1071 (*ent*1071), enterocin Q (*ent*Q), mundticin KS (*ent*KS), enterocin X (*ent*X), bacteriocin 31 (*ent*31) and enterocin AS48 (*ent*AS48), and were amplified using PCR primers (Table 1). The PCR reactions were performed as previously mentioned in a thermocycler (Esco Technologies, USA). Gene amplification was conducted with an initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 seconds, matching the oligonucleotide (Thermo Fisher Scientific, USA) (Table 1) for 30 seconds, 72 °C for 30 seconds, and a final extension at 72 °C for 5 min. Amplicons were analyzed on a 1.0% agarose gel (Merck, Germany).

Hemolytic activity

The hemolytic activity of the enterococci isolates was performed on blood agar supplemented with 5% sheep blood (Newprov, Brazil). Ten microliters of a culture of overnight enterococci were deposited on the blood agar plates. The plates were incubated at 37 °C for 48 h. Clear colonies were considered hemolytic. Each determination was carried out in duplicates.

Determination of bacteriocin production and antimicrobial activity

Cell-free supernatants (CFS) of the isolates were previously selected and assayed as described by Tomé *et al.* (2009) with modifications. The strains were cultured in deMan, Rogosa, and Sharpe (MRS) medium (Neogen Culture Media, USA) overnight, adjusted to 1.0×10^{8} CFU ml^{-1} in MRS (pH 6.2) and maintained at 180 rpm for 18 h at 37 °C. The cultures were centrifuged at 5000 rpm for 15 min, the supernatant was adjusted to pH 6.5 with NaOH (1N) and membrane filtrated with a 0.22 µm filter (Merck, Germany). The CFS of the isolates were used for subsequent experiments. In an agar well diffusion assay (AWDA), 30 µL of the CFS were deposited in 5 mm wells on BHI agar containing *L. innocua* 12612 (1×10^{8} CFU ml^{-1}). Finally, the plates were incubated for 24

h at 37 °C. Inhibition halo ≥ 2 mm was considered positive for bacteriocin production. Each condition was tested in duplicate.

Effect of heat and enzymes on enterocin activity against *L. innocua* 12612

The thermostability of enterocin was evaluated by the treatment of CFS at 80 °C for 10 min and 100 °C for 20 min. To determine the sensitivity of the antimicrobial components against proteolytic enzymes, the selected CFS were treated with α-chymotrypsin (50 mg/ml), protease (50 mg/ml) and trypsin (20 mg/ml) enzymes (Merck, Germany) in addition to the catalase enzyme (Merck, Germany) at a final concentration of 1 mg ml^{-1} (Garriga *et al*., 1993). Then, the antagonistic activity of the treated CFSs was evaluated by the AWDA assay. Each condition was tested in duplicate.

Bacteriophage activity

A portion of the inhibition halo against *L. innocua* 12612 was retired from the plate and added to 3 ml of BHI broth, and then macerated with sterile tips. The suspension was maintained at room temperature for 1 h, and aliquots of 100 µL of the suspension and 100 µL of *L. innocua* culture was added to 4 ml of BHI soft agar, into which was poured into BHI agar plates overlapping, and incubated at 37 °C for 24 h. The formation of plaques is considered indicative of bacteriophage activity (LEWUS et al., 1991). Each condition was tested in duplicate.

Quantification of antibacterial activity by arbitrary unit (AU)

To quantify the antibacterial activity of CFS against *L. innocua* 12612, the protocol described by (Bromberg et al., 2006) was used with modifications. For this experiment, CFS at 1:2 (v/v) dilutions were deposited on microplates using MRS. Then, 100 µL of each dilution were deposited on a new microplate with 100 µL of the indicator bacterium (10^8 CFU ml^{-1}) and incubated at 37 °C for 24 h, and bacterial growth was measured by optical density (OD) in a spectrophotometer (Bio Tek, USA) (600 nm) every 3 h. Each condition was tested in triplicate. The unit of arbitrary activity (AU ml^{-1}) was defined as the reciprocal of the last dilution that showed growth compared to the control (bacteria without CFS) multiplied by 100. The OD values were evaluated using one-way ANOVA and Tukey's test, and $p < 0.05$ was considered a statistically significant difference between the antimicrobial activity of an isolate and the control.

Partial purification of enterocins

CFSs that showed antimicrobial activity against *L. innocua* 12612 were submitted to partial purification of enterocins. Partial purification of the enterocins followed the protocol described by Foulquié-Moreno *et al.* (2003), using 40% saturated ammonium sulfate (Merck, Germany), followed by shaking at 4 °C for 18 h. Quantitative determination (AU ml^{-1}) of the partially purified enterocins was conducted as previously described.

Mode of action

The antimicrobial effect of partially purified enterocins was evaluated using *L. innocua* 12612 as described by Foulquié Moreno et al. (2003) with modifications. The indicator bacteria was adjusted to 1.5 x 10^8 CFU ml^{-1} in MRS medium at an OD of 600 nm at 37 °C. A volume of 10 ml of culture medium was distributed into tubes, and 0.5 ml of partially purified enterocin was added, except for the control, which only contained ultrapure water (MilliQ). The optical density and cell count (CFU ml^{-1}) were measured at 10 min, 1, 2, and 4 h of incubation time. The cell counts were determined on BHI agar medium.

Spectrum of antimicrobial activity

CFSs selected by the previous experiments against *L. innocua* 12612 were used to evaluate their activity spectrum against several indicator bacteria. For this experiment, the antagonistic activity of CFS was evaluated by a diffusion technique in agar as previously described. The determination of the antimicrobial spectrum was performed quantitatively against *L. innocua* 12612 and qualitatively against other bacteria.

Results

Among the 76 enterococci isolates, 11 (14%) isolates presented antagonistic activity against *L. innocua* 12612, and 9 of these isolates had at least one enterocin-encoding gene (Table 2). As expected, no hemolytic activity was observed since no clear zones were observed around the colonies.

Determination of the antimicrobial spectrum of CFS was performed quantitatively against *L. innocua* 12612 and qualitatively against the other microorganisms (Table 3).

From the data obtained, 7 enterococci were selected to obtain CFS, since the Ent5A, Ent5B, Ent5C and Ent12h isolates did not inhibit the indicator bacteria (Table 3).

All CFS were thermally stable at both evaluated temperatures since they had the same halo measurements relative to the untreated control. The loss of the antibacterial activity of CFSs after treatment with α-chymotrypsin, protease and trypsin suggests that the enterocins contained in these CFSs have proteinaceous characteristics. Treatment with catalase showed that the CFSs formed inhibition halos, discarding the action of hydrogen peroxide. In relation to the bacteriophage activity, no plaque zones were observed, suggesting that the antimicrobial activity observed against *L. innocua* 12612 by CFS is not due to the action caused by bacteriophage.

In the quantification of antibacterial activity by arbitrary unit (AU), the 7 selected CFSs were used, containing enterocins in diluted and partially purified forms (Table 4). For that, times 6 and 12 hours were used to determine the AU values, since they were more pronunced ($p < 0,05$) even though it was observed that at 3 hours there was inhibition by the CFS and continued until 24h (data not shown). It was observed that in CFSs, there was an increase in UA values for most CFSs, and for some, the values remained the same for the two time points that were evaluated. The highest AU values were observed for the CFSs from Efm20, Efm22, Efm24, and Efs27 with 6400 UA ml^{-1} at 12 h. When comparing the enterocins contained in CFSs with the partially purified CFSs, increases, maintenance and, in some cases, loss of activity against *L. innocua* 12612 were observed. At 6 h, the partially purified enterocin of the isolates Efm20, Efm22 and Efm25 presented increased AU values than when diluted in CFSs. In contrast, at 12 h, there was maintenance or loss of the activity of enterocins when they were partially purified. Both the enterocins of Efs18h and Ent22h showed a loss of activity after partial purification.

The antibacterial effect of partially purified enterocins from food was shown to be similar against *L. innocua* 12612 (Figure 1). The analysis showed a bactericidal effect of the enterocins analyzed since there was no decrease in the optical density as consequence of cellular lysis (bacteriolytic activity).

In relation to the spectrum of CFS activity against the 33 indicator bacteria were observed against inhibition of growth against the *Listeria* spp, *E. faecalis* and *B. subtilis* (Tabela 3). Among the *Listeria* that were studied, only *L.* monocytogenes CDC 4555 and *Listeria* sp (from dairy) were not inhibited by any of the selected CFSs. In contrast, all *L. innocua* (3/3), *L. monocytogenes* (4/5) and *L. ivanovii* (1/1) were inhibited by all food and environmental enterocins (CFS Efs18h). Notably, the evaluated enterocins were able to

inhibit bacteria of clinical origin. There was no antibacterial activity of the enterocin when in contact its own producing cell.

Discussion

Enterococci have been attracting interest in recent decades, mainly for their applications in food, since they are able to produce bacteriocins, with characteristics and a spectrum of inhibition that are favorable for these applications. In this study, a collection of 76 isolates from different sources was investigated for enterocin production. The preselection of enterococci that were mainly enterocin producers as well as the characterization of enterocins, were preliminary assayed against *L. innocua* 12612. The selected isolates were evaluated for their antimicrobial spectrum against other indicator bacteria from several sources.

Of the strains selected as potential enterocin producers, none showed hemolytic activity under the conditions tested. Hemolysin production may increase the risk of enterococcal infections (Valenzuela *et al.*, 2008), which is undesirable in probiotic candidates.

Five enterocin genes, which included *ent*A, *ent*B, *ent*P, *ent*1071 and *ent*X, were detected in this study. The structural enterocin genes *ent*A, *ent*B, *ent*P and *ent*X were the most frequently observed, and they appeared in all enterococci from food. Genes for enterocin L50A/B, Q, K, 31 and AS-48 were not found in any of the enterococci evaluated. Foulquié-Moreno *et al.* (2003) have observed the genomes of *E. faecalis* and *E. faecium*, which indicated the presence of *ent*A, *ent*B and *ent*P genes. The genes *ent*A and *ent*B are used by the same carrier responsible for externalizing enterocin, and they are controlled by the same regulatory system (Nes *et al.*, 2007; Ogaki *et al.*, 2015) and are commonly found together (Hassan *et al.*, 2012). Many enterocins relate to anti-listerial activity with the presence of class IIa bacteriocins, such as enterocin A and P (Klaenhammer *et al.*, 1993; Aymerich *et al.*, 1996; Ennahar *et al.*, 2000). Hassan *et al.* (2012) also observed the presence of the *ent*1071 gene but identified the *ent*L50A/B gene, which differs from the results in this study. The presence of four enterocin genes in strains from food indicates the potential of these isolates to produce various enterocins.

It was observed of the 11/76 preselected enterococcal strains against *L. innocua* 12612, four strain (Ent5A, EntB, Ent5C and Ent12h) from environment did not inhibit activity against this bacterium in CFS. This could be due to the absence or the low amount

of genes related to the production of enterocin, since the Ent5B and Ent5C isolates did not present structural enterocin gene and the other isolates Ent5A and Ent12h isolates showed only one encoding enterocin gene.

Enterocins are commonly classified as class II bacteriocins and are characterized as small, non-lantibiotics with a strong anti-listerial effect, which are desired characteristics for their use in foods (Giraffa, 2002). By characterizing the enterocins in this study, it was observed that only those from foodborne strains were thermostable and of a proteinaceous nature, with the inhibition of hydrogen peroxide activity through the use of catalase. Characteristics observed by Du Toit *et al.* (2000), Tomé *et al* (2009), Hassan *et al.* (2012), and Banwo *et al.* (2013) as well as not possible to observe bacteriophage activity.

When determining the AU of enterocins, it was observed that even without precipitation, there were high values of AU, indicating that the enterocins identified in this study had high bactericidal activity. When comparing culture for 6 and 12 h, there was an increase in the UA values (800 to 6400 AU.ml^{-1}) of some enterocins. After partial purification of these enterocins, it was observed that there was an increase in the AU at 6 h (6400 UA ml^{-1}), while at 12 h, there was maintenance or a slight loss of activity (3200 AU ml^{-1}) for some isolates; however, at 3 h, there was inhibition by CFSs that continued until 24 h. Foulquié-Moreno et al. (2003), Javed *et al.* (2011) and Hassan *et al.* (2012) observed an increase in the activity of inhibition of enterocins after partial purification of CFSs.

These data are a good indication of the action of these enterocins over time since their goal as food biopreservatives is to remain active as long as possible for the safety of food products. In addition, their susceptibility to proteases demonstrates that they are easily digested by enzymes in the gastrointestinal tract without affecting the normal microbiota.

The effect of the activity of enterocins as bactericidal or bacteriolytic was evaluated by partially purified enterocins with ammonium sulfate. In the 10 minutes to 4 hours period, it was possible to observe that *L. innocua* 12612 was very sensitive, except for the enterocin obtained from the isolates from the environment, in which the Efs18h effect was observed after 2h. The partially purified enterocins demonstrated bactericidal activity against *L. innocua* 12612. The enterocins of the environmental strains were not as active compared to those from food, which may be associated with a lower amount of structural enterocin genes by these strains. Foulquié-Moreno et al. (2003) observed both

bactericidal and bacteriolytic effects of enterocins against *L. innocua*. Although several enterocins differ in their molecular structures, the mode of bactericidal action is similar (Gálvez *et al*., 1998).

The activity spectrum of enterocins that were characterized and selected against *L. innocua* 12612 was evaluated against 33 other indicator bacteria. The enterocins showed anti-listerial activity against *L. innocua*, *L. monocytogenes*, *L. ivanovii*, *E. faecalis* 29112 and *B. subtilis*. The anti-listerial activity of CFSs from *E. faecalis* and *E. faecium* has been observed in reports by several authors, including Giraffa *et al*. (1995) (2002), Aymerich et al. (1996), Tomé *et al*. (2009), Khan *et al*. (2010), Hassan *et al*. (2012), Banwo *et al*. (2013) and Jaouani *et al*. (2014).

It was observed that these enterocins inhibited at least 6 indicator bacteria, whereas those from food inhibited the growth of 13 bacteria, while the environmental enterocins (Efs18h and Ent22h) demonstrated activity against 9 and 6 indicator bacteria, respectively. Probably the greater inhibition of indicator bacteria by CFS from food is due to the greater amount of genes harbored by these isolates when compared to those from environment.

The anti-listerial activity of enterocins is very valuable since these bacteria can cause foodborne diseases such as listeriosis, gastroenteritis and bacteremia (Dalton *et al*., 1997; Vázquez-Boland *et al*., 2001; Guillet *et al*., 2010). Among *Listeria*, *L. monocytogenes* is the best known for its pathogenicity, but *L. ivanovii* has been well characterized by infection in ruminants (Vázquez-Boland *et al*., 2001). In contrast, although it is considered a sporadic occurrence, an infection caused by *L. ivanovii* in immunocompromised humans has been observed (Lessing *et al*., 1994; Snapir *et al*., 2006; Guillet *et al*., 2010). In addition, it was observed that food enterocins presented antibacterial activity against all clinical enterococci. No inhibitory activity of enterocins against gram-negative bacteria was observed, which is corroborated by observations by Tomé *et al.* (2009), Javed *et al.* (2011), and Banwo *et al*. (2013), and is likely due to the presence of the outer membrane that hinders the entry of enterocins. In contrast, Jaouani *et al*. (2014) observed the activity of enterocins against gram-negative bacteria. Notably, there was no inhibition of bacterial growth when in contact its produced enterocin, showing that there is immunity in the producing cells. Immunity of bacteriocin-producing cells is conferred by a peptide expressed along with bacteriocin (Ennahar *et al* 2000; Cotter *et al*., 2013; Benz and Meinhart, 2014, Ogaki *et al*., 2015).

Contrary to Jaouani *et al*. (2014), in this study, there was a correlation between the presence of enterocin structural genes as well as the values of arbitrary units and the activity spectrum of bacteriocinogenic strains. The enterocins evaluated in this study, especially those from food enterococci, showed characteristics of interest as well as potential activity against clinical isolates and species known as contaminants and pathogens in food, which make them candidates as food biopreservatives, thus helping to increase the shelf life of food as well as the food supply and human safety.

Conclusions

The antibacterial capacity of enterococci has been well studied due to the search for alternative forms of antimicrobials and food biopreservatives in the pharmaceutical and food industries, respectively. The antibacterial activity observed by the supernatants of enterococcal cultures against important bacteria in the contamination and pathogenicity of food as well as against bacteria of clinical origin makes these isolates promising candidates in an alimentary and/or pharmaceutical context.

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Table 1. Information about primers used in PCR reactions for detection of enterocin structural genes.

Target gene	**Sequence (5´→3´)**	**Annealing temperature**	**Amplicon size**	**References**
Enterocin A	GGTACCACTCATAGTGGAAA CCCTGGAATTGCTCCACCTAA	55	138 pb	Özdemir *et al*. (2011)
Enterocin B	CAAAATGTAAAAGAATTAAGTACG AGAGTATACATTTGCTAACCC	56	201 pb	De Vuyst *et al.* (2003)
Enterocin P	GCTACGCGTTCATATGGTAAT TCCTGCAATATTCTCTTTAGC	55	87pb	Özdemir *et al*. (2011)
Enterocin *L*50A/B	ATGGGAGCAATCGCAAAATTA TAGCCATTTTTCAATTTGATC	55	274 pb	Özdemir *et al*. (2011)
Enterocin 1071 A,B	GGGGAGAGTCGGTTTTTAG ATCATATGCGGGTTGTAGCC	50	243 pb	Martin *et al*. (2006)
Bacteriocin 31	CCTACGTATTACGGAAATGGT GCCATGTTGTACCCAACCATT	50	122 pb	Du Toit *et al*. (2000)
Enterocin AS48	ATATTGTTAAATTACCAA GAGGAGTATCATGGTTAAAGA	50	185 pb	Du Toit *et al*. (2000)
Enterocin X	CCTCTTAATCATTAACCATAC GTTTCTGTAAAAGAGATGAAAC	50	500 pb	Edalatian *et al*. (2012)
Enterocin Q	GAAGAAATTTTTTCCCATGGC CTTCTTAAAAATGGTATCGCAA	55	95 pb	Citti *et al*. (2002)
Mundticin KS	CTACGGTAATGGAGTCTCATG CATCTGCATACAGGCTATACC	50	275 pb	Edalatian *et al*. (2012)

Table 2. Presence of enterocin structural genes in enterococcal strains.

Origin of Isolate	**Identification** PCR	**Strain**	**Structural genes of enterocins**[a]									
			*ent*A	*ent*B	*ent*P	*L50*A/B	*ent*1071	*ent*Q	*ent*KS	*ent*X	*ent*31	*AS48*
Food	*E. faecium*	Efm20	+	+	+	-	-	-	-	+	-	-
	E. faecium	Efm22	+	+	+	-	-	-	-	+	-	-
	E. faecium	Efm24	+	+	+	-	-	-	-	+	-	-
	E. faecium	Efm25	+	+	+	-	-	-	-	+	-	-
	E. faecalis	Efs27	+	+	+	-	-	-	-	+	-	-
Environment	*Enterococcus*	Ent5A	-	+	-	-	-	-	-	-	-	-
	Enterococcus	Ent5B	-	-	-	-	-	-	-	-	-	-
	Enterococcus	Ent5C	-	-	-	-	-	-	-	-	-	-
	Enterococcus	Ent12h	-	-	-	-	-	-	-	+	-	-
	E. faecalis	Efs18h	-	-	-	-	+	-	-	-	-	-
	Enterococcus	Ent22h	-	-	-	-	+	-	-	-	-	-
	Gene frequency		5	6	5	0	2	0	0	6	0	0

[a]Enterocin A, *ent*A; enterocin B, *ent*B; enterocin P, *ent*P; enterocin LB50A/B, *entL50*A/B; Enterocin 1071, *ent*1071; Enterocin Q, *ent*Q; Enterocin Mundticin, *ent*KS; Enterocin X, *ent*X; Enterocin 31, *ent*31 and Enterocin AS48, *ent*AS48. (+) indicates the presence of a gene, and (-) indicates the absence of a gene.

Table 3. Inhibitory spectrum of CFS from enterococci against indicator bacteria.

Indicator bacteria (34)/ origin	Cell-free supernatant						
	Food					Environment	
	E_{fs}20	E_{fs}22	E_{fs}24	E_{fs}25	E_{fs}27	E_{fs}18h	E_{nt}22h
ATCC							
L. innocua CLIP 12612	+	+	+	+	+	+	+
L. innocua CLIST 2050	+	+	+	+	+	+	+
L. innocua CLIST 2052	+	+	+	+	+	+	+
L. monocytogenes 2032	+	+	+	+	+	+	-
L. monocytogenes CLIST 2044	+	+	+	+	+	+	-
L. monocytogenes CLIST 2048	+	++	+	+	+	+	+
L. monocytogenes 2032	+	+	+	+	+	+	-
L. monocytogenes CDC 4555	-	-	-	-	-	-	-
L. ivanovii CLIST 2056	++	++	++	++	++	+	+
E. faecalis 29212	+	+	+	+	+	-	-
S. aureus 25925	-	-	-	-	-	-	-
S. aureus 6538	-	-	-	-	-	-	-
E. coli 49 LT	-	-	-	-	-	-	-
S. Typhimurium 14028	-	-	-	-	-	-	-
Clinical							
E. faecalis 10766	+	+	+	+	+	-	-
*Enterococcus*14524	+	+	+	+	+	+	+
E. faecium 4c	+	+	+	+	+	-	-
Water							
E. coli Lon 156	-	-	-	-	-	-	-
E. coli Lon 164	-	-	-	-	-	-	-
E. coli 2012 Rol 18	-	-	-	-	-	-	-
Dairy							
Bacillus sp	-	-	-	-	-	-	-
Legionella massiliensis	-	-	-	-	-	-	-
Bacillus licheniformes	-	-	-	-	-	-	-
Bacillus sporothermodurans	-	-	-	-	-	-	-
Bacillus pumilus	-	-	-	-	-	-	-
Bacillus circulans	-	-	-	-	-	-	-
Bacillus borstelensis	-	-	-	-	-	-	-
Bacillus subtilis	+	+	+	+	+	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-
Listeria sp	-	-	-	-	-	-	-
Bacillus cereus	-	-	-	-	-	-	-
Animal							
Staphylococcus sp	-	-	-	-	-	-	-
Staphylococcus sp	-	-	-	-	-	-	-
Klebsiella spp	-	-	-	-	-	-	-
Total	13	13	13	13	13	9	6

Inhibitory activity: (+) $2 \leq x \leq 10$; (++) $10 \leq x \leq 18$; no inhibitory activity: (-) $x \leq 1$

Table 4. Activity of enterocins in CFSs and partially purified CFSs.

Strain	**CFS (AU.ml^{-1})**		**CFS** precipitated with ammonium sulfate **(AU.ml^{-1})**	
	6 h	12 h	6 h	12 h
Efm20	3200	6400	6400	6400
Efm22	800	6400	6400	3200
Efm24	6400	6400	6400	6400
Efm25	800	3200	6400	3200
Efs27	6400	6400	6400	6400
Efs18h	800	1600	0	0
Ent22h	400	400	0	0

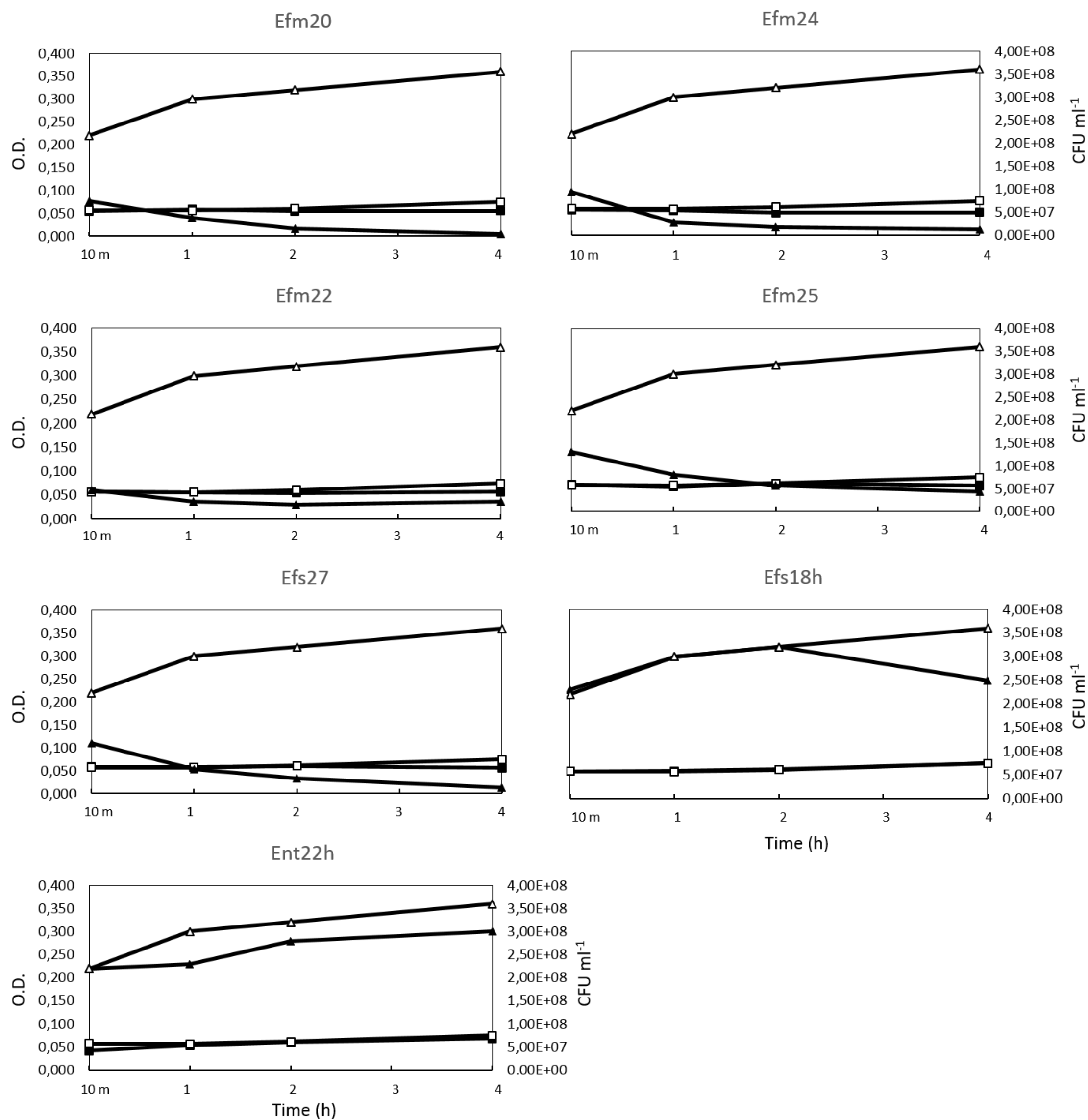

Figure 1. Mode of inhibition of cell-free supernatants precipitated with ammonium sulfate at an optical density (■, OD) of 600 nm and the number of viable cells (▲, UFC ml^{-1}) of *Listeria innocua* 12612. Open symbols correspond with the control.

ARTIGO 2

Inhibitory activity of enterocin produced by *Enterococcus* spp. against developing and preformed biofilms of *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria innocua*.

ABSTRACT

The biofilm forming ability of *Listeria* spp. is a concern to the food industry and health sectors. The aim of this study was to verify the inhibitory activity of cell-free supernatant (CFS) from four enterocin-producer enterococci (*Enterococcus faecium* 20, 22 and 24; and *Enterococccus faecalis* 27) on developing biofilm and preformed biofilm of *Listeria* species. Eight *Listeria* strains were evaluated for the production of biofilm on polystyrene surface at 30 and 37 ºC. Three strains exhibiting high biofilm formation ability, *L. monocytogenes* 2032, *L. innocua* 2050 and *L. ivanovii* 2056, were selected for the analysis of the inhibitory effect of CFS on biofilm biomass (crystal violet staining) and biofilm viability (quantified by XTT-reduction). The ability of *L. monocytogenes*, *L. ivanovii* and *L. innocua* to form biofilm at 30 °C and 37 °C was significantly reduced ($p<0.05$) in the presence of all CFS tested. Biomass of *Listeria* spp. preformed biofilm was also inhibited by the addition of all four CFS, where the highest reduction was observed for biofilm of *L. innocua* formed at 37 °C, with reduction of up to 90.5% (activity of CFS_{24}). CFS-containing enterocin of *E. faecium* 22 (CFS_{22}), *E. faecium* 24 (CFS_{24}) and *E. faecalis* 27 (CFS_{27}) were effective at decreasing the viability of developing biofilms and preformed biofilm of all three *Listeria* species. Concerning preformed biofilm, the highest reduction in viability was observed for *L.monocytogenes* (up to 98.7%). These data corroborated with data obtained from biofilm structure and presence of non-viable cells by labeling with propidium iodide. The enterocin activity found in CFS studied here may have the potential to prevent biofilm formation and to reduce cell viability of preformed biofilms of *Listeria* spp.

KEYWORDS: enterococci; anti-biofilm; *Listeria monocytogenes*; *Listeria ivanovii*; *Listeria innocua*.

Introduction

The genus *Listeria* is comprised of 17 species (Orsi and Wiedmann 2016) where two species are known to be pathogenic, *L. monocytogenes* and *L. ivanovii* (Orsi and Wiedmann 2016). *L. monocytogenes* is a food-borne pathogen involved in several listeriosis outbreaks, a severe human disease with high mortality rates among susceptible individuals (EFSA 2017). For this reason, *L. monocytogenes* is one of the major concerns in the food industry (Carpentier and Cerf 2011). *L. monocytogenes* can be found in a wide variety of raw and processed foods (Gandhi and Chikindas 2007; Ferreira et al. 2014; Buchanan et al. 2017). In Brazil, a descriptive data concerning a listeriosis outbreak suggested that a common source of contamination was placed in a hospital kicthen (Martins et al. 2010). *L. ivanovii* is known to cause disease predominately in ruminants, however, it has been considered a potential opportunistic pathogen of humans (Snapir et al. 2006; Guillet et al. 2010). Some strains of *L. ivanovii* exhibit ability to invade human epithelial cells comparable to *L. monocytogenes* whilst some wild-type isolates had higher invasion efficiencies than a clinical *L. monocytogenes* reference strain (Alvarez-Ordonez et al. 2015). The species *L. innocua*, originally considered a non-pathogenic *Listeria* species, has been shown to cause a fatal human disease (Perrin et al. 2003). Further, *L. innocua* has been shown to have ability to invade Caco-2 cells at same levels as *L. monocytogenes* (den Bakker et al. 2010), supporting that some *L. innocua* may be pathogenic to humans although at rare frequencies.

Along the food chain, apart from *L. monocytogenes*, that has received special research attention, *L. innocua* is the most frequently isolated *Listeria* species, being considered another important foodborne bacteria pathogen (Chambel et al. 2007; Gebretsadik et al. 2011). When *L. monocytogenes* is present in a food production environments it can persist for extended periods, presumably preserved by biofilms (Orgaz et al. 2013).

Biofilms are organized surface attached sessile communities of bacteria that can be found at practically any solid–liquid interface in industrial, environmental and clinical settings (Costerton et al. 1995). In food processing environments, the formation of biofilms on inert surfaces by microorganisms is of concern as it represents a potential source of contamination, leading to serious hygienic problems and economic losses due to food spoilage. For instance, the persistence of certain *L. monocytogenes* strains in processing equipment poses a major challenge for the food industry (Carpentier and Cerf

2011). The ability of *L. ivanovii* isolates to form biofilm is also a concern to the food industry (Nyenje et al. 2012). Further, biofilm formation by *Listeria* spp. has harmful consequences in medicine. For instance, the biofilm forming ability of *Listeria* spp. associated with human infections, makes them effective in colonizing surfaces within the host thus responsible for persistence infections (Osman et al. 2016).

Among the advantages to an organism forming a biofilm, including protection from disinfectants and antibiotics making them more difficult to be eliminated and treated. There are several evidences that biofilm mode of life of *L. monocytogenes* leads to increased resistance to sanitizers when compared to planktonic cells, making their elimination an enormous challenge (Ferreira et al. 2014, Buchanan et al. 2017; Camargo et al. 2018).

Several strategies to eliminate or control biofilms on the abiotic surface have emerged, such as the use of essential oils (Laird et al. 2012; Adukwu et al. 2012), extracts of mushrooms (Bolocan et al. 2017) and bacteriocins (Camargo et al. 2016). Lactic acid bacteria (LAB) and their bacteriocins have been reported for their antimicrobial activity against *L. monocytogenes* (Gálvez et al. 2007, Pimentel-Filho et al. 2013, 2014; Camargo et al. 2016). Bacteriocins are ribosomally synthesized peptides with antimicrobial activity, usually acting on the bacterial cytoplasmic membrane, by increasing its permeability (Gálvez et al. 2007). *Enterococcus* genus produce a great number of bacteriocins designed as enterocins.

Although there have been significant research advances concerning microbial biofilm, biofilm control is still an open and central research field, particularly control of *Listeria* spp. biofilms. Thus, the aim of this study was to verify the inhibitory activity of cell-free supernatant (CFS) of enterococci on developing and preformed biofilm of *Listeria* spp., including *L. innocua* and *L. ivanovii* that are largely unexplored.

Materials and Methods

Bacterial isolates and growth conditions

Four enterococci isolates denoted Ent+ (*Enterococcus faecium* 20, 22 and 24; and *Enterococcus faecalis* 27) previously described as bacteriocin-producing isolates, harboring the enterocin-encoding genes, with listerial activity against *Listeria* spp.

(unpublished data) were used in this study. As indicator bacteria we utilized eight strains (*Listeria innocua* CLIP 12612, 2050, 2052*; Listeria monocytogenes* 2032, 2048, 2044, 4555 and *Listeria. ivanovii* 2056). The isolates belong to the Laboratory of Basic and Applied Microbiology of Federal Technological University of Paraná-Londrina, Brazil. Stock cultures were maintained at -20°C in BHI (Brain Heart Infusion) (Acumedia-Neogen) broth supplemented with 20% (v/v) glycerol (Gibco). Fresh cultures were prepared by inoculation of 20 µl of the frozen stock into 3 ml of BHI broth incubated for 18 to 24 h at 37°C.

Preparation of cell-free supernatant (CFS)

Ent+ isolates were cultured in Man-Rogosa-Sharpe (MRS) (Acumedia- Neogen) broth medium at 37 ºC for 18 h. Cultures were adjusted to 0.5 McFarland standard in MRS broth at pH 6.2 following incubation at 37 ºC, 180 rpm for 24 h. The respective CFS was obtained by centrifugation at 12,000xg for 15 min, neutralization with 1 M NaOH to a pH of 6.5, and filtered through a 0.22-µm filter. The CFS was stored at -20˚C for further use.

Biofilm Formation Assay

All eight indicator bacteria (*L. innocua* 12612, 2050, 2052*; L. monocytogenes* 2032, 2048, 2044, 4555 and *L. ivanovii* 2056) were tested for biofilm-forming ability on polystyrene surface. The experiments were performed in BHI broth, according to Camargo et al. (2016). The overnight cultures of bacteria were adjusted to 0.5 McFarland standard and 200 µL transferred to a 96- well polystyrene microtiter. After incubation of 24 h, at 30 ºC and at 37 ºC, culture media were discarded, and the wells were washed with NaCl (0.85%) three times to remove non-attached cells. Cells were fixed by addition of 200 µL of methanol (Synth) for 15 min. Then, crystal viotet (1.0% solution) was added and after 15 min, the plates were washed with NaCl (0.85%). After drying, 33% acetic acid for 20 min was added to solubilize the crystal violet. The absorbance was measured at 600 nm ($O.D._{600nm}$). For each isolate, the experiment was performed in triplicate with three replicate wells per microtitre plate. Isolates that exhibit the highest biofilm biomass, e.g., values of $O.D._{600nm} > 0.4$, at both tested temperatures, were selected for further experiments.

Activity of CFS from Ent+ enterococci isolates on biofilm formation by *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria innocua*

The activity of supernatants on biofilm formation was quantified in a microtiter plate assay. Briefly, overnight culture of the indicator bacteria were adjusted to 0.5 McFarland standard in BHI and 100 µL transferred to a 96- well polystyrene microtitre with addition of 100 µL of CFS of each *Enterococcus* isolates in each well. For the control, 100 µL of MRS broth was added to each well. Plates were incubated at 30 ºC and at 37 ºC for 24 h. The inhibitory activity of CFS from enterococci on developing biofilm was determined based on determination of biofilm biomass. For this, after incubation culture media were discarded, and the wells were washed with NaCl (0.85%) three times to remove non-attached cells. Cells were fixed by addition of 200 µL of methanol (Synth) for 15 min. Then, crystal viotet (1.0% solution) was added and after 15 min, the plates were washed with NaCl (0.85%). After drying, 33% acetic acid for 20 min was added to solubilize the crystal violet. The absorbance was measured at 600 nm ($O.D._{600nm}$).

Anti-biofilm activity was also measured based on biofilm viability, by the employment of the method based on the reduction of the tetrazolium salt XTT [2,3-bis (2-methyloxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxaniline] by dehydrogenase enzymes of metabolic active cells in biofilms, according to Alonso et al. (2017). Briefly, a final volume of 10 ml of a premixed solution of XTT/menadione in proportion 10 ml/1 µL was prepared before each experiment. Then, 100 µL were added to each well, incubated in dark at 37 °C for 3h and the O.D. measured at 490 nm in a spectrophotometer (Biotek EL808).

Anti-biofilm activity was presented as percentage of biofilm reduction compared to control (biofilm without CFS exposure). For each strain, the experiment was performed in triplicate with three replicate wells per microtitre plate.

Activity of CFS from Ent+ enterococci isolates on preformed biofilm of *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria innocua*

For this, the biofilm was formed at the same temperature and incubation time as describe above. After biofilm formation, the culture media were discarded and 200 µL of CFS of each *Enterococcus* isolates was added, followed incubation for 24 h, at the respective biofilm formation temperatures.

The inhibitory activity of CFS from enterococci on preformed biofilm was determined based on biofilm biomass and biofilm viability as described above. Anti-biofilm activity

was presented as percentage of biofilm reduction compared to control (biofilm without CFS exposure). For each isolate, the experiment was performed in triplicate with three replicate wells per microtitre plate.

Fluorescence microscopy analysis

Fluorescence microscopy analysis was done to observe cell death of the *Listeria* spp. following addition of CFS-containing enterocin on developing biofilm and on preformed biofilm on coverslips (13mm). The coverslips were washed twice with PBS and incubated for 15 min at room temperature in the dark with 30 μg/ml of fluorophores propidium iodide (PI) (Sigma-Aldrich, Germany) in PBS, as described elsewhere (Johson and Criss, 2013). After incubation the staining solution was aspirated, the coverslips were washed in PBS and biofilms were observed using an epifluorescence microscope (Zeiss, Germany). The cells with reddish color were considered not viable.

Statistical analysis

Data were evaluated using one-way ANOVA and Tukey's test considering $p < 0.05$, to establish significant differences between the antimicrobial activity of the isolates and the control.

Results

Biofilm Formation Assay

The biofilm forming capacity was confirmed in all *Listeria* spp. isolates tested on the polystyrene microtiter plates at both incubation temperatures 30 ºC and 37 ºC (Figure 1). Tree isolates, *L. monocytogenes* 2032, *L. ivanovii* 2056 and *L. innocua* 2050, showed higher total biomass ($O.D._{600nm} > 0.4$) at both temperatures, compared to others. Based on the results obtained, these isolates were selected for the analysis of anti-biofilm activity of CFS-containing enterocin.

Reduction of total biomass of developing biofilm and preformed biofilm of *Listeria* spp. exposure to CFS-containing enterocin produced by of *Enterococcus* spp. (Ent+ isolates)

Cell-free supernatant from four enterocin-producer enterococci, *E. faecium* 20 (CFS20), *E. faecium* 22 (CFS22), *E. faecium* 24 (CFS24) and *E. faecalis* 27 (CFS27) were tested against developing biofilm and preformed biofilm of the selected *Listeria* spp. indicator bacteria.

The ability of *L. monocytogenes*, *L. ivanovii* and *L. innocua* to form biofilm was significantly reduced ($p<0.05$) in the presence of all CFS at both incubation temperatures (30 °C and 37 °C) compared to control biofilm formation (Table 1). The lowest reduction of biofilm biomass was observed for *L. innocua* 2050 with reduction percentage of 51.4% and 46.7%, at 30 °C and 37 °C, respectively. The largest reduction of biofilm formation was shown for *L. ivanovii* and *L. monocytogenes* at both temperatures (Table 1). Further, for *L. innocua*, the CFS from *E. faecium* 22 (CFS_{22}) exhibited higher inhibition of biofilm formation at 30 °C (85.1%) while the CFS from *E. faecium* 24 (CFS_{24}) showed higher inhibition of biofilm formation at 37 °C (66.3%) compared to others CFS tested.

Biomass of *Listeria* spp. preformed biofilm was inhibited by the addition of all four CFS producing enterococci, however at distinct levels (Table 1). The highest reduction of biofilm biomass was observed for biofilm formed at 37 °C, with reduction of up to 90.5% (activity of CFS_{24} against *L. innocua*). On the other hand, for biofilm formed at 30 °C, the inhibitory activity was variable. The lowest reduction of biofilm biomass was observed for *L. ivanovii*, compared to others *Listeria* species. Of note, cell-free culture supernatant of *E. faecium* 22 showed a significant ($p<0.05$) inhibition of *L. ivanovii* preformed biofilm (51.6%) compared to others CFS tested. Interesting, significant ($p<0.05$) inhibition (81.4%) was also observed for biofilm formed at 37 °C (Table 1).

Effect of CFS-containing enterocin produced by *Enterococcus* spp. (Ent+ isolates) on viability of developing biofilm and preformed biofilm of *Listeria* spp.

CFS-containing enterocin of *E. faecium* 22 (CFS_{22}), *E. faecium* 24 (CFS_{24}) and *E. faecalis* 27 (CFS_{27}) were effective at decreasing the viability (metabolic activity) of developing biofilms and formed biofilm of all three *Listeria* species tested (Figure 2A, B). In general, CFS of *E. faecium* 20 (CFS_{20}) was less effective in reduction viability of listerial biofilms.

As shown in Figure 2A, for *L.monocytogenes* the CFS from *E. faecium* 22 (CFS_{22}) and the CFS from *E. faecalis* 27 (CFS_{27}) exhibited higher reduction ($p<0.05$) in cells viability during biofilm formation at 30 °C (54.4%) and at 37 °C (77.2%),respectively, compared

to others CFS tested. Overall, highest reduction in viability was observed for preformed *L.monocytogenes* that reached up to 98.7% (Figure 2B).

L.ivanovii biofilm viability was less affected by CFS exposure during biofilm formation compared to others *Listeria* species (Figure 2A). Besides, the CFS from *E. faecium* 22 (CFS_{22}) exhibited higher reduction in cells viability during biofilm formation at 37 °C (33.3%), while the addition of CFS_{20} had none effect on biofilm viability (Figure 2A). Reduction in viability was high for preformed *L.ivanovii* biofilm that reached up to 75% (Figure 2B).

CFS-containing enterocin from *E. faecium* 20 (CFS_{20}), *E. faecium* 22 (CFS_{22}), *E. faecium* 24 (CFS_{24}) and *E. faecalis* 27 (CFS_{27}) were high effective at decreasing the viability of developing biofilms of *L. innocua* (Figure 2A). Differently, viability reduction was less expressive of cells from preformed biofilm (Figure 2B).

Fluorescence microscopy studies

The images obtained by the fluorescence microscopy confirmed the reducing viability of development biofilm and preformed biofilm of *Listeria* spp. exposed to CFS-containing enterocin (Fig. 3A, B). In the absence of enterocin (control) it can be observed a reduced amount of dead cells (reddish color) compared to treated biofilms (Fig. 3A,B).

The addition of CFS in developing biofilm resulted in a biofilm structure composed of sparsed cells compared to control biofilm (absence of CFS) (Fig. 3A). While the addition of CFS on preformed biofilm resulted in grouping of cells and a larger number of dead cells within the biofilm, mainly for *L. monocytogenes* and *L. ivanovii* (Fig. 3B).

Discussion

Listeria spp. have a considerable economic impact for society and the food industry. Biofilm formation by some *Listeria* species is one of the main problems for disinfection in the food industry and in other industrial and health sectors. For instance, *L. monocytogenes* mature biofilm can act as a source of contamination for food products due to the detachment of biofilm cells from the biofilm surface (Poimenidou et al. 2009).

It is known that bacteriocins are “friendly” antimicrobial agents, because they do not have any known associated public health risk (Camargo et al. 2018). For example, nisin is a bacteriocin produced by *Lactococcus lactis* strains and is approved for use in food

preservation in many countries; however, several other bacteriocins have shown the potential for future applications in food systems. It has been used as a preservative in the dairy and meat industries to control pathogens such as *L. monocytogenes* (Gandhi and Chikindas, 2007).

Highlighting the potential of bacteriocins against biofilms, recent studies have evaluated the effectiveness of enterocins against *L. monocytogenes* biofilms (Camargo et al. 2018); differently, few studies are found regarding *L. ivanovii* and *L. innocua*. Anti-biofilm activities of several bacteriocins against *L. monocytogenes* were recently assessed (Bolocan et al. 2017). According to these authors, bacteriocins were effective at inhibiting formation of *L. monocytogenes* biofilms and decreasing the viability of biofilms already formed (Bolocan et al. 2017).

The *Enterococcus* spp. Ent+ isolates used in this study for production of cell-free supernatants harbor enterocin encoding genes, including the enterocin of class IIa that has specific activity against *Listeria* spp. Further, the anti-listerial substances in CFS obtained from these enterococci isolates were previously characterized as exhibiting proteinaceous nature with assigned them most probably to bacteriocins, named enterocins (unpublished data). It is interesting to emphasize that bacteriocins with anti-listerial activity, could open new possibilities to prevent adhesion and thus, to control biofilm formation. For this reason, in the present study we evaluate whether CFS-containing enterocin were capable of preventing *Listeria* spp. biofilm formation as well as the inhibitory effect on preformed biofilms.

Overall, the reduction of biofilm biomass conferred by CFS-containg enterocin was more pronounced for development biofilm compared to that observed for preformed biofilm of *Listeria* species (Table 1). Thus, our data showed that CFS-containing enterocin were effective at inhibiting formation of biofilms of *L. monocytogenes*, *L. ivanovii* and *L. innocua*. Besides, our data also showed that CFS-containing enterocin were effective at decreasing the viability of developing biofilms and preformed biofilm of all three *Listeria* species tested (Fig. 2A,B).

Caballero Gómez et al. (2012) had previously described the reduction of *L. monocytogenes* adhesion and biofilm production by the enterocin AS-48. Winkelströter et al. (2011) observed the reduction of the adhesion of *Listeria* sp. to the stainless steel surface with co-culture with *Lactococcus sakei* 1 and its CFS containing sakacina 1.

Camargo et al. (2016) showed the capacity of CFS containing bacteriocins from *E. faecium* to prevent *L. monocytogenes* biofilm formation by inhibition; however, the CFS failed to eliminate biofilms already formed.

Studies with enterococcal CFS-containing enterocins or purified enterocin against listerial biofilms have been restricted to *L. monocytogenes* species (Caballero Gómez et al. 2012; Camargo et al. 2016; Al Atya et al. 2016). Our data revealed promising results regarding CFS-containing enterocins against *L. innocua*, another important foodborne bacteria, and the ruminants pathogen *L. ivanovii*. Of note, CFS_{22} showed a significant inhibition of biomass of preformed biofilm of *L. ivanovii*, compared to others CFS tested, suggesting that the enterocin produced by *E. faecium* 22 may have potential to control *L. ivanovii* biofilm formed at two temperatures 30 °C and 37 °C.

Decrease in biofilm viability had correlation with total biomass decrease, particularly for preformed biofilm. These findings were confirmed by fluorescence microscopy images which revealed poor biofilm formation, also characterized by the presence of dead cells. In contrast, biofilm formed without CFS was dense and with greater presence of cell viability.

In general, concerning the structure of the biofilm, the reduction of the *Listeria* biofilm caused by the addition of CFS before and after biofilm formation, revealed more sparse cells and a cell grouping, respectively. Pimentel- Filho et al. (2014) reported that bacteriocins change the hydrophobicity of polystyrene surface, indicating a difficulty for the bacteria to get in close contact with the surface. Thus, bacteriocin appears to reduce the size of its structure, exerting an effect on the disintegration of the biofilm cells.

Conclusions

All of the enterocins produced by *Enterococcus* isolates inhibited biofilm production by *L. monocytogenes*, *L. ivanovii* and *L. innocua* according to the observed inhibition of developing biofilm and preformed biofilm. For pathogenic species *L.monocytogenes* and *L.ivanovii* the effect of CFS-containing enterocin on viability was more pronounced on preformed biofilm compared to that observed on developing biofilm. The reduction of *Listeria* spp. biofilm on the abiotic surface may be a potential approach to mitigate the contamination of industrial facilities. In fact, the results of this study contribute to the

enrichment of the literature data and may have important implications for the development and implementation of new strategies of control of *Listeria* biofilm development.

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Disclosure statement

The authors declare that they have no conflict of interest.

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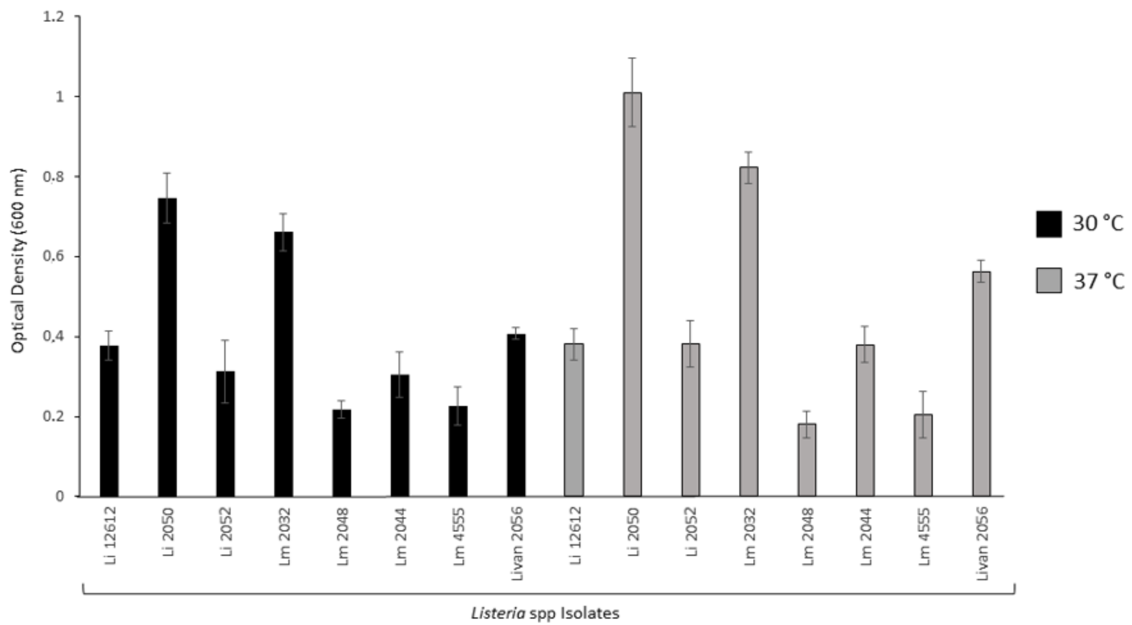

Fig. 1 Total biofilm biomass of *Listeria* spp. on polystyrene surface. Li12612, *Listeria innocua* CLIP 12612; Li2050, *Listeria innocua* 2050; Li2052, *Listeria innocua* 2052; Lm 2032, *Listeria monocytogenes* 2032; Lm 2048, *Listeria monocytogenes* 2048; Lm 2044, *Listeria monocytogenes* 2044; Lm 4555, *Listeria monocytogenes* 4555, Livan 2056, *Listeria ivanovii* 2056. Bars represent the mean and standard deviations of three independent experiments.

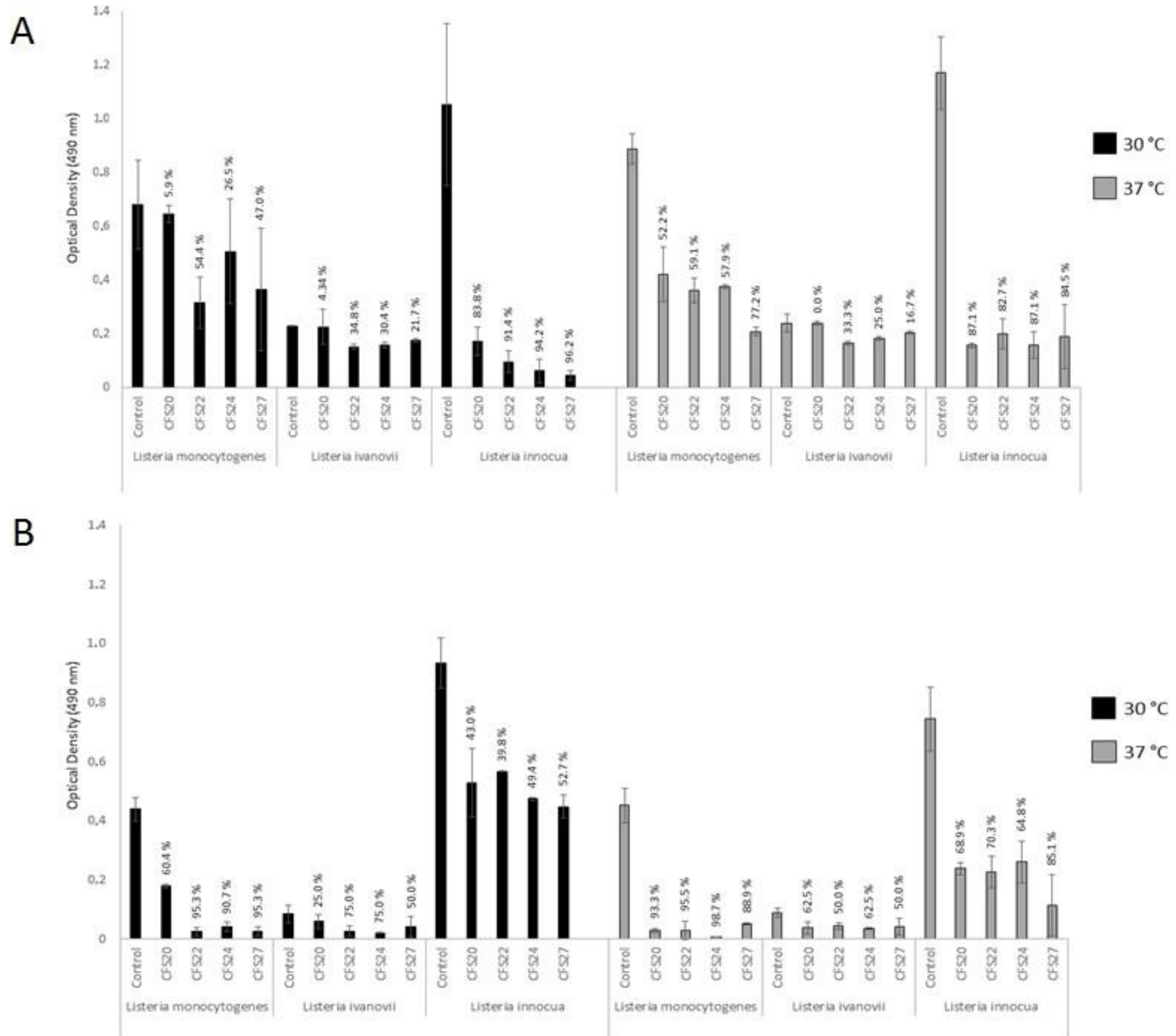

Fig. 2 Effect of cell-free supernatant (CFS) from *E. faecium* 20 (CFS20), *E. faecium* 22 (CFS22), *E. faecium* 24 (CFS24) and *E. faecalis* 27 (CFS27) on developing biofilm (A) and preformed biofilm (B) cells viability (metabolic activity). Bars represent the standard deviations of three independent experiments.

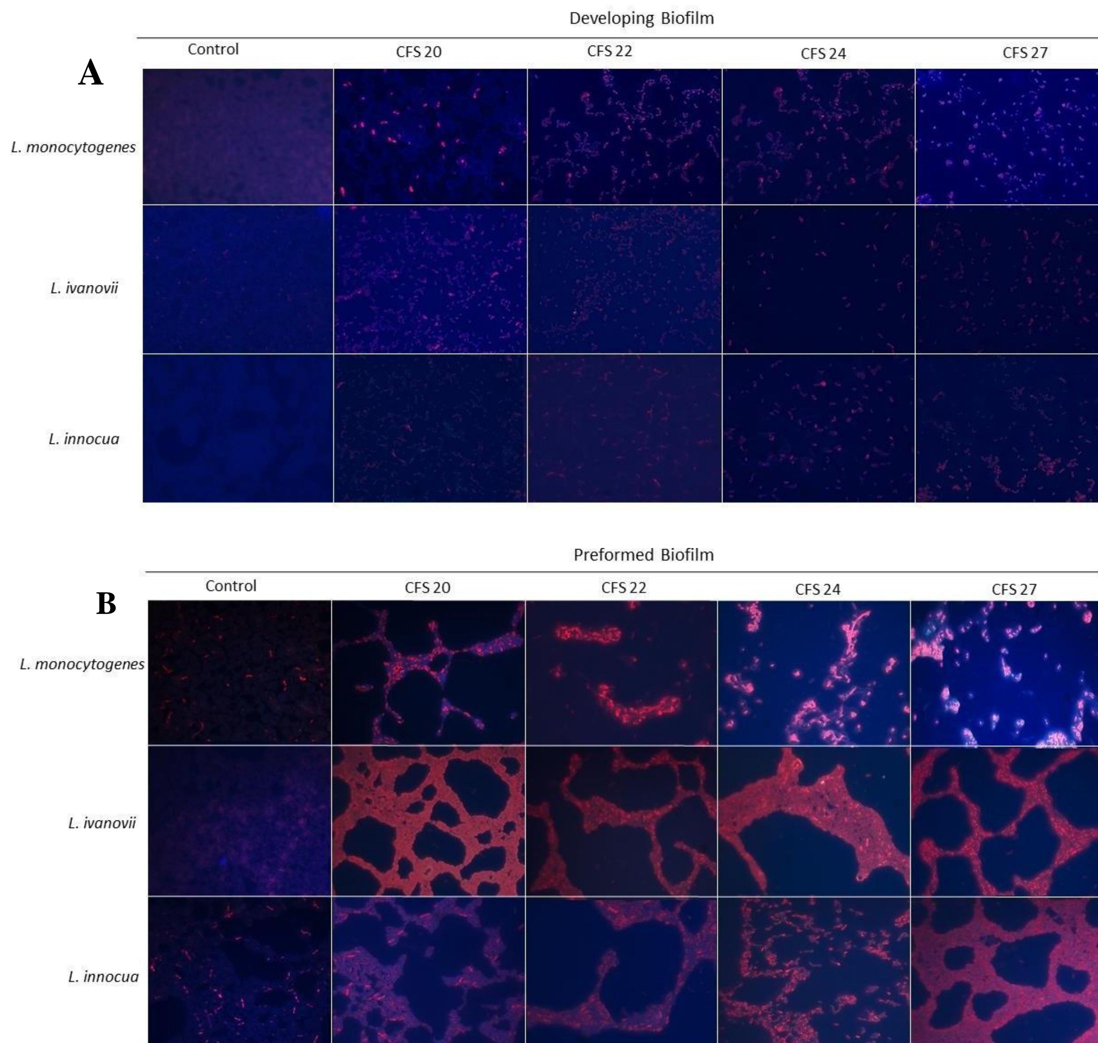

Fig. 3 Fluorescence microscopy images 1000x. Anti-biofilm activity of cell-free supernatant (CFS). (A) developing biofilm, (B) preformed biofilm, stained with propidium iodate. *L. monocytogenes* 2032, *L.ivanovii* 2056 and *L. innocua* 2050; Control; CFS20; CFS22; CFS24 and CFS27. The reddish fluorescence labelled cells with damaged membranes.

Table 1. Effect of cell-free supernatant (CFS) of enterococci on biofilm formation and preformed biofilm of *Listeria monocytogenes, Listeria ivanovii* and *Listeria innocua* on polystyrene surfaces expressed as total biomass.

Strain/CFS	biofilm formation				preformed biofilm			
	30ºC		37ºC		30ºC		37ºC	
	Control/test	Reduction	Control/test	Reduction	Control/test	Reduction	Control/test	Reduction
L.mono	0.146 ± 0.011a		0.178 ± 0.079a		0.453 ± 0.145a		0.365 ± 0.161a	
CFS_{20}	0.043 ± 0.015b	70.5%	0.053 ± 0.015b	70.2%	0.190 ± 0.063b	58.1%	0.099 ± 0.000b	72.8%
CFS_{22}	0.029 ± 0.021b	80.1%	0.023 ± 0.005b	87.1%	0.155 ± 0.060b	65.8%	0.080 ± 0.045b	78.1%
CFS_{24}	0.038 ± 0.008b	74.0%	0.040 ± 0.012b	77.5%	0.143 ± 0.046b	68.4%	0.132 ± 0.025b	63.8%
CFS_{27}	0.052 ± 0.027b	64.4%	0.064 ± 0.022b	64.0%	0.097 ± 0.015b	78.6%	0.126 ± 0.018b	63.5%
L.ivanovii	0.223 ± 0.032a		0.396 ± 0.140a		0.289 ± 0.029a		0.543 ± 0.059a	
CFS_{20}	0.034 ± 0.020b	84,7%	0.088 ± 0.052b	77.8%	0.249 ± 0.019ab	13.8%	0.187 ± 0.004b	65.6%
CFS_{22}	0.028 ± 0.022b	87.4%	0.572 ± 0.024b	85.6%	0.140 ± 0.010c	51.6%	0.101 ± 0.022c	81.4%
CFS_{24}	0.033 ± 0.026b	85.2%	0.058 ± 0.031b	85.3%	0.221 ± 0.043ab	23.5%	0.142 ± 0.005bc	73.8%
CFS_{27}	0.043 ± 0.044b	80.7%	0.096 ± 0.045b	75.7%	0.213 ± 0.024b	26.3%	0.142 ± 0.039bc	73.8%
*Li*2050	1.125 ± 0.244a		2.008 ± 0.119a		0.691 ± 0.012a		1.159 ± 0.106a	
CFS_{20}	0.547 ± 0.098b	51.4%	0.872 ± 0.170bc	56.6%	0.311 ± 0.040b	55.0%	0.142 ± 0.033b	87.7%
CFS_{22}	0.168 ± 0.029c	85.1%	1.070 ± 0.242b	46.7%	0.385 ± 0.043b	44.3%	0.161 ± 0.053b	86.1%
CFS_{24}	0.484 ± 0.124bc	56.9%	0.676 ± 0.217c	66.3%	0.349 ± 0.117b	49.5%	0.110 ± 0.027b	90.5%
CFS_{27}	0.266 ± 0.07bc	76.3%	0.708 ± 0.184bc	64.7%	0.400 ± 0.048b	42.1%	0.180 ± 0.056b	84.5%

L.monoc (*Listeria monocytogenes* 2032); *L.ivanovii* (*Listeria ivanovii* 2056); Li2050 (*Listeria innocua* 2050). *E. faecium* 20 (CFS20), *E. faecium* 22 (CFS22), *E. faecium* 24 (CFS24) and *E. faecalis* 27 (CFS27). Mean ±standard deviations of three independent experiments. Means followed by the same letter in each column are not significantly different according to Tukey test ($p < 0.05$).

4. CONCLUSÃO

Os enterococos selecionados para a produção de bacteriocinas apresentaram genes estruturais para enterocinas, bem como não apresentaram atividade hemolítica. Ao caracterizar os sobrenadantes livre de células (CFS) de enterococos contra *Listeria innocua* 12612 foi possível determinar que o composto antibacteriano ocorreu pela presença de enterocinas e de forma bactericida. Nos CFS de enterococos que abrigaram maiores quantidades de genes codificadores de enterocinas resultaram em maiores valores de unidade arbitrária e espectro de atividade contra o crescimento de bactérias patogênicas de alimento, bem como de isolados clínicos. Esses dados sugerem que a maior quantidade de genes estruturais de enterocinas esteja relacionada com a maior atividade antibacteriana. A atividade antibacteriana dos CFS contendo enterocina mostrou uma maior ação contra *Listeria* spp., importante patógenos alimentar. Além disso, foi possível observar que esses CFS contendo enterocina apresentaram atividade anti-biofilme de três espécies de *Listeria* (*Listeria monocytogenes, Listeria ivanovii* e *Listeria innocua)* tanto no desenvolvimento, quanto no biofilme pré-formado em superfícies abióticas, bem como reduziu a quantidade de células viáveis. Esses dados contribuem para o enriquecimento da literatura, uma vez que os enterococos aqui estudados apresentam-se como potenciais candidatos para sua aplicação no contexto alimentícios e farmacêutico, bem como possibilitam novas estratégias no controle de biofilme de *Listeria* spp. na indústria alimentícia, uma vez que o biofilme dessas bactérias em processamento de alimento apresenta um grande risco para a saúde pública.