VALÉRIA QUINTANA CAVICCHIOLI

CHARACTERIZATION OF BACTERIOCINOGENIC *Enterococcus hirae* AND *Pediococcus pentosaceus* ISOLATED FROM ARTISANAL CHEESE AND THEIR BACTERIOCINS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 26 de julho de 2018.

Luana Martins Perin

Luciano dos Santos Bersot

Svetoslav Dimitrov Todorov
(Coorientador)

Hilário Cuquetto Mantovani

Luís Augusto Nero
(Orientador)

“Sua tarefa é descobrir o seu trabalho e, então, com todo o coração, dedicar-se a ele”.

Buda

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CONTEÚDO

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

CAPÍTULO 4

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

CAPÍTULO 1

CAPÍTULO 3

CAPÍTULO 4

RESUMO

CAVICCHIOLI, Valéria Quintana, D.Sc., Universidade Federal de Viçosa, julho de 2018. **Caracterização dos isolados bacteriocinogênicos *Enterococcus hirae* e *Pediococcus pentosaceus* obtidos de queijo artesanal e suas bacteriocinas.** Orientador: Luís Augusto Nero. Coorientador: Svetoslav Dimitrov Todorov.

Os produtos lácteos possuem uma microbiota autóctone bastante diversificada, na qual o grupo das Bactérias Ácido Lácticas (BAL) é de notável relevância devido às suas características benéficas, tecnológicas e bioconservantes, atraindo o interesse para sua utilização em diversos segmentos biotecnológicos, em especial na indústria de alimentos. O objetivo deste trabalho foi isolar e identificar BAL bacteriocinogênicas de queijos artesanais, caracterizando aspectos ligados à produção e purificação das bacteriocinas, inocuidade, potencial benéfico dos isolados e propriedades inibitórias contra *Listeria* spp. As cepas bacteriocinogênicas *Enterococcus hirae* ST57ACC e *Pediococcus pentosaceus* ST65ACC foram isoladas a partir da técnica de tripla camada e identificadas por metodologias fenotípicas e moleculares. As bacteriocinas produzidas por *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC demostraram estabilidade em ampla faixa de pH e temperatura, e foram inativadas após tratamento com enzimas proteolíticas, comprovando sua natureza proteica. Tratamentos com EDTA, SDS, NaCl e Tween 80 não afetaram a atividade das bacteriocinas. Os sobrenadantes de ambos os isolados foram capazes de inibir *Listeria innocua* e diversas cepas de *L. monocytogenes* pertencentes à diferentes sorogrupos e obtidas de fontes distintas, inibindo completamente o desenvolvimento de *L. monocytogenes* após 12 h. Em co-culturas das cepas bacteriocinogênicas com a cepa indicadora *L. monocytogenes* 422 em leite desnatado, observou-se que *E. hirae* ST57ACC foi capaz de controlar a multiplicação do patógeno após 48 h. *E. hirae* ST57ACC e *P. pentosaceus* não apresentaram resultados positivos para 25 genes relacionados a bacteriocinas conhecidas, indicando que podem produzir novas bacteriocinas. As cepas de *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC foram também avaliadas quanto ao seu potencial benéfico e segurança: ambos os isolados permaneceram viáveis após tratamento em condições gastrointestinais simuladas, exibindo altos níveis de auto e co-agregação com *L. monocytogenes* e níveis variados de hidrofobicidade, demonstrando que *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC podem prevenir potencialmente o estabelecimento de infecções pelo patógeno. Por meio da metodologia de agar-spot, avaliou-se a possibilidade de

interferência de 33 medicamentos comerciais, de diferentes grupos sobre a multiplicação de *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC, revelando que apenas antiinflamatórios e medicamentos contendo loratadina e cloridrato de propranolol apresentaram atividade inibitória sobre as cepas. Testes fenotípicos para determinação da susceptibilidade antimicrobiana demonstraram que *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC foram resistentes à vancomicina, oxacilina e sulfa/trimetoprim dentre os 11 antibióticos testados pelo método de disco difusão. Com relação à PCR, poucos genes relacionados à resistência a antibióticos foi foram identificados. Nenhum dos isolados amplificou genes de produção de aminas biogênicas e nem apresentou produção das mesmas. A expressão de diferentes elementos do sistema de transporte ABC e metabolismo de açúcares foi identificada para ambos os isolados. Variações na proporção de inóculo não influenciaram a taxa de multiplicação de *E. hirae* ST57ACC nem de *P. pentosaceus* ST65ACC, no entanto, a produção de bacteriocinas foi detectada apenas 9 horas após a inoculação das cepas, quando inoculadas nas proporções de 5% e 10%. Adicionalmente, verificou-se que a densidade celular das cepas bacteriocinogênicas esteve correlacionada à produção de bacteriocinas em sistemas de fermentação tradicional e fermentação com controle de pH a 5,5 e agitação. *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC foram capazes de se multiplicar e produzir bacteriocinas na presença de xilo-oligossacarídeos após 6 horas de incubação, porém em níveis reduzidos quando comparados ao cultivo em meio MRS. Por fim, as bacteriocinas produzidas por *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC foram purificadas a partir de diferentes metodologias. A bacteriocina produzida por *P. pentosaceus* ST65ACC foi purificada em duas etapas, com rendimento final de 101,33 revelando-se um peptídeo com massa molecular de 3,5 a 8,5 kDa, determinado por SDS-PAGE. Em contrapartida, um protocolo de três etapas foi empregado na purificação da bacteriocina produzida por *E. hirae* ST57ACC, com rendimento final de 3,05. Adicionalmente, uma fração semi-purificada foi testada com a linhagem celular HT-29, demonstrando que a bacteriocina não apresenta efeitos citotóxicos contra células humanas, sendo considerada segura neste aspecto. Os dados obtidos neste trabalho indicam que os isolados *E. hirae* ST57ACC e *P. pentosaceus* ST57ACC podem ser considerados importantes ferramentas biotecnológicas na produção de bacteriocinas de interesse ao controle de *L. monocytogenes* e na biopreservação de alimentos.

ABSTRACT

CAVICCHIOLI, Valéria Quintana, D.Sc., Universidade Federal de Viçosa, July, 2018. **Characterization of bacteriocinogenic *Enterococcus hirae* and *Pediococcus pentosaceus* isolated from artisanal cheese and their bacteriocins.** Advisor: Luís Augusto Nero. Co-advisor: Svetoslav Dimitrov Todorov.

Dairy products present a rich and diverse autochthonous microbiota, in which Lactic Acid Bacteria (LAB) are relevant, due to their beneficial, technological and biopreservative features, attracting the interest for their biotechnological application, in food industry, pharmaceutic area and human and veterinary medicine fields. The aim of this study was to isolate and to identify bacteriocinogenic LAB from artisanal cheeses, characterizing some aspects linked to bacteriocin production and purification, safety and beneficial potential of the isolates, as well as their inhibitory properties against *Listeria* spp. Bacteriocinogenic strains *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC were isolated by using the triple-layer technique and identified by phenotypical and molecular methods. Bacteriocins produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were stable in a wide range of pH and temperature, losing their activity after treatment with proteolytic enzymes, confirming their proteinaceous nature. Treatments with EDTA, SDS, NaCl and Tween 80 did not affect bacteriocin activity. Cell-free supernatants from both isolates were able to inhibit *Listeria innocua* and several *L. monocytogenes* strains, from different serogroups obtained from diverse sources, eliminating *L. monocytogenes* after 12 h. In co-culture experiments conducted in skimmed milk with the bacteriocinogenic isolates and the target strain *L. monocytogenes* 422, *E. hirae* ST57ACC controlled the target strain growth after 48 h. *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC did not present positive results for 25 known bacteriocin related genes, indicating that they might express new bacteriocins. *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC were also evaluated for their beneficial and safety features: both isolates remained viable after treatment replicating gastrointestinal conditions, showing high levels of auto and co-aggregation with *L. monocytogenes* and diverse levels of hydrophobicity, demonstrating that *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC might prevent the establishment of infections caused by this pathogen. Interference of 33 commercial drugs from different groups on growth of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC was tested by agar-spot method, revealing that only anti-inflammatories and drugs

containing loratadine and propranolol hydrochloride influenced the growth of bacteriocinogenic strains. Phenotypical tests employed to determine antibiotic susceptibility have shown that *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were resistant to vancomycin, oxacillin and sulfa/trimethoprim out of 11 antibiotics tested by disk-diffusion test, nonetheless low number of antibiotic resistance genes was observed by PCR analysis. None of the isolates amplified biogenic amines encoding genes neither presented phenotypical evidence of their production. Expression of different ABC transporters linked to bacteriocin export and sugar metabolism was detected, for both isolates. Changes in inoculum size did not influenced the growth of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC; however, bacteriocin production was affected, and bacteriocins were detected only after 9 h with inoculation at 5% and 10% of bacteriocinogenic strains. Additionally, it was observed that cell density of both bacteriocinogenic strains was linked to bacteriocin production in traditional and pH at 5.5 and agitation controlled fermentation continuous. *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were capable to grow and produce bacteriocins in the presence of xylo-oligossacharides after 6 h of incubation, but in lower levels than those obtained with cultivation in MRS broth. Finally, *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were purified from different methods. The bacteriocin produced by *P. pentosaceus* ST65ACC was purified in two-steps, with final yield of 101.33, recognized as a 3.5 to 8.5 kDa peptide, determined by Tricine-SDS-PAGE. In contrast, a three-step-protocol was used to purify the bacteriocin produced by *E. hirae* ST57ACC, with final yield of 3.05. Moreover, a semi-purified fraction of *E. hirae* ST57ACC bacteriocin was tested in HT-29 cell-line, demonstrating no-cytotoxic effects in human cells, which means the bacteriocin can be considered safe in this aspect. Obtained data from this study indicate that *E. hirae* ST57ACC and *P. pentosaceus* ST57ACC may be considered as important biotechnological tools for bacteriocin production to control *L. monocytogenes* and as biopreservatives in food.

INTRODUÇÃO

Os produtos de origem animal possuem uma microbiota autóctone bastante diversificada, composta por micro-organismos provenientes dos próprios animais e do ambiente de produção. Bactérias Ácido Lácticas (BAL) representam um grupo importante dessa microbiota e apresentam grande relevância para a indústria de alimentos devido ao seu metabolismo particular, que permite o desenvolvimento de efeitos benéficos e tecnológicos interessantes à sua utilização. Neste sentido, os produtos de origem animal representam uma notável fonte na obtenção de cepas de BAL.

Além da participação ativa no desenvolvimento de atributos sensoriais e tecnológico específicos em produtos nos quais ocorrem naturalmente ou são intencionalmente adicionadas, BAL são ainda capazes de produzir substâncias antimicrobianas com importância para a biopreservação em sistemas alimentares. Entre as substâncias produzidas por BAL, as bacteriocinas são de interesse particular para a indústria de alimentos, devido a sua atuação no controle e inibição de micro-organismos deteriorantes e patogênicos, que podem ser veiculados por alimentos específicos. Por serem considerados antimicrobianos naturais, existe uma tendência na utilização de bacteriocinas na promoção da inocuidade de alimentos, especialmente em produtos prontos para consumo. O potencial antimicrobiano das bacteriocinas se estende ainda ao seu aproveitamento em outros setores, como nos segmentos veterinário e farmacêutico, especialmente no contexto de resistência aos antibióticos tradicionais enfrentado na atualidade.

Independente do propósito de uso, a seleção de BAL bacteriocinogênicas e de suas bacteriocinas deve ser realizada com cautela, visando a garantia da segurança em seu uso. Consequentemente, a avaliação de aspectos relacionados à segurança e virulência não deve ser negligenciada durante o processo de seleção de uma nova cepa. Do mesmo modo, a caracterização adequada das bacteriocinas e dos fatores envolvidos em sua biossíntese, transporte e estabilidade em diferentes condições permite a otimização de fatores ligados à sua produção e obtenção, bem como o direcionamento de sua utilização.

As características promissoras de BAL bacteriocinogênicas e suas bacteriocinas na biopreservação de alimentos e seu potencial uso em outras áreas

como agentes antimicrobianos alternativos, encoraja o desenvolvimento de estudos que busquem a obtenção de novos isolados bacteriocinogênicos e a caracterização de suas bacteriocinas. Deste modo, o objetivo deste trabalho foi isolar e identificar BAL bacteriocinogênicas de queijos artesanais, caracterizando aspectos ligados à produção e purificação das bacteriocinas, segurança e potencial benéfico dos isolados, assim como avaliar as propriedades inibitórias das cepas e bacteriocinas contra *Listeria* spp.

REVISÃO BIBLIOGRÁFICA

1. Bactérias ácido láticas de importância em alimentos

Bactérias ácido lácticas (BAL) constituem um amplo grupo de micro-organismos que compõem naturalmente a microbiota autóctone de diversos alimentos, incluindo o leite cru e seus derivados. Atualmente, 15 gêneros compõem este grupo: *Aerococcus, Atopobium, Brochotrix, Carnobacterium, Enterococcus, Tetragenococcus, Vagococcus, Lactobacillus, Pediococcus, Leuconostoc, Oenococcus, Weissella, Lactococcus e Streptococcus* (Kleerebezem, et al., 2017, Lahtinen et al., 2011).

Os micro-organismos reunidos como BAL compartilham diversas características morfológicas, metabólicas e fisiológicas. São cocos ou bacilos Gram-positivos, ácido e aerotolerantes, não formadores de esporos, não móveis (exceto *Vagococcus* spp. e algumas espécies de *Lactobacillus*) (Sharpe et al., 1973, Collins et al., 1989, Nielsen et al., 2007, Chao et al., 2008), oxidase e catalase negativos, embora algumas espécies possam apresentar atividade de pseudocatalase quando cultivadas em meios ricos em Ferro, como ágar sangue (Singh et al., 2007). Entretanto, a principal característica que agrupa todos estes gêneros é a produção de ácido láctico a partir da fermentação de carboidratos (Leroy and De Vuyst, 2004, Lahtinen et al., 2011). Dois mecanismos são reconhecidos neste processo: homo e heterofermentativo. O mecanismo homofermentativo resulta apenas na produção de ácido láctico, enquanto o processo heterofermentativo gera diversos produtos finais além do ácido láctico, como dióxido de carbono, etanol ou acetato (Leroy and De Vuyst, 2004, Lahtinen et al., 2011).

A humanidade tem explorado há longa data o potencial de BAL na produção de alimentos, em função de sua habilidade em conferir características sensoriais e tecnológicas específicas a alimentos fermentados (Balciunas et al., 2013). Além destas funcionalidades, BAL possuem ainda naturalmente a capacidade de inibir micro-organismos patogênicos e deteriorantes presentes nos alimentos (Leroy and De Vuyst, 2004, Davidson et al., 2013). A interferência de BAL sobre esses organismos pode ocorrer por meio da competição por nutrientes, sítios de ligação ou produção de substâncias antagonistas, dentre as quais se destacam as bacteriocinas (Gálvez et al., 2007, Lahtinen et al., 2011, Balciunas et al., 2013).

1.1. Pediococcus spp. e Enterococcus spp. como BAL de importância em alimentos

O gênero *Pediococcus* compreende onze espécies: *P. acidilactici, P. argentinicus, P. cellicola, P. claussenii, P. damnosus, P. ethanolidurans, P. inopinatus, P. parvulus, P. pentosaceus, P. siamensis* e *P. stilesii* (Franz et al., 2014). Como as demais BAL, são micro-organismos Gram-positivos, catalase e oxidase-negativos, desenvolvendo-se condições microaerofilia (Porto et al., 2017). Não apresentam motilidade e não são capazes de formar cápsulas ou esporos. À microscopia, apresentam-se morfologicamente em cocos isolados, em pares, ou tétrades, uma vez que o plano de divisão celular pode ocorrer alternadamente em sentido perpendicular, característica que difere este gênero das demais BAL. As bactérias pertencentes a este gênero são homofermentativas, produzindo ácido láctico como produto final da fermentação de glicose, pela via Embden–Meyerhof (Franz et al., 2014).

Pediococcus spp. são amplamente distribuídos no ambiente e compartilham seu habitat com muitas outras BAL (Porto et al., 2017). Seu isolamento já foi descrito em diferentes nichos ecológicos, incluindo vegetais, frutas, pólen, bebidas fermentadas, produtos lácteos e cárneos e amostras clínicas humanas (Franz et al., 2014, Porto et al., 2017). Este gênero apresenta grande importância para a indústria de alimentos devido ao seu papel chave no desenvolvimento de características sensoriais desejáveis, controle do processo de fermentação e como bioconservantes de alimentos. *P. acidilactici* e *P. pentosaceus* são as espécies de maior relevância em alimentos fermentados e podem fazer parte da microbiota autóctone ou serem adicionadas como culturas starter na fermentação de diversos alimentos, como picles, azeitonas, e produtos cárneos, como salsichas e salames (Franz et al., 2014).

Considerando a limitação do gênero em fermentar lactose, sua utilização em produtos lácteos é mais restrita (Porto et al., 2017). Entretanto, alguns trabalhos apontam que *Pediococcus* spp. podem desenvolver atributos sensoriais desejáveis durante o processo de maturação de queijos, sugerindo que as cepas que possuam a particularidade de utilizar a lactose possam ser empregadas com sucesso na fermentação de produtos lácteos (Arqués et al., 2015).

Enterococcus spp. é o terceiro maior gênero de BAL, após *Streptococcus* e *Lactobacillus* (FRANZ *et al*., 2011). Até a década de 1980, *Enterococcus* spp. eram caracterizados como pertencentes aos estreptococos do grupo D, segundo a classificação de Lancefield. Em 1984, Schleifer and Kilpper-Bälz (1984), a partir de estudos de hibridização DNA-RNA**,** demonstraram uma relação distinta o suficiente dos outros estreptococos, justificando sua transferência para um gênero separado.

Atualmente, 37 espécies de *Enterococcus* são relatadas na literatura, enquadrando-se em sete grupos de espécies com base na similaridade do gene rRNA

16S (Gilmore et al., 2014). O número exato de espécies de *Enterococcus* descritas pode sofrer variação ao longo do tempo, pela reclassificação de espécies ou descoberta novos táxons. Aproximadamente metade das espécies de *Enterococcus* foi recentemente descrita (Franz et al., 2011).

Este gênero compõe uma grande proporção de bactérias autóctones associadas ao trato gastrointestinal de mamíferos, e após a sua liberação nas fezes humanas ou dejetos animais são hábeis a colonizar diferentes nichos, devido à grande capacidade de resistir ou se multiplicar em ambientes extra-entéricos, justificando seu caráter ubiquitário. São, portanto, encontrados em animais de sangue quente, podendo ainda estar presentes no solo, águas superficiais e plantas (Moreno et al., 2006). Por meio da contaminação ambiental, podem colonizar alimentos crus, multiplicando-se facilmente, devido à capacidade de sobreviver a condições ambientais adversas, como ampla faixa de pH, temperatura e salinidade (Moreno et al., 2006, Franz et al., 2011). Isso significa que estas bactérias podem resistir a condições normais de produção e processamento de alimentos, persistindo até os produtos finais. Dentre as espécies isoladas de produtos lácteos, as mais frequentes são: *E. faecium*, *E. faecalis* e *E. durans*, podendo ser encontrados, com menor frequência, *E. hirae* e *E. casseiflavus* (Franz et al., 2011).

Quando adicionadas intencionalmente aos alimentos, algumas espécies de *Enterococcus* têm a vantagem de contribuir com as propriedades sensoriais, como maturação e desenvolvimento de aromas específicos, assim como sua ação biopreservante, resultante da produção de bacteriocinas de espectro de ação diversificado contra importantes patógenos veiculados por alimentos (Moreno et al., 2006, Franz et al., 2011, Nes et al., 2014).

2. Bacteriocinas

As bacteriocinas de BAL são definidas como compostos proteicos ribossomalmente sintetizados, liberados extracelularmente e que possuem atividade antimicrobiana, principalmente contra espécies de bactérias estreitamente relacionadas, na qual a célula produtora expressa imunidade específica. Estes peptídeos podem variar quanto ao espectro de atividade, modo de ação, origem genética, massa molecular e propriedades bioquímicas (Cotter et al., 2005, Dobson et al., 2012, Snyder and Worobo, 2014).

A atividade antimicrobiana das bacteriocinas pode ocorrer contra micro-organismos estreitamente relacionados ou não. A inibição de espécies Gram-positivas é frequentemente relatada, envolvendo antagonismo contra espécies patogênicas e deteriorantes de alimentos (Achemchem et al., 2012, Settanni et al., 2014, Zhu et al., 2014, Martinez et al., 2015). Algumas bacteriocinas também podem apresentar atividade contra bactérias Gram-negativas de importância em alimentos, como *Escherichia coli*, *Salmonella* Thyphimurium, *Campylobacter jejuni* e *Pseudomonas aeruginosa*, especialmente quando previamente submetidas a algum tipo de tratamento, como alta pressão e choque osmótico (Stern et al., 2006, Gong et al., 2010, Woraprayote et al., 2015). Ainda, a inibição de fungos, vírus e protozoários já foi relatada (Todorov et al., 2010, Belguesmia et al., 2013, Al Kassaa et al., 2014, Amer et al., 2014, Abengózar et al., 2017, Cavicchioli et al., 2018).

Diversas classificações têm sido propostas desde a descoberta das bacteriocinas. A classificação de Cotter et al. (2005) propõe três classes, de acordo com a estrutura química e mecanismo de ação. A classe I é denominada lantibióticos, reunindo pequenos peptídeos (<5 kDa) termoestáveis, modificados pós-

traducionalmente, formando aminoácidos modificados, denominados lantionina e metilantioninas. Dentro desta classe, as bacteriocinas ainda são divididas em Ia e Ib. A subclasse Ia inclui peptídeos longos, flexíveis, carregados positivamente que atuam formando poros na membrana plasmática da bactéria alvo. A subclasse Ib reúne peptídeos esféricos, rígidos e neutros ou carregados negativamente que atuam nas reações enzimáticas essenciais de bactérias sensíveis. A classe II é composta por peptídeos não modificados, de até 10 kDa. Em geral, os peptídeos dessa classe possuem uma estrutura helicoidal anfifílica que permite sua inserção na célula alvo, seguida pela despolarização da membrana e morte celular por meio da formação de poros. Este grupo de bacteriocinas também é dividido em três subclasses. A subclasse IIa é composta por peptídeos semelhantes à pediocina, como a pediocina PA-1/ACH, os quais possuem ampla atividade antimicrobiana contra *Listeria* spp. Estes peptídeos são caracterizados por conter uma região N-terminal altamente conservada (pediocina-box YGNGVXC) e atuam na célula-alvo formando poros e dissipando a força próton-motriz. Já a subclasse IIb é composta por bacteriocinas que necessitam de dois componentes para sua atividade antibacteriana e requerem um complexo sistema de transporte, uma vez que são sintetizadas sem peptídeo líder. Por fim, a subclasse IIc compreende peptídeos circulares enquanto a classe III agrupa bacteriocinas maiores e termolábeis, que atuam na célula alvo por meio da hidrólise do envelope celular.

A produção de bacteriocinas ocorre inicialmente como um mecanismo de resposta a estímulos ou estresses ambientais, como competição microbiana e escassez de nutrientes (Savadogo et al., 2006, Nes et al., 2016). Geralmente, as bacteriocinas são sintetizadas como pré-peptídeos inativos com uma sequência precursora na região N-terminal, que é transportada à superfície celular e catalisada

na sua forma ativa (Hetrick and van der Donk, 2017, Porto et al., 2017). A regulação da produção de bacteriocinas por cepas produtoras é intermediada pela ativação da transcrição de genes que podem ter localização cromossomal ou em elementos móveis, como plasmídeos e transposons (Ennahar et al., 2000, Drider et al., 2006, Perez et al., 2015). De modo geral, a síntese de bacteriocinas envolve a ativação de quatro genes: (1) o gene responsável pela produção do pré-peptídeo; (2) o gene de imunidade específica que protege a célula produtora; (3) o gene que codifica proteínas do transportador ABC, responsável por secretar a bacteriocina, e (4) o gene que codifica uma peptidase acessória ao transportador ABC, essencial para a secreção da bacteriocina. Esses genes, normalmente estão organizados em um ou dois operons (Nes et al., 2007b). As enterocinas A e B, por exemplo, possuem genes organizados em dois operons, referentes aos loci *entA* e *entB* (Nes et al., 2007a). O sistema responsável pela regulação da síntese de bacteriocinas é composto por três componentes: um peptídeo indutor, histidina quinase e um regulador da resposta. O peptídeo indutor é sintetizado ribossomalmente em baixas concentrações pelas células bacterianas. Ao atingir determinada densidade populacional, o peptídeo indutor ativa a histidina quinase, promovendo sua auto-fosforilação, que é transferido ao regulador da resposta. Em sua forma fosforilada, o regulador de resposta ativa a transcrição dos genes ligados à produção de bacteriocina e sua regulação (Nes et al., 2007b). Este é um mecanismo regulado por *quorum sensing*, uma vez que a comunicação bacteriana resultante da alta densidade populacional, estimula a síntese do peptídeo indutor, num sistema de *feedback* positivo (Ennahar et al., 2000, Drider et al., 2006, Nes et al., 2007b).

Para os lantibióticos, pertencentes à classe I, a regulação é feita pela própria bacteriocina, que age como peptídeo indutor, retroalimentando positivamente a

produção de bacteriocinas (Todorov, 2009, Balciunas et al., 2013). O pró-peptídeo desta classe possui resíduos de serina, treonina e cisteína, que sofrem modificação pós-traducional (Chen and Hoover, 2003). Os pré-peptídeos são constituídos por uma sequência-líder de 14 a 30 aminoácidos na região N-terminal, que é removida durante a maturação da proteína. As etapas que envolvem a maturação compreendem a síntese do pré-peptídeo, clivagem em local de processamento específico e remoção da sequência N-terminal, e liberação para o exterior da célula (Ennahar et al., 2000). Com base na biossíntese, os lantibióticos podem ser subdivididos em dois grupos: no grupo I, no qual o pré-peptídeo sofre desidratação pela enzima LanB e, posteriormente, formação de uma ligação tio-éter pela enzima LanC. Em seguida, o pré-peptídeo sofre modificações pela serina protease LanP e é translocado em sua forma madura através da membrana, pelas proteínas LanT do transportador tipo ABC. Já no grupo II, o pré-peptídeo é extensivamente modificado por uma única enzima LanM e o processamento é concomitante à translocação pela enzima LanT (Chen and Hoover, 2003).

As bacteriocinas da Classe II são sintetizadas como pré-peptídeos tipo dupla glicina. Na região N-terminal dos pré-peptídeos, há dois resíduos de glicina conservados, que têm como funções: impedir a bacteriocina de ser biologicamente ativa 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; em seguida, os pré-peptídeos tipo dupla-glicina são exportados através da membrana citoplasmática por meio de um sistema dependente de ATP, denominado transportador ABC. O transportador ABC é uma protease, cujo domínio proteolítico usualmente reside na região N-terminal da proteína; portanto, tem por função a remoção da sequência-líder e a translocação do peptídeo maduro através da

membrana citoplasmática. Transportadores do tipo ABC também podem ser responsáveis pela translocação de diferentes substratos, como íons, carboidratos, aminoácidos, vitaminas, lipídeos e antibióticos, além de moléculas maiores, como oligossacarídeos, oligopeptídeos e proteínas de elevada massa molecular (Ennahar et al., 2000, Cotter et al., 2005, Drider et al., 2006, Nes et al., 2016).

A presença ou ausência da extensão N-terminal no pré-peptídeo determina o mecanismo de secreção das bacteriocinas da Classe II. Reconhece-se que as enterocinas L50A, L50B, Q e a bacteriocina LsbB são os poucos exemplos de bacteriocinas Classe II que são sintetizadas sem extensão N-terminal, sendo seus mecanismos de secreção desconhecidos (Herranz and Driessen, 2005). Outras exceções, como as bacteriocinas circulares, distinguem-se das demais por suas porções N e C terminal estarem covalentemente ligadas de forma circular; porém, também são sintetizadas na forma de pré-peptídeos lineares, que são clivados para sua ativação (Gabrielsen et al., 2014). A maioria das bacteriocinas de Classe II contém o sinal tipo dupla-glicina; porém, outras apresentam um pré-peptídeo do tipo Sec-dependente, transportado pela via Sec-translocase, como, por exemplos, as bacteriocinas divergicina A (*Carnobacterium divergens*), acidocina B (*Lactobacillus acidophilus*), bacteriocina 31 (*E. faecalis*) e enterocina P (*E. faecium*) (Herranz and Driessen, 2005). Os pré-peptídeos do tipo Sec-dependente da bacteriocina 31, por exemplo, possuem uma sequência com três resíduos de aminoácidos básicos (Arg/Lys-Lys-Lys) próximos à região N-terminal e ao sítio de clivagem Val-X-Ala (nas posições 23-21), o qual contém um resíduo ácido (Asp ou Glu) na posição 22 (Cintas et al., 1997). O movimento dos pré-peptídeos do tipo Sec através da membrana plasmática é mediado por um complexo de proteínas multiméricas citosólicas e presentes na membrana, chamado de translocase. A translocação de

proteínas para o exterior das células é acionada por meio de hidrólise de ATP e por força próton-motriz (Herranz and Driessen, 2005).

2.1. Bacteriocinas produzidas por Pediococcus spp. *e Enterococcus* spp.

A identificação e caracterização de bacteriocinas produzidas por BAL com aplicação biotecnológica tem sido alvo de inúmeras pesquisas nos últimos anos. Nesse cenário, *Pediococcus* spp. e *Enterococcus* spp. destacam-se como importantes gêneros de BAL capazes de produzir estas substâncias.

P. acidilactici e *P. pentosaceus* destacam-se como as principais espécies bacteriocinogênicas entre *Pediococcus* spp., relacionadas à produção de: pediocina AcH, por *P. acidilactici* H, E, F, e M (Bhunia et al., 1988, Ray et al., 1989, Kim et al., 1992), pediocina PA-1/ACH, por *P. acidilactici* PAC 1.0 (Gonzalez and Kunka, 1987), pediocina JD, por *P. acidilactici* SJ-1(Schved et al., 1993), pediocina 5, por *P. acidilactici* UL5 (Huang et al., 1996), pediocina A, por *P. pentosaceus* FBB-61 (Fleming et al., 1975), pediocina N5p, por *P. pentosaceus* (Saad et al., 1995), pediocina ST18, por *P. pentosaceus* (Todorov and Dicks, 2005) e pediocina SA-1 por *P. acidilactici* NRRL B5627 (Anastasiadou et al., 2008). *P. damnosus* também é relatada como uma espécie bacteriocinogênica, pela produção de pediocina PD-1 (Green et al., 1997, Porto et al., 2017).

As bacteriocinas produzidas por *Pediococcus* spp. são genenericamente denominadas pediocinas e a maioria enquadra-se na classe IIa. Alguns peptídeos desta classe ainda recebem a denominação *pediocin-like*, devido ao relato do primeiro peptídeo antimicrobiano desta classe, pediocina PA-1/ACH, ter sido obtido de um isolado de *Pediococcus* (Bhunia et al., 1988, Rodríguez et al., 2002). Os

peptídeos deste grupo são conhecidos pela alta conservação da sequência hidrofílica N-terminal, denominada *pediocin box*, contendo duas cisteínas unidas por uma ponte dissulfeto, na sequência -Y-G-N-G-V-X_1-C-X_2-K/N-X_3-X_4-C-, onde $X_{1–4}$ representa resíduos carregados ou não (Papagianni and Anastasiadou, 2009). Inicialmente, acreditava-se que estas bacteriocinas poderiam ser produzidas apenas por bactérias do gênero *Pediococcus*, porém sua produção por espécies de outros gêneros já foi demonstrada (Aymerich et al., 1996, Ennahar et al., 1996, Le Marrec et al., 2000, Papagianni and Anastasiadou, 2009). Do mesmo modo, bacteriocinas diferentes da pediocina podem ser produzidas por espécies de *Pediococcus* spp. Um exemplo é a bacteriocina produzida por *P. pentosaceus* IE-3, que possui massa molecular de 1,7 kDa e não apresenta similaridade com as sequências de pediocina conhecidas (Singh et al., 2014).

As bacteriocinas produzidas por *Pediococcus* spp. tem atraído interesse devido às características interessantes sob o ponto de vista industrial, como termoestabilidade, atividade em ampla faixa de pH, efetividade em baixas concentrações e amplo espectro antimicrobiano. A pediocina ST18 produzida por *P. pentosaceus* ST18 isolado da bebida fermentada boza, manteve a atividade bacteriocinogênica após tratamento térmico a 121°C por 30 minutos (Todorov and Dicks, 2005). De modo similar, a manutenção da atividade após exposição a 100°C por 2 horas foi observada para a pediocina ST44AM, isolada de *P. pentosaceus* (Todorov and Dicks, 2009). Os mesmos autores verificaram ainda a manutenção da atividade bacteriocinogênica após seis meses de produção e estocagem a 25 °C, valorizando o potencial de aplicação destas bacteriocinas em produtos tratados termicamente no processamento industrial (Todorov and Dicks, 2005, 2009).

Uma característica comum entre as pediocinas é a sua eficácia contra *Listeria monocytogenes*, sendo comum o uso do termo bacteriocinas anti-listeria e relatos da inibição deste patógeno por pediocinas ou por cepas bacteriocinogênicas de *Pediococcus* spp. são frequentes na literatura. *P. pentosaceus* 05-10, isolado de um produto vegetal fermentado tradicional da China, foi caracterizado como produtor de bacteriocina por Huang et al. (2009). A bacteriocina, de massa molecular de aproximadamente 6,5 kDa apresentou efeito bactericida contra *L. monocytogenes* 54002. Presuntos pré-tratados com a pediocina 05-10 apresentaram contagens significativamente reduzidas de *L. monocytogenes* comparadas à amostras não tratadas, durante estocagem por 10 dias a 4 °C (Huang et al., 2009).

A cepa *P. acidilactici* HA-6111-2 produtora de pediocina PA-1/AcH, isolada de Alheira, um produto cárneo embutido tradicional de Portugal, foi testada como biopreservante na produção de Alheira inoculada com um coquetel de cepas de *L. inoccua*, utilizada em alguns estudos como sentinela do comportamento de *L. monocytogenes* (Albano et al., 2007, Albano et al., 2009). Foi observada redução da população de *Listeria* spp. abaixo do limite de detecção (1,5 log UFC/g), sem efeitos no desenvolvimento de BAL naturalmente presentes no produto e sem alterações de pH, destacando o potencial bioprotetor da cepa em alimentos fermentados, como atestado pelos autores (Albano et al., 2009). Kingcha et al. (2012) observaram decréscimo significativo de *L. monocytogenes* ATCC 19115 em Nham, uma linguiça suína tradicional da Tailândia, quando inoculada com *P. pentosaceus* BCC 3772 bacteriocinogênico. Um decréscimo de 3,2 log UFC/g na população de *L. monocytogenes* foi observado após 18 – 24h de fermentação, comparado à população inicial. A redução de *L. monocytogenes* foi atribuída à produção de uma bacteriocina

com 100% de identidade de aminoácidos com a pediocina comercial PA-1/AcH (Kingcha et al., 2012).

No trabalho de Nieto-Lozano et al. (2010), linguiças fermentadas foram incubadas com *L. monocytogenes* CECT4031 e com a cepa bacteriocinogênica *P. acidilactici* MCH14. As contagens de *L. monocytogenes* foram reduzidas em 2 ciclos logarítmicos após 60 dias de fermentação, em comparação ao controle, que não foi adicionado da cepa bacteriocinogênica. Os mesmos autores demonstraram ainda inibição *L. monocytogenes* CECT4031 e *Clostridium perfringens* CECT 376 em outro tipo de embutido avaliado, com redução de aproximadamente 2 log de UFC/g pela adição de pediocina PA-1/ACH purificada, após 60 dias de estocagem a 10°C, quando comparado às amostras controle correspondentes (Nieto-Lozano et al., 2010).

Pediococcus spp. são apontados ainda como probióticos em alguns estudos. Rodríguez et al. (2002) constataram que *P. acidilactici* BA28 produtor de pediocina retardou o crescimento e a colonização estomacal por *H. pylori* em camundongos, além de reverter o processo infeccioso causado pelo patógeno. Dabour et al. (2009) avaliaram a diferença do efeito protetor frente à infecção por *L. monocytogenes* na administração intra-gástrica de pediocina e na administração oral de *P. acidilactici,* constatando que a bacteriocina exerce maiores efeitos benéficos quando comparada à administração oral *P. acidilactici* em camundongos.

Com relação à *Enterococcus* spp., diversas bacteriocinas produzidas por espécies provenientes de ambientes distintos têm sido caracterizadas. *E. faecium* e *E. faecalis* são descritas como as principais espécies produtoras, e em menor frequência, *E. mundtii*, *E. avium*, *E. hirae* e *E. durans* (Nes et al., 2014).

Genericamente denominadas enterocinas, as principais bacteriocinas sintetizadas pelo gênero incluem as enterocinas A, B, P, L50 e AS-48 (Franz et al.,

2011, Nes et al., 2014). A primeira enterocina purificada homogeneamente foi a enterocina AS-48, definida como um peptídeo cíclico e produzida por *E. faecalis* S-48 (Gálvez et al., 1989). Desde então, o número de novas enterocinas caracterizadas a partir de fontes diversas tem aumentado consideravelmente. A enterocina A, pertencente à classe IIa, consiste num peptídeo de 47 aminoácidos, com massa molecular de 4,8 kDa (Aymerich et al., 1996). A enterocina B, também incluída na classe IIa, apresenta massa molecular de 5,5 kDa em um peptídeo constituído por 53 aminoácidos (Casaus et al., 1997). Assim como algumas enterocinas, a enterocina B possui amplo espectro de ação, atuando contra importantes patógenos de alimentos, como *S. aureus, C. perfringens* e *L. monocytogenes* (Franz et al., 2007). As enterocinas A e B podem ser produzidas simultaneamente por cepas de *E. faecium* (Franz et al., 2007). A enterocina B apresenta peptídeo sinal, contudo, os genes relacionados à sua secreção não são encontrados em seu operon. Com isso, acredita-se que esta bacteriocina seja transportada pelas mesmas proteínas envolvidas no transporte da enterocina A (Franz et al., 2007).

A enterocina P é constituída por 44 aminoácidos e apresenta massa molecular de 4,5 kDa. O gene responsável pela produção desta bacteriocina localiza-se no cromossomo bacteriano (Herranz et al., 1999). Em seu espectro de ação, inclui a inibição de algumas BAL como *Enterococcus* spp., *Lactococcus* spp. *Lactobacillus* spp., *Pediococcus* spp., além de *B. cereus, C. botulinum, C. perfringens, L. monocytogenes, S. aureus, P. aeruginosa, Aspergillus niger* e *Fusarium equiseti* (Ben Braïek et al., 2017).

A enterocina L50 é codificada por um plasmídeo de 50Kb presente em algumas cepas de *E. faecium*. Esta bacteriocina é constituída por dois peptídeos, denominados L50A e L50B, com 44 e 43 aminoácidos, respectivamente, e apresenta massa

molecular de 5,2 KDa. A síntese da bacteriocina L50 inclui a expressão de dois genes estruturais em *tandem*, algumas proteínas acessórias e quatro genes de alta homologia ao ABC transporters-48EFGH, que participa da imunidade celular à sua própria bacteriocina (Franz et al., 2007). Essa bacteriocina apresenta atividade antimicrobiana contra *Enterococcus* spp., *Lactobacillus* spp., *Pediococcus pentosaceus*, além de bactérias patogênicas como *B. cereus* e *L. monocytogenes* (Cintas et al., 1997, Rahmeh et al., 2018).

A utilização de *Enterococcus* spp. bacteriocinogênicos em alimentos é relatada por diversos autores, influenciando positivamente características tecnológicas dos produtos, assim como na inibição de controle de micro-organismos patogênicos e deteriorantes. A adição de *E. faecium* F58 como cultura *starter* na elaboração de queijo frescal experimentalmente contaminado com uma cultura patogênica de *L. monocytogenes* resultou na redução de até 4 log UFC/g da concentração inicial do patógeno (Achemchem et al., 2006). Os autores observaram ainda que a eliminação completa de *L. monocytogenes* ocorreu quando os queijos foram inoculados com a cultura patogênica 12 horas após a adição da cultura *starter* (Achemchem et al., 2006).

Em estudo realizado com queijo Cottage, a adição 80 UA/mL da enterocina RM6, produzida pelo isolado *E. faecalis* OSY-RM6, reduziu em 4 log UFC/g a população de *L. monocytogenes* intencionalmente adicionada ao queijo, após 30 minutos, sem detecção de células viáveis após 26 horas (Huang et al., 2013).

A inibição de *L. monocytogenes* em queijos frescais por cepas bacteriocinogênicas de *Enterococcus* spp. foi também observada por Pingitore et al. (2012). *Enterococcus mundtii* CRL35 e *E. faecium* ST88Ch foram co-inoculados com a cepa *L. monocytogenes* 426 em queijo Minas frescal e avaliados quanto à

capacidade de controlar o patógeno em condições de estocagem refrigerada a 8 ºC. Nos queijos contendo *E. mundtii* CRL35, o crescimento de *L.monocytogenes* 426 foi inibido até 12 dias a 8°C, evidenciando o efeito bacteriostático da cultura bacteriocinogênica. Já a cepa *E. faecium* ST88Ch demonstrou ser menos efetiva, controlando o patógeno por apenas 6 dias em estocagem a 8 °C (Pingitore et al., 2012).

Além da atividade bactericida ou bacteriostática contra *L. monocytogenes* observadas na utilização de diferentes isolados de *Enterococcus*, Ye et al. (2018) verificaram que o isolado *E. faecium* B1 foi capaz de modular negativamente a expressão de seis importantes genes de virulência de *L. monocytogenes*. Entre os genes avaliados, destacam-se *hylA, inlA* e *actA*, envolvidos nos processos de escape do fagossomo, invasão das células hospedeiras e disseminação célula a célula, respectivamente, na patogenia da listeriose. Os autores observaram que em co-culturas de *E. faecium* B1 houve redução da capacidade de *L. monocytogenes* de invadir células Caco-2 (Ye et al., 2018).

3. Potencial benéfico de BAL bacteriocinogênicas

LAB apresentam diversas propriedades interessantes à produção e conservação de alimentos, como desenvolvimento de características sensoriais particulares e produção de substâncias antimicrobianas que contribuem na inocuidade e extensão da vida de prateleira dos produtos. Diversas BAL empregadas como cultura *starter*, especialmente na produção de produtos lácteos podem apresentar também propriedades probióticas.

Probióticos são definidos como micro-organismos vivos, que administrados em quantidades adequadas conferem benefícios à saúde do consumidor (WHO, 2002, Nanno et al., 2011, Reid et al., 2015). Diversos efeitos positivos são descritos a partir do consumo regular de probióticos, embora os mecanismos de ação não sejam completamente esclarecidos. Os benefícios envolvem inibição de patógenos, imunomodulação, redução dos níveis de colesterol sérico, redução da pressão arterial, auxílio no metabolismo de lactose, produção de vitaminas e possíveis efeitos anticarcinogênicos e antitóxicos (de Vrese et al., 2001, Bertazzoni Minelli et al., 2004, Hlivak et al., 2005, Fabian et al., 2008, Kabeerdoss et al., 2011, Shida et al., 2011, Tompkins et al., 2014).

BAL têm sido utilizadas por séculos na produção de produtos fermentados e sua relação com o desenvolvimento de efeitos benéficos faz com que estes micro-organismos sejam os principais representantes probióticos. *Lactobacillus* e *Bifidobacterium* são os principais gêneros relacionados à atividade probiótica. Entretanto, o potencial de outros gêneros pertencentes ao grupo das BAL tem sido bastante explorado, especialmente com relação à *Enterococcus* e *Pediococcus* (Bhardwaj et al., 2010, Osmanagaoglu et al., 2010, Pieniz et al., 2014, Dubey et al., 2016).

Para ser considerada probiótica, uma cepa deve passar por uma série de avaliações e atender a alguns requisitos mínimos, estabelecidos pela FAO e WHO (2002). O primeiro desafio após a ingestão é atravessar as barreiras biológicas do trato gastrointestinal para alcançar seu local de ação. Estas barreiras incluem as condições ácidas do ambiente estomacal e a presença de sais biliares e enzimas intestinais. Considerando a sobrevivência a esta etapa, a capacidade de competição por nutrientes com a microbiota autóctone do intestino e a habilidade de aderir e se

desenvolver na superfície mucosa por meio de propriedades hidrofóbicas auxiliam na colonização intestinal e são importantes para o estabelecimento neste nicho (Corr et al., 2009, Kaushik et al., 2009).

Testes específicos adicionais devem ser realizados, a fim de que se conheçam as potencialidades de uma cepa potencialmente probiótica. A agregação de BAL é uma característica relevante na avaliação de uma cepa probiótica. A auto-agregação pode resultar na formação de biofilmes, facilitando a permanência intestinal, enquanto a co-agregação com patógenos é importante na eliminação de cepas não desejáveis no trato gastrointestinal (Todorov and Dicks, 2008, Furtado et al., 2014). Essas propriedades são codificadas por genes relacionados à expressão de proteínas de superfície, sendo, portanto, estirpe-específicas (Kleerebezem et al., 2003).

LAB bacteriocinogênicas também podem desempenhar funções probióticas se forem capazes de sobreviver às condições gastrointestinais. De acordo com Dobson et al. (2012), as bacteriocinas podem contribuir na atividade probiótica atuando como peptídeos colonizadores, inibidores ou ainda como peptídeos sinalizadores (Figura 1), portanto a produção destas substâncias representa um critério de seleção importante.

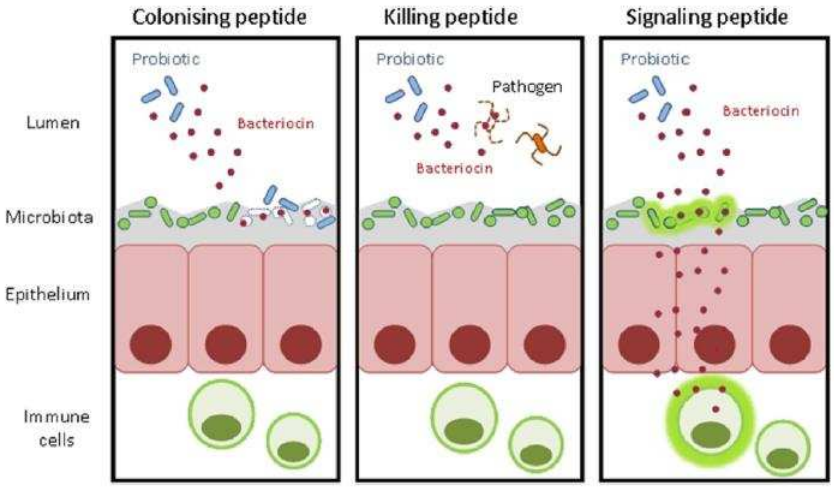

Figura 1: Mecanismos de contribuição das bacteriocinas na atividade probiótica. Fonte: Dobson et al. (2012).

Na atuação como peptídeos colonizadores, as bacteriocinas podem facilitar a introdução e/ou dominância da cepa probiótica produtora em um nicho pré-colonizado pela microbiota autóctone (Majeed et al., 2011). A alta densidade populacional neste ambiente facilita o contato entre as células e a dispersão de substâncias antimicrobianas, representando uma vantagem competitiva sobre as cepas sensíveis (Gillor et al., 2009, Dobson et al., 2012).

Cepas bacteriocinogênicas também podem atuar modulando o desenvolvimento de espécies que não sejam sensíveis. Na administração de *E. faecium* KH24 produtor de bacteriocina na concentração de 10^8 UFC/dia em camundongos durante 12 dias, observou-se que a população de *Lactobacillus* spp. foi significativamente maior quando comparada à população de camundongos que recebeu a mesma concentração de *E. faecium* KH24 não bacteriocinogênico, durante o mesmo período. Os autores sugerem que *Enterococcus* spp. bacteriocinogênicos podem contribuir no controle da microbiota autóctone de maneira positiva e concluíram que a cepa de *E. faecium* KH24 poderia ser explorada como probiótica (Bhardwaj et al., 2010).

De modo alternativo, as bacteriocinas podem atuar como peptídeos inibidores, eliminando cepas competidoras e micro-organismos patogênicos. Muitos estudos têm demonstrado que cepas bacteriocinogênicas são capazes de inibir patógenos *in vitro*, no entanto, alguns trabalhos evidenciam a inibição gastrointestinal de patógenos importantes, como *L. monocytogenes*. *L. salivarius* UCC 118, foi efetivo contra a infecção por *L. monocytogenes* em camundongos, como demonstrado por Corr et al. (2007). A inibição foi atribuída à produção de uma bacteriocina composta por dois peptídeos, denominada Abp 118, uma vez que a administração da cepa não

bacteriocinogênica isogênica derivada falhou na proteção dos animais contra a infecção (Corr et al., 2007).

Por fim, as bacteriocinas podem atuar como peptídeos sinalizadores em cepas probióticas, tanto por meio de *quorum sensing* e interações com comunidades microbianas quanto na sinalização de células do sistema imune do hospedeiro (Czárán et al., 2002, Di Cagno et al., 2007, Majeed et al., 2011, Dobson et al., 2012). Em bactérias Gram-positivas, diversos peptídeos, incluindo algumas bacteriocinas, funcionam como agentes de sinalização (Sturme et al., 2002). Neste sentido, propõe-se que algumas bacteriocinas podem desempenhar dupla função, atuando como inibidores quando presentes em altas concentrações e como compostos sinalizadores, quando em baixas concentrações (Fajardo and Martínez, 2008).

A ligação entre bacteriocinas e seu impacto no sistema imune é relativamente recente. Em diferentes trabalhos publicados em 2010 pelo mesmo grupo de pesquisa foram identificados diversos genes em *L. plantarum* relacionados à influência na resposta imune de células dendríticas e células mononucleares de sangue periférico, respectivamente (Meijerink et al., 2010, van Hemert et al., 2010). A deleção destes genes do genoma de *L. plantarum* WCFSI resultou em mudanças consideráveis no perfil de citocinas, e observou-se que a maioria dos genes deletados estavam envolvidos na produção ou secreção de bacteriocinas. Ambos os autores sugerem que as bacteriocinas produzidas por *L. plantarum* podem modular a resposta imune de modo semelhante aos peptídeos antimicrobianos secretados no trato gastrointestinal (Meijerink et al., 2010, van Hemert et al., 2010).

Consumidores de produtos probióticos podem estar sob o tratamento de doenças, e os efeitos benéficos dos probióticos podem ser prejudicados por possíveis interações com os medicamentos utilizados por estes consumidores. Em doenças

crônicas, nas quais o tratamento geralmente é feito durante períodos prolongados, pode haver acumulação intestinal destas substâncias, afetando a viabilidade de bactérias probióticas. Sobretudo, deve-se destacar que a interação entre antimicrobianos ou outros medicamentos com micro-organismos probióticos no trato gastrointestinal é dependente da concentração que estes medicamentos atingem neste ambiente, por isso, o estabelecimento da Concentração Mínima Inibitória (MIC) é importante para que se possam avaliar adequadamente essas interações (Todorov and Dicks, 2008, Furtado et al., 2014).

Furtado et al. (2014) avaliaram a interação de diversos medicamentos com a cepa potencialmente probiótica *Lactococcus lactis* subsp. *lactis* DF04Mi. Antiarrítmicos contendo amidarona e anti-inflamatórios não esteroidais contendo diclofenaco de potássio ou ibuprofeno como princípio ativo foram fortemente inibitórios para *L. lactis* subsp. *lactis* DF04Mi. O MIC determinado para estes medicamentos variou de 0,16 mg/mL para diclofenaco de potássio e 7,5 mg/mL para ibuprofeno, sendo inferiores às doses recomendadas de 150 mg e 600 mg, respetivamente. De modo similar, De Paula et al. (2015) observaram que alguns analgésicos, anti-inflamatórios não esteroidais e hipertensivos interferiram no desenvolvimento de *Leuconostoc mesenteroides* SJRP55, apresentando também MICs abaixo das concentrações comumente utilizadas por essas drogas. Nesse sentido, estudos mais detalhados dos fatores que interferem na inibição de probióticos por medicamentos devem ser realizados, levando em consideração as concentrações que os mesmos podem atingir no trato gastrointestinal.

4. Segurança de BAL como culturas biopreservantes ou probióticas

Em virtude de um longo histórico de utilização na produção de alimentos fermentados, BAL são reconhecidas como micro-organismos seguros, recebendo o *status* GRAS "Generally Recognized as Safe" nos Estados Unidos ou QPS "Qualified Presumption of Safety" na Europa. Entretanto, as pesquisas têm demonstrado que LAB podem apresentar algumas características indesejáveis, potencialmente danosas à saúde dos consumidores. Deste modo, apesar do crescente interesse na utilização de LAB, é preciso ter cautela na seleção de novas cepas com propriedades biotecnológicas.

No caso de *Enterococcus* spp., apesar das distintas propriedades benéficas e tecnológicas, sua utilização em alimentos é paradoxal. Inicialmente considerados patógenos comensais e de mínimo impacto clínico, *Enterococcus* spp. emergiram nas duas últimas décadas como agentes etiológicos importantes em infecções nosocomiais adquiridas por pacientes imunossuprimidos e internados em unidades de terapia intensiva (Ventola, 2015). *E. faecalis* e *E. faecium* representam até 80% e 20% dos isolados clínicos, respectivamente e a proporção de infecções por *E. faecium* tem aumentado, principalmente devido a um número crescente de cepas resistentes à antibióticos (Ventola, 2015, Prieto et al., 2016).

A patogenicidade de *Enterococcus* spp. é relacionada a presença de fatores de virulência e determinantes de resistência a antibióticos, que acredita-se ser mais dependente da espécie do que da fonte de isolamento. Franz et al. (2001) verificaram que a presença de genes de virulência é maior em *E. faecalis* do que em *E. faecium*, representando 78,7% e 10,4%, respectivamente. Ainda, a presença de tais genes já foi verificada em outras espécies do gênero, como *E. casseliflavus, E. durans, E. hirae, E. avium, E. cecorum, E. gallinarum, E. malodoratus. E. raffinosus* e *E. mundtii* (Semedo et al., 2003, Poeta et al., 2005, Jackson et al., 2015, İspirli et al.,

2017). Os principais fatores de virulência do gênero envolvem o potencial de aderência a tecidos, invasão e formação de abscessos, modulação da resposta imune e secreção de toxinas; codificados por genes como *esp* (codifica proteínas de superfície), *agg e asa* (codificam substâncias agregativas), *efa* (codifica adesinas de superfície), *gel* (codifica a enzima gelatinase) e *cyl* (codifica citolisina) (Eaton and Gasson, 2001, Franz et al., 2011, Anderson et al., 2016, Chajęcka-Wierzchowska et al., 2017). Outro importante fator de virulência é a β-hemolisina, uma enzima que é capaz de lisar eritrócitos humanos e de animais como equinos e coelhos (Eaton and Gasson, 2001).

Isolados de *Enterococcus* spp. de origem alimentar contendo genes de virulência já foram identificados. Perin et al. (2014) avaliaram a presença e expressão de alguns genes ligados à produção de fatores de virulência em *Enterococcus* spp. isolados de leite de cabra. Os autores verificaram que a maioria dos isolados testados apresentaram amplificação dos genes *asa*, *cylA*, *esp*,*efa*A e *gel*. Contudo, apenas três, dentre 18 isolados avaliados, foram capazes de expressar produção de gelatinase. Os autores verificaram ainda que a temperatura pode atuar modulando a expressão de genes de virulência (Perin et al., 2014).

Majhenič et al. (2005), em um trabalho avaliando determinantes de virulência em *Enterococcus* spp. provenientes de queijo Tolmin, típico da Eslovênia, observaram a alta prevalência de isolados contendo genes *agg*, *efA*fs, *cyl*A, *cyl*B, *esp* e *gel*E. A expressão destes genes, contudo, não foi avaliada e os autores destacam que o consumo deste tipo de queijo na Europa central não relaciona-se a ocorrência de casos clínicos (Majhenič et al., 2005).

Medeiros et al. (2014) compararam cepas isoladas de materiais de origem clínica e de alimentos, como vegetais e carnes cruas, leite pasteurizado e queijos,

demonstrando que um grande número de isolados de origem alimentar possui em seus genomas os mesmos fatores de virulência encontrados em isolados clínicos. A pesquisa envolvendo isolados de *E. faecalis* (57 de origem clínica e 55 provenientes de alimentos) revelou que os isolados de origem clínica continham genes de citolisina (*cyl*A), substância agregativa (*agg*) e proteína enterocócicas de superfície (*esp*) com maior frequência. Por outro lado, a presença dos genes de gelatinase (*gel*E) e proteína de ligação ao colágeno (*ace*) estavam presentes em níveis similares, sugerindo sua ampla ocorrência em ambos os ambientes.

Embora infecções por isolados de *Enterococcus* spp. provenientes de alimentos nunca tenham sido relatadas, esses isolados e outras cepas de BAL portadoras de genes de virulência e resistência a antimicrobianos, caracterizam-se como possíveis reservatórios naturais em alimentos, e o seu consumo pode representar uma rota de transferência de traços de resistência a antibióticos, influenciando o surgimento de linhagens multirresistentes (Nero et al., 2015, Chajęcka-Wierzchowska et al., 2017). A transferência de genes presentes em BAL a cepas patogênicas intestinais é ainda mais preocupante, por dificultar a terapia antibiótica em infecções que poderiam ser facilmente combatidas.

Enquanto as informações sobre virulência e resistência em *Enterococcus* spp. são abundantes na literatura, nota-se que para *Pediococcus* spp. dados similares são limitados ou inexistentes. Por razões de segurança, é essencial que estudos detalhados sejam conduzidos com BAL a serem empregadas como co-culturas e culturas starter em alimentos, para minimizar os riscos de emergência de resistência.

A resistência a antimicrobianos pode ser inerente ao gênero bacteriano ou espécie, mas também pode ser adquirida através de troca de material genético, mutações e incorporação de novos genes (Mathur and Singh, 2005, Ammor et al.,

2007, Hummel et al., 2007, Verraes et al., 2013). Os perfis de resistência e sensibilidade são bastante variáveis entre gêneros e espécies, e podem ser influenciados ainda pela origem da cepa. BAL são intrinsecamente resistentes a alguns antimicrobianos, geralmente cefalosporinas, alguns β-lactâmicos e aminoglicosídeos (Ammor et al., 2007, Danielsen et al., 2007). A determinação da resistência intrínseca aos aminoglicosídeos é associada à presença dos genes *aac(6')Ie-aph(2'')Ia*, *aac(6')-aph(2'')* e *ant(6)* (Rojo-Bezares et al., 2006, Ammor et al., 2007). Algumas cepas de *Pediococcus* são ainda naturalmente resistentes a glicopeptídeos (vancomicina e teicoplanina), a estreptomicina, canamicina, tetraciclina, doxiciclina, ciprofloxacina, sulfas e associações com trimetoprim e ao metronidazol, devido à ausência da enzima hidrogenase, que é o alvo deste antimicrobiano (Ammor et al., 2007, Haakensen et al., 2009). Em *Enterococcus* spp., a resistência à vancomicina não é considerada intrínseca, sendo relacionada à presença de alguns genes, dentre os quais *vanA* e *vanB*, podem estar localizados em plasmídeos transferíveis.

Além da possibilidade de carrear genes de resistência e virulência, outros fatores de segurança devem ser ponderados na seleção de cepas biopreservantes ou probióticas, como a capacidade de produção de aminas biogênicas. As aminas biogênicas são compostos nitrogenados de baixa massa molecular, formadas pela desaminação ou descarboxilação de certos aminoácidos, como parte do metabolismo de micro-organismos. Fatores como o tempo e temperatura de estocagem dos produtos podem influenciar na produção destas substâncias e o consumo de alimentos contendo altas concentrações de aminas biogênicas pode causar diversos efeitos tóxicos (Perin et al., 2014). A severidade dos sintomas depende da quantidade

ingerida, susceptibilidade individual, e nível de detoxificação intestinal destes compostos (Ladero et al., 2015).

Em produtos fermentados, LAB estão presentes em abundância e representam os principais produtores de aminas biogênicas, podendo afetar a qualidade e a segurança nestes alimentos (Ladero et al., 2012). Neste sentido, a pesquisa fenotípica e de genes ligados à produção de aminas biogênicas é importante, a fim de se evitar a utilização de cepas reconhecidamente produtoras como ferramentas biotecnológicas de preservação dos alimentos.

5. Purificação de bacteriocinas produzidas por BAL

Muitas espécies de BAL possuem propriedades bacteriocinogênicas e a purificação das bacteriocinas produzidas é útil para o conhecimento da estrutura, mecanismo de ação e outras características que contribuem para sua caracterização. No entanto, a purificação destas substâncias geralmente envolve protocolos complexos e de custo elevado, fazendo com que a purificação em escala industrial seja o principal obstáculo para sua aplicação (Garsa et al., 2014, Kaškonienė et al., 2017).

Bacteriocinas produzidas por BAL são tipicamente catiônicas, hidrofóbicas e amplamente variadas quanto à massa molecular, composição de aminoácidos, ponto isoelétrico e modificações pós-traducionais de certos aminoácidos (De Vuyst and Leroy, 2007, Garsa et al., 2014). Esta heterogeneidade de características impede o desenvolvimento de um protocolo universal de purificação, justificando a ampla gama de procedimentos para isolamento e purificação desenvolvidos. A escolha do método de purificação depende de uma série de fatores, como a natureza da

bacteriocina, tempo de processo, pureza e rendimento desejado, e muitas vezes a obtenção de um protocolo para a purificação de determinada substância acontece por tentativa e erro (Garsa et al., 2014).

Considerando-se que geralmente as bacteriocinas são liberadas extracelularmente após sua síntese, a maioria das estratégias de purificação envolve uma etapa inicial de concentração, como precipitação com sulfato de amônio, precipitação ácida ou com solventes orgânicos (De Vuyst and Leroy, 2007). A precipitação com sulfato de amônio é frequentemente o método de escolha, devido ao baixo custo e facilidade de manuseio (Saavedra et al., 2004). Sua ação é baseada no princípio da redução de solubilidade das proteínas e precipitação em altas concentrações de sal no fenômeno de *salting-out*. A utilização da precipitação com sais pode ainda melhorar a estabilidade da conformação nativa da proteína (Wingfield, 2001). Os processos de purificação de amilovorina L, produzida por *L. amylovorus* DCE 471, de enterocinas produzidas pelas cepas RZS C5, RZS C13 e FAIR-E 406 de *E. faecium* e do lantibiótico macedocina, produzido por *S. macedonicus* ACA-DC 198, foram realizados a partir da precipitação em sulfato de amônio, seguida de extração e precipitação em solventes como metanol e clorofórmio e HPLC (Callewaert et al., 1999, Georgalaki et al., 2002, Balciunas et al., 2013). Embora estes procedimentos sejam essencialmente utilizados com o objetivo de reduzir o volume a ser trabalhado, o grau de pureza obtido com a precipitação com sulfato de amônio geralmente não é satisfatório (Pingitore et al., 2007, Garsa et al., 2014). Por consequência, diferentes métodos cromatográficos têm sido utilizados em etapas subsequentes para remoção de proteínas provenientes do meio de cultura, do metabolismo celular e outros componentes indesejáveis, permitindo o refinamento dos resultados.

Devido às propriedades catiônicas das bacteriocinas, a cromatografia de troca iônica é muito difundida em protocolos de purificação, em uso isolado ou associado à cromatografia por interação hidrofóbica, gel-filtração e fase reversa (Garsa et al., 2014). Diferentes estratégias podem ser utilizadas na purificação de bacteriocinas e a combinação de métodos pode fornecer melhores resultados em relação ao rendimento e grau de pureza, dependendo das perspectivas de uso do material.

As etapas finais de purificação geralmente consistem na aplicação de métodos de alta resolução e capazes de fornecer alto grau de pureza. A cromatografia líquida de alta pressão em fase reversa (RP-HPLC) é descrita em diversos trabalhos como metodologia recomendada para este fim, uma vez que as bacteriocinas são usualmente resistentes aos diferentes ácidos orgânicos utilizados como fase móvel e as altas pressões empregadas neste processo (Pingitore et al., 2007). A RP-HPLC tem como princípio a separação pela adsorção diferenciada de peptídeos ou proteínas à fase estacionária e a dessorção seletiva pela fase móvel. A fase estacionária localiza-se no interior da coluna, sendo constituída por resinas orgânicas ou sílica contendo grupamentos apolares, que atraem as moléculas da solução alvo a ser purificada. A fase móvel é aplicada em gradiente crescente de concentração de solventes orgânicos, como acetonitrila, metanol e isopropanol. Deste modo, as proteínas ou peptídeos polares são eluídos com uma menor concentração do solvente da fase móvel enquanto as substâncias mais apolares são eluídas quando a concentração do solvente é maior (Silva Junior, 2004).

Os métodos convencionais de purificação geralmente consistem em diversas etapas consecutivas, geralmente de custo elevado, demoradas, que fornecem baixos rendimentos e não são reprodutíveis em escala industrial. Além disso, os baixos níveis de recuperação destes métodos resultam em perda significativa da substância

alvo (Pingitore et al., 2007, Kaškonienė et al., 2017). Com o intuito de superar as limitações dos métodos convencionais, alguns pesquisadores desenvolveram métodos rápidos para a purificação de bacteriocinas.

Uteng et al. (2002) desenvolveu um método rápido de purificação para uma bacteriocina do tipo pediocina e outros peptídeos antimicrobianos catiônicos a partir de um meio de cultura complexo, aplicando a cultura bacteriana diretamente sobre uma coluna de troca catiônica seguida por uma coluna reversa de baixa pressão. A combinação destes métodos permitiu a purificação da bacteriocina em menos de duas horas, com rendimento de 80% (Uteng et al., 2002). O tempo de processamento curto foi um grande avanço comparado à purificação da mesma cultura a partir da precipitação de sulfato de amônio, cromatografia de troca catiônica, cromatografia de interação hidrofóbica e cromatografia de fase reversa, que permitiram um rendimento de apenas 10% (Uteng et al., 2002).

A utilização de um método alternativo foi proposta por Simha et al. (2012), a partir da imobilização de *P. pentosaceous* NCDC 273 produtor de pediocina PA-1/ACH em uma matriz de alginato com quitosana e goma xantana, durante a fermentação da cultura. Esta abordagem eliminou a primeira etapa de centrifugação para obtenção do sobrenadante da cultura. A precipitação com sulfato de amônio foi usada em seguida, e o uso de alta velocidade durante a agitação magnética permitiu melhores resultados do que a centrifugação na obtenção do pellet nesta etapa, com 134,4% de rendimento (Simha et al., 2012).

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OBJETIVOS

Objetivo Geral

Isolar e identificar bactérias ácido láticas (BAL) bacteriocinogênicas de queijos artesanais, caracterizando aspectos ligados à produção e purificação das bacteriocinas, segurança e potencial benéfico dos isolados, assim como avaliar as propriedades inibitórias das cepas e bacteriocinas contra *Listeria monocytogenes*.

Objetivos Específicos

- ✓ Isolar culturas de BAL bacteriocinogênicas de queijos artesanais;
- ✓ Realizar a identificação fenotípica e molecular das cepas de BAL isoladas e pesquisa de genes relacionados a produção de bacteriocinas;
- ✓ Avaliar as características de produção, espectro de ação e estabilidade das bacteriocinas produzidas por BAL isoladas;
- ✓ Verificar a capacidade de inibição de *Listeria monocytogenes* pelas cepas e bacteriocinas produzidas;
- ✓ Avaliar o potencial benéfico das cepas de BAL quanto à capacidade de colonização e controle de infecções por *Listeria monocytogenes*, bem como a interação das cepas com medicamentos usualmente utilizados;
- ✓ Caracterizar molecular e fenotipicamente aspectos de segurança das cepas de BAL, relacionados à resistência antimicrobiana, atividade virulenta e produção de aminas biogênicas;
- ✓ Avaliar interferências do inóculo no crescimento das cepas e produção de bacteriocinas;
- ✓ Comparar a produção de bacteriocinas em sistemas tradicionais e controlados de fermentação;
- ✓ Detectar a expressão de elementos do sistema de transporte ABC ligados ao transporte de bacteriocinas e metabolismo de açúcares;
- ✓ Purificar as bacteriocinas produzidas pelas cepas de BAL e verificar as características de segurança das bacteriocinas purificadas quanto à citotoxicidade.

Title page

Interpretative summary

Novel Bacteriocinogenic *Enterococcus hirae* and *Pediococcus pentosaceus* Strains with Anti-Listerial Activity Isolated from Brazilian Artisanal Cheese. Cavicchioli. The present study describes the production of potentially novel bacteriocins by two strains naturally present in an artisanal raw milk cheese produced in Brazil. The antimicrobial potential of the strains highlights their relevance as potential candidates as biopreservatives agents in the dairy industry.

Novel Bacteriocinogenic *Enterococcus hirae* and *Pediococcus pentosaceus* Strains with Anti-Listerial Activity Isolated from Brazilian Artisanal Cheese

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ABSTRACT

Bacteriocin producers *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC were isolated from raw milk artisanal cheeses and characterized. Their bacteriocins were tolerant to temperatures ranging from 4°C up to 100 °C and also in sterilization conditions (121 °C for 15 min). Aditionally, the tested bacteriocins remained the activity after to be exposed to pH ranging from 2.0 to 10.0 during 2 h. The activity of bacteriocins was affected by proteolytic enzymes, but remained stable after treatment with EDTA, SDS, NaCl, skim milk and Tween 80. Cell-free supernatants were capable of inhibiting *Listeria innocua* and several strains of *L. monocytogenes* obtained from different sources and belonging to different serotypes. Additionally, the growth of *L. monocytogenes* 211 and *L. monocytogenes* 422 treated with bacteriocins was completely inhibited over a period of 12 h. Co-cultures of bacteriocinogenic strains and *L. monocytogenes* 422 in skimmed milk showed that *E. hirae* ST57ACC was able to control the growth of the pathogen in this matrix, after 48h. None of the selected isolates presented positive results on a screening panel for 25 bacteriocin related genes, indicating that both strains might express novel bacteriocins.

Key Words: bacteriocin, cheese, *Pediococcus*, *Enterococcus*

INTRODUCTION

Artisanal cheeses are produced in different Brazilian regions by local small farmers that normally use raw milk in their production (Brant et al., 2007). In general, such cheeses are subjected to ripening, when their autochthonous lactic acid bacteria (LAB) may produce several substances with antimicrobial activity, such as organic acids, hydrogen peroxide, diacetyl, CO_2 and bacteriocins (Deegan et al., 2006, Favaro et al., 2015). A variety of LABs can be found in milk and cheeses, and some have been recognized as bacteriocins producers and used for controlling pathogens, in different food products (dos Santos et al., 2015, Favaro et al., 2015). *Listeria monocytogenes* is considered one of the most important foodborne pathogens, due to high mortality rates associated with listeriosis (Swaminathan and Gerner-Smidt, 2007). The occurrence of *L. monocytogenes* in cheeses is a especial cause of concern, once these products are often consumed without any processing, and its persistence in cheese manufacturing plants in Brazil have already been reported (Barancelli et al., 2014).

Some studies conducted in Brazil have already demonstrated the presence of bacteriocin producers from raw milk and cheeses, characterizing such products as important sources of novel strains with bacteriocinogenic potential in (Ortolani et al., 2010, Moraes et al., 2012, Perin et al., 2012, Tulini et al., 2013, dos Santos et al., 2015). Bacteriocins are low molecular weight polypeptides/proteins, released extracellularly, genetically encoded and defined as ribosomally synthesized (Klaenhammer, 1988, Vuyst and Vandamme, 1994). After binding to surface receptors or entering host cells, bacteriocins can act in the target cell via pore formation, the degradation of cellular DNA and the inhibition of peptidoglycan

synthesis (Vuyst and Vandamme, 1994, Heu et al., 2001). Bacteriocins can be used as biopreservatives via their direct addition to the food product, or by the addition of bacteriocinogenic strains that will produce these peptides *in situ* (Gálvez et al., 1998, Deegan et al., 2006). This application is particularly interesting in raw milk cheeses, where adding bacteriocinogenic strains is relevant to inhibit spoilage and foodborne pathogens, improving their safety (Ross et al., 2000, Favaro et al., 2015). These aspects determine a constant demand for novel bacteriocinogenic strains to be used in dairy industries (Beshkova and Frengova, 2012). However, prior to using novel LAB strains as biopreservatives in foods, it is necessary to extensively characterize their technological, safety and virulent features, in order to allow their proper usage in food (Favaro et al., 2015).

The present study aimed to isolate bacteriocinogenic LAB from an artisanal cheese produced in a specific Brazilian region, and to characterize the antimicrobial activity of selected strains and their bacteriocins, aiming the future use by dairy and food industries.

MATERIAL AND METHODS

LAB Selection from Artisanal Cheese

Cheese Samples, LAB Isolation and Identification. Four samples of artisanal cheeses, produced by non-pasteurized cow milk in the region of Nova Venécia, Espírito Santo state, Brazil, were subject to homogenization at a 1:10 ratio with saline solution (0.85% NaCl, w/v). The obtained suspensions were ten-fold diluted in saline solution and plated on the surface of multiple plates containing 10 mL of de Man, Rogosa and Sharpe agar (MRS, BD - Becton, Dickinson and Company,

Franklin Lakes, NJ, USA) and incubated at 37 °C for 24 h. The triple-layer method, described by Todorov et al. (2010) and test organisms (*Listeria monocytogenes* 211, *L. monocytogenes* 422, *L. monocytogenes* 506, *Lactobacillus sakei* ATCC 15521 or *Enterococcus faecalis* ATCC 19443) was performed in order to pre-select for the potential producers of antimicrobials LAB. Briefly, colonies on MRS agar plates were overlaid with a second layer of agar. Then, plates with less than 50 colonies were overlaid with 10 mL of semi-solid BHI agar (BD) containing active growing cells from test organisms (approximately 10^6 CFU/mL) and at 37 ◦C for 24 h. Colonies with evident inhibition zones were selected for isolation and examined for purity, Gram staining, catalase and oxidase reaction and the production of antimicrobial compounds, as described in the next section. All cultures used in this study (Bacteriocinogenic LAB and test organisms) were stored in MRS or BHI (BD) added with 20% glycerol (v/v) at -80ºC.

Total genomic DNA of selected isolates was extracted by using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA). The extracted DNA was quantified by a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and isolates were differentiated by RAPD-PCR, using primers OPL-01 (GGC ATG ACC T), OPL-14 (GTG ACA GGC T) and OPL-20 (TGG TGG ACC A) and by rep-PCR (using the primer GTG_5). Amplification reactions were performed according to Todorov et al. (2010) for RAPD-PCR as follows: 45 cycles of 1 min at 94 °C, and 1 min at 36 °C, followed by an increase to 72 °C over 2 min. Extension of the amplified product was at 72 °C for 5 min. Conditions for rep-PCR were: 5 min at 95°C, 30 cycles of 30 s at 95°C; 30 s at 40°C and 8 min at 65°C, and final extension of 16 min at 65°C, according to Perin and Nero (2014). The amplified products were separated by electrophoresis in 1.4% (w/v) agarose gels in 0.5x TAE buffer at 100 V

for 2 h. Gels were stained in TAE buffer containing GelRed (Biotium Inc., Hayward, CA, USA). Banding patterns were analyzed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium).

The identification of isolates was performed by amplifying 16S rRNA genes from the genomic DNA with the universal primers 8F (CAC GGA TCC AGA CTT TGA TYM TGG CTC AG) and 1512R (GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT) (Felske et al., 1997). The amplified fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands). Purified fragments were sequenced at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of São Paulo, Brazil and compared to sequences in GenBank using BLAST, the Basic Local Alignment Search Tool.

As recommended by Robredo et al. (1999) and Costa et al. (1993), the presence of genes related to the identification of *E. durans* (AAC AGC TTA CTT GAC TGG ACG C and GTA TTG GCG CTA CTA CCC GTA TC) and *E. faecium* (GCG GTA GCA GCG GTA GAC CAA G and GCA TTT GGT AAG ACA CCT ACG) was tested by PCR.

Bacteriocin Production Test and Spectrum of Activity. Individual colonies which presented an inhibitory zone in the previous test were subjected to determination of the proteinaceous nature of the antimicrobial compound, as previously described by dos Santos et al. (2015). Selected isolates were grown on MRS (BD) at 37°C for 24h and cell-free supernatant was obtained by centrifugation at 10,000 × *g*, for 10 min. The pH of the supernatant was adjusted to 6.0-6.5 with 1M NaOH and treated for 10 min at 80°C. Agar-spot test using *L. monocytogenes* 211, *L. monocytogenes* 422, *L. monocytogenes* 506, *Lb. sakei* ATCC 15521 or *E. faecalis* ATCC 19443 at 10^5 CFU

mL^{-1} final concentration was performed and inhibition zones larger than 2 mm diameter were considered as positive results.

Isolates that were identified as bacteriocinogenic were grown as described above. Then, their cell-free supernatant were obtained and treated as described above, and used to assess the inhibitory spectrum of isolates considering as targets strains of *L. monocytogenes* (different serological groups) and LAB (Table 1). Culture conditions (growth medium and incubation temperature) and the origin of the test microorganisms used were specified in Table 1.

Bacteriocin Genes. DNA extracted from selected strains were also subjected to PCR reactions in order to check for the presence of the following bacteriocins: enterocin A, enterocin P, enterocin B, enterocin L50B, pediocin PA-1/ACH, nisin, plantaricin W, plantaricin NC8, plantaricin A, plantaricin S, sakacinGA1, sakacin GA2, sakacin X, sakacin A, sakacin Q, sakacin G, sakacin P, sakacin Tα, sakacin Tβ, lactacin 481, lactacin A, lactacin M, lactacin 3147, lactacin GQ, and leuconocin 972.The PCR reactions were prepared using primers at a concentration of 10 pM μL^{-1} and the conditions described previously (Aymerich et al., 1996, Cintas et al., 1998, Du Toit et al., 2000, Barbosa et al., 2014, Furtado et al., 2014, Barbosa et al., 2015, Favaro et al., 2015, Barbosa et al., 2016). For each PCR reaction, the annealing temperatures were adjusted according to the specification of the primers used. The amplified products were separated by electrophoresis on agarose gels in 0.5x TAE buffer. Agarose gels were stained in 0.5x TAE buffer in the presence of GelRed (Biotium) and visualized on LPIX transilluminator (Loccus Biotecnologia, São Paulo, SP, Brazil).

Bacteriocins Production by Selected LAB

The Effect of Enzymes, pH, Temperature and Detergents on the Stability of Bacteriocins. The cell-free supernatants from the selected isolates, obtained as described above, were treated with 0.1 mg mL^{-1} (final concentration) of selected proteolytic enzymes, α-amylase and lipase (Table 2) at 37°C for 1 h, followed by the deactivation of enzymes by thermal treatment at 98°C for 3 min. Antimicrobial activity was determined as described above against *L. monocytogenes* 211, *L. monocytogenes* 422, *L. monocytogenes* 506, *Lb. sakei* ATCC 15521 and *E. faecalis* ATCC 19443. Cell-free supernatants without any enzyme treatments served as a control.

In addition, cell-free supernatants were tested for the effects of pH, temperature and selected detergents and chemicals on the stability of the antimicrobial substance/s produced (Table 2) as described by Todorov and Dicks (2006). The effect of pH on the bacteriocins was determined by adjusting the cell-free supernatants from pH 2.0 to 10.0 with sterile 1 N HCl or 1N NaOH. After 2h of incubation a 30 °C, the samples were readjusted to pH 6.5 and the activity was tested as described before. The effect of temperature on the bacteriocins was tested by cooling or heating the cell-free to 4°C, 20°C, 25°C, 30°C, 37°C, 45°C, 60°C, 80°C and 100°C, respectively. Residual bacteriocin activity was tested after 15, 30, 60 and 120 min at each of these temperatures as previously described. Additionally, the activity was tested after exposure the bacteriocins to121°C for 15 min. The effect of detergents and chemicals (EDTA, SDS, NaCl, skim milk, Tween 80) on the bacteriocins was tested by adding 1% (w/v, final concentration) to the cell-free supernatants. Untreated cell-free supernatants and surfactants at the latter concentrations served as

controls. All treated supernatants were incubated at 37 °C for 3 h and then the activity was tested as previously described. For these tests, *L. monocytogenes* 211, *L. monocytogenes* 422, *L. monocytogenes* 506, *Lb. sakei* ATCC 15521, and *E. faecalis* ATCC 19443 were used as target organisms and non-treated cell-free supernatants were used as a control.

Inhibitory Activity of Cell-Free Supernatant

Effect of Cell-Free Supernatant on Actively Growing Cultures. One hundred milliliters of BHI broth was inoculated with 1% (v/v) overnight culture of *L. monocytogenes* 211 and *L. monocytogenes* 422, respectively, and incubated at 37°C for 3 h. Then, 20 mL of filter-sterilized cell-free supernatant containing bacteriocins of selected LAB was added to the culture and changes in OD_{600} recorded every hour for 12 h. The cell counts of *L. monocytogenes* 211 and *L. monocytogenes* 422 were recorded by pour-plating aliquots of the cultures obtained on BHI supplemented with 2% agar and incubated at 37°C for 48 h. Results were expressed as CFU mL^{-1}.

Co-Culture with Listeria monocytogenes in Milk. After optimization of bacteriocin production, overnight cultures of selected bacteriocinogenic LAB strains were individually inoculated onto 10% (w/v) reconstituted skimmed milk at concentrations of 2.0% (v/v), as equivalents of approximately 10^7 CFU mL^{-1}. Mixed cultures were incubated at 37°C for 48 h; at 3 h intervals, aliquots were obtained and pH was measured by using pH meter (W3B pH meter, BEL), *Listeria* counts were obtained by surface plating of aliquots onto Chromogenic Listeria agar (Oxoid) followed by incubation at 35°C for 48 h, and LAB counts were obtained by pour-plating of aliquots on MRS agar (BD) followed by incubation at 37°C for 24 h; microbial counts were expressed as log CFU mL^1. In parallel, aliquots were tested for the

presence of bacteriocins, as described above, using *L. monocytogenes* 422 as the target organism.

RESULTS AND DISCUSSION

LAB Selection from Artisanal Cheese

Cheese production is a traditional part of the Brazilian Dairy industry and artisanal practices. The safety of such products is an important issue, as artisanal cheeses are widely consumed by the population and alternative methods to assure their safety are required. The Brazilian Ministry of Health and the Brazilian Ministry of Agriculture, Livestock and Food Supply have established production, quality and safety standards for these cheeses, in order to ensure consumers' health (Brasil, 2001, 2011). Different quality assurance tools can be adopted by dairy industries in order to guarantee the safety and quality of end products (Cusato et al., 2012, Cusato et al., 2014), and the usage of bacteriocinogenic strains and/or their bacteriocins can improve these aspects.

The cheese samples included in this study presented LAB populations ranging from 7.36×10^4 to 2.21×10^6 CFU g^{-1}. However, after adding the agar overlay to the target organisms, only one sample presented colonies with inhibitory activity. Following this step, 84 colonies were selected due to the inhibitory activity and tested for the production of bacteriocins. Based on the results obtained, two isolates both Gram positive cocci, presented bacteriocinogenic activity, and were catalase and oxidase negative.

Based on the RAPD-PCR and rep-PCR profiles, it was possible to determine that both isolates were different strains. After16S rRNA analysis, isolates were identified as *E. hirae* (namely ST57ACC) and *Pediococcus pentosaceus* (namely ST65ACC). As 16S rRNA usually not provides accurate data for the identification of *Enterococcus* spp. strains (Costa et al., 1993, Robredo et al., 1999), additional PCR targeting specific genes for *E. durans* and *E. faecium* were performed, generating negative results, thereby confirming the previous result obtained indicating that isolate ST57ACC belongs to the species *E. hirae*.

The presence of *Enterococcus* spp. in raw milk products is not surprising, particularly in cheeses. Different *Enterococcus* spp., including bacteriocin producers, were previously isolated from artisanal cheeses from different regions of Brazil, as well as worldwide (Pingitore et al., 2012, Rehaiem et al., 2012, Tuncer et al., 2013, dos Santos et al., 2015, Kırmacı et al., 2015).

The presence of *P. pentosaceus* was a surprising fact, related to the observation that strain ST65ACC was not able to use lactose in its metabolite pathway (data not shown). However, presence of *Pediococcus* species have been identified in many cheeses made in different regions (Gerasi et al., 2003, Gurira and Buys, 2005, El-Baradei et al., 2007). Carafa et al. (2015) characterized the LAB from the traditional Mountain Malga cheese, which is made in small farms in Italy by using raw cow's milk and spontaneous fermentation; they found that *P. pentosaceus* was among the dominant species. In addition, the antimicrobial activity of *Pediococcus* species isolated from cheeses has already been reported, and its inhibitory activity against *Listeria* species was previously demonstrated.

Based on screening for the presence of different bacteriocin genes, none of the selected isolates presented positive results for the targets considered in this study.

These results indicate that both strains probably express novel bacteriocins/different from these included in the gene screening panel. The presence of genes for bacteriocin production is frequently incorrectly interpreted as evidence for the expression of these genes.

Bacteriocins produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were able to inhibit all of the tested *L. monocytogenes* strains (99 in total) and 2 strains of *L. innocua* used in this study (Table 1). It is important to underline that the *L. monocytogenes* tested were from different serological groups and isolated from various ecological niches, including food processing environments. In addition to *L. monocytogenes*, both of the studied bacteriocins expressed only a limited activity against LAB tested as sensitive strains. These results are relevant for food industries, as this indicates that the bacteriocins produced by these strains are not active against potential starter cultures added in fermented products, highlighting their potential as biopreservatives.

Bacteriocins Production by Selected LAB

After contact with proteolytic enzymes, both cell-free supernatants lost their activity, pointing protein nature of the antimicrobial compounds produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC (Table 2). Moreover, the presence of α-amylase or lipase did not affect the bacteriocin activity (Table 2). This result indicates that the antimicrobial activity is not related to the presence of a glycolytic or lipolytic moiety of the bacteriocin molecule.

As for most bacteriocins and other small proteins, both of the studied bacteriocins presented significant stability after treatment for up to 2h at different temperatures (Table 2). This finding was previously described in other bacteriocins

produced by *Enterococcus* spp. and *Pediococcus* spp. (Gurira and Buys, 2005, Altuntaş et al., 2014, Cavicchioli et al., 2015). The thermal stability of bacteriocins is considered a positive feature, allowing them to be applied for biopreservation in products that will be exposed to pasteurization or other thermal treatments in order to ensure the safety and extended shelf life of food products. In addition, in the case of potential medical (human and veterinary) application of bacteriocins, sterile applied products are requested, and the stability of bacteriocins after exposure to sterilization processes is essential.

When cell-free supernatants produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were corrected to different pH values (from 2.0 to 10.0), only a low level reduction of antimicrobial activity was observed at pH 9.0 and 10.0 for both bacteriocins. In the rest of the experiments, no changes in the antimicrobial activity were recorded compared to the non-treated cell-free supernatants. Such results are not unusual, since the stability of most of the known bacteriocins, including nisin, was previously reported at both neutral and acidic pH (Barbosa et al., 2014, Barbosa et al., 2015).

It was observed that EDTA, SDS, NaCl, skimmed milk, and Tween 80 had no effect on the stability of bacteriocins produced by *E. hirae* ST57ACCand *P. pentosaceus* ST65ACC, as similarly reported by other authors (Gálvez et al., 1998, Ivanova et al., 2000, Todorov and Dicks, 2005).

Inhibitory Activity of Bacteriocins

The growth of *L. monocytogenes* 211 and *L. monocytogenes* 422 treated with cell-free supernatants containing bacteriocins produced by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, was completely inhibited over a period of 12 h (Fig. 1).

Over the same period, controls of *L. monocytogenes* 211 and *L. monocytogenes* 422 reached a growth of approximately 10^7 CFU mL^{-1} (Fig. 1). The cell count of *L. monocytogenes* 211 and *L. monocytogenes* 422 in treated samples was lower than the detection threshold.

When both strains were cultured in skimmed milk at 25, 30 and 37 °C, similar growth results were recorded at 12, 24 and 36 h. However, bacteriocin production was higher at 37 °C (data not shown). These results were considered a preliminary test for choosing the ideal temperature and incubation time for the study of bacteriocin production by *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC in skimmed milk. Results for the co-cultures of selected LAB strains and *L. monocytogenes* in milk are presented in Fig. 2. Cell numbers of *L. monocytogenes* 422 decreased from log 4.37 CFU mL^{-1} (time 0) to log 1.48 CFU mL^{-1} at 36 h and to undetectable levels after 48 h, highlighting the high anti-listerial activity of bacteriocin produced by *E. hirae* ST57ACC in these matrixes. *Enterococcus* spp. are important in the field of food microbiology, mainly due to its contribution in different fermentation processes, with its functionality being seen in dairy and meat products (Giraffa, 2003, Pingitore et al., 2012). According to Achemchem et al. (2006), co-culture experiments with *E. faecium* F58 and *L. monocytogenes* in goat's milk did not eliminate this pathogen, but promoted reductions in its populations of 1 to 4 log. In addition, when *L. monocytogenes* contamination was reported after 12 h of *E. faecium* F58 growth in milk, the pathogen was eliminated after 130 h of co-culture. In another experiment, it was shown that *E. faecalis* EJ97 was also able to produce enterocin during co-cultivation with *L. monocytogenes* in skimmed milk; however, its capacity to control the pathogen was limited to populations of 10^3 CFU mL^{-1} or lower (García et al., 2003). In our experiment, it was demonstrated that *E.*

hirae ST57ACC was able to eliminate *L. monocytogenes* in milk after 48 h of inoculation, demonstrating their potential for the biocontrol of this pathogen in milk (Fig. 2).

Regarding the counts of control and co-cultures of *P. pentosaceus* ST65ACC and *L. monocytogenes* 422, the results were the opposite. The cell numbers of *L. monocytogenes* 422 increase from log 3.94 CFU mL^{-1} (time 0) to log 7.26 CFU mL^{-1} after 36 h and log 7.11 CFU mL^{-1} after 48 h (Fig. 2). Thereby, *P. pentosaceus* did not show anti-listerial activity in the tested milk matrix, probably due to the limitation of the fermentation of lactose and low levels of bacteriocins detected. However, if it is used in other products (such as meat or fermented fruit products), there may be a different inhibitory profile, which should be evaluated in future studies.

Production of cheeses is a combination of microbiological and biochemical processes, and it can be simplified as precipitation of milk proteins and maturation processes. Starter and non starter cultures have specific roles in the organoleptic characteristics of cheeses (Ross et al., 2000), but special attention is given to their antimicrobial potential in order to prevent foodborne pathogen grown and spoilage (Favaro et al., 2015). Considering these goals, search for autochthonous LAB from different cheeses, capable of producing antimicrobial substances as bacteriocins, can be an answer for controlling spoilage and pathogenic microorganisms, assuring quality and safety in dairy products. Many examples of bacteriocinogenic LAB isolated from cheeses were already reported as tools for controlling spoilage and pathogenic microbiota (Ross et al., 2000, dos Santos et al., 2015, Favaro et al., 2015). However, selecting novel bacteriocinogenic LAB strains can be considered an interesting approach to find alternatives for biopreservation to be used by dairy and other food industries (Beshkova and Frengova, 2012). Moreover, these LAB strains

need to be carefully examined for their technological and biopreservation properties, in addition to their safety considering the presence of virulence factors, antibiotic resistance and production of biogenic amines.

CONCLUSIONS

In the present work, bacteriocinogenic *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were isolated from raw milk cheese, showing strong anti-listerial activity with bacteriocins resistant to different conditions that can be found in food processing. These isolates can have a potential impact on the development of new industrially important cultures to control *L. monocytogenes* in the manufacture of food products. As these peptides can be new bacteriocins, future studies are required to identify them.

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Table 1. Inhibition zones (mm) presented by bacteriocins produced by *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC, against different target microorganisms.

Target	Detail	Origin	Serotype	inihbition zone (mm)	
				ST57ACC	ST65ACC
L. monocytogenes	A70	Round	4b	13	18
	A71	Round	4b	12	17
	A72	Round	4b	12	18
	A73	Round	4b	10	19
	A74	Round	4b	13	20
	A75	Round	4b	12	18
	A512	Chuck	4b	18	26
	A522	Chuck	4b	15	24
	A536	Loin	4b	18	25
	A537	Loin	4b	18	26
	A549	Hand	4b	14	19
	A554	Hand	4b	14	24
	A593	Hand	4b	14	20
	A4	Chuck	1/2b	11	18
	A5	Chuck	1/2b	13	19
	A6	Chuck	1/2b	12	18
	A7	Chuck	1/2b	13	20
	A8	Chuck	1/2b	12	18
	A9	Chuck	1/2b	11	18
	A10	Chuck	1/2b	12	18
	A11	Chuck	1/2c	12	17
	A12	Chuck	1/2c	13	20
	A19	Hand	1/2c	14	18
	A20	Hand	1/2c	11	18
	A45	Chuck	1/2c	14	17
	A46	Chuck	1/2c	14	17
	A78	Chuck	1/2c	12	17
	A79	Chuck	1/2c	14	17
	A80	Chuck	1/2c	12	17
	A81	Chuck	1/2c	12	15
	A82	Chuck	1/2c	12	18
	A83	Chuck	1/2c	12	19
	A139	Table	1/2c	12	18
	A140	Table	1/2c	13	18
	A142	Table	1/2c	12	18
	A170	Hand	1/2c	12	18
	A171	Hand	1/2c	14	15
	A172	Table	1/2c	14	15
	A173	Table	1/2c	13	15
	A186	Table	1/2c	12	18
	A187	Table	1/2c	12	17
	A188	Table	1/2c	15	18

A189	Table	1/2c	13	18
A190	Hand	1/2c	11	18
A191	Hand	1/2c	13	17
A192	Hand	1/2c	12	17
A193	Hand	1/2c	12	17
A210	Hand	1/2c	14	18
A232	Table	1/2c	12	16
A233	Table	1/2c	11	20
A234	Table	1/2c	13	17
A367	Chuck	1/2c	11	18
A368	Chuck	1/2c	10	18
A371	Chuck	1/2c	14	18
A372	Chuck	1/2c	12	18
A373	Chuck	1/2c	12	17
A505	Loin	1/2c	13	17
A506	Loin	1/2c	12	17
A507	Loin	1/2c	13	14
A508	Loin	1/2c	14	16
A509	Chuck	1/2c	12	14
A510	Chuck	1/2c	17	22
A511	Chuck	1/2c	16	24
A517	Hand	1/2c	18	24
A518	Hand	1/2c	20	22
A519	Loin	1/2c	14	20
A520	Loin	1/2c	16	22
A521	Chuck	1/2c	17	22
A523	Chuck	1/2c	15	23
A524	Chuck	1/2c	15	21
A525	Chuck	1/2c	14	21
A526	Loin	1/2c	18	22
A527	Loin	1/2c	10	21
A528	Loin	1/2c	19	23
A529	Loin	1/2c	15	16
A530	Hand	1/2c	16	22
A531	Hand	1/2c	13	20
A532	Hand	1/2c	18	22
A534	Chuck	1/2c	16	20
A535	Chuck	1/2c	19	20
A539	Loin	1/2c	18	21
A540	Loin	1/2c	13	18
A550	Hand	1/2c	12	18
A581	Round	1/2c	13	17
A582	Round	1/2c	18	18
D110	Tenderizer	1/2c	15	20
D58	Tenderizer	1/2c	14	18
D121	Grinder	1/2c	14	18

	D210	Tenderizer	1/2c	8	14
	D123	Grinder	1/2c	14	16
	D206	Grinder	1/2c	0	0
	D184	Knife	1/2c	12	18
	D136	Hands	1/2c	15	19
	L101	chicken		12	18
	L211	chicken		10	18
	L409	chicken		0	0
	L703	chicken		12	18
	L422	chicken		18	21
	L506	chicken		19	22
L. innocua D102		Table		17	20
L. innocua D107		Grinder		14	20
E. faecium 159		goat cheese		0	14
E. faecium ATCC 19443		reference		10	16
E. faecium ET05		salmon		0	0
E. faecium ET12		salmon		0	0
E. faecium ET88		salmon		0	18
E. mundtii CRL35		cheese		15	15
Enterococcus spp. 13Lb1		goat milk		0	15
Enterococcus spp. 1En2		goat milk		0	15
L. curvatus ET06		salmon		0	0
L. curvatus ET30		salmon		0	0
L. curvatus ET31		salmon		0	0
L. delbrueckii ET32		salmon		0	0
L. fermentum ET35		salmon		0	0
L. plantarum LP08AD		donkey milk		0	0
L. sakei ATCC 15521		reference		15	20
L. sakei MK02R		rocket salad		0	0
L. sakei ST152Ch		salpicao		0	0
L. mesenteroides UCV		baby food		0	0
P. acidilactici ET34		salmon		0	0
P. pentosaceus A3		cheese		0	0
St. infantarius L13		goat milk		20	24
St. mucosae L21		goat milk		0	0

Listeria spp. were cultured in BHI at 37°C
Lactic acid bacteria were cultured in MRS broth at 37°C.

Table 2. Effect of proteolytic enzymes, temperature and pH on activity of the bacteriocin produced by *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC

Treatment		*E. hirae* ST57ACC	*P. pentosaceus* ST65ACC
Enzyme	Proteinase K	-	-
	Trypsin	-	-
	Pepsin	-	-
	α-chymotrypsin	-	-
	Protease Type XIV	-	-
	α-amylase	+	+
	lypase	+	+
Temperature	4 – 100 °C (60 min)	+	+
	121º C (15 min)	+	+
pH	2.0 – 6.0	+	+
	8.0	+	+
	10.0	+	+

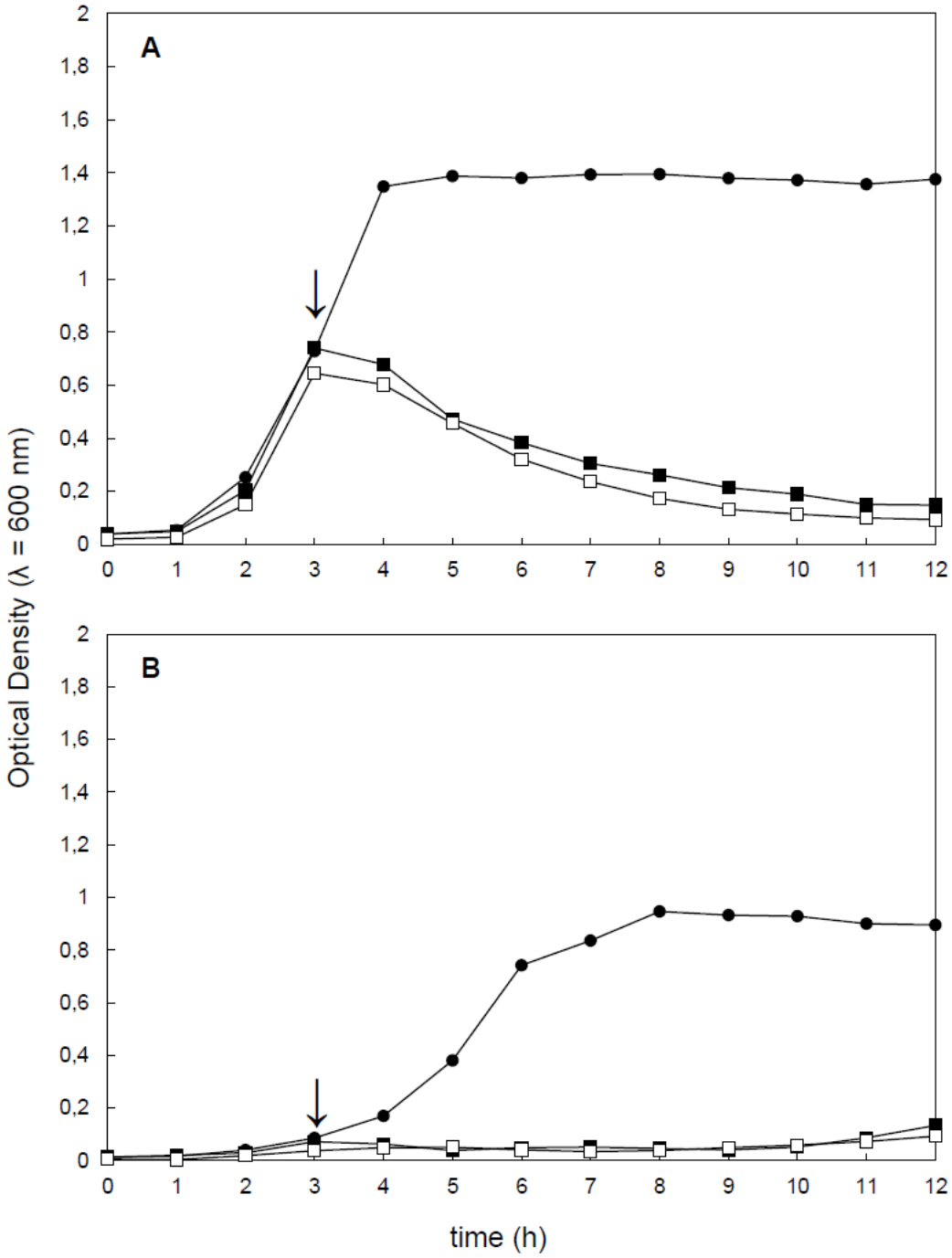

Figure 1. Effect of bacteriocins produced by *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC on *L. monocytogenes* 211 (A) and *L. monocytogenes* 422 (B) growth, by optical density (λ= 600 nm). Black circles: *L. monocytogenes* growth control (no bacteriocins added); Black squares: *L. monocytogenes* growth with bacteriocin from *E. hirae* ST57ACC; White squares: *L. monocytogenes* growth with bacteriocin from *P. pentosaceus* ST65ACC. Arrows indicate the moment of cell-free supernatant containing bacteriocins adding.

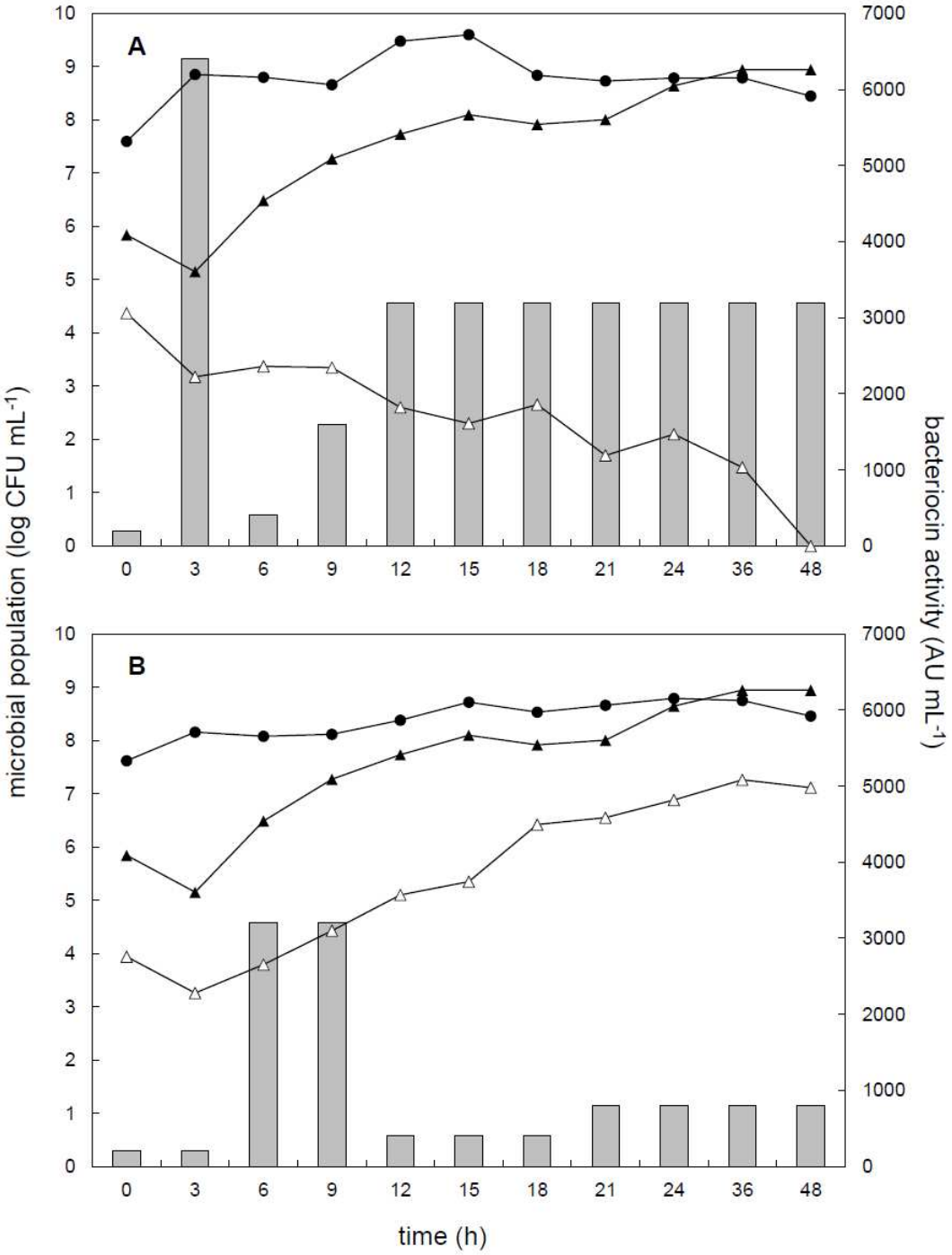

Figure 2. Growth curves and bacteriocin production of *Enterococcus hirae* ST57ACC (A) and *Pediococcus pentosaceus* ST65ACC (B) co-cultured with *Listeria monocytogenes* 422 in skimmed milk 10%. Black circles: *E. hirae* ST57ACC (A) and *P. pentosaceus* ST65ACC (B) growth (log CFU/mL); Black triangles: *L. monocytogenes* 422 growth without bacteriocin added (log CFU/mL); White triangles: *L. monocytogenes* 422 growth with bacteriocin added (log CFU/mL); Grey bars: bateriocin production (AU/mL).

CAPÍTULO 2 - Potential control of *Listeria monocytogenes* by bacteriocinogenic *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC strains isolated from artisanal cheese

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Title page

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Potential control of *Listeria monocytogenes* by bacteriocinogenic *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC strains isolated from artisanal cheese

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Abstract

Bacteriocinogenic *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC strains, previously isolated from artisanal cheese, were evaluated for their safety with the aim to determine whether they could be used as beneficial strains, especially in the control of *Listeria monocytogenes*. Both isolates survived simulated gastrointestinal conditions and showed high levels of auto- and co-aggregation with *L. monocytogenes*, although the hydrophobicity of cells varied. Using the agar-spot test with 33 commercial drugs from different groups, only anti-inflammatory drugs and drugs containing loratadine and propranolol hydrochloride were able to affect the growth of the tested strains. Both strains were resistant to 3 out of 11 antibiotics tested by the disk-diffusion method, and low frequencies of antibiotic resistance-encoding genes were observed by PCR analysis. Neither tested strains presented biogenic amine-related genes, nor produced these substances. Aside from some antibiotic resistance characteristics, the tested strains were considered safe as they lack other virulence-related genes. *Enterococcus hirae* ST57ACC and *P. pentosaceus* ST65ACC both presented beneficial properties, particularly their ability to survive gastrointestinal conditions and to aggregate with *L. monocytogenes*, which can facilitate the elimination of this pathogen. Further studies should be conducted to better understand these interactions.

Keywords: antibiotic resistance, beneficial strains, bacteriocins, LAB, *Listeria monocytogenes*

Introduction

Human listeriosis is one of the most severe foodborne diseases worldwide. The infection is caused by *Listeria monocytogenes* and usually affects at-risk populations with impaired cell-mediated immunity, including elderly people, pregnant woman, newborns and immunocompromised adults [1]. However, healthy children and adults are occasionally infected, and it is estimated that transient asymptomatic intestinal carriage of *Listeria* occurs twice per year in healthy adults [2]. Different clinical manifestations have been noted, including gastroenteritis, septicemia, meningitis or meningoencephalitis and abortion [3]. Listeriosis morbidity can be considered low worldwide, but its mortality rates reach up to 30%, highlighting this disease as a public health concern [4].

The main contamination route for *L. monocytogenes* transmission is by ingestion of contaminated food, including raw milk and dairy products, vegetables, raw seafood, meat and ready-to-eat products [3]. *L. monocytogenes* is a widespread pathogen in nature, and due to its ability to survive and multiply in harsh conditions, it can persist in food handling environments, reaching ready-to-eat products by cross-contamination during or after processing steps [5,6]. These characteristics make the rigorous control of *L. monocytogenes* essential to prevent its multiplication in food products and, consequently, reduce the transmission risk of this pathogen.

The development of biopreservation technologies utilising 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 [7]. Bacteriocins are ribosomally synthesised antimicrobial peptides produced by Gram-

negative and Gram-positive bacteria [8]. These bacteriocins can act as antagonists, most often against closely related organisms [9,10,7]. The control of *L. monocytogenes* by LAB and their bacteriocins has been widely studied [11-13]. Beyond their direct influence on protecting against microbial contamination of food, several studies have demonstrated that bacteriocinogenic LAB have the potential to control infections due to their effect on microbial interactions in the human intestinal environment [9,14]. However, the use of LAB for this purpose requires confirmation of the safety of particular strains, as well as their ability to survive the harsh gastrointestinal conditions and their virulent potential, in order to ensure the safety of consumers.

Enterococcus and *Pediococcus* species are commonly part of the microbiota of fermented foods due to their important role in the technological aspects of cheese maturation and contribution to the sensorial characteristics of these foods [15,16]. *Enterococcus* and *Pediococcus* are known to be able to produce different antimicrobial substances. They are considered a biopreservative tool to control the growth of spoilage-related and pathogenic bacteria, especially *L. monocytogenes* [17,18].

In a previous study, *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC were described as bacteriocinogenic strains with strong activity against *Listeria* spp. from different serogroups [19]. Considering the antimicrobial potential of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, this study aimed to characterise these strains in regard to their potential beneficial roles as tools to control *L. monocytogenes* development and colonisation, and also to characterise their virulence.

Materials and Methods

Strains and culture conditions

Enterococcus hirae ST65ACC and *P. pentosaceus* ST57ACC used in the present study were isolated from artisanal cheeses, and were previously characterised as bacteriocinogenic by Cavicchioli et al. [19]. *Listeria monocytogenes* L711, L422 and L637 were used as target strains in the present study.

Stock cultures were maintained in de Man, Rogosa and Sharpe (MRS) broth (Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA), and *L. monocytogenes* isolates were maintained in brain heart infusion (BHI) (Oxoid Ltd., Basingstoke, England). All cultures were kept at -20°C in MRS broth or BHI supplemented with 20% (w/v) glycerol. The isolates were streaked onto their respective agar media to obtain single colonies. These were cultivated overnight at 37°C in each respective broth for use in subsequent tests.

Colonisation ability

Resistance to conditions simulating the gastrointestinal tract

Cultures of *E. hirae* ST65ACC and *P. pentosaceus* ST57ACC were diluted until a turbidity similar to tube 1 from the McFarland scale (approximately 3×10^8 CFU/mL) and diluted 10-fold in NaCl 0.85% (w/v). Selected dilutions were plated on MRS agar (BD) and incubated at 37°C for 48 h, when colonies were enumerated and the results expressed as log CFU/mL at time 0. Resistance to the simulated

gastrointestinal conditions was assessed according to Santos et al. [20]. Briefly, 6 mL aliquots of test culture cell suspensions were diluted in 10 mL of an artificial gastric fluid consisting of a sterile electrolyte solution (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$ and 1.2 g/L $NaHCO_3$; pH 2.5) supplemented with 0.3% pepsin (all chemicals from Sigma-Aldrich, St. Louis, MO, USA) and incubated for 1 h at 37°C under continuous agitation (150 rpm). Populations of both tested cultures were enumerated as described above, then 2 mL of gastric-simulated cultures were diluted in 8 mL of an artificial duodenal secretion (pH 7.2) consisting of 6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl, 0.5% bile salts and 0.1% pancreatin (all chemicals from Sigma-Aldrich) and incubated for 3 h at 37°C under continuous agitation (150 rpm), after which populations of tested cultures were enumerated as described above.

The survival rates (SR) of tested strains after gastric- and enteric-simulated passage were calculated considering both steps using the equation provided by Santos et al. [20] based on their log populations: SR (%) = [log CFU N/log CFU N_0] × 100, where N_0 and N are the population values before and after the assay, respectively. The tests were performed in duplicate.

Cell surface hydrophobicity

Cell surface hydrophobicity of the tested strains was assessed as described by Santos et al. [21]. Briefly, overnight cultures of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were centrifuged at 7000 × *g* for 5 min at 4°C, washed twice with NaCl 0.85% (w/v) and resuspended in the same solution until a turbidity of 1.0 was reached, measured by the optical density (OD) at λ = 560 nm (Spectrophotometer UV-M51; BEL Engineering s.r.l., Monza, Italy), which corresponded to the absorbance at time 0 (A0). N-hexadecane (Sigma-Aldrich) was added to the cell

suspension at a ratio of 1:5, and the mixture was homogenised for 2 min by vortex. After 1 h of incubation at 37°C, the OD of the aqueous layer (A) was measured at λ = 560 nm. Cell surface hydrophobicity was calculated according to the equation provided by Santos et al. [21]: % H = [(A0 - A)/A0] × 100, where A0 and A are the absorbance values before and after extraction with the organic solvent, respectively. This test was performed in duplicate.

Auto-aggregation and co-aggregation with Listeria monocytogenes

For the auto-aggregation test, *E. hirae* ST65ACC and *P. pentosaceus* ST57ACC were grown in MRS broth (BD) for 24 h at 37°C, centrifuged at 7000 × *g* for 10 min at 20°C, then washed twice with 0.85% NaCl (w/v). Cultures were then diluted in 0.85% NaCl (w/v) until a turbidity of 0.3 was reached, with the OD measured at λ = 660 nm (OD_0; UV-M51, BEL). Cultures were incubated at 37°C for 60 min then centrifuged at 300 × *g* for 2 min at 20°C, after which the ODs at λ = 660 nm were recorded (OD_{60}; UV-M51, BEL). Auto-aggregation was determined based on the equation proposed by Todorov et al. [22]: % auto-aggregation = [(OD_0 - OD_{60})/OD_0] × 100. Experiments were conducted in duplicate.

Co-aggregation was assessed with *L. monocytogenes* L711, L422 and L637. Aliquots of cultures of the target strains were transferred to BHI (Oxoid) and incubated at 37°C for 24 h. *E. hirae* ST65ACC and *P. pentosaceus* ST57ACC cells were obtained as described above and diluted in 0.85% NaCl (w/v) until a turbidity of 0.3 was reached, measured by the OD at λ = 660 nm (UV-M51, BEL). Then, 750 µL of each bacteriocinogenic LAB suspension was mixed with 750 µL of prepared cultures of each target strain and the OD was measured at λ = 660 nm (OD0; UV-M51, BEL). Combined cultures were incubated at 37°C for 60 min, then the supernatant was

obtained by centrifugation at 3000 × g for 2 min at 20°C and the OD was measured at λ = 660 nm (OD_{60}; UV-M51, BEL). Co-aggregation was calculated based on the equation proposed by Todorov et al. [22]: % co-aggregation = $[(OD_0 - OD_{60})/OD_0] \times 100$. Experiments were conducted in duplicate.

Effect of commercial drugs on growth of the bacteriocinogenic strains

The growth of *E. hirae* ST65ACC and *P. pentosaceus* ST57ACC in the presence of 33 commercial drugs from 19 distinct groups was tested according to Paula et al. [23]. Drugs were solubilized in Milli-Q water (Merck KGaA, Darmstadt, Germany) 24 h prior to the tests in order to obtain solutions at different concentrations (Supplementary Table 1). Overnight cultures of *E. hirae* ST65ACC and *P. pentosaceus* ST57ACC were inoculated in 15 mL MRS agar (BD) to reach a final concentration of 10^6 CFU/mL. After solidification, 10 µL of the solubilized drugs were spotted on to the surface of the agar and incubated at 30°C for 24 h. Inhibition zones around the spotted drugs were checked, and those which presented inhibition zones larger than 2 mm in diameter were tested to determine the minimal inhibitory concentration (MIC). For this test, a serial two-fold dilution of the drugs was prepared in Milli-Q water (Merck), and 10 µL of each dilution was spotted on to the surface of the MRS agar plates, which were prepared as previously described. The plates were incubated at 30°C for 24 h and observed for the presence of inhibition zones around the spotted drug. The MIC corresponded to the highest dilution that resulted in an inhibition halo at least 2 mm in diameter.

Safety characteristics

Antibiotic resistance

Enterococcus hirae ST65ACC and *P. pentosaceus* ST57ACC were tested to assess their susceptibility to 11 antibiotics (ampicillin, penicillin G, oxacillin, clindamycin, erythromycin, imipenem, rifampicin, chloramphenicol, tetracycline, trimethoprim/sulfamethoxazole and vancomycin), selected based on the recommendations of the Clinical and Laboratory Standards Institute, as well as the concentrations tested by the disk diffusion method [24]. Cultures in the exponential phase in MRS broth (BD) were diluted in 0.85% NaCl (w/v) until a turbidity similar to tube 0.5 of the McFarland scale was reached. Diluted cultures were swabbed on to the surface of the Mueller-Hinton agar (Oxoid) and antibiotic disks (Oxoid) were added (three disks per plate). After incubation at 37°C for 24 h, halos around each antimicrobial disk were measured and the results were classified as susceptible or resistant according to the diameters of the halos [24].

Phenotypic virulence factors

To identify the virulence activity related to haemolysis and the production of hydrolytic enzymes (lipase, gelatinase and DNAse), cultures of *E. hirae* ST65ACC and *P. pentosaceus* ST57ACC were subjected to phenotypic tests according to Perin et al. [35].

Haemolytic activity was assessed by streaking the cultures onto trypticase soy agar (Oxoid) supplemented with 5% (v/v) of defibrinated horse blood and incubating at 37°C for 24 h. The pattern of haemolysis exhibited by each isolate was classified according to the degree of destruction of erythrocytes as total or β-haemolysis, partial or α-haemolysis, and absent or γ-haemolysis.

Lipase production was assessed by spotting 1 μL of the 18-h cultures on to plates containing Luria Bertani (LB) agar (10% tryptone, 5% yeast extract and 10% NaCl; pH 7.0) supplemented with 0.2% (w/v) $CaCl_2$ (Sigma-Aldrich) and 1% (v/v) Tween 80 (Sigma-Aldrich) and incubated at 37°C for 48 h. The formation of clear halos around the colonies was recorded as lipase production.

Gelatinase production was identified by spotting 1μL aliquots of the 18-h cultures on to the surface of LB agar supplemented with 3% (w/v) of gelatine (BD) and incubated at 37°C for 48 h. After incubation, the plates were maintained at 4°C for 4 h, after which the hydrolysis of gelatine was recorded by the formation of opaque halos around the colonies.

DNAse activity was assessed by spotting 1-μL aliquots of the 18-h cultures on to the surface of DNAse methyl green agar (BD) and incubating at 37°C for 48 h. Positive results were identified by the formation of clear halos around the colonies. All tests were performed in duplicate.

Enterococcus hirae ST57ACC and *P. pentosaceus* ST65ACC were also subjected to the protocol described by Bover-Cid, Holzapfel [36] to detect the production of biogenic amines. The bacteriocinogenic strains were cultivated in MRS broth (BD) supplemented with 0.005% (w/v) pyridoxal-5-phosphate (Sigma-Aldrich), to which each of the biogenic amine precursors, including tyrosine free base (for tyramine), histidine monohydrochloride (for histamine), ornithine monohydrochloride (for putrescine) and L-lysine (for cadaverine; all from Sigma-Aldrich), were added at 0.1% (w/v) to induce the production of decarboxylase. After five consecutive passages in these media, each culture was streaked on to MRS decarboxylase agar supplemented with 1% (w/v) of each amino acid precursor of the biogenic amines, performed as described above and according to Joosten, Northolt [37]. The plates

were incubated at 37°C for 4 days, and positive results were recorded by a change in colour of the medium from yellow to purple. Tests were performed in duplicate.

Genotypic virulence determinants

Total DNA from *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were obtained using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. All PCR reactions were performed using the GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, CA, USA). Both isolates were tested for the presence of 41 genes related to safety, including antimicrobial resistance, virulence and biogenic amine production (Supplementary Table 2). The amplified products were separated by electrophoresis in 1.5% (w/v) agarose gels in 0.5× TBE buffer containing GelRed (Biotium Inc., Hayward, CA, USA) at 100 V and visualized on a LPIX transilluminator (Loccus Biotecnologia, São Paulo, SP, Brazil).

Results

Colonisation ability

Both tested bacteriocinogenic strains were able to resist and survive when subjected to simulated gastric and/or intestinal conditions, with the SR of their log populations ranging from 79.1% (*E. hirae* ST57ACC after gastric and enteric conditions) to 92.6% (*P. pentosaceus* ST65ACC after gastric condition; Table 1). The population of *E. hirae* ST57ACC decreased from 7.8 to 6.2 log CFU/mL, while *P. pentosaceus* ST65ACC decreased from 7.6 to 6.2 log CFU/mL, demonstrating their resistance to the simulated gastrointestinal conditions (Table 1).

The results obtained for cell surface hydrophobicity, auto-aggregation and co-aggregation with *L. monocytogenes* strains are presented in Table 2. Both bacteriocinogenic strains presented similar results for cell hydrophobicity of about 40%, but the aggregation rates varied according to strain, where *E. hirae* ST57ACC aggregation rates varied from 17.3% (with *L. monocytogenes* L711) to 51.6% (auto-aggregation), while *P. pentosaceus* ST65ACC aggregation rates varied from 48.1% (with *L. monocytogenes* L711) to 71.8% (auto-aggregation; Table 2). For both strains, *L. monocytogenes* L711 presented a lower aggregation capacity when compared to *L. monocytogenes* L422 and L637.

Both strains were able to survive in the presence of most of the tested commercial drugs (Table 3). Among the evaluated drugs, the growth of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC were affected by only five and three drugs, respectively. The strains were inhibited by anti-inflammatory drugs containing diclofenac potassium, aceclofenac, nimesulide or ibuprofen (Table 3). Moreover, *E. hirae* ST65ACC was inhibited by the antihistamine loratadine and the antihypertensive propranolol. Diclofenac potassium showed the lowest MIC among all evaluated drugs of 0.5 and 0.12 mg/mL for *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, respectively. In contrast, the MIC of Ibupril, containing ibuprofen, was highest (120 mg/mL) against *P. pentosaceus* ST65ACC. For propranolol hydrochloride, the MIC observed against *E. hirae* ST57ACC was 8 mg/mL, while for loratadine, the MIC observed against the same strain was 2 mg/mL.

Safety characteristics

Both strains evaluated in this study were sensitive to the majority of tested antibiotics, presenting low frequencies of antibiotic resistance-encoding genes (Table

4). From the three target genes evaluated for erythromycin resistance (*ermA*, *ermB* and *ermC*), only *E. hirae* ST57ACC amplified a fragment corresponding to *ermB*. Despite the presence of the *ermB* gene in this isolate, a correlation with phenotypic resistance was not observed (Table 4). For *P. pentosaceus* ST65ACC, amplicons corresponding to *bcrB*, *tetO* and *vatE* genes, related to bacitracin, tetracycline and streptogramin resistance, respectively, were detected. Vancomycin-encoding genes were detected in both strains. In *E. hirae* ST57ACC, *vanC1* and *vanC2* were observed, while *vanA* and *vanC1* were amplified in *P. pentosaceus* ST65ACC. In both cases, phenotypic resistance to vancomycin was observed, as well as for sulfa/trimethoprim and oxacillin (Table 4).

Regarding virulence factors, phenotypic tests showed that neither *E. hirae* ST57ACC nor *P. pentosaceus* ST65ACC produced gelatinase, DNAse or lipase, and did not induce haemolysis. Considering the 15 virulence-related genes assessed in this study, *E. hirae* ST57ACC presented amplification only for the *fsrB* gene, while *P. pentosaceus* ST65ACC amplificated only *ccf* out of the three genes tested, which is related to sex pheromones.

Biogenic amine production was not detected in the phenotypic tests performed with *E. hirae* ST57ACC and *P. pentosaceus* ST57ACC, and genes related to the expression of histamine, tyramine and putrescine were absent.

Discussion

The ability to survive and remain viable after passage through the gastrointestinal tract is the most important feature when searching for beneficial strains. Hydrophobicity and auto-aggregation are also relevant and desirable traits, as they

are required for the initial steps of intestinal adhesion and are also related to the ability of the bacteria to adsorb toxic substances like mycotoxins [38]. Hydrophobic interactions precede the subsequent adhesion processes mediated by specific mechanisms involving cell surface proteins and lipoteichoic acids, while auto-aggregation is an important criterion for biofilm formation, which can aid in colonisation of the intestine and help bacteria bind effectively to the intestinal epithelium, thereby preventing pathogen adhesion [39,40] . The ability of *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC to survive passage through the simulated gastrointestinal tract was demonstrated in this study (Table 1), in addition to their hydrophobic and auto-aggregation potential (Table 2). Due to these characteristics, these strains can be considered beneficial microorganisms.

Another characteristic considered positive for a beneficial strain is its ability to interact with pathogens and prevent gastrointestinal infections. Co-aggregation of beneficial strains with pathogens and the close proximity between them facilitates interactions among these microorganisms, leading to elimination of the pathogens through a variety of mechanisms including the production of antimicrobial compounds such as bacteriocins [41]. In a previous study, both *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC showed strong bacteriocinogenic activity against *L. monocytogenes* [19]. The bacteriocins produced by these isolates were able to inhibit the growth of *L. monocytogenes* after 12 h, and *E. hirae* ST57ACC was also found to efficiently reduce the population of this pathogen in skim milk [19]. Considering the earlier findings and the results from this work with *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC (Table 2), it is possible to conclude that these isolates have potential for the control of *L. monocytogenes*. As the pathogenesis of *L. monocytogenes* begins with binding to intestinal epithelial cells, beneficial strains

may interact with the pathogen before this adhesion process, preventing invasion of the host intestine. Moreover, close contact between *L. monocytogenes* and beneficial isolates allows the bacteriocins produced by these strains to promptly act on the target cells prior to being inactivated by the proteolytic enzymes present in the intestinal environment.

Drugs commonly used by patients for the treatment of different diseases may affect the viability of live cultures delivered as beneficial organisms in food products or as prescribed probiotic cultures. In this sense, it is important to evaluate possible interactions between drugs and beneficial strains with the aim to avoid negative interactions. Anti-inflammatory drugs containing diclofenac potassium and its analogues have been previously reported to interfere with the viability of beneficial LAB [42,23,43]. In the same way, inhibition by medications containing loratadine, propranolol hydrochloride and other similar antiallergics and antihypertensives were also reported to be associated with LAB inhibition [42,23,44]. It is important to note that the effectiveness of these drugs depends on the amount of the active compound that reaches the gastrointestinal tract, and correct evaluation of possible interactions between drugs and strains depends on determining the MIC of these compounds [42]. Depending on the period of treatment, drugs may accumulate in the gastrointestinal environment and, as a result, concentrations sufficient to inhibit beneficial cultures can be easily reached, affecting their viability. Only a few drugs considered in this study interfered with the growth of the bacteriocinogenic strains *P. pentosaceus* ST65ACC and *E. hirae* ST57ACC, although this occurred at low MICs when compared to the common dosages indicated for humans (Table 3 and Supplementary Table 1), demonstrating their poor potential for inhibiting the viability of the tested strains. Although the inhibitory doses recorded for these drugs were also lower than

the daily minimum recommendations, factors such as interactions with foods and enzymes should be studied *in vivo* to clarify the interactions between these medications and potentially beneficial cultures.

As LAB can acquire and play a role in the transfer of antibiotic resistance elements to pathogenic bacteria, the food chain is considered an important route for introducing these elements into the gastrointestinal tract. There is current concern regarding the identification of LAB isolates that present antibiotic resistance genes in foods, as they can act as reservoirs and transfer these genes to pathogenic bacteria [45]. Both strains evaluated in this work were sensitive to the majority of tested antibiotics, presenting low frequencies of antibiotic resistance-related genes, and correlation with phenotypic results was rarely observed (Table 4).

Vancomycin-encoding genes were detected in both strains. This is important as vancomycin is considered an antibiotic of last resort when most other antibiotics fail in treating infections caused by Gram-positive bacteria. Resistance to this antibiotic is recognized as an intrinsic trait of *Pediococcus* spp., and is commonly reported [46,47]. In contrast, vancomycin-resistant enterococci represent the main source of infections in humans and carriers of transferable vancomycin resistance markers [48]. While *vanA* and *vanB* are normally associated with plasmid DNA, *vanC, vanD, vanE* and *vanG* are encoded on the bacterial chromosome [28]. *Pediococcus* spp. is described as intrinsically resistant to several groups of antibiotics, including β-lactams, cephalosporins, aminoglycosides, glycopeptydes, streptomycin, kanamycin, tetracyclines, doxycycline and sulfa (with or without trimethoprim) [47,46], and phenotypic resistance to some of these antibiotics was observed in the present study (Table 4). However, studies related to genetic markers in *Pediococcus* spp. are scarce, and this appears to be the first report of the presence of *bcrB*, *tetO* and *vatE*

genes in *P. pentosaceus* isolated from raw milk cheese. Investigations into the role of these genes in resistance and their contribution to acquired resistance need to be studied further, as well as the presence of other genes related to antibiotic resistance that could explain the differences between results of phenotypic and genotypic assays.

Virulence factors are usually surveyed in enterococcal species, and virulent activity has previously been reported in dairy isolates [35,49]. In this study, phenotypic tests were negative for both tested strains, and only the presence of the *fsrB* gene was detected in *E. hirae* ST57ACC. The *fsr* operon has previously been identified in *E. faecalis*, *E. faecium*, *E. durans*, *E. hirae* and *E. dispar*, indicating that these genes may be widespread among species of the *Enterococcus* genus [50,49]. Genes belonging to this operon (*fsrA*, *fsrB* and *fsrC*) in association with *gelE* are responsible for gelatinase activity [49]. The expression of *gelE* is dependent on cell density and is positively regulated by the *fsr* operon. This means that the expression of all *fsr* genes is required in order to regulate *gelE* and its consequent positive phenotype [49]. Concerning sex pheromones, these have been well characterized in *Enterococcus* spp., known to play a role in the exchange of genetic information by conjugation. However, although sex pheromones are not considered to be virulence factors *per se*, their production in enterococci may favour the dissemination of virulence determinants and promote the acquisition of antibiotic resistance and other linked traits from other enterococci, thereby leading to increased virulence [51]. While the presence of sex pheromones has not yet been reported in *Pediococcus* spp., and its role in this genus is currently unknown, *P. pentosaceus* ST65ACC was found to harbor *ccf* in this study. Further characterization of this activity and its possible

contribution to virulence or participation in horizontal gene transfer needs to be investigated.

The production of biogenic amines by LAB are variable, and are usually associated with the presence of genes that are generally strain-specific [20,52] and to specific growth conditions that promote decarboxylase synthesis [35]. Ladero et al. [53] suggested that in the *Enterococcus* genus, the production of tyramine is specific to *E. faecalis*, *E. faecium* and *E. durans*, putrescine is specific to *E. faecalis*, and no biogenic amine is linked to *E. hirae*. The absence of biogenic amine production and target genes related to this characteristic further corroborates the safety of *E. hirae* ST65ACC and *P. pentosaceus* ST57ACC and supports their use as beneficial or biopreservative strains.

Enterococcus hirae ST57ACC and *P. pentosaceus* ST65ACC both presented interesting beneficial properties, especially due to their ability to survive gastrointestinal conditions and aggregate with *L. monocytogenes*, which can help in the control of this pathogen. Also, the tested strains presented very low frequencies of virulence markers and showed no production of biogenic amines, indicating that they could be considered suitable for industrial applications. However, the tested strains presented some antibiotic resistance characteristics, which represent a current concern worldwide. Further studies should investigate how to minimize the hazards linked to antibiotic resistance in *E. hirae* ST57ACC and *P. pentosaceus* ST65ACC, as well as provide a deeper understanding of the interactions between these strains and *L. monocytogenes* to assess the use of these bacteria or their bacteriocins as alternatives to control this foodborne pathogen.

Conflict of Interest

Authors declare that they have no conflict of interest.

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Ethical approval

None animal was used in any of the described experiments.

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Table 1. Populations (log CFU/mL) and survival rates (%) based on the log populations of the bacteriocinogenic strains *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC after simulating conditions of the gastrointestinal tract.

Tested strain	Population (log CFU/mL)			Survival rate (%)*		
	Initial (T_0)	After gastric phase (T_1)	After enteric phase (T_2)	Gastric phase	Enteric phase	Entire assay
E. hirae ST57ACC	7.8 ± 0.11	7.1 ± 0.07	6.2 ± 0.03	91.0	86.9	79.1
P. pentosaceus ST65ACC	7.6 ± 0.17	7.0 ± 0.12	6.2 ± 0.09	92.6	88.7	82.2

* Survival rates calculated as described in Material and Methods section, and based on equation proposed by Santos et al., 2015.

Table 2. Cell surface hydrophobicity (%), auto-aggregation (%) and co-aggregation with *Listeria monocytogenes* strains (%) of the bacteriocinogenic strains *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC.

Tested strain	Cell surface hydrophobicity (%)*	Auto-aggregation (%)*	Co-aggregation (%) with*		
			L. monocytogenes L711	*L. monocytogenes* 422	*L. monocytogenes* L637
E. hirae ST57ACC	41.0 ± 0.13	51.6 ± 0.01	17.3 ± 0.01	42.5 ± 0.06	48.1 ± 0.02
P. pentosaceus ST65ACC	46.5 ± 0.04	71.8 ± 0.02	48.1 ± 0.05	71.7 ± 0.08	71.0 ± 0.02

* Values were calculated as described in Material and Methods section, based on equations proposed by Santos et al. (2015) and Todorov et al. (2008).

Table 3. Effect of commercial drugs on growth of *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC in MRS agar, presented as diameter of inhibition zones (mm) and Minimal Inhibitory Concentration (MIC, in mg/mL).

Medical group	Commercial name	Inhibition*	
		E. hirae ST57ACC	*P. pentosaceus* ST65ACC
Analgesic and antipyretic	Anador	0	0
	Paracetamol	0	0
	Paracetamol	0	0
	Saridon	0	0
Analgesic and antispasmodic	Buscopan composto	0	0
Analgesic, anti-inflammatory and muscle relaxant	Doralflex	0	0
Anti-acid	Sonrisal	0	0
Antiallergic	Loratadina	6 (2)	0
Antiasmatic	Aminofilina	0	0
	Fumarato de cetotifeno	0	0
Antidiarrheic	Lopedium	0	0
Antiemetic	Bromoprida	0	0
Anti-flu	Perfenol	0	0
	Trimedal	0	0
Antihistaminic	Allergosan	0	0
	Dramin	0	0
Antihypertensive	Cloridrato de propanolol	10 (8)	0
	Lisinopril	0	0
	Press Plus	0	0
Anti-inflammatory	Aceclofenaco	15 (20)	14 (20)
	Ácido mefenâmico	0	0
	Diclofenaco de potássio	11 (0.5)	20 (0.12)
	Ibupril	0	12 (120)
	Nisoflan	30 (20)	0
Antitussive	Libexin	0	0
Anti-ulcers	Pratiprazol	0	0
Antivertiginous and vasodilatory	Cinarizina	0	0
Digestive stimulant	Hepatilon	0	0
Laxative	Dulcolax	0	0
Mucolytic	Acetilcisteína	0	0
	Fluteína	0	0
Vitamin complex	Complexo Ômega A-Z	0	0
	Dayvit	0	0

* Inhibition presented as diameter of inhibition zones (mm), followed by recorded MIC (mg/mL). Concentrations of the tested commercial drugs are detailed in the Supplementary Table 1.

Table 4. Antibiotic resistance profiles of *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC evaluated by disk diffusion method and PCR. Inhibition zones in disk diffusion method are expressed in millimeters (mm) and strains were classified as susceptible (S) or resistant (R), according to CLSI (2016).

Antimicrobial group	Antimicrobial (µg per disk)	*E. hirae* ST57ACC		*P. pentosaceus* ST65ACC	
		Disk	PCR*	Disk	PCR*
Chloramphenicol	Chloramphenicol (30)	30 (S)	None	30 (S)	None
Glicopeptides	Vancomycin (30)	0 (R)	*vanC1*, *vanC2*	0 (R)	*vanA*, *vanC1*
Tetracyclines	Tetracycline (30)	22 (S)	None	22 (S)	*tetO*
Macrolides/Lincosamides	Erytromycin (15)	28 (S)	*ermB*	28 (S)	*vatE*
	Clindamycin (2)	28 (S)		30 (S)	
Bacitracin	Not tested	-	None	-	*bcrB*
Sulfonamides	Trimethoprim/sulfamethoxazole (25)	0 (R)	Not tested	0 (R)	Not tested
Rifamicin	Rifampicin (5)	25 (S)	Not tested	22 (S)	Not tested
β-Lactam	Ampicillin (10)	21 (S)	Not tested	22 (S)	Not tested
	Imipenem (10)	30 (S)	Not tested	32 (S)	Not tested
	Oxacillin (1)	0 (R)	Not tested	0 (R)	Not tested
	Penicilin G (10**)	25 (S)	Not tested	28 (S)	Not tested

* Tested genes: *bcrB*, *bcrD*, *bcrR* (bacitracin resistance), *ermA*, *ermB*, *ermC* (erythromycin resistance), *tetK*, *tetL*, *tetM*, *tetO*, *tetS* (tetracycline resistance), *vanA*, *vanB*, *vanC1*, *vanC2* (vancomycin resistance), *catA* (chloranphenicol resistance), *vatE* (streptogramin resistance). **Concentration of antimicrobial expressed as Units.

Supplementary Table 1. Medical groups, commercial names, active substances/composition, dosages and tested concentrations of commercial drugs selected in the present study to assess their antimicrobial activity against the bacteriocinogenic strains *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC.

Medical group	Commercial name	Active substance/composition	Dosage*	Tested concentration (mg/mL)
Analgesic and antipyretic	Anador	Dypirone sodium	500	100
	Paracetamol	Paracetamol	750	150
	Paracetamol	Paracetamol	500	50
	Saridon	Caffeine	50	10
		Paracetamol	250	50
		Propranolol hydrochloride	150	30
Analgesic and antispasmodic	Buscopan composto	Butylscopolamine	10	2
		Dipyrone sodium	250	50
Analgesic, anti-inflammatory and muscle relaxant	Doralflex	Caffeine	50	10
		Dipyrone sodium	300	60
		Orphenadrine citrate	35	7
Anti-acid	Sonrisal	Acetylsalicylic acid	325	65
		Citric acid	1,575	315
		Sodium acid carbonate	1,700	340
		Sodium carbonate	400	80
Antiallergic	Loratadina	Loratadin	10	2
Antiasmatic	Aminofilina	Aminophylline	100	20
	Fumarato de cetotifeno	Ketotifen fumarate	0.2	0.2
Antidiarrheic	Lopedium	Loperamide	2	0.4
Antiemetic	Bromoprida	Bromopride	10	2
Anti-flu	Perfenol	Chlorpheniramine maleate	4	0.8
		Paracetamol	400	80
		Phenylephrine hydrochloride	4	0.8
	Trimedal	Ascorbic acid	40	4
		Dimethindene maleate	0.5	0.05
		Paracetamol	500	50

		Phenylephrine hydrochloride	2	0.2
		Ruthoside	15	1.5
Antihistaminic	Allergosan	Chloropyramine	25	5
	Dramin	Dimenhydrinate	50	10
		Pyridoxine hydrochloride	10	2
Antihypertensive	Cloridrato de propanolol	Propranolol hydrochloride	40	8
	Lisinopril	Lisinopril	20	4
	Press Plus	Amlodipine besylate	2.5	0.5
		Benazepril hydrochloride	10	2
Anti-inflammatory	Aceclofenaco	Aceclofenac	100	20
	Ácido mefenâmico	Mefenamic acid	500	100
	Diclofenaco de potássio	Diclofenac potassium	50	10
	Ibupril	Ibuprofen	600	120
	Nisoflan	Nimesulide	100	20
Antitussive	Libexin	Prenoxdiazine hydrochloride	100	20
Anti-ulcers	Pratiprazol	Omeprazole	20	4
Antivertiginous and vasodilatory	Cinarizina	Cinarizine	75	15
Digestive stimulant	Hepatilon	*Peumus boldus*	67	13.4
Laxative	Dulcolax	Bisacodyl	5	1
Mucolytic	Acetilcisteína	Acetylcysteine	100	20
	Fluteína	Acetylcysteine	600	120
Vitamin complex	Complexo Ômega A-Z	Ascorbic acid	22.5	1.125
		Biotin	15**	0.75
		Calcium chloride	125	6.25
		Cholecalciferol	2.5**	
		Cholesterol	1.5	0.075
		Chrome	17.5**	0.875
		Copper sulphate	450**	22.5
		Cyanocobalamin	1.2**	0.06
		Ferrous oxide	7	0.35
		Folic acid	120**	6
		Iodine	65**	3.25

	Magnesium oxide	32.5	1.625
	Manganese chloride	1.15	0.0575
	Molybdenum	22.5**	1.125
	Niacin	8	0.4
	Omega 3	60	3
	Omega 6	40	2
	Pantothenic acid	2.5	0.125
	Phytometadione	32.5**	1.625
	Retinyl acetate	300**	15
	Riboflavin	0,6*	0.03
	Selenium	17	0.85
	Thiamine hydrochloride	0.6	0.03
	Tocopherol acetate	5	0.25
	Zinc oxide	3.5	0.175
Dayvit	Ascorbic acid	90	18
	Calcium	162	32.4
	Cyanocobalamin	9**	1.8
	Fructose 1,6 diphospate calcium	80	16
	Iodine	150**	30
	Iron	27	5.4
	Magnesium	100	20
	Nicotinamide	20	4
	Phosphor	125	25
	Pyridoxine hydrochloride	3	0.6
	Racealfatochoferol acetate	30*	6
	Retinyl acetate	500*	100
	Riboflavin	2.6	0.52
	Selenium	100**	20
	Thiamine nitrate	2.25	0.45
	Zinc	15	3

* Commercial dosage of selected drugs in International Units (IU), except in indicated with **, in micrograms (µg).

Supplementary Table 2. Primers and conditions used for the detection of genes implicated in antibiotic resistance, virulence and biogenic amines production in *Enterococcus hirae* ST57ACC and *Pediococcus pentosaceus* ST65ACC

Target	Gene*	Primer	Amplicon (bp)	Reference
Antibiotic resistance	*bcrB*	AAAGAAACCGACTGCTGATA	489	[1]
		GCTTACTTGTATAGCAGAGA		
	bcrD	GCGAAGCGTTTAAGGAAATG	482	[1]
		TGGCACAGCAAGAAAGAATG		
	bcrR	TAACGCAGGAACAACTTGC	461	[1]
		CAAAGCGGTAATGGTGAGG		
	ermA	TCTAAAAAGCATGTAAAAGAA	645	[2]
		CTTCGATAGTTTATTAATATTAG		
	ermB	GAAAAGTACTCAACCAAATA	639	[2]
		AGTAACGGTACTTAAATTGTTTA		
	ermC	TCAAAACATAATATAGATAAA	642	[2]
		GCAAATATTGTTTAAATCGTCAAT		
	tetK	TTAGGTGAAGGGTTAGGTCC	348	[3]
		GCAAACTCATTCCAGAAGCA		
	tetL	CATTTGGTCTTATTGGATCG	696	[3]
		ATTACACTTCCGATTTCGG		
	tetM	GTTAAATAGTGTTCTTGGAG	656	[3]
		CTAAGATATGGCTCTAACAA		
	tetO	CAATATCACCAGAGCAGGCT	634	[3]
		GATGGCATACAGGCACAGAC		
	tetS	TGGAACGCCAGAGAGGTATT	667	[3]
		ACATAGACAAGCCGTTGACC		

	vanA	TCTGCAATAGAGATAGCCGC	377	[4]
		GGAGTAGCTATCCCAGCATT		
	vanB	GCTCCGCAGCCTGCATGGACA	529	[4]
		ACGATGCCGCCATCCTCCTGC		
	vanC1	GGTATCAAGGAAACCTC	822	[5]
		CTTCCGCCATCATAGCT		
	vanC2	CTCCTACGATTCTCTTG	439	[5]
		CGAGCAAGACCTTTAAG		
	catA	GGATATGAAATTTATCCCTC	648	[3]
		CAATCATCTACCCTATGAAT		
	vatE	ACGTTACCCATCACTATG	282	[6]
		GCTCCGATAATGGCACCGAC		
Virulence	*ace*	GAATTGAGCAAAAGTTCAATCG	1,008	[4]
		GTCTGTCTTTTCACTTGTTTC		
	asa1	GCACGCTATTACGAACTATGA	375	[7]
		TAAGAAAGAACATCACCACGA		
	ccf	GGGAATTGAGTAGTGAAGAAG	543	[8]
		AGCCGCTAAAATCGGTAAAAT		
	cob	AACATTCAGCAAACAAAGC	1,405	[8]
		TTGTCATAAAGAGTGGTCAT		
	cpd	TGGTGGGTTATTTTTCAATTC	782	[8]
		TACGGCTCTGGCTTACTA		
	cylA	ACTCGGGGATTGATAGGC	688	[7]
		GCTGCTAAAGCTGCGCTT		
	efaA	GCCAATTGGGACAGACCCTC	688	[4]
		CGCCTTCTGTTCCTTCTTTGGC		

	esp	AGATTTCATCTTTGATTCTTG	510	[7]
		AATTGATTCTTTAGCATCTGG		
	fsrA	ATGAGTGAACAAATGGCTATTTA	740	[9]
		CTAAGTAAGAAATAGTGCCTTGA		
	fsrB	GGGAGCTCTGGACAAAGTATTATCTAACCG	566	[9]
		TTGGTACCCACACCATCACTGACTTTTGC		
	fsrC	ATGATTTTGTCGTTATTAGCTACT	1,343	[10]
		CATCGTTAACAACTTTTTTACTG		
	gelE	TATGACAATGCTTTTTGGGAT	213	[7]
		AGATGCACCCGAAATAATATA		
	hyl	ACAGAAGAGCTGCAGGAAATG	276	[7]
		GACTGACGTCCAAGTTTCCAA		
	int-Tn	TGACACTCTGCCAGCTTTAC	579	[5]
		CCATAGGAACTTGACGTTCG		
Biogenic amines	*hdc*1	AGATGGTATTGTTTCTTATG	367	[11]
		AGACCATACACCATAACCTT		
	*hdc*2	AAYTCNTTYGAYTTYGARAARGARG	534	[11]
		ATNGGNGANCCDATCATYTTRTGNCC		
	tdc	GAYATNATNGGNATNGGNYTNGAYCARG	924	[11]
		CCRTARTCNGGNATAGCRAARTCNGTRTG		
	odc	GTNTTYAAYGCNGAYAARCANTAYTTYGT	1,446	[11]
		ATNGARTTNAGTTCRCAYTTYTCNGG		

**bcr*B, *bcr*D, *bcr*R (bacitracin resistance), *erm*A, *erm*B, *erm*C (erythromycin resistance), *tet*K, *tet*L, *tet*M, *tet*O, *tet*S (tetracycline resistance), *van*A, *van*B, *van*C1, *van*C2 (vancomycin resistance), *cat*A (chloranphenicol resistance), *vat*E (streptogramin resistance), *asa*1(aggregationsubstance), *ccf*, *cob*, *cpd* (related to sex pheromones), *cyl*A (cytolisin), *efa*A (endocardites antigen), *esp* (enterococcal surface protein), *fsr*A, *fsr*B, *fsr*C (related to gelatinase activity), *gel*E(gelatinase), *hyl*(hyaluronidase), *int-Tn* (transposom related), *hdc*1, *hdc*2 (histidine decarboxylase), *tdc* (tyrosine decarboxylase) and *odc* (ornithinedecarboxylase).

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CAPÍTULO 3 – Physiological and molecular insights of bacteriocin production by *Enterococcus hirae* ST57ACC from Brazilian artisanal cheese

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Title page

Physiological and molecular insights of bacteriocin production by *Enterococcus hirae* ST57ACC from Brazilian artisanal cheese

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Abstract

The bacteriocinogenic *Enterococcus hirae* ST57ACC recently isolated from a Brazilian artisanal cheese was subjected here for additional analyses in order to gain novel insights on the parameters guiding its bacteriocin production, and their impact on ABC transporters expression. Besides these physiological and molecular aspects, the purified bacteriocin was evaluated for its cytotoxicity against the human colon adenocarcinoma cell line, HT-29. Here we reveal that differences in the inoculum size has no impact on the growth of *E. hirae* ST57ACC; however, the bacteriocin was only produced after 9 h of growth when the strain was inoculated at 5% or 10% (v/v), and we establish that biomass was linked to the bacteriocin production in batch fermentation. Furthermore, expression of different ABC transporters corresponding to the bacteriocin transport and sugar metabolism were identified. In terms of adverse effects, when a semi-purified fraction of the bacteriocin and the cell-free supernatant were tested against HT-29, total cell viability observed was 125.84% and 123.74%, respectively, arguing on the absence of cytotoxic effect on human cells. This manuscript correlated data regarding a non-cytotoxic bacteriocin production by *E. hirae* ST57ACC to its physiological parameters and gene expression.

Keywords: *Enterococcus hirae*; bacteriocin; ABC transporter; cytotoxicity

1. Introduction

Enterococcus spp. are known to be largely distributed in the environment, as they have been isolated from humans, animals, soil, surface waters, plants, and vegetables [1,2]. At medical level, *Enterococcus* is known as an important opportunistic pathogen, especially the species *E. faecalis* and *E. faecium*, causing a wide variety of infections, whereas in food production these microorganisms are associated with the development of sensory characteristics in fermented products delineating a rich and diverse metabolic pathways [2,1,3,4]. These microorganisms are endowed with antagonistic properties attributed to their abilities to produce a variety of antimicrobial compounds as organic acids and bacteriocins [5,6].

Regarding the bacteriocins, these antimicrobial peptides are synthesized by both Gram-negative and Gram-positive bacteria [7]. They are active against bacteria that cause spoilage in foods, and for this reason they are clearly considered as potential bio-preservative agents, with the ability to improve food safety and quality [8,7]. Bacteriocins from *Enterococcus* spp., called enterocins, are overall well characterized among the other ones produced by lactic acid bacteria (LAB). Remarkably, there has been a growing interest in their antimicrobial potential for applications in both human and veterinary medicines [6,9,10].

E. hirae ST57CC was recently isolated from a Brazilian artisanal cheese and characterized as a bacteriocin-producing strain [11]. The virulome and resistome aspects concluded that *E. hirae* ST57CC is a safe bacteriocinogenic strain [12]. Considering the inhibitory potential of *E. hirae* ST57CC, this study aimed at highlighting its physiological parameters guiding the production of its bacteriocin(s)

and their association with ABC transporters expression. To complete this study, cytotoxicity of purified peptide was measured against human cell line.

2. Material and Methods

2.1. Bacteriocin-producing strain *E. hirae* ST57ACC

E. hirae ST57ACC was previously isolated from a Brazilian artisanal cheese and identified by 16S rDNA sequencing (Cavicchioli et al., 2017). The strain was maintained in de Man, Rogosa and Sharpe broth (MRS, Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA) supplemented with glycerol 20 % (v/v) and stored at −20 °C. Before the tests, an aliquot of 100 µL of the stored culture was transferred to 5 mL of MRS broth (BD) and incubated at 37 °C for 18 h.

2.2. Optimization of culture conditions and bacteriocin production

2.2.1. Inoculum size effect on bacteriocin production

MRS broth (100 mL, BD) was inoculated with *E. hirae* ST57ACC at 1%, 2%, 5% and 10% (v/v) and incubated at 37°C. Samples were collected at different intervals of time (0, 3, 6, 9, 18, and 24 h) and analyzed by spectrophotometry at 600 nm in order to determine the optical density (Beckman Coulter®, Brea, CA, USA) and the pH (Hanna Instruments, Vöhringen, Germany). Bacteriocin production was detected using a qualitative assay previously described by Todorov [13]. In summary, *E. hirae* ST57ACC was centrifuged (12,000 × *g*, 10 min, 4 ºC) and the pH of the cell-free supernatant (CFS) was adjusted with 1 M NaOH to a pH between 6.0 - 6.5. The

supernatant was heat treated (10 min at 80°C), and then 10 µL of the treated CFS was spotted onto the surface of a Brain Heart Infusion (BHI) agar plate supplemented with 1% agar (BD), which has been previously inoculated with a culture of the target *E. faecium* ATCC 19443 strain (at 10^6 colony forming units per mL, CFU/mL). The plates were then incubated at 37°C for 24 h. Inhibition halos larger than 2 mm were considered positive, indicating bacteriocin production. This experiment was conducted in duplicate.

2.2.2. Growth dynamics

MRS broth (BD) was inoculated with *E. hirae* ST57ACC at 5% (v/v), transferred to sterile flasks (500 mL) for conventional growth, and then to a bioreactor (3,000 mL, Infors-HT, Bottmingen, Switzerland) for batch fermentation. Conventional growth was performed at 37°C and batch fermentation was performed at 37°C with agitation (50 rpm), this was to maintain homogenous culture conditions. After reaching a pH of 5.5, NaOH was constantly added to keep the pH stable at pH 5.5. For both growth conditions, samples were taken after 0, 6, 12, and 24 h of incubation, and subjected to spectrophotometry and pH measuring, as described above. Bacteriocin production was measured using a quantitative assay, as described previously by Campos et al. [14] and Todorov [13]. In brief, CFS was obtained and treated as described above and then diluted two-fold in 10 mM phosphate buffered saline (PBS) at pH 6.5. Aliquots (10 µL) of the diluted CFS were spotted onto the surface of BHI agar (BD), previously plated with the target strain *E. faecium* ATCC 19443 (at 10^6 CFU/mL). The plates were then incubated at 37°C for 24 h. Bacteriocin activity was expressed as arbitrary units per mL (AU/mL), corresponding to the reciprocal of the highest dilution having a detectable halo of inhibition (higher than 2 mm).

2.3. Expression of ABC-transporter related genes

A culture of *E. hirae* ST57ACC was subjected to RNA extraction using the GeneMATRIX Universal RNA Purification Kit (EURx Ltd., Gdansk, Poland) according to manufacturer´s instructions. The obtained RNA was used for measuring the expression of the genes associated with ABC-transporter system, using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Brea, CA, USA). The house-keeping gene *kanR* was used as an external control. Details of primers sequences, gene functions and length of products are presented in Supplementary Table.

Reverse transcriptase (RT) reactions were prepared with a final volume of 20 µL, containing 3 µL of DNase/RNase free water, 4 µL of 5x RT buffer, 5 µL*kanR* RNA, 1 µL RT enzyme (GenomeLab™ GeXP Start Kit, Beckman Coulter), 2 µL of primers (10 pmol/mL) and 5 µL of RNA. Reactions placed in a thermal cycler (VWR International Ltd, Leicestershire, UK) and the following protocol was run: 1 min at 48°C, 60 min at 42°C, 5 min at 95°C, and held at 4°C. Each experiment included a RT-negative control and a no-template control (NTC).

PCR samples were prepared to a final volume of 20 µL, containing 4 µL 5x PCR buffer, 4 µL 25 mM $MgCl_2$, 0.7 µL Thermo-Start DNA Polymerase (GenomeLab™ GeXP Start Kit, Beckman Coulter), 2 µL of primers plex (10 pmol/mL, Supplementary Table) and 9.3 µL of cDNA samples from the RT plate. PCRs were conducted in a thermal cycler (VWR, Radnor, PA, USA) under the following

conditions: 10 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 68°C, and then the reactions were held at 4°C.

Then, the GeXP fragment and data analyses followed the manufacturer instructions (Beckman Coulter, Brea, CA, USA). Aliquots of 1 µL from the PCR products were added to the appropriate wells of a 96-well sample microplate. DNA size standard 400 (0.5 mL, GenomeLab™ GeXP Start Kit, Beckman Coulter) was added to 38.5 µL of sample loading solution, thoroughly mixed and added to the 96-well sample microplate. The PCR product was separated based on the fragment size by capillary gel electrophoresis (GenomeLab™ GeXP Genetic Analysis System, Beckman Coulter). The strength of the dye signal was measured after normalization to *kanR* RNA. The signals were normalized using the GeXP express profiler software (Beckman Coulter). Results were expressed as either absence (-) or different degrees of gene expression (+ weak, < 100 units; ++ medium, 100-300 units; +++ high, > 300 units).

2.4. Bacteriocin purification

E. hirae ST57ACC was grown in BHI broth (BD), to avoid the interference of proteins from MRS broth, at 37°C for 18 h. The culture was centrifuged at 4°C and 10,000 × *g*, the pH of the CFS corrected with 3M NaOH to pH 6.5 and then heated to 80°C for 10 min. The resin CM Sephadex® C-25 (GE Healthcare Life Sciences, Milwaukee, WI, USA) was washed with 3 column volumes (20 mL) of distillated water and then charged with 40 mL of CFS. The resin-bound bacteriocin was eluted with 2 column volumes in a gradient of 0.5 M, 1.5 M, and 4 M NaCl until complete elution. Active fractions were further subjected to solid phase extraction on C-18 columns (GE Healthcare Life Sciences), using 20%, 60%, and 80% acetonitrile

(ACN) solutions containing 0.1% trifluoroacetic acid (TFA). The samples were then evaporated in a Speed-Vac (Savant, Thermo Fisher Scientific, Waltham, MA, USA). Further purification of the bacteriocin was performed using reversed-phase high-performance liquid chromatography (RP-HPLC), eluted using the following linear gradient of ACN, 0.1% TFA and water (at 0 min 80 % water, 20% [ACN/0.1% TFA] to 40 min 20% water, 80% [ACN/0.1% TFA]). After each purification step, the protein concentration was measured using a bicinchoninic acid assay (Sigma-Aldrich, St. Louis, MI, USA) and the antibacterial activity measured using the quantitative assay as described above; the target strain was *Listeria monocytogenes* 162, previously obtained from food [15].

2.4. Bacteriocin cytotoxicity

A cytotoxicity test was performed on HT-29 cells, grown in 96-well tissue culture plates for 48–72 h, with 5% CO_2, and in Dulbecco's Modified Eagle Medium (DMEM). The active fraction obtained by solid phase extraction (corresponding to the fraction eluted by 60% ACN) and the CFS were tested. Non-diluted aliquots of samples were added to the cell culture monolayer and incubated for 24 h at 37°C. After incubation, the supernatants were removed, and the HT-29 monolayer was washed twice with DMEM medium containing antibiotics and fetal bovine serum. In addition, the CCK-8 assay (Dojindo Molecular Technologies, Rockville, MD, USA), based on the reduction of the tetrazolium salt by active mitochondria, was used to assess cell viability of the treated HT-29 cells. Plates were then read at 450 nm in a microplate reader spectrophotometer (Xenius, Safas, Monaco). 0.1% Triton X-100 was used as a cytotoxic control.

3. Results

3.1. Optimization of culture conditions and bacteriocin production

The growth of *E. hirae* ST57ACC with different inoculum concentrations is presented in Fig. 1. During the first 9 hours of growth, the optical density (OD) appeared proportional to the inoculum size (Fig. 1). Then, from 9 to 24 h, the OD values registered were similar, discarding the effect of the initial inoculum (Fig. 1). To be noted, that changes in pHs were overall similar throughout the experiment, decreasing from 6 to 4, after 24 h of growth (Fig. 1). Bacteriocin production was, nevertheless, detected only after 9 h of growth when the culture was inoculated with 5% or 10% of *E. hirae* ST57ACC . The production of bacteriocin remained detectable in such conditions up to 24 h of growth (Fig. 1). Usually the inoculum size has no effect on bacterial growth, however, bacteriocin production could be affected. Regarding these findings, the initial inoculum of 5% of the bacteriocin-producing strain was then standardized in this study.

To gain more insights on the bacteriocin production of *E. hirae* ST57ACC, the growth dynamics of this bacteriocinogenic strain was established, as indicated on Fig. 2, upon its classical growth in MRS medium as well as under fermentation conditions using a bioreactor. The data analyses indicated that bacteriocin production was associated to the cell density under both growth conditions. It should be pointed out that *E. hirae* ST57ACC cultivated in the bioreactor, after 24 h of fermentation at a controlled pH of 5.5, levels of bacteriocin production were similar to those obtained from cultivation in flasks (Fig. 2).

3.2. Expression of ABC-transporter related genes

Here we established different expression levels of the genes, involved in the ABC transporter system from *E. hirae* ST57ACC. Except, the sugar ABC transporter ATP-binding protein and alcohol dehydrogenase, all genes tested were upregulated, including the bacteriocin ABC-transporter (Table 1). Higher levels of expression were noticed for glucose-6-phosphate 1-dehydrogenase (Table 1). For malate/lactate dehydrogenase, bacteriocin ABC-transporter ATP binding and permease protein and ABC transporter ATP-binding protein, levels between 100 and 300 units of expression were observed (Table 1).

3.3. Bacteriocin purification and cytotoxicity

The bacteriocin produced by *E. hirae* ST57ACC was purified, as previously described for the purification of enterocin DD14, with some modifications [16] consisting in the removal of the overnight incubation of the CFS and the Sephadex matrix. Higher bacteriocin activity was obtained in the elution with 1.5 M NaCl comparatively to other eluents used (Table 2). The use of solid-phase extraction increased the specific activity of the bacteriocin by 15-fold when compared to the cell-free supernatant, and almost ten-fold when compared to gel filtration (Table 2). However, the specific activity after RP-HPLC was reduced (Table 2). Three major peaks were observed in the RP-HPLC analysis, with retention times of 8.5, 10.5 and 18.5 min. These samples were individually collected to evaluate their antimicrobial activity. An inhibition zone was only observed around the spot corresponding to the

8.5 min retention time, suggesting that the bacteriocin produced by *E. hirae* ST57ACC was eluted in a single peak.

Regarding cytotoxic effects, the semi-purified fraction obtained by elution with 60% ACN during solid-phase extraction and the cell-free supernatant were tested, presenting 125.84% and 123.74% of cell viability, respectively, compared to cells treated with Triton X100, as positive control. Non-treated cells were used as negative control to compare cell viability.

4. Discussion

In our study, the growth of *E. hirae* ST57ACC was not influenced by the size of the inoculum, however, bacteriocin production was observed only when high proportions of inoculum size were used (Fig. 1). Still, similar OD values were observed for conventional and batch fermentation, while for batch fermentation, where pH was controlled, the lag phase was longer. Additionally, higher levels of bacteriocin production were observed when *E. hirae* ST57ACC was cultivated in batch fermentation (Fig. 2), indicating that this approach could be useful in industrial scale to obtain more bacteriocin. Bacteriocin production and cell-density in LAB are substantially influenced by different factors including, pH, temperature, and media composition [17,18]. Lv et al. [19] reported that bacteriocin production in LAB is following a scheme of primary metabolite growth-associated kinetics, occurring during the exponential growth phase and ceases once the stationary had been reached. These data indicate that bacteriocin production is, indeed, dependent on the total biomass [19]. However, the relationship between bacteriocin production and growth also depends on the strain used. In fact, a correlation between the peptide production and cell-density is evident in some cases, whereas in other cases

bacteriocin production starts only when stationary phase had been reached [20,21]. It should be mentioned that a high cell density does not necessarily lead to higher bacteriocin yield, as observed in this study, as it is possible that low bacteriocin production per gram of cells can occur.

There is a complex relationship between the environmental conditions and bacteriocin production. Production in *E. mundtii* CRL1656 has been studied. Indeed, it was reported that the biomass and level of agitation did not affect bacteriocin production or the strain growth, although 5% inoculum significantly reduced the length of the lag phase [22]. Furthermore, Herranz et al. [23] observed that the production of enterocin P was strongly dependent on pH during continuous cultivation of *E. faecium* P13. Moreover, bacteriocins production were optimally produced at a pH of 6.0, lower than that for optimal growth of the producer (pH 7.0) [23]. Therefore, optimization of the culture conditions can lead to a more effective recovery of bacteriocins. This strategy could be considered for a large-scale production of bacteriocin and industrial applications.

ABC transporters form one of the largest protein super-families and they act secreting and/or uptaking a range of different compounds across the membrane, requesting the energy resulting from ATP hydrolysis in this process [24]. Considering that LAB generate their primary energy via glycolysis, upregulation of ABC transporters linked to sugar metabolism is an essential feature for this bacterial group and expression of glycolysis enzymes and genes associated with sugar metabolism have been previously observed [25]. ABC transporters are also related to the release of most of class IIa bacteriocin, where the leader peptide is processed by the peptidase domain of the N-terminal region in an ATP-dependent manner for bacteriocin translocation [26,27]. It has been recognized that the same ABC

transporter system can be simultaneously involved in secretion of bacteriocins and quorum sensing signaling molecules, according to environmental conditions in Gram-positive bacteria [28]. Bifunctional transporters are also involved in the transport of other peptide products across bacterial species and this ability of intercommunication can confer fitness advantages to LAB during bacteriocin production and secretion in vivo, contributing to competitive colonization [29,24]. Besides, different transport systems may result in most efficient bacteriocin production and in LAB. The effects of combination of SunT transporter and double glycine leader sequence were studied for mesentericin Y105 production, revealing that association of different systems resulted in increased production levels compared to the general secretion and replacement of original double glycine type by the sec-dependent signal result in considerable bacteriocin production [30,31]. Efforts have been made to increase bacteriocin production and improve their production on a large scale and the understanding of varied transport systems and mechanisms involved in its regulation, may help to develop different strategies for efficient industrial bacteriocin production by.

Bacteriocin produced by *E. hirae* ST57ACC was successfully purified in a three-step protocol in this study and previous results about bacteriocin encoding genes have shown that *E. hirae* ST57ACC does not harbor enterocin A, enterocin P, enterocin B neither enterocin L50B genes, and the bacteriocin produced by this strain might be a novel bacteriocin [11]. Purified bacteriocins can be a strategy to improve safety and quality in food products as well as it can facilitate their use in human and veterinary medical industries. Bacteriocins produced by *Enterococcus* spp. have been purified previously using a range of different chromatographic methods [16]. For example, a final recovery of 1.6% of enterocin DD14 produced by *E. faecalis* 14, using cation-

exchange and size-exclusion chromatographic methods was achieved, along with successful purification of enterocin AS-48 from *E. faecalis* subsp. *liquefaciens* A-48-32, with a final recovery of 74.95% [20]. The purification process has always been considered the main bottleneck for industrial applications of bacteriocins, as bacteriocin yields tend to be low, most likely due to the high number of steps involved in the purification [32,33]. The recovery and purification of bacteriocins produced by LAB is usually achieved using salt precipitation from culture supernatants, as well as a combination of gel filtration, ion exchange chromatography, hydrophobic interaction chromatography, and RP- HPLC [33]. These methods produce satisfactory results on a small scale, but are usually of low yield, very expensive, difficult to handle, time-consuming, and unsuitable when tested on a large scale [20]. The yields can be variable depending on the method used, and it is has been previously shown that RP-HPLC contributes the most to the loss of bacteriocin activity [34].

Here, despite low purification yields, sufficient amounts of bacteriocin were obtained to evaluate its cytotoxicity against a human colon adenocarcinoma cell line, HT-29. After treatment with both the CFS and a semi-purified fraction of bacteriocin, HT-29 cells were found to remain viable, indicating no cytotoxic effect. Most bacteriocins produced by *Enterococcus* spp. have been shown to present low cytotoxicity against eukaryotic cells [35,36,16]. However, it has been reported that mild toxicity can occur when higher concentrations of bacteriocins are used [37,38]. Different toxicities can be observed depending on differences in the membrane composition, metabolic activity of target cells, exposure time, and the cytotoxicity assay employed [37,39,38,40]. Despite cytotoxic effects being considered a positive characteristic for treatment of carcinogenic cells, for the use as a bio-preservative agent in food or

pharmaceutical preparations to treat infections, cytotoxicity is not desirable. Therefore, for these reasons the safety of each bacteriocin needs to be performed to clarify their potential use in these industries.

In this study, *E. hirae* ST57ACC was found to express different ABC transporters, including a bacteriocin transporter, which may help to identify the bacteriocin produced by this strain. There were no disadvantages observed when the strain was grown in a bioreactor, suggesting that cultivation of *E. hirae* ST57ACC in industrial fermenters may be a strategy for implementing large-scale production of this bacteriocin. Furthermore, with no cytotoxic effects observed on a human cell line, this bacteriocin may be considered safe for use as a potential bio-preservative tool in the future.

Conflict of Interest

Authors declare that they have no conflict of interest.

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Ethical approval

All authors of this paper have read and approved the final version submitted. The contents of this manuscript have not been copyrighted or published previously. No

procedures performed in these studies have been conducted in human participants and/or animals.

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Table 1. Expression of genes related to ABC transport system by the strain *Enterococcus hirae* ST57ACC.

ABC transport system function	expression*
efflux ABC transporter, ATP-binding and permease protein	+
sugar ABC transporter, ATP-binding protein	-
malate/lactate dehydrogenase	++
multiple sugar ABC transporter, ATP-binding protein	+
bacteriocin ABC-transporter, ATP-binding and permease protein	++
ABC transporter ATP-binding protein	++
glucose-6-phosphate 1-dehydrogenase	+++
alcohol dehydrogenase	-

* Expression levels measured by using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Brea, CA, USA). -: absence of signal, +: weak (less than 100 units), ++: medium (between 100 and 300 units), +++: high expression (more than 300 units).

Table 2. Purification of bacteriocin produced by *E. hirae* ST57ACC from a 40-mL cell-free supernatant (CFS) by gel-filtration (GF), Solid-phase extraction (SFE) and RP-HPLC.

step	volume (mL)	protein concentration (mg/mL)	bacteriocinogenic activity (AU/mL)	total activity (AU)	total protein (mg)	specific activity (AU/mg)	purification (fold)
CFS	40	7.82	6,400	256,000	312.8	818.41	1
GF	20	1.23	1,600	32,000	24.6	1,300.81	1.58
SFE	1	0.26	3,200	3,200	0.26	12,307.69	15.03
RP-HPLC	1	0.04	100	10	0.004	2,500	3.05

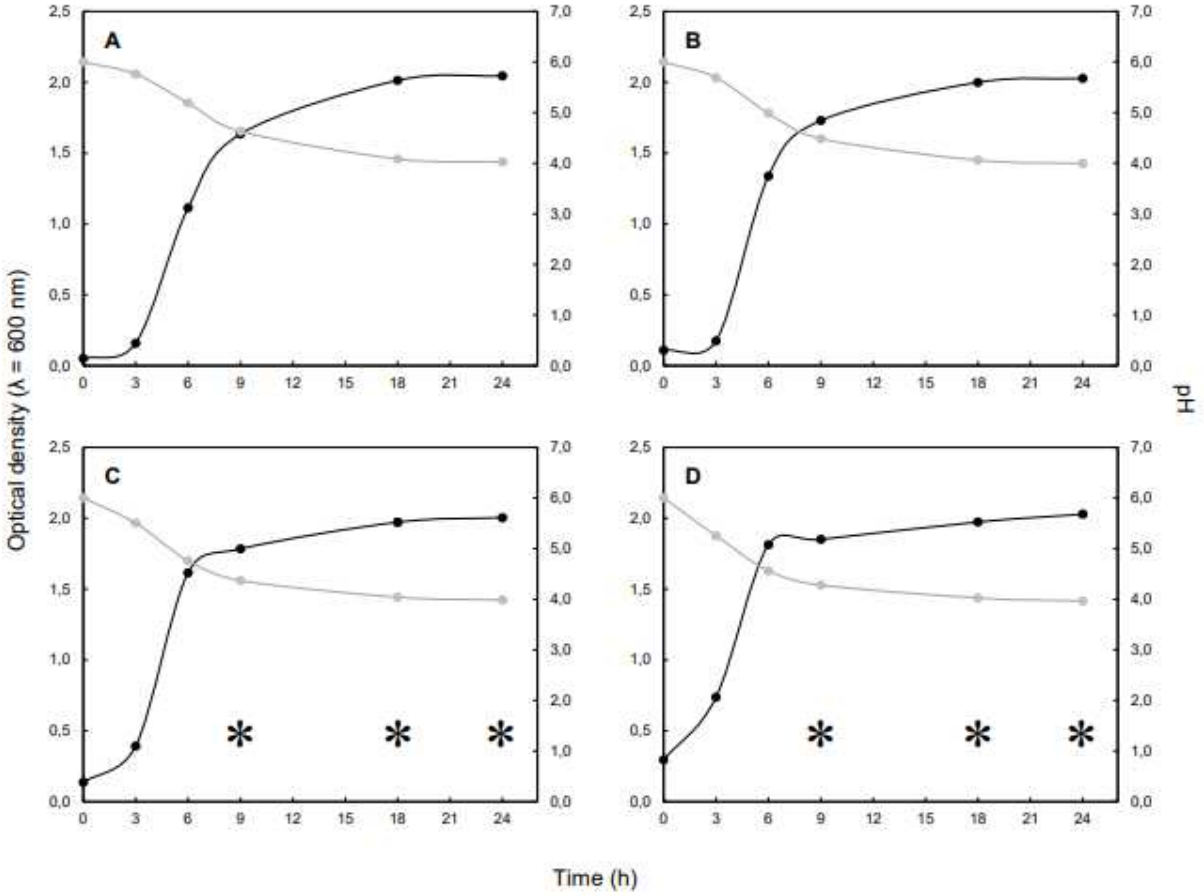

Figure 1. Variation of Optical density (black points) and pH (grey points) values of *Enterococcus hirae* ST57ACC inoculated in the Man, Rogosa and Sharpe broth (BD) at 1 (A), 2 (B), 5 (C) and 10 (D) % and incubated at 37 °C for 24 h. Mean values and standard errors.

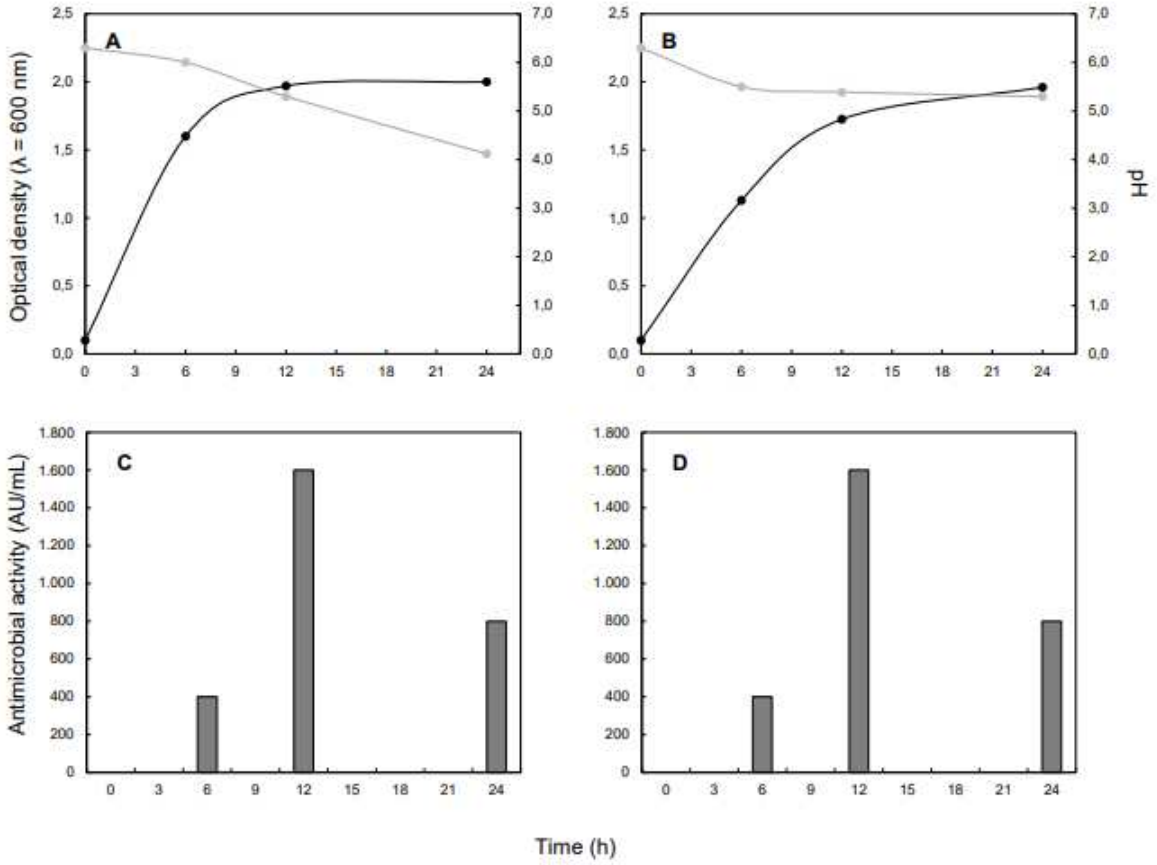

Figure 2. Variation of biomass and pH (black and grey points, respectively, in graphs A and B) values and bacteriocin production (grey bars, graphs C and D) of *Enterococcus hirae* ST57ACC inoculated in the Man, Rogosa and Sharpe broth (BD) at 5% and incubated at 37 °C for 24 h by conventional culture (A, C) and in bioreactor (B, D). Mean values and standard errors.

Supplementary Table. Primers used in the study expression of ABC transporters related genes by *Enterococcus hirae* ST57ACC (Ananieva et al., 2014).

primer	sequence	function	length of the product (bp)
zj316_2428 F	AGGTGACACTATAGAATACGGTTCCGTCGAACCTAACA	efflux ABC transporter, ATP-binding and permease protein	347
zj316_2428 R	ATAAGCGGTTGTCAGGCGAAGTACGACTCACTATAGGGA		
LBP_cg0987 F	AGGTGACACTATAGAATATCAACGGCAACGAGTAGCTT	sugar ABC transporter, ATP-binding protein	280
LBP_cg0987 R	TGGACCTGACCAGATTGTGCGTACGACTCACTATAGGGA		
dhL1 F	AGGTGACACTATAGAATATCTGCGGCAAAGTACCCAAT	malate/lactate dehydrogenase	151
dhL1 R	GCCGGATTATTCGCAAGCAGGTACGACTCACTATAGGGA		
msmK1 F	AGGTGACACTATAGAATATCCGGTCGAATTCCGAAGAC	multiple sugar ABC transporter, ATP-binding protein	450
msmK1 R	GTACGGGATGCACCGATCTTGTACGACTCACTATAGGGA		
plnG F	AGGTGACACTATAGAATATTGCCCTTTTCTTTGCACCG	bacteriocin ABC-transporter, ATP-binding and permease protein	540
plnG R	CCCACCACTGCCAATGTACTGTACGACTCACTATAGGGA		
JDM1_2227 F	AGGTGACACTATAGAATACGGTCCAAATTTGTTGCCGT	ABC transporter ATP-binding protein	200
JDM1_2227 R	TTAGGGATGGAGGCTGTGGAGTACGACTCACTATAGGGA		
gluc F	AGGTGACACTATAGAATATCTTTGGCGGTACTGGTGAC;	glucose-6-phosphate 1-dehydrogenase	120
gluc R	GTACGACTCACTATAGGGATGTTGGGCAATCGTACCGAA		
Aldeh F	AGGTGACACTATAGAATACAATTGGCTCGGCCATTACG	alcohol dehydrogenase	430
Aldeh R	GTACGACTCACTATAGGGACCATTTGCTGCCGATCTTCG		

CAPÍTULO 4 – Production and purification of a bacteriocin produced by the bacteriocinogenic strain *Pediococcus pentosaceus* ST65ACC isolated from a Brazilian artisanal cheese

Valéria Quintana Cavicchioli et al.

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Title page

Production and purification of a bacteriocin produced by the bacteriocinogenic strain *Pediococcus pentosaceus* ST65ACC isolated from a Brazilian artisanal cheese

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Abstract

Pediococcus pentosaceus stand out as one of the main bacteriocin producers among *Pediococcus* spp. and their bacteriocins are known for the activity in a wide range of temperature and pH, as well as high inhibition of important foodborne pathogens, especially *Listeria monocytogenes*. *P. pentosaceus* ST56ACC was isolated from a Brazilian artisanal cheese and its bacteriocinogenic potential and safety traits have been characterized previously. This study focused on going through some specific growth and bacteriocin production parameters, considering the expression of ABC transporters linked to sugar metabolism and bacteriocin expression, influence of inoculum size, culture conditions in bioreactor and conventional fermentation for bacteriocin production and utilization of xylooligosaccharides (XOS) as alternative carbon source. In addition, purification considering different approaches were performed. The expression of different ABC transporters corresponding to bacteriocin transport and sugar metabolism was observed. Inoculum size did not influence the growth of *P. pentosaceus* ST65ACC, although detection of bacteriocin only occurred after 9 h of incubation when the strain was inoculated at 5% and 10%. Cell density was linked to bacteriocin production in batch fermentation, and *P. pentosaceus* ST65ACC was able to grow and to produce bacteriocin (after 6 h) in the presence of XOS. Precipitation with 70% of Ammonium Sulphate allowed higher protein content and bacteriocin activity, compared to evaporation and ultrafiltration as initial steps for purification. Further purification of bacteriocin by RP-HPLC analysis presented an active peak at 20 minutes of elution (70 % acetonitrile), compatible with the high hydrophobicity described for bacteriocins produced by Lactic Acid Bacteria. Bacteriocin produced by *P. pentosaceus* ST65ACC was purified at a final yield of 0.4% and revealed to be a peptide with molecular weight between 3.5 and 8.5kDa, determined by tricine-SDS-PAGE. ABC transporter system is enrolled in expression of a bacteriocin produced by *P. pentosaceus* ST65ACC, and 70% ammonium sulphate was the approach that provided the better yeld of purification.

Keywords: *Pediococcus pentosaceus*, bacteriocin, ABC transporters, purification

1. Introduction

Lactic Acid Bacteria (LAB) are widely used by food industries in the production of fermented products and as tools for food safety, acting as starter cultures, biopreservative agents or probiotics. Many LAB strains are already known by their status of Generally Regarded As Safe (GRAS), being the focus of several studies and application in food safety and quality, human and animal health and pharmaceutical industry, due to their ability to produce bacteriocins (Cotter et al., 2013). Bacteriocins are antimicrobial peptides produced by Gram-negative and Gram-positive bacteria, active towards spoilage and pathogenic microorganisms, generally closely related to the producer organisms (Cotter et al., 2013; Drider and Rebuffat, 2011). Unlike antibiotics, bacteriocins are ribosomally synthesized molecules with encoding genes organized in same operon, including maturation, production, immunity and secretion genes (Drider and Rebuffat, 2011).

Pediococcus strains are intensively applied as starter cultures in the industrial fermentation of meat and vegetables (Huang et al., 2009; Kingcha et al., 2012; Nanasombat et al., 2017). In cheeses, *Pediococcus* strains may occur as part of the non-starter LAB, contributing to ripening (Carafa et al., 2015; Morales-Estrada et al., 2016; Portilla-Vázquez et al., 2016). *P. acidilactici* and *P. pentosaceus* stand out as the main bacteriocin producers (Altuntaş et al., 2014; Millette et al., 2008; Papagianni and Anastasiadou, 2009; Porto et al., 2017) and different strains were characterized as able to produce different bacteriocins, including pediocin AcH (Bhunia et al., 1988; Kim et al., 1992; Ray et al., 1989), pediocin PA-1/ACH, (Gonzalez and Kunka, 1987), pediocin JD (Schved et al., 1993), pediocin 5 (Huang et al., 1996), pediocin A, (Fleming et al., 1975), pediocin N5p (Saad et al., 1995),

pediocin ST18 (Todorov and Dicks, 2005) and pediocin SA-1 (Anastasiadou et al., 2008).

Bacteriocins produced by *Pediococcus* spp. are described as active in a wide range of temperature and pH, effective in low concentration and powerful inhibitors of important foodborne pathogens, such as *Listeria monocytogenes* and *Clostridium perfringens* (Albano et al., 2009; Kingcha et al., 2012; Nieto-Lozano et al., 2010; Todorov and Dicks, 2005, 2009). These characteristics, in association with the increased demand by the consumers for products containing natural preservatives, bring about the potential use of these bacteriocins and strains as natural preservatives.

P. pentosaceus ST65ACC was isolated from a Brazilian artisanal cheese and characterized as a bacteriocinogenic strain (Cavicchioli et al., 2017). Beneficial properties and lack of antimicrobial resistance and virulent aspects were also studied, indicating an interesting biotechnological potential of this strain (*Cavicchioli et al., 2018, under review*). Considering these previous information, the present study aimed to characterize some aspects related to production, expression and purification of the bacteriocin produced by *P. pentosaceus* ST65ACC.

2. Material and Methods

2.1. *Pediococcus pentosaceus* ST57ACC bacteriocin expression

P. pentosaceus ST65ACC was previously isolated from a Brazilian artisanal cheese and identified by 16S rRNA sequencing (Cavicchioli et al., 2017). The strain was stored in de Man, Rogosa and Sharpe broth (MRS, Becton, Dickinson and Company

- BD, Franklin Lakes, NJ, USA) supplemented with 20 % (v/v) glycerol and kept at −20 °C. Aliquots of 100 µL of the stored culture was transferred to 5 mL of MRS broth (BD) and incubated at 37 °C for 18 h, for at least 2 consecutive times.

A culture of *P. pentosaceus* ST65ACC was subjected to RNA extraction by using the GeneMATRIX Universal RNA Purification Kit (EURx Ltd., Gdansk, Poland) and assessed for measuring the expression of genes enrolled in the ABC transporter system by using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Brea, CA, USA). Supplementary Table presents the primers sequences, gene functions and expected fragment sizes for the target genes. The housekeeping gene *kanR* was considered as external control.

Reverse transcriptase (RT) reactions included: 3 µL of DNase/RNase free water, 4 µL of RT buffer 5×, 5 µL KAN^r RNA, 1 µL RT enzyme (all from GenomeLab™ GeXP Start Kit, Beckman Coulter), 2 µL of reverse primers (at 10 pmol/mL, Supplementary Table) and 5 µL of RNA (final volume of 20 µL). Reactions conditions were 1 min at 48 °C, 60 min at 42 °C, 5 min at 95 °C, and hold at 4 °C, in a thermal cycler (VWR International Ltd, Leicestershire, UK). Each experiment included a RT-negative control and a no-template control (NTC).

Then, PCR reactions were prepared containing: 4 µL of PCR buffer 5×, 4 µL of 25 mM $MgCl_2$, 0.7 µL of Thermo-Start DNA Polymerase (all from GenomeLab™ GeXP Start Kit, Beckman Coulter), 2 µL of forward primers plex (10 pmol/mL, Supplementary Table) and 9.3 µL of cDNA samples from the RT plate (20 µL final volume). Reactions conditions were 10 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 68 °C, and hold at 4 °C (thermal cycler VWR).

GeXP procedures and data analyses were conducted based on the recommendations of the manufacturer (Beckman Coulter): 0.5 mL DNA size standard 400

(GenomeLab™ GeXP Start Kit, Beckman Coulter) was mixed to 38.5 µL of sample loading solution, and the mix solution was transferred to wells of a 96-well microplate, previously added with 1 µL aliquots of the PCR products. The PCR product was separated based on fragment size by capillary gel electrophoresis (GenomeLab™ GeXP Genetic Analysis System, Beckman Coulter), and the strength of the dye signal was measured after normalization to KAN^r RNA. The signals were normalized in GeXP express profiler software (Beckman Coulter), and the results were expressed as absence (-) or different degrees of gene expression (+: weak, < 100 units; ++: medium, 100-300 units; +++: high, > 300 units), based on reading of the dye signal.

2.2. Growth conditions and bacteriocin production

A culture of *P. pentosaceus* ST65ACC was prepared as described above, inoculated at 1%, 2%, 5% and 10% (v/v) in flasks containing 100 mL of MRS broth (BD) and incubated at 37 °C. After 0, 3, 6, 9, 18 and 24 hours of incubation, aliquots of the cultures were subjected to spectrophotometry at 600 nm (Beckman Coulter®, Brea, CA, USA) to estimate the optical density (OD) and pH measuring. Also, aliquots were tested for bacteriocin production by a qualitative assay, as described by Todorov (2008): cultures were centrifuged at 12,000 × g and pH of the obtained cell-free supernatants (CFS) corrected to pH 6.0 to 6.5 with 1M NaOH, and heated at 80 °C for 10 min; then, 10 µL aliquots of CFS were spotted on the surface of a brain heart infusion agar (BHI, BD) previously inoculated with *E. faecium* ATCC 19443 (final concentration of 10^6 colony forming units per mL, CFU/mL), and incubated at 37 °C for 24 h; inhibition halos bigger than 2 mm were considered as positive results,

indicating bacteriocin production. This experiment was conducted in two independent repetitions.

P. pentosaceus ST55ACC was cultured in static conditions (in flasks) and in bioreactor with continuous agitation. *P. pentosaceus* ST65ACC was added to MRS broth (BD) at 5% (v/v), and distributed to sterile flasks (500 mL) for conventional growth and to a bioreactor (3,000 mL, Infors-HT, Bottmingen, Switzerland) for continuous fermentation. Both growth conditions were performed at 37 °C, and continuous fermentation included agitation at 50 rpm, and correction of pH to 5.5 with 1 M NaOH. Aliquots of the cultures were obtained after 0, 6, 12 and 24 h of incubation from both experimental models, and subjected to spectrophotometry to estimate the OD and to pH measuring, as described above. The quantitative assay described by Todorov (2008) was considered to measure the bacteriocin production: CFS was obtained and treated as described above and serially two-fold diluted in 10 mM phosphate buffered (K_2HPO_4 / KH_2PO_4) at pH 6.5; then, aliquots of 10 µL of dilutions were spotted on the surface of BHI agar (BD) previously inoculated with *E. faecium* ATCC 19443 (at 10^6 CFU/mL final concentration), and incubated at 37 °C; bacteriocin activity was expressed as arbitrary units per mL (AU/mL), corresponding to the reciprocal of the highest dilution having a detectable halo of inhibition (higher than 2 mm).

A modified MRS base was prepared without the carbohydrate source, supplemented with xylooligosaccharides (XOS, Longlive Bio-technology Co., Ltd, China) at 2%, and inoculated with cultures of *P. pentosaceus* ST65ACC at 5%, being incubated at 37 °C. *P. pentosaceus* ST65ACC was also inoculated in MRS (BD), as control. Aliquots of cultures were obtained at 0, 6, 9, 12 and 24 h of incubation, subjected to

spectrophotometry, pH and bacteriocin production measuring, as described above. The assay was conducted in two independent repetitions.

2.3. Bacteriocin purification

P. pentosaceus ST65ACC was cultured in 3 liters of MRS for 18 h at 37 °C. CFS was obtained and treated as described above. In order to determine optimal condition for bacteriocin purification, different approaches were investigated. As initial step of bacteriocin purification, CFS was subjected to three different approaches: one fraction of CFS (1,000 mL) was subjected to evaporation, one fraction (1,000 mL) was ultrafiltrated by using a 10 kDa cutoff cartridge filter (Pall Corporation, New York, USA) and one part (1,000 mL) was precipitated with 70% of ammonium sulphate. Protein concentration (mg/mL) was measured by Bradford assay (Bradford, 1976) and bacteriocinogenic activity was measured by a quantitative assay as described above. Obtained pellet after ammonium sulphate precipitation was dialyzed against water and resolved in 30 mL of 10 mM phosphate buffered (K2HPO4 / KH2PO4) at pH 6.5. Further bacteriocin purification was performed by RP-HPLC using a PrepNucleosil C18 (250 mm x 10 mm) column. Column was activated and equilibrated prior to material loading. Separation of the proteins was performed applying a linear gradient of following mobile phases: A (0.1% trifluoroacetic acid in ultrapure water) and B (0.1% trifluoroacetic acid in acetonitrile), in gradient from 10% up 80% of phase A; elution of the peptides was monitored spectrophotometrically at 220 nm, at a flow rate 2.0 ml/min, for 30 minutes. After each purification step, protein concentration and bacteriocinogenic activity were measured, as described above.

The molecular weight of the partially purified bacteriocin at 70% amonium sulphate was determined by Tricine-SDS-PAGE, according to Schägger and Von Jagow (1987): the acrylamide gels (Stacking gel 4%, Diffusion gel 15%) were loaded in duplicate with aliquots of the samples (15 µL) and the electrophoresis was conducted at constant voltage of 90 V until the tracking dye reached the bottom of the stacking gel, then changed to 150 V during migration via diffusion gel. Amersham™ Rainbow Marker (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used as low molecular weight marker. After electrophoresis, the gels were divided in two parts: one half was fixed and stained with silver, and the other half was over-laid with BHI (BD) supplemented with 1% (w/v) agar, inoculated with approximately 10^6 CFU/mL of *E. faecium* ATCC 19443 and incubated at 37 °C for 24 h, in order to detect the antimicrobial activity of the bacteriocin produced by *P. pentosaceus* ST65ACC.

3. Results and Discussion

Genes enrolled in ABC transport system and sugar metabolism of *P. pentosaceus* ST65ACC were expressed in different levels, as show in Table 1. With exception of *msmK*, which codifies a multiple sugar ABC transporter and ATP-binding protein, all tested genes were upregulated (Table 1). The diversity of ABC transporters and sugar metabolism genes can reflect the nutrient-rich lifestyle during specific niche adaptations of LAB (Pfeiler and Klaenhammer, 2007). Expression of bacteriocin ABC transporter by *P. pentosaceus* ST65ACC indicates the efficient conversion of the nutrients for cell growth and bacteriocin production in tested culture conditions. Class IIa bacteriocins depend on ABC transporter system in different steps of their

expression: this system is enrolled in the recognition and cleavage of the double-glycine motif of the leader peptide and in the translocation of the peptide outside the producer cell (Sushida et al., 2018).

Independently of the initial inoculum load of the strain, *P. pentosaceus* ST65ACC presented a similar growth profile in MRS broth at 37 °C (Figure 1). Cell density production was proportional to inoculums size from 0 up to 6 h of incubation, and similar levels were observed at 9 h until the end of the experiment (Figure1). pH values ranged from 6 at the beginning of growth to 4 after 24 of incubation, independently of the initial inoculum load of bacteriocinogenic strain (Figure 1). Bacteriocin production was influenced by inoculum concentration and it was detected only when the strain was inoculated at initial levels of 5 and 10%, and after 9 h up to 24 h of incubation (Figure 1, C and D). Considering the results obtained in this step of the study, the initial inoculum at 5% of the bacteriocinogenic strain was standardized in further tests.

Others studies of growth and bacteriocin production have shown that inoculum size is an important parameter to be evaluated in LAB strain cultures. Himelbloom et al. (2001) reported that production of an antilisterial bacteriocin by *Carnobacterium piscicola* strain A9b was unaffected by the inoculum size when cultivated in BHI and MRS without glucose at 25°C. In contrast, no bacteriocin production was observed in All Purpose Tween (APT) when a 0.001% inoculum was used, whereas a 1% inoculum resulted in bacteriocin production of 160 AU/ml (Himelbloom et al., 2001). Optimization of bacteriocin production by *Lactobacillus plantarum* LPCO10 was also explored by Leal-Sánchez et al. (2002) in a series of experiments, where glucose and NaCl concentrations in the culture medium, inoculums size, aeration of the culture, and growth temperature were statistically combined in an experimental

design. These authors observed that the best conditions for bacteriocin production were obtained with temperatures ranging from 22 to 27°C, salt concentration from 2.3 to 2.5%, and *L. plantarum* LPCO10 inoculum size ranging from $10^{7.3}$ to $10^{7.4}$ CFU/mL, fixing the initial glucose concentration at 2%, with no aeration of the culture. Under these optimal conditions, about 3.2×10^4 times more bacteriocin per liter of culture medium was obtained in comparison with *L. plantarum* LPCO10 cultivated at 30°C in MRS medium (Leal-Sánchez et al., 2002). These data highlight that inoculums size cannot be neglected and it may be taken in account together with other variables in studies of bacteriocin production optimization.

Bacteriocin production was linked to cell density when cultivated by conventional growth and in a bioreactor, demonstrating similar growth dynamics (Figure 2). Nevertheless, higher levels of bacteriocin were detected when the strain was cultivated in bioreactor, after 24 h of fermentation (Figure 2 D). Successful production of bacteriocins in continuous fermentation systems has been reported for different LAB strains (Abriouel et al., 2003; Garsa et al., 2014; Liu et al., 2005; Papagianni and Sergelidis, 2015). The increasing in cell density during cultivation on bioreactor, as well as the constant pH maintenance, must have led to high bacteriocin production in this system: bacteriocin production is usually proportional to bacterial growth, with rate of production directly linked to cell density, once bacteriocins are primary metabolites (Abbasiliasi et al., 2017). Papagianni and Sergelidis (2015) observed that pediocin SM-1 biosynthesis was remarkably affected by pH and biomass of *P. pentosaceus* Mees 1934: at the optimal pH growth (6.5), the bacteriocin production increased in 25% when compared to cultivation of *P. pentosaceus* Mees 1934 at pH 6.8 (Papagianni and Sergelidis, 2015). Turgis et al. (2016) demonstrated that *P. acidilactici* MM33 was able to produce a high level of

bacteriocin when cultivated in culture media at pH ranging from 5.0 to 6.0. pH can interfere in the microbial growth in culture media, as well as in the bacteriocin production, adsorption and desorption to the cell membrane (Biswas et al., 1991). Also, pH is directly enrolled in the regulation of the processes of post-translational modification and secretion of bacteriocins (Parente and Ricciardi, 1994), highlighting that the control of such variable is important to optimize bacteriocin production.

P. pentosaceus ST65ACC was also able to grow in MRS modified with XOS and to produce bacteriocins (Figure 3). Maximum OD of the bacteriocinogenic culture was observed in MRS (control), after 12 h of incubation; in MRS supplemented with XOS, the maximum growth was about 1.3 mg/mL, after 24 h (Figure 3 A and B). pH from MRS dropped from 6.0 to approximately 4.0, when in MRS modified with XOS, pH ranged from 6.0 to approximately 5.0. Bacteriocin was produced in both culture media after 6 h of incubation, with maximum levels after 9 h: *P. pentosaceus* ST65ACC produced bacteriocins at 1,600 AU/mL from 9 to 24 of incubation, while in MRS modified with XOS the production varied from 800 to 200 AU/mL (Figure 3, C and D). XOS are non-digestible oligosaccharides that have recently received increasing attention as potential prebiotic candidates (Ananieva et al., 2014; Viborg et al., 2013). Utilization of XOS and other prebiotic oligosaccharides are usually strain-specific in LAB and its metabolism is better characterized in traditional probiotic strains, such as *Lactobacillus* and *Bifidobacterium* (Ananieva et al., 2014; Korakli et al., 2002). It seems to be the first report about utilization and bacteriocin production by using XOS as carbon source by *Pediococcus* strains. It is proposed that the metabolism of specific sugars can induce in somehow the production of antimicrobial substances, but the exact mechanisms remains unclear (Ananieva et al., 2014).

Three different approaches were employed and compared for the first step of purification of bacteriocin produced by *P. pentosaceus* STA65CC: when the CFS was evaporated, bacteriocinogenic activity was 3,200 AU/mL and protein concentration was 0.89 mg/mL; when the CFS was subjected to ultrafiltration, bacteriocinogenic activity was 3,200 AU/mL and protein concentration was 0.78 mg/mL; finally, by precipitation with 70% ammonium sulphate, bacteriocinogenic activity was 12,800 AU/mL and protein concentration was 1 mg/mL. Analysis of the three applied approaches pointed that ammonium sulphate can be considered as a more appropriate for the purification of bacteriocin produced by *P. pentosaceus* ST65ACC. Precipitation of bacteriocins with salts is a well applied method employed in the first step of bacteriocin purification, reported as a successful strategy to purify bacteriocins produced by *P. pentosaceus* (Todorov and Dicks, 2005). Considering the high antimicrobial recovery obtained with ammonium sulphate precipitation, further steps of purification were performed with this material. After dialysis against water, the obtained material was used to perform protein separation on tricine-SDS-PAGE gel. Weak bands were observed in the gel stained with Comassie Blue (Figure 4A), while the bands were more evident when the gel was stained with silver (Figure 4B). However, it is well known that silver staining is a more sensitive approach compared to Comassie Blue staining and is recommended in experiments where proteins were present in very low concentrations. Based on the antimicrobial assay, an inhibition zone was observed between 3.5 and 8.5 kDa, corresponding to the molecular weight of the bacteriocin produced by *P. pentosaceus* ST65ACC (Figure 4C). Previous results about bacteriocin encoding genes have shown that *P. pentosaceus* ST65ACC does not harbor pediocin PA-1/ACH gene (Cavicchioli et al., 2017). Bacteriocin produced by However, pediocins bacHA6111-

2 and bacHA-5692-3, produced by two different *P. acidilactici* strains, present their size between 3.5 and 6.5 kDa, as reported by Albano et al. (2007), while the partially purified bacteriocin bacALP57, produced by *P. pentosaceus*, presented molecular weight below 6.5 kDa, by using Tricine-SDS-PAGE (Pinto et al., 2009), suggesting that the bacteriocin produced by *P. pentosaceus* ST65ACC present similar molecular mass to bacteriocins studied by these authors.

The bacteriocin produced by *P. pentosaceus* ST65ACC was purified at a final yield of 0.4% after RP-HPLC, compared to CFS (Table 2). When RP-HPLC was performed loading precipitated and dialyzed material obtained from CFS of *P. pentosaceus* ST65ACC, three major peaks eluted at 20, 23 and 24 minutes were detected (Figure 5). Antimicrobial activity test of collected material was pointed that peak of future interest was that eluted at 20 minutes, corresponding to 70% of acetonitril. This result is in agreement with high hydrophobicity nature of most of known bacteriocins produced by LAB (Parada et al., 2007; Rodríguez et al., 2002; Uteng et al., 2002).

Purification of bacteriocins is a big challenge and factors such as cost, time-consuming procedures and low yields are frequently reported (Kaškonienė et al., 2017). However, the objective of the purification needs to be clearly stated: the proper assessment of potential application of bacteriocins must be conducted with highly purified substances. In an attempt to overcome these hindrances, different strategies have been investigated in the purification of bacteriocins produced by *Pediococcus* strains. Millette et al. (2008) purified a peptide produced by *P. acidilactici* MM33 by using cation exchange chromatography followed by elimination of salts by Sep-Pak Plus column in tandem and obtained a final yield of 50.7%. This approach eliminated the RP-HPLC step, reducing the cost of the process

(Millette et al., 2008), however, bacteriocin can be considered only as a semi-purified, since obtained material was not a single protein. A culture of *P. pentosaceous* NCDC 273 was immobilized in alginate-xanthan gum gel beads covered with chitosan, eliminating two-centrifugation steps, precipitated with ammonium sulphate and further purified using cation-exchange chromatography and RP-HPLC, resulting in yield of 134.4% of pediocin PA-1/ACH (Simha et al., 2012). More recently, Sadishkumar and Jeevaratnam (2018) reported the purification of an antilisterial bacteriocin produced by *P. pentosaceus* KJBC11 isolated from Idli batter, by a procedure involving cell-adsorption and desorption technique, gel permeation chromatography, hydrophobic interaction chromatography and RP-HPLC successively, obtaining 15% of recovery. Modifications and improvements on purification protocols may help to make bacteriocins suitable for use in analytical investigations.

4. Conclusion

The presented study described that ABC transporter system is enrolled in the expression of the bacteriocin produced by *P. pentosaceus* ST65ACC, which is not influenced substantially by its initial inoculum size and fermentation process; XOS also did not influenced its bacteriocin production. The better approach for purification of the bacteriocin produced by *P. pentosaceus* ST65ACC was the precipitation with 70% ammonium sulphate, resulting in a peptide with 3.5 to 8.5 kDa of molecular size.

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Table 1. Expression of genes related to ABC transport system by the bacteriocinogenic strain *Pediococcus pentosaceus* ST65ACC.

gene/protein	function	expression*
zj316_2428	efflux ABC transporter, ATP-binding and permease protein	+
LBP_cg0987	sugar ABC transporter, ATP-binding protein	++
dhL	malate/lactate dehydrogenase	++
msmK	multiple sugar ABC transporter, ATP-binding protein	-
plnG	bacteriocin ABC-transporter, ATP-binding and permease protein	+++
JDM1_2227	ABC transporter ATP-binding protein	++
gluc	glucose-6-phosphate 1-dehydrogenase	+++
Aldeh	alcohol dehydrogenase	++

* Expression levels measured by using the GenomeLab™ GeXP Genetic Analysis System(Beckman Coulter, Brea, CA, USA). -: absence of signal, +: weak (less than 100 units), ++: medium (between 100 and 300 units), +++: high expression (more than 300 units).

Table 2. Purification of bacteriocin produced by *Pediococcus pentosaceus* ST65ACC.

step	volume (mL)	protein concentration (mg/mL)	bacteriocinogenic activity (AU/mL)	total activity (AU)	total protein (mg)	specific activity (AU/mg)	purification (fold)	yield (%)
CFS	1,000	7.6	3,200	3,200,000	7,600	421.05	1	100
Ammonium sulphate (70%)	100	3.04	12,800	1,280,000	304	4,210.53	10	40
RP-HPLC	1	0.03	12,800	12,800	0.03	426,666.67	101.33	0.4

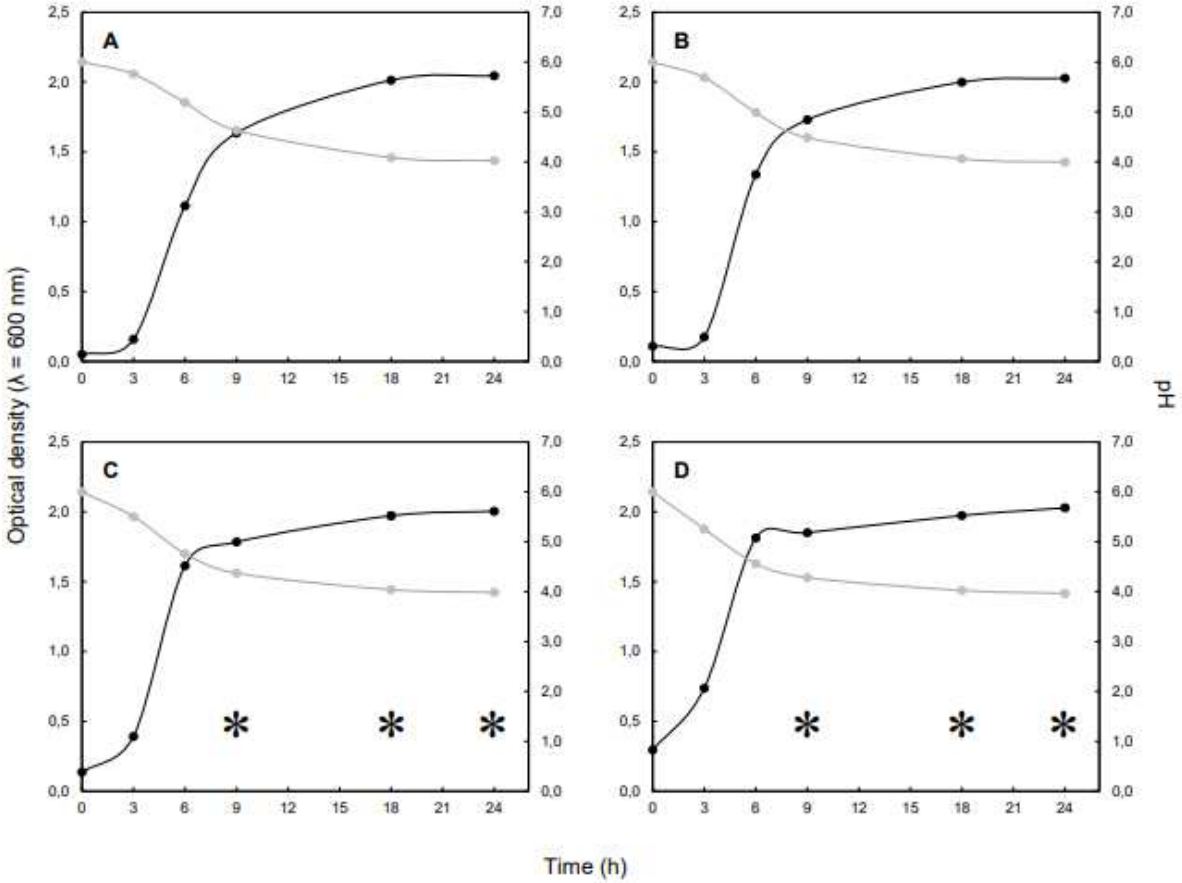

Figure 1. Variation of Optical density (black points) and pH (grey points) values of *Pediococcus pentosaceus* ST65ACC inoculated in the Man, Rogosa and Sharpe broth (BD) at 1 (A), 2 (B), 5 (C) and 10% (D) and incubated at 37 °C for 24 h. Antimicrobial activity is indicated by asterisks (*). Mean values and standard errors.

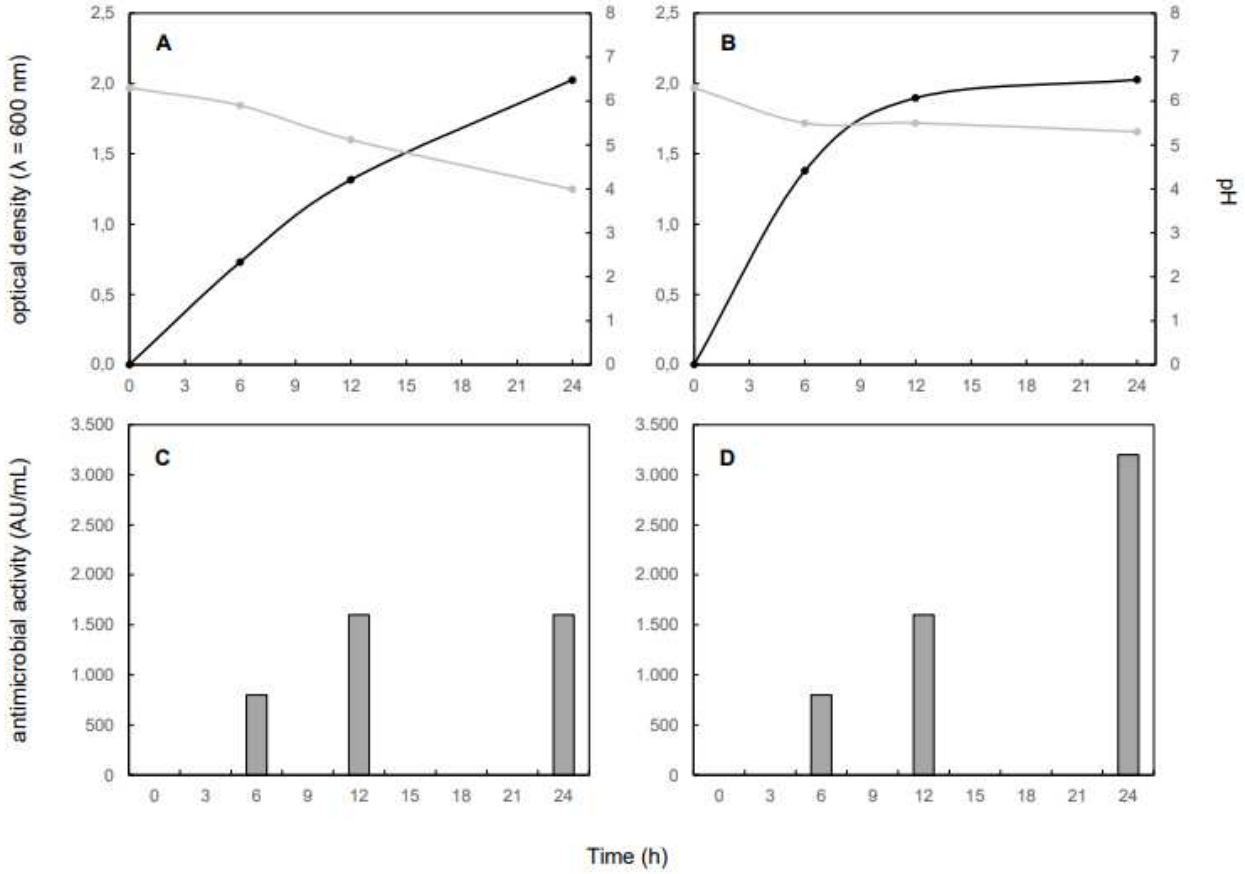

Figure 2. Variation of optical density and pH (black and grey points, respectively, in graphs A and B) values and bacteriocin production (grey bars, graphs C and D) of *Pediococcus pentosaceus* ST65ACC inoculated in the Man, Rogosa and Sharpe broth (BD) at 5% and incubated at 37 °C for 24 h by conventional culture (A, C) and in bioreactor (B, D).

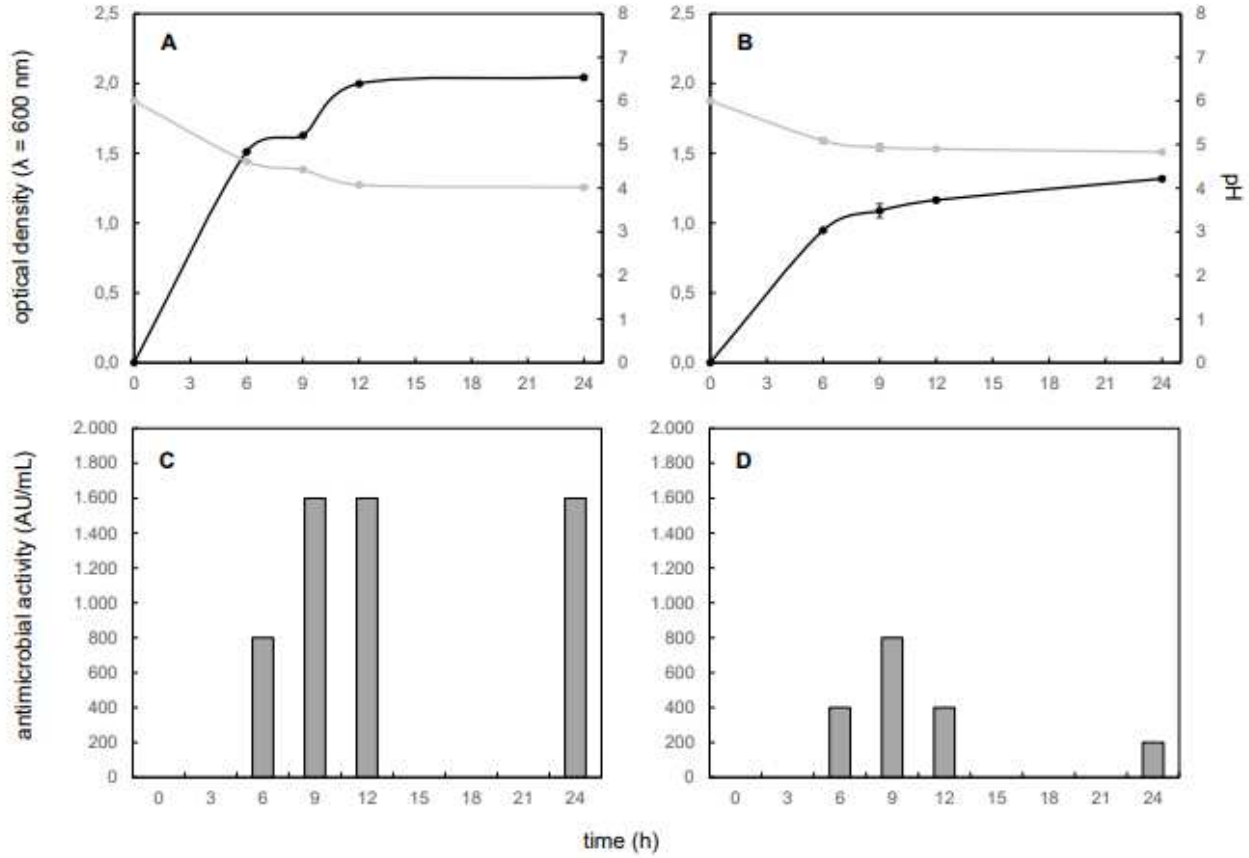

Figure 3. Variation of biomass and pH (black and grey points, respectively, in graphs A and B) values and bacteriocin production (grey bars, graphs C and D) of *Pediococcus pentosaceus* ST65ACC inoculated in the Man, Rogosa and Sharpe broth (MRS, BD) (A, C) and modified MRS supplemented with XOS (B, D) at 5% and incubated at 37 °C for 24 h. Mean values and standard errors.

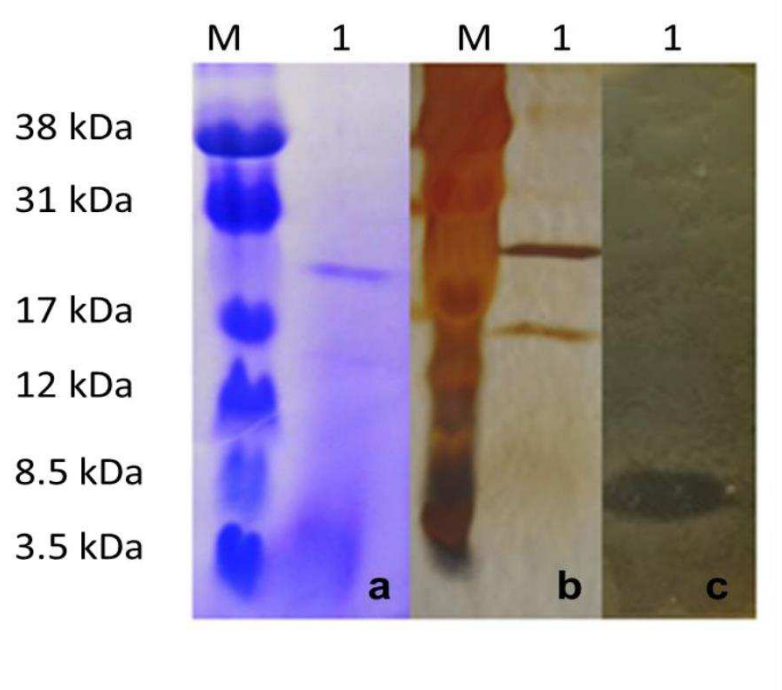

Figure 4: Tricine-SDS–PAGE of bacteriocin produced by *Pediococcus pentosaceus* ST65ACC stained with Comassie Blue (a) and Silver (b). The gels were overlaid with active growing cells of *Enterococcus faecium* ATCC 19443. Inhibition zones indicated the position of the active peptide bands (c). M: Marker, 1: Bacteriocin from *P. pentosaceus* ST65ACC precipitated with 70% Ammonium Sulphate .

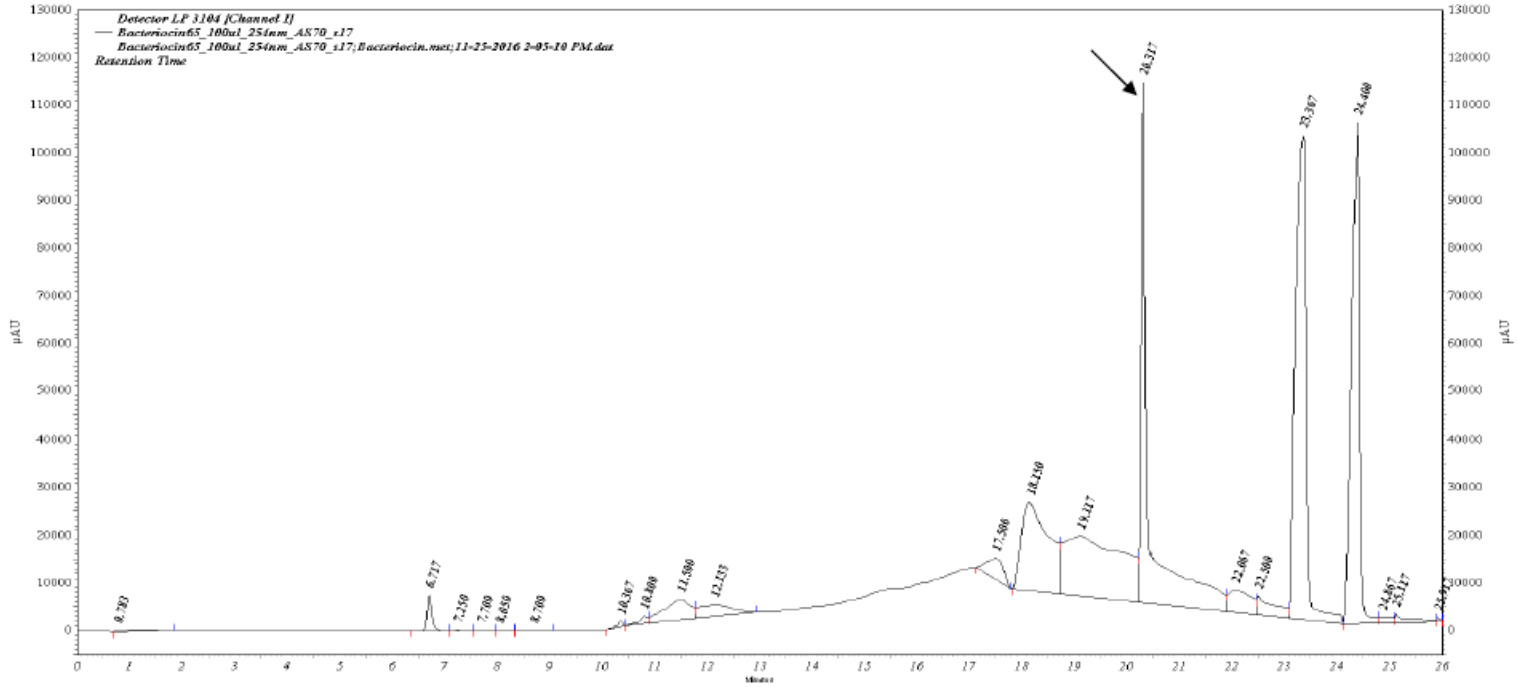

Figure 5: RP-HPLC elution profile of purified bacteriocin produced by *Pediococcus pentosaceus* ST65ACC. The black arrow indicates the peak with antimicrobial activity against *Enterococcus faecium* ATCC 19443

Supplementary Table. Primers used in the study expression of ABC transporters related genes by *Pediococcus pentosaceus* ST65ACC.

gene/protein	primer	sequence	function	length of the product (bp)
zj316_2428	zj316_2428 F zj316_2428 R	AGGTGACACTATAGAATACGGTTCCGTCGAACCTAACA ATAAGCGGTTGTCAGGCGAAGTACGACTCACTATAGGGA	efflux ABC transporter, ATP-binding and permease protein	347
LBP_cg0987	LBP_cg0987 F LBP_cg0987 R	AGGTGACACTATAGAATATCAACGGCAACGAGTAGCTT TGGACCTGACCAGATTGTGCGTACGACTCACTATAGGGA	sugar ABC transporter, ATP-binding protein	280
dhl	dhL1 F dhL1 R	AGGTGACACTATAGAATATCTGCGGCAAAGTACCCAAT GCCGGATTATTCGCAAGCAGGTACGACTCACTATAGGGA	malate/lactate dehydrogenase	151
mamK1	msmK1 F msmK1 R	AGGTGACACTATAGAATATCCGGTCGAATTCCGAAGAC GTACGGGATGCACCGATCTTGTACGACTCACTATAGGGA	multiple sugar ABC transporter, ATP-binding protein	450
plnG	plnG F plnG R	AGGTGACACTATAGAATATTGCCCTTTTCTTTGCACCG CCCACCACTGCCAATGTACTGTACGACTCACTATAGGGA	bacteriocin ABC-transporter, ATP-binding and permease protein	540
JDM1_2227	JDM1_2227 F JDM1_2227 R	AGGTGACACTATAGAATACGGTCCAAATTTGTTGCCGT TTAGGGATGGAGGCTGTGGAGTACGACTCACTATAGGGA	ABC transporter ATP-binding protein	200
gluC	gluc F gluc R	AGGTGACACTATAGAATATCTTTGGCGGTACTGGTGAC; GTACGACTCACTATAGGGATGTTGGGCAATCGTACCGAA	glucose-6-phosphate 1-dehydrogenase	120
Aldeh	Aldeh F Aldeh R	AGGTGACACTATAGAATACAATTGGCTCGGCCATTACG GTACGACTCACTATAGGGACCATTTGCTGCCGATCTTCG	alcohol dehydrogenase	430

CONCLUSÕES

- ✓ *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC isolados de queijos artesanais foram identificados como bacteriocinogênicos e a ausência dos principais genes envolvidos na produção de bacteriocinas pode ser um indicativo de que estas cepas sejam capazes de produzir novas bacteriocinas;

- ✓ As bacteriocinas produzidas por *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC foram estáveis a diferentes condições que podem ser utilizadas durante o processamento de alimentos, indicando seu potencial de utilização como biopreservantes;

- ✓ Os isolados *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC e suas bacteriocinas apresentam interessante potencial na inibição e controle de *L. monocytogenes in vitro* e em leite;

- ✓ Os isolados bacteriocinogênicos obtidos neste estudo apresentam capacidade de sobrevivência a condições similares àquelas encontradas no trato gastrointestinal, com propriedades de auto-agregação e co-agregação à *L. monocytogenes*, que sugerem seu potencial para utilização como cepas benéficas*;*

- ✓ A ausência de determinantes genéticos de produção de aminas biogênicas e baixa frequência de genes relacionados à resistência a antibióticos observada *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC demonstra que os isolados podem ser considerados seguros para sua utilização em alimentos;

- ✓ A expressão de diferentes fatores do sistema de transporte ABC relacionados ao transporte de bacteriocinas e metabolismo de açúcares foi identificada, apontando que bacteriocinas produzidas por *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC são secretadas por este mecanismo;

- ✓ A produção de bacteriocinas por *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC é influenciada pela biomassa e período de incubação;

- ✓ *E. hirae* ST57ACC e *P. pentosaceus* ST65ACC são capazes de se multiplicar e produzir bacterinas na presença de XOS;

- ✓ A bacteriocina produzida por *E. hirae* ST57ACC não apresenta efeitos citotóxicos à células humanas da linhagem HT-29;

- ✓ A bacteriocina produzida por *P. pentosaceus* ST65ACC é um peptídeo com peso molecular de 3,5 a 8,5 kD;

- ✓ Os isolados *E. hirae* ST57ACC e *P. pentosaceus* ST57ACC podem ser considerados importantes ferramentas biotecnológicas na produção de bacteriocinas de interesse ao controle de *L. monocytogenes* e na biopreservação de alimentos.