NATÁLIA PARMA AUGUSTO DE CASTILHO

BACTERIOCINOGENIC POTENTIAL OF LACTIC ACID BACTERIA ISOLATES FROM ARTISANAL FERMENTED MEAT PRODUCTS

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*.

Viçosa
Minas Gerais - Brasil
2018

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
C352b
2018

Castilho, Natália Parma Augusto de, 1987-
Bacteriocinogenic potential of lactic acid bacteria isolates from artisanal fermented meat products / Natália Parma Augusto de Castilho. – Viçosa, MG, 2018.
xiii, 136 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.
Orientador: Luís Augusto Nero.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Bacteriocinas. 2. Bactérias ácido láctico. 3. Carne - Microbiologia. 4. *Listeria monocytogenes. 5.* Embutidos (Alimentos) - Microbiologia . I. Universidade Federal de Viçosa. Departamento de Veterinária. Programa de Pós-Graduação em Medicina Veterinária. II. Título.

CDD 22. ed. 636.08969201

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APROVADA: 16 de agosto de 2018.

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AGRADECIMENTOS

A Deus por me guiar e estar sempre comigo.

Aos meus pais por compreenderem minhas escolhas e me apoiarem em todo momento.

Ao Rafael, pelo amor, amizade, companheirismo e principalmente paciência ao longo desses quatro anos de doutorado.

Ao meu orientador Nero, por sempre confiar em mim, pelo apoio nos momentos de insegurança, pela oportunidade de realizar um sonho e pelo exemplo de dedicação e inteligência.

Ao meu co-orientador Slavi, por todo o carinho e amizade e pelo auxilio e orientação na realização dos experimentos.

Ao professor Leandro, pela amizade e auxílio na realização da parte mais que mais “deu errado” ao longo desses anos.

Ao professor Luciano, por todo e carinho e atenção durante a realização de parte do projeto na Universidade Federal do Paraná.

Aos professores que aceitaram a participar da banca, por toda ajuda para a melhoria do trabalho.

A todos os professores do Departamento de Veterinária, pelos ensinamentos.

Aos técnicos da Preventiva, especialmente Dagô e Luís, por todo carinho e boa vontade para ajudar na rotina do laboratório.

A Rose, por todo carinho e pelos conselhos.

Aos amigos do InsPOA, em especial, Anderson, Bruna, Cibeli, Danilo, Tom, João, Ju, Luana, Mili, Rafaela, Valéria, Rodrigo, Frida e Mallu, pela amizade e pelos momentos descontraídos e animados fora do laboratório.

Aos amigos em geral, especialmente Gustavo, Monique e Idelvânia, pela amizade, compreensão, apoio, conversas e muitas risadas.

Aos amigos do Laboratório de Imunoquímica e Glicobiologia, DBG-UFV, especialmente, Clara, Paloma, Alessandra, Daniel e Paula, pela parceira e pelos momentos de descontração com pão de queijo e café.

Aos amigos do LACOMA, pela receptividade e ajuda durante a realização do experimento na Universidade Federal do Paraná.

A Universidade Federal de Viçosa e o Programa de Pós Graduação em Medicina Veterinária.

Ao CNPq, FAPEMIG e CAPES, pela bolsa de estudos e apoio financeiro para a realização desse projeto.

A todos que de alguma forma contribuíram para a realização de um sonho.

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RESUMO

CASTILHO, Natália Parma Augusto, D.Sc., Universidade Federal de Viçosa, Agosto de 2018. **Potencial bacteriocinogênico de bactérias ácido láticas isoladas de produtos artesanais cárneos fermentados.** Orientador: Luís Augusto Nero. Coorientador: Svetoslav Dimitrov Todorov.

O objetivo do presente trabalho foi isolar e caracterizar bactérias ácido láticas (BAL) presentes em embutidos cárneos fermentados artesanais através de técnicas fenotípicas e genotípicas, buscando a seleção de isolados com potencial bacteriocinogênico. BAL isoladas foram obtidas de embutidos cárneos fermentados artesanais e foram caracterizadas quanto a sua atividade bacteriocinogênica; após o isolamento e identificação foram obtidos 5 diferentes isolados: *Lactobacillus curvatus* 12 e 36; *Lactococcus garvieae* 32 e *Weissella viridescens* 23 e 31 e realizada a detecção de genes de bacteriocinas; devido aos resultados encontrados em relação ao espectro de atividade e efeito de substâncias químicas e temperatura na atividade inibitória, três isolados foram selecionados (*L. curvatus* 12 e 36 e *W. viridescens* 23) para as demais análises para avaliar o potencial bacteriocinogênico. Os isolados selecionados apresentaram multiplicação distinta e a produção de bacteriocinas foi mais evidente em *L. curvatus* 12 e *W. viridescens* 23. As bacteriocinas produzidas foram adsorvidas pelas cepas produtoras em diferentes níveis. As bacteriocinas parcialmente purificadas mantiveram sua atividade inibitória após a eluição com 60% de isopropanol. Os padrões de lise celular foram semelhantes para todos os isolados testados. A detecção de β-galactosidase indicou desestabilização da permeabilidade da membrana celular das BAL isoladas. As bacteriocinas produzidas por *L. curvatus* 12 foram purificadas através do HPLC e foram identificados 4 diferentes sequências de peptídeos. Os isolados de BAL selecionados foram capazes de produzir bacteriocinas com alta atividade inibitória contra *L. monocytogenes*, indicando sua potencial aplicação na indústria de alimentos como bioconservadores. Para a avaliação da presença de genes de virulência, resistência a antibióticos e probióticos foram utilizados os cinco isolados anteriormente identificados. Todos os isolados testados foram positivos para *mub*, enquanto *EF226-cbp*, *EF1249-fbp* e*EF2380-maz* foram detectados em pelo menos um isolado; nenhum isolado apresentou os genes *map*, *EFTu* ou *prgB*. Os isolados testados apresentaram resultados variados em relação aos genes de virulência e nenhum isolado apresentou os genes *gelE*, *cylA*, *efsA*, *cpd*, *int-Tn* ou *sprE*. Os genes de resistência aos

antibióticos também apresentaram resultados variados. Os isolados de BAL apresentaram alguns aspectos benéficos, além da produção de bacteriocinas, porém a presença de genes de virulência e resistência a antibióticos é um problema ao utilizar esses isolados como culturas *starter* ou bioconservadores em alimentos. Considerando o potencial inibitório dessas cepas, uma alternativa seria o uso de suas bacteriocinas após procedimentos de semi-purificação ou purificação. Linguiça frescal foi preparada e inoculada com diferentes combinações de *L. curvatus* 12 (BAL bacteriocinogênica), *L. sakei* ATCC 15521 (BAL não bacteriocinogênica), *L. monocytogenes*, nisina e a bacteriocina parcialmente purificada produzida por *L. curvatus* 12 e estocadas a 7 °C durante 10 dias. As análises microbiológicas foram realizadas nos dias 1, 4, 7 e 10 e as análises físico-químicas (controle) nos dias 1 e 10. No geral, as contagens de BAL não apresentaram diferenças significativas entre os tratamentos e ao longo do período de estocagem ($p > 0.05$). As contagens de *L. monocytogenes* em linguiça frescal inoculada com o patógeno e a BAL bacteriocinogênica variaram de 1.0 a 2.0 log UFC/g, sendo significativamente diferente da linguiça inoculada apenas com *L. monocytogenes* ($p < 0.05$). Nisina e a bacteriocina parcialmente purificadas também determinaram uma redução nas contagens de *L. monocytogenes* quando comparado com o tratamento que foi inoculado somente o patógeno, variando de 1.0 a 3.0 log UFC/g ($p > 0.05$). Esses resultados indicam que BAL bacteriocinogênica foi capaz de determinar uma redução significativa na contagem de *L. monocytogenes* em linguiça frescal estocada a 7 °C.

ABSTRACT

CASTILHO, Natália Parma Augusto, D.Sc., Universidade Federal de Viçosa, August, 2018. **Bacteriocinogenic potential of lactic acid bacteria isolates from artisanal fermented meat products.** Advisor: Luís Augusto Nero. Co-advisor: Svetoslav Dimitrov Todorov.

The aim of the study was isolate and characterized lactic acid bacteria (LAB) from artisanal fermented meat products through phenotypic and genotypic methodologies, searching for the selection of isolates with bacteriocinogenic potential. LAB isolates were obtained from artisanal fermented meat products and were characterized to their bacteriocinogenic activity; after isolation and identification were obtained 5 different isolates: *Lactobacillus curvatus* 12 and 36; *Lactococcus garvieae* 32 and *Weissella viridescens* 23 and 31 and the detection of bacteriocins related genes were performed; *L. curvatus* 12 and 36 e *W. viridescens* 23 were selected for the other analysis to evaluate the bacteriocinogenic potential. The selected isolates showed distinct growth and bacteriocin production was more evident in *L. curvatus* 12 and *W. viridescens* 23. The bacteriocins produced were adsorbed by the producing strains at different levels. Partially purified bacteriocins maintained their inhibitory activity after elution with 60% isopropanol. Cell lysis patterns were similar for all isolates tested. Detection of β-galactosidase indicated destabilization of the cell membrane permeability of the isolated BAL. The bacteriocins produced by *L. curvatus* 12 were purified by HPLC and four different peptide sequences were identified. The selected BAL isolates were able to produce bacteriocins with high inhibitory activity against *L. monocytogenes*, indicating their potential application in the food industry as bioconservatives. For the evaluation of the presence of virulence genes, resistance to antibiotics and probiotics, the five isolates previously identified were used. All isolates tested were positive for mub, while *EF226-cbp*, *EF1249-fbp* and *EF2380-maz* were detected in at least one isolate; none isolated showed *map*, *EFTu* or *prgB*. The isolates tested presented variable results in relation to the virulence genes and no isolates showed the genes *gelE*, *cylA*, *efsA*, *cpd*, *int-Tn* or *sprE*. Antibiotic resistance genes were also detected at different patterns. LAB isolates presented some beneficial aspects besides the production of bacteriocins, but the presence of virulence genes and resistance to antibiotics is a problem when using these isolates as starter or bioconservative cultures in foods. Considering the inhibitory potential of these strains, an alternative would be

the use of their bacteriocins after semi-purification or purification procedures. Fresh sausage was prepared and inoculated with different combinations of *L. curvatus* 12 (bacteriocinogenic BAL), *L. sakei* ATCC 15521 (non bacteriocinogenic BAL), *L. monocytogenes*, nisin and partially purified bacteriocin produced by *L. curvatus* 12 and stored at 7 ° C for 10 days. Microbiological analyzes were performed on days 1, 4, 7 and 10 and physical-chemical analyzes (control) on days 1 and 10. In general, LAB counts did not show significant differences between treatments and throughout the storage period ($p > 0.05$). The counts of *L. monocytogenes* in fresh sausage inoculated with the pathogen and the bacteriocinogenic LAB varied from 1.0 to 2.0 log CFU/g, being significantly different from the sausage inoculated with only *L. monocytogenes* ($p < 0.05$). Nisin and partially purified bacteriocin also determined a reduction in the counts of *L. monocytogenes* when compared to the treatment that was inoculated only the pathogen, ranging from 1.0 to 3.0 log CFU/g ($p > 0.05$). These results indicate that the bacteriocinogenic LAB was able to determine a significant reduction in the count of *L. monocytogenes* in fresh sausage stored at 7 ° C.

INTRODUÇÃO

Atualmente a indústria de alimentos enfrenta um grande desafio: os consumidores buscam cada vez mais produtos que possuem uma longa vida de prateleira, que sejam minimamente processados e com o mínimo possível de conservantes químicos. Nesse contexto, as bacteriocinas são uma opção atraente, pois podem solucionar pelo menos uma parte desse problema. As bacteriocinas são produzidas por vários micro-organismos envolvidos na produção de alimentos, geralmente são estáveis a tratamentos térmicos e podem inibir os principais micro-organismos patogênicos e deteriorantes que causam problemas em alimentos processados.

Vários métodos de preservação veem sendo utilizado para evitar a toxiinfecção alimentar e a deterioração dos alimentos. Essas técnicas incluem tratamento térmico, redução do pH e da atividade de água e adição de conservantes. Embora estes métodos tenham se mostrado bem-sucedidos, existe uma procura crescente por produtos naturais, microbiologicamente seguros, proporcionando aos consumidores elevados benefícios para a saúde (Deegan et al., 2006). Nos dias de hoje as bacteriocinas têm sido amplamente utilizadas, principalmente em relação à preservação de alimentos. Assim, o uso de bacteriocinas na indústria de alimentos tem sido extensivamente investigado. Entre as bacteriocinas produzidas por BAL, a nisina tem se mostrado altamente eficaz contra agentes microbianos que causam toxinfecções alimentares e micro-organismos deteriorantes (Deegan et al., 2006; Moll et al., 1999).

Entretanto, para uma bacteriocina ser legalizada para sua utilização em alimentos existem alguns critérios importantes, tais como: a cepa produtora de bacteriocina deve possuir o *status* GRAS (Generally Recognized As Safe); a bacteriocina deve possuir

um amplo espectro de inibição frente a vários micro-organismos patogênicos e deteriorantes ou a atividade antagonista frente a um patógeno específico; a bacteriocina deve ser estável a tratamentos térmicos e não deve apresentar risco associado à saúde do consumidor (Holzapfel et al., 1995). O uso de bacteriocinas tem demostrado importante potencial na bioconservação de carnes, produtos lácteos, alimentos enlatados, peixes, bebidas alcóolicas, saladas, produtos de ovos, produtos de padaria, dentre outros, sendo utilizada isoladamente no produto, na incorporação em filmes plásticos ou na superfície de alimentos (Chen and Hoover, 2003; Ryan et al., 2001).

As bacteriocinas podem ser adicionadas nos alimentos de diferentes formas. Em alimentos fermentados, por exemplo, as bacteriocinas podem ser adicionadas *in situ* por culturas bacterianas que podem substituir total ou parcialmente as culturas *starter* utilizadas na produção desses alimentos, ou então pode-se adicionar no alimento as bacteriocinas purificadas ou semi-purificadas. Apesar das bacteriocinas com amplo espectro de inibição serem as mais procuradas para a bioconservação em alimentos, outros fatores, incluindo pH ótimo, solubilidade e estabilidade, são importantes determinantes da escolha de um inibidor a ser aplicado em um determinado alimento, ou tendo como objetivo o controle de um micro-organismo alvo. Assim, estudos *in situ* com o objetivo de avaliar o real comportamento das bacteriocinas e cepas produtoras em condições naturais na matriz alimentar são fundamentais.

Com base nessas informações, o objetivo da pesquisa foi isolar BAL com potencial bacteriocinogênico de produtos cárneos artesanais, caracterizar as condições de produção e inibição de bacteriocinas pelas cepas bacteriocinogênicas selecionadas, purificar e identificar as bacteriocinas, avaliar a presença de genes de virulência, resistência a antibióticos e probióticos da coleção de BAL bacteriocinogênica e avaliar

as interações da BAL bacteriocinogênica e suas bacteriocinas com *L. monocytogenes* em linguiça frescal.

REVISÃO BIBLIOGRÁFICA

1. Hábitos alimentares

Atualmente, os consumidores vêm buscando cada vez mais produtos com o mínimo de conservantes químicos e que sejam minimamente processados, levando à indústria de alimentos a enfrentar um desafio em fornecer produtos mais naturais possíveis e seguros aos consumidores. Durante o processamento de alimentos existem várias etapas que podem servir como porta de entrada para micro-organismos indesejáveis, como os patógenos. Geralmente os patógenos possuem alta capacidade de adaptação e conseguem sobreviver, se multiplicar e produzir compostos tóxicos nos mais diferentes ambientes. Com isso, surtos envolvendo micro-organismos patogênicos veiculados por alimentos de origem animal são relatados com frequência (Havelaar et al., 2010).

No entanto, vários métodos de preservação vêm sendo utilizado para evitar a intoxicação alimentar e a deterioração dos alimentos. Essas técnicas incluem tratamento térmico (pasteurização, esterilização por aquecimento), redução do pH e da atividade de água (acidificação, desidratação) e adição de conservantes. Embora estes métodos tenham mostrado ser bem sucedidos, existe uma procura crescente por produtos naturais, microbiologicamente seguros, proporcionando aos consumidores elevados benefícios para a saúde (Deegan et al., 2006). Com o intuito de atender essa demanda, apesar de antimicrobianos sintéticos já serem aceitos em diversos países, atualmente a tendência é o estudo a respeito do uso de conservantes naturais na produção de alimentos, podendo também possibilitar o desenvolvimento de novos

produtos para os consumidores (Arqués et al., 2011; Cleveland et al., 2001; Negi, 2012).

As culturas *starter* de bactérias ácido láticas (BAL) utilizadas nas indústrias de alimentos são capazes de produzir vários compostos antimicrobianos e também substâncias de natureza proteica que conseguem inibir bactérias deteriorantes e patogênicas (Holzapfel et al., 1995; Klaenhammer, 1988). As BAL são bactérias heterogêneas capazes de fermentar açúcares em ácido lático, promovendo uma acidificação do produto. Esses micro-organismos são naturalmente encontrados em vários alimentos e em alguns produtos são adicionadas com o objetivo de melhorar as características sensoriais, como textura e sabor, aumentar a segurança microbiológica, aumentar a vida útil e até mesmo melhorar a qualidade nutricional do produto (Djadouni and Kihal, 2012). Algumas BAL estão relacionadas à biopreservação de alimentos devido à capacidade de inibir a multiplicação de micro-organismos deteriorantes e patogênicos, por meio de competição e/ou produção de substâncias antimicrobianas, tais como as bacteriocinas (Chen and Hoover, 2003).

O uso de bacteriocinas na indústria de alimentos, principalmente em produtos lácteos, ovos, vegetais e carne, têm sido extensivamente investigados. Entre as bacteriocinas produzidas por BAL, a nisina A, assim como a sua variante natural nisina Z, têm mostrado ser altamente eficaz contra agentes microbianos que causam toxinfecções alimentares e micro-organismos deteriorantes (Deegan et al., 2006; Moll et al., 1999). Os embutidos cárneos curados e fermentados condizem com as tendências atuais de consumo da população, uma vez que são produtos prontos para o consumo, possuem caráter nutritivo, são produtos fáceis de conservar, podem ser usados em acompanhamento em preparações culinárias ou individualmente e, além disso, apresentam-se de variados sabores e formas (Macedo et al., 2008).

2. Culturas láticas em produtos cárneos

Embutidos cárneos são produtos cárneos preparados ou processados, em que as propriedades originais da carne fresca foram alteradas por tratamentos físicos, químicos ou biológicos, ou pela combinação desses tratamentos. Geralmente, o processo envolve cortes das carnes, adição de condimentos e/ou especiarias e aditivos (Pardi et al., 1993). De acordo com o Regulamento de Inspeção Industrial e Sanitária de Produtos de Origem Animal (RIISPOA), embutido cárneo é definido como todo produto elaborado com carne ou órgãos comestíveis curados ou não, condimentado, podendo ou não ser cozido, defumado, dessecado, e contido em envoltório natural ou artificial (BRASIL, 2017).

BAL constitui uma parte relevante da microbiota inicial em carnes e suas populações se multiplicam facilmente durante o armazenamento refrigerado, mesmo quando estes produtos são embalados sob atmosfera modificada ou a vácuo, e após o processamento de produtos fermentados (Toldrá, 2010). As BAL autóctones usualmente encontrados em carnes são: *Carnobacterium piscicola*, *C. divergens*, *Lactobacillus sakei*, *L. curvatus*, *L. plantarum*, *Leuconostoc mesenteroides*, *L. gelidum* e *L. carnosum* (Toldrá, 2010). *Weissella halotolerans, W. hellenica* e *W. viridescens* também são comumente associados a carnes e produtos cárneos (Collins et al., 1993).

A produção de um produto cárneo fermentado é realizada em duas diferentes etapas, a fermentação e a maturação. A fermentação ocorre durante aproximadamente 7 dias e é nesta fase que ocorre o desenvolvimento da acidificação e formação de cor (Pardi, 2006). Nesta fase, BAL homofermentativas produzem ácido lático e contribuem para a inibição de micro-organismos indesejáveis, e com a perda de água ocorre uma redução na atividade de água do produto final. Durante a maturação, ocorre

uma maior desidratação do produto, assim como hidrólise enzimática das proteínas e gorduras, gerando compostos com importante papel nas características sensoriais do embutido cárneo fermentado (Lücke, 1994).

A fermentação é de extrema importância, pois este processo é responsável pelo desenvolvimento do sabor característico de um embutido cárneo, e o uso de culturas *starter* tem sido feita com o objetivo de melhorar a conservação do produto, através da redução do pH e produção de substâncias antimicrobianas, como por exemplo, as bacteriocinas. Além disso, a fermentação é responsável pelo desenvolvimento do sabor ácido característico de produtos cárneos fermentados e aceleram o processo de maturação. Vários micro-organismos têm sido usados na fabricação de produtos cárneos fermentados, com o objetivo de fornecer produtos com qualidade sanitária adequada (Bernardi et al., 2010).

Bactérias isoladas da microbiota autóctone de produtos cárneos artesanais são as mais promissoras para serem utilizadas como culturas *starter*. BAL conseguem se adaptar bem ao meio cárneo e conseguem controlar os micro-organismos deteriorantes e patogênicos, devido à produção de vários compostos antimicrobianos, dentre eles, diacetil, ácidos orgânicos e, principalmente, as bacteriocinas (Hugas and Monfort, 1997).

3. Bacteriocinas

3.1. Histórico e definição

O estudo das bacteriocinas teve início em 1925, quando Gratia observou pela primeira vez a produção de colicina através de uma cepa virulenta de *Escherichia coli*

(Gillor et al., 2008). Nesta época, os estudos estavam focados apenas em micro-organismos Gram negativos, porém essas bactérias eram associadas de forma negativa aos alimentos. Apesar disso, o estudo com colicinas permitiu o desenvolvimento de metodologias de isolamento e detecção de outras classes de bacteriocinas (Lewus et al., 1991). Mais tarde, Jacob et al. (1953) utilizaram o termo bacteriocinas para denominar proteínas com efeito bactericida, as quais eram caracterizadas pela biossíntese letal, pela sua ligação específica a determinados receptores celulares e pelo espectro restrito de atividade (Chen and Hoover, 2003). Desta forma, as famílias de novas bacteriocinas passaram a ter o nome da espécie ou do gênero da bactéria produtora adicionada com o sufixo "cin" (Cascales et al., 2007).

Atualmente, as bacteriocinas são definidas como peptídeos antimicrobianos que são sintetizados nos ribossomos e conseguem atuar tanto contra bactérias Gram positivas quanto Gram negativas, sendo que a bactéria produtora de bacteriocina possui um mecanismo de resistência específico que a protege de sua própria bacteriocina (Chen and Hoover, 2003; Cotter et al., 2013). As bacteriocinas que são produzidas por BAL podem ser de espectro largo ou estreito, no entanto, geralmente a atividade é dirigida contra micro-organismos Gram positivos. A atividade contra bactérias Gram negativas foi demonstrada, mas apenas em situações em que a integridade da membrana externa foi comprometida (Cotter et al., 2005).

Segundo estudos genéticos e bioquímicos, diversas bacteriocinas já foram caracterizadas, tais como lactocinas (Upreti and Hinsdill, 1975), lactococcinas (Holo et al., 1991), plantaricinas (Nissen-Meyer et al., 1993), pediocinas (Cintas et al., 1995), enterocinas (Aymerich et al., 1996), entre outras. Porém, somente a nisina é autorizada pela Organização Mundial de Saúde (OMS) a ser utilizada em alimentos destinados a consumo humano desde 1969, sendo a única bacteriocina comercializada e usada como

conservante de alimentos em de mais de 40 países, incluindo o Brasil (Chen and Hoover, 2003; Cleveland et al., 2001).

3.2. Classificação

As bacteriocinas produzidas por BAL podem ser classificadas em diferentes grupos com base em sua estrutura, tais como propriedades físico-químicas, número de peptídeos que compõem sua estrutura ativa, similaridade entre sequência primária e sequência líder, e também podem ser agrupadas de acordo com seu modo de ação (Aymerich et al., 1996). Estas bacteriocinas já foram agrupadas de diferentes formas, porém ainda não existe um consenso quanto a sua classificação.

Klaenhammer (1993) definiu a classificação dessas bacteriocinas em quatro grandes grupos. A classe I é formada pelos lantibióticos, que são um grupo de pequenos peptídeos, sintetizados nos ribossomos e que sofrem uma extensa modificação pós-traducional. Esse grupo possui resíduos de β-metil lantionina e lantionina, bem como outros aminoácidos, tais como didehidroalanina e didehidrobutirina. A classe II possui peptídeos de 4 a 6 KDa, sintetizados nos ribossomos, termoestáveis e não sofrem extensa modificação pós-traducional, exceto a clivagem do peptídeo durante o transporte para fora da célula. A classe II é subdividida em IIa, IIb e IIc. As bacteriocinas pertencentes à classe III são peptídeos termolábeis e são maiores que 30KDa. Já a classe IV é formada por complexos de bacteriocinas que possuem lipídeos essenciais ou porções de carboidratos ligados a proteínas. Alguns autores excluíram a classe IV (Nes et al., 2007) por não terem sido caracterizadas ou subdividiram a classe II de acordo com o número de resíduos de cisteína (Van Belkum and Stiles, 2000).

Cotter et al. (2005) sugeriram uma classificação simplificada, sendo a classe I os lantibióticos (bacteriocinas que possuem a lantionina), a classe II os não lantibióticos (bacteriocinas que não possuem a lantionina) e a classe III as bacteriolisinas (peptídeos líticos). A classe IV (bacteriocinas com porções não protéicas) não foi incluída nessa classificação. Segundo essa classificação, as bacteriocinas circulares são pertencentes à classe II de peptídeos não modificados, a subclasse IIc. Entretanto, tem-se sugerido uma nova classe (IV ou V) que englobe essas bacteriocinas, uma vez que estas se distinguem das outras produzidas por bactérias Gram positivas, e por ser um grupo único devido à sua homogeneidade (Gabrielsen et al., 2014).

Devido às várias divergências em relação à classificação das bacteriocinas, uma nova classificação foi recentemente proposta por (Cotter et al., 2013). Considerando essa nova classificação, as bacteriocinas produzidas por bactérias Gram positivas são separadas das produzidas por Gram negativas. Com isso, as bacteriocinas produzidas por Gram positivas são divididas em dois grupos (classe I e II), excluindo as classes III e IV. A classe III seria incluída na classe IIc e a classe IV seria extinta, pois a designação de bacteriocina corresponderia apenas aos pequenos peptídeos ribossomicamente sintetizados, não incluindo proteínas antimicrobianas grandes. De acordo com essa nova classificação, a classe I engloba as bacteriocinas que sofrem extensas modificações pós-traducionais e a classe II corresponde as bacteriocinas que não sofrem ou sofrem modificações modestas pós-traducionais, com formação de pontes dissulfeto e adição ou circularização de N-formilmetionina. As bacteriocinas produzidas por bactérias Gram negativas, seriam classificadas em dois grupos, sendo um de peptídeos pequenos, como por exemplo, as microcinas, e um segundo grupo de peptídeos grandes, como por exemplo, as colicinas. Trabalhos recentes já mostram

autores utilizando essa nova classificação das bacteriocinas (Ogaki et al., 2015; Yang et al., 2014).

3.3. Produção nos alimentos

A atividade das bacteriocinas produzidas por diferentes BAL não é uniforme e nem constante, e depende da composição química e das condições físico-químicas dos alimentos e principalmente do pH, adsorção a células ou proteínas, atividade de proteases e outras enzimas (Schillinger et al., 1996). As condições ótimas para o desenvolvimento da cepa bacteriocinogênicas não coincidem necessariamente com as condições ótimas de produção de bacteriocinas. Os suplementos de meios específicos que podem afetar acentuadamente a produção de bacteriocinas por bactérias Gram positivas incluem extrato de levedura, glicose e íons de magnésio (Heng et al., 2007).

Além de interagir com os componentes dos alimentos, as bacteriocinas podem ser adversamente afetadas por condições de processamento e armazenamento, tais como pH e temperatura do produto. Segundo Drosinos et al. (2005), o pH ideal para a produção de bacteriocinas por cepas do gênero *Leuconostoc* (5.5) não corresponde ao pH ótimo de multiplicação microbiana (6.5). Devido a sua estabilidade máxima em condições ácidas, a atividade da nisina é aumentada quando utilizada em alimentos ácidos. Por isso, aplicações eficazes da nisina requerem que o pH do alimento seja inferior a 7, a fim de assegurar solubilidade satisfatória, estabilidade durante o processamento e período de armazenamento.

Determinadas concentrações de NaCl podem reduzir a multiplicação de BAL e, consequentemente, a produção de bacteriocinas, além de proteger as bactérias alvo, como por exemplo *Listeria monocytogenes* (Hugas et al., 2002). Sarantinopoulos et al.

(2002) observaram redução na atividade da bacteriocina após a adição de 2% de NaCl. Nilsen et al. (1998) atribuíram esse fenômeno à interferência de NaCl no fator de produção que liga o indutor ao receptor.

A eficiência da atividade inibitória das bacteriocinas também está relacionada com o nível de contaminação dos alimentos pelo micro-organismo alvo. Se a contaminação inicial é relativamente alta, a eficiência da bacteriocina é baixa e incapaz de prevenir o desenvolvimento de micro-organismos contaminantes. Rilla et al. (2004) investigaram a ação da bacteriocina produzida por *Lactococcus lactis* subsp. *lactis* IPLA 729 contra *Staphylococcus aureus* em duas concentrações diferentes (1.8×10^4 e 7.2×10^6 CFU/mL). Após o período de incubação, estes autores não detectaram *S. aureus* na amostra mais diluída, enquanto a outra amostra apresentou uma contagem ainda maior (5.0×10^4 CFU/mL).

3.4. Organização genética de operons e papel dos diferentes genes na produção e expressão

Em geral, a produção de bacteriocinas ocorre na forma de um pré-peptídeo inativo e os genes relacionados à produção desta substância ativa estão localizados em um operon. Os genes envolvidos na produção de bacteriocina são: gene estrutural (codifica a produção da pré-bacteriocina), gene de resistência (localizado ao lado do gene estrutural e que confere resistência à célula produtora), gene que codifica um transporte ABC (responsável pela externalização da bacteriocina) e um gene que codifica uma proteína acessória (não pertencente ao transporte ABC, mas necessária para a excreção da bacteriocina) (Nes et al., 1996). Alguns autores acreditam que a produção de bacteriocinas possa estar relacionada com a presença de plasmídeos

bacteriocinogênicos, apesar de vários genes que codificam bacteriocinas da classe IIa tenham sido localizados em fragmentos de cromossomos (Aymerich et al., 1996; Hühne et al., 1996).

O pré-peptídeo formado contém uma sequência de 18 a 27 aminoácidos, sendo duas glicinas na região N-terminal. As funções dessa sequência de aminoácidos são evitar que a bacteriocina se torne biologicamente ativa dentro da célula produtora e servir como sinal de reconhecimento para o sistema de transporte, que envolve as proteínas do sistema de transporte ABC e uma proteína acessória. Além disso, as duas glicinas na sequência de aminoácidos são responsáveis pelo reconhecimento da pré-bacteriocina (Moll et al., 1999; Nes et al., 1996).

A sequência líder mantém a bacteriocina em uma forma inativa dentro da célula produtora, facilita a interação com o transportador e, provavelmente no caso dos lantibióticos, tem um papel de reconhecimento pela maquinaria de modificação (Chen et al., 2001; Neis et al., 1997; van der Meer et al., 1994). Essa sequência líder é normalmente clivada durante a exportação por um sistema de transporte de bacteriocina ou, menos frequentemente, pela via de secreção geral da célula, e em seguida ocorre a separação desta sequência tornando a molécula biologicamente ativa (Havarstein et al., 1995). Os genes que codificam os pré-peptídeos estruturais estão geralmente intimamente associados a genes que codificam produtos envolvidos na regulação, exportação, auto resistência e modificação.

As proteínas de resistência estão relacionadas com a proteção total da cultura produtora contra os efeitos da sua própria bacteriocina, porém é sugerida a existência de uma proteção parcial contra outras bacteriocinas de mesma classe (Eijsink et al., 1998). Segundo Nes et al. (2007), as bactérias produtoras de bacteriocinas possuem um mecanismo de resistência que a protege da ação de suas próprias bacteriocinas,

com o intuito de evitar a lise da própria célula e de células análogas. Essa proteção é dada através de um peptídeo de resistência expresso junto às bacteriocinas (Abee et al., 1995; Nes et al., 2007). Neste caso, quando a bacteriocina é produzida pela bactéria, a proteína de resistência se liga ao receptor evitando que a bateriocina se ligue a este e forme poros na membrana citoplasmática, o que causaria a lise celular (Nes et al., 2007).

Os sistemas de regulação da produção de bacteriocinas são formados por um sistema com três componentes diferentes: uma histidina proteína quinase (HPK), um regulador de resposta (RR) e um fator de indução (IF), o qual é requisitado como um sinal para a indução da transcrição de genes alvo (Kuipers et al., 1998). O peptídeo indutor é sintetizado em baixos níveis no ribossomo como um pré-peptídeo, que será clivado e secretado no meio externo através do transportador. Quando este composto chega à determinada concentração, ele ativa a histidina quinase transmembrana, que leva a uma auto-fosforilação do resíduo de histidina, transferindo para proteína reguladora de resposta um fosfato. Este regulador fosforilado ativa a transcrição da bacteriocina, além dos componentes que formam o sistema regulador, começando um feedback positivo (Nes and Eijsink, 1999). A regulação da produção da classe I das bacteriocinas, tais como nisina e subtilina, é realizada pela própria bacteriocina, que funciona como feromônio para induzir sua produção em níveis altos (Kleerebezem and Quadri, 2001). Alguns autores acreditam que acontece um aumento gradual do IF como consequência da multiplicação celular (Nes et al., 1996). Através do aumento da biomassa microbiana, o IF passa a atuar como iniciador do processo. Acredita-se que este sistema regulatório da produção das bacteriocinas seja capaz de mediar uma resposta para um sinal no meio onde a substância está sendo produzida, propondo que fatores ambientais sejam capazes de afetar a ligação do IF à HPK, induzindo a

fosforilação requerida para a ativação do RR. No entanto, pouco se sabe sobre os mecanismos e a extensão da implicação de fatores ambientais neste sistema (Nilsen et al., 1998).

As proteínas envolvidas na modificação, exportação e regulação de bacteriocinas são muitas vezes codificadas pelos genes presentes próximos ao gene que codifica a proteína precursora (Lee et al., 2008). Estes genes podem estar localizados no plasmídeo, cromossomo ou transposon (Rodríguez et al., 2002). A produção de bacteriocinas é um processo específico de uma espécie e as diferenças na localização do gene biosintético, gene de resistência e de transporte pode resultar na forma variante da bacteriocina e lhe conferir novas propriedades (Cotter et al., 2005; Quadri et al., 1994).

3.5. Modo de ação

Quase todas as bacteriocinas são capazes de interagir com os lipídeos aniônicos presentes na membrana plasmática das bactérias-alvo, sendo ativas na grande maioria das vezes contra bactérias Gram positivas, já que estes micro-organismos possuem um elevado teor de lipídeos aniônicos na membrana plasmática (Guilhelmelli et al., 2013; Zacharof and Lovitt, 2012). A maioria das bacteriocinas formam poros devido à permeabilização da membrana, o que vai promover a dissipação da força próton motora (PMF) e a inibição do transporte de aminoácidos. A PMF está relacionada em vários processos que ocorrem na membrana citoplasmática, como por exemplo, a síntese de ATP e o acúmulo de metabólitos e íons. Algumas bacteriocinas conseguem inibir as bactérias Gram negativas, entretanto, estas precisam transpor a membrana externa da parede celular e assim conseguem alcançar a membrana plasmática da

célula-alvo para então atuarem. As bacteriocinas em contato com as membranas plasmáticas conseguem interferir na síntese de proteínas, RNA e DNA, tais como algumas microcinas (Mcc), como, por exemplo, a MccB17 que inibe a DNA-girase, a MccC7 que inibe a asparil-RNAt sintase e a MccJ25 que inibe a RNA polimerase. Neste caso existem também exceções, como por exemplo, a MccE4492 que consegue atuar através da formação de poros (Cotter et al., 2013).

A classe I das bactericiocinas (lantibióticos) é conhecida por possuírem um amplo espectro de ação e, na maioria das vezes, formam poros instáveis, mas algumas moléculas de ancoragem que estão presentes na membrana-alvo podem funcionar como receptores e com isso, aumentam a condutividade e a estabilidade dos poros (Moll et al., 1999). Além disso, alguns membros desta classe (nisina, por exemplo) tem um modo de ação duplo. Neste caso, os lipídeos II presentes na membrana-alvo são os principais transportadores de subunidade de peptideoglicano do citoplasma para a parede celular e a nisina consegue se ligar ao lipídeo II impedindo a correta síntese da parede celular. Outro ponto importante é que o lipídeo II é capaz de atuar como uma molécula de ancoragem a que a nisina se liga para conseguir se inserir na membrana e então formar os poros. A lacticina 3147 é um lantibiótico que possui dois peptídeos e apresenta sua atividade distribuída nos dois peptídeos, enquanto a mersacidina se liga ao lipídeo II sem formar poros na membrana (Cotter et al., 2005). A nisina é capaz de apresentar pelo menos três tipos de atividade antimicrobiana além da formação de poros na membrana-alvo: consegue inibir a biossíntese da parede celular, a atividade de enzimas autolíticas e a germinação de esporos (Moll et al., 1999).

As bacteriocinas pertencentes à classe II são termoestáveis e possuem um espectro de atividade restrito, sendo que os receptores na membrana da célula-alvo é

que determinam sua especificidade de ligação. Geralmente, essas bacteriocinas tem uma estrutura helicoidal anfifílica, o que lhes permite inserir na membrana da célula-alvo, conduzindo à despolarização por dissipação da PMF, com isso, ocorre um desequilíbrio do conteúdo intracelular (Cotter et al., 2005; Moll et al., 1999). O espectro de inibição dessas bacteriocinas é bastante estreito, limitando a espécies ou estirpes relacionadas com os produtores. Consequentemente, as bacteriocinas da classe II são principalmente ativas contra bactérias Gram positivas de baixo G + C, tais como BAL, *Listeria*, *Enterococcus* e *Clostridium* (Héchard and Sahl, 2002). Entre as bacteriocinas da classe II descritas até agora, mais de vinte possuem grandes semelhanças na sua estrutura primária, bem como uma atividade anti-*Listeria*. Essas bacteriocinas que são agrupadas na subclasse IIa, em particular, possuem uma parte N-terminal altamente conservada incluindo YGNGV, uma ponte dissulfeto e vários resíduos conservados (Nes and Holo, 2000). Exemplos dessa subclasse de bacteriocinas incluem pediocina PA-1, bavaricina MN, divercina V41, mesentericina Y105, leucocina A e enterocina P (Héchard and Sahl, 2002).

Um exemplo do mecanismo de ação da classe II é o que ocorre com a lactococcina. Os monômeros de lactococcina se ligam a um receptor de membrana (manose-fosfotransferase – Man PTS), se inserem e começam a formar os poros (Cotter et al., 2013). Entretanto, estudos recentes mostraram que as bacteriocinas da subclasse IIc (garvicina ML) e subclasse IId (lactococcina LsbB) possuem recepetores de membrana diferentes de Man-PTS (Cotter, 2014). As bacteriocinas subclasse IIc (enterocina AS-48, garvicina e a cicularina) tem uma estrutura globular compacta composta por porções helicoidais repetidas ao redor de um núcleo hidrofóbico. Essa característica as tornam muito estáveis e resistentes a maiores variações de pH, altas temperaturas e ação proteolítica (Gabrielsen et al., 2012). Estudos recentes observaram

que a atividade antimicrobiana de bacteriocinas cíclicas (garvicina ML) está intimamente relacionada ao receptor de membrana maltose ABC transportador, que pode atuar como permease ou molécula de ancoragem para a ligação e a atividade do peptídeo (Cotter, 2014; Gabrielsen et al., 2012). Já bacteriocinas da subclasse IId (como a lactococcina LsbB) possuem metalopeptidades zinco-dependentes presentes na membrana plasmática das células-alvo como receptor (Uzelac et al., 2013). Finalmente, as bacteriocinas pertencentes à classe III (bacteriolisinas) como, por exemplo, a lisostafina, é capaz de funcionar diretamente sobre a parede celular de bactérias-alvo, causando então a lise celular (Cotter et al., 2005).

4. Produção e purificação de bacteriocinas

4.1. Identificação da produção – ensaios

A obtenção de bacteriocinas puras é um passo importante para permitir a caracterização e análise detalhada de seus mecanismos inibitórios (Epand and Vogel, 1999). A descoberta de novas cepas produtoras de bacteriocinas, bem como o isolamento de quantidades suficientes de bacteriocinas puras, são igualmente importantes quando se deseja obter uma investigação mais profunda. A identificação de uma bacteriocina é um processo lento e tedioso, em que as bactérias produtoras de bacteriocinas são isoladas, rastreadas e identificadas a nível molecular. Além disso, a bacteriocina precisa ser purificada, identificada, caracterizada e testada extensivamente quanto à inibição do desenvolvimento de outras bactérias (Migaw et al., 2014).

O primeiro passo para o desenvolvimento de estudos de distribuição e detecção de atividade antagonista de substâncias antimicrobianas é a detecção de cepas produtoras de bacteriocinas. Existe um grande número de técnicas de purificação de bacteriocinas devido ao fato dessas moléculas serem extremamente heterogêneas. Com o intuito de purificar esses compostos, são necessários estudos de produção e grandes volumes de cultivo. A produção das bacteriocinas pode ser influenciada pelas condições de incubação, temperatura, tempo, pH e a composição dos meios de cultura. Para cada micro-organismo produtor de bacteriocina devem ser determinadas as condições ótimas de produção. Devido à grande diversidade de bacteriocinas, não existe um protocolo geral ou um método único para a purificação (Motta and Brandelli, 2002; Rojo-Bezares et al., 2007).

4.2. Métodos de purificação

Para se realizar a purificação de bacteriocinas, o primeiro passo é a obtenção do sobrenadante livre de células da cultura produtora de bacteriocina, seguido por um processo que envolve a concentração do peptídeo (Venema et al., 1997). A concentração do peptídeo é feita por técnicas que permitem a separação de frações de acordo com a sua natureza físico-química ou com o seu tamanho. As técnicas mais utilizadas incluem: filtração por diálise ou ultrafiltração (Muriana and Klaenhammer, 1991; Parente and Hill, 1992; Schillinger and Lücke, 1989); precipitação de proteínas utilizando sais como o sulfato de amônio ou ácidos (Holo et al., 1991); extração de proteínas com uso de solventes orgânicos (butanol e etanol) (Piva and Headon, 1994); e, além disso, o sobrenadante livre de células pode também ser concentrado por liofilização. Entretanto, essas técnicas não eliminam compostos indesejáveis do meio

de cultura que interferem no processo de purificação. O uso de sulfato de amônio é preferível, pois este sal precipita reversivelmente às proteínas sem um aumento considerável da temperatura do meio, entretanto o ácido tricloroacético, metanol, etanol e acetona também podem ser usados para a extração (Schillinger and Lücke, 1989). A etapa de concentração do sobrenadante é realizada com o intuito de diminuir o volume da amostra inicial e para recuperar bacteriocinas, porém não é muito seletivo. Para obter bacteriocinas de alta pureza é necessário realizar a separação de outros compostos proteicos através de técnicas baseadas nas propriedades e características físico-químicas da bacteriocina. Geralmente, os peptídeos são purificados depois de serem submetidos a uma sequência de métodos cromatográficos (Casaus et al., 1997). Independentemente do tipo de processo de purificação que é utilizado, o rendimento reduz durante o processo, sendo necessário otimizar as condições experimentais para a produção da bacteriocina (Cintas et al., 2001).

A vantagem da etapa de precipitação antes da purificação é o aumento na concentração da proteína no meio. As proteínas precipitadas são dissolvidas em tampão ou água deionizada e separadas através do uso de várias metodologias, incluindo as cromatografias líquida de alta performance e fase reversa, de gel-filtração, de troca iônica ou de interação hidrofóbica, utilizadas de forma isolada ou combinadas. Métodos de purificação de bacteriocinas que usam protocolos complexos e demorados possuem a desvantagem de obter baixo rendimento de proteínas (Carolissen-Mackay et al., 1997; Drider et al., 2006; Gautam and Sharma, 2009). A instabilidade das bacteriocinas aumenta de acordo com o seu grau de pureza. O problema desta instabilidade é atribuído, possivelmente, a alterações no sítio catalítico como a perda de cofatores ou modificações covalentes (Héchard et al., 1992).

As técnicas cromatográficas consistem na partição do soluto entre uma fase estacionária e uma fase móvel. Geralmente, a fase estacionária é sólida e formada por uma matriz de partículas empacotadas em uma coluna. Já a fase móvel possui a mistura de componentes que serão separados e passarão através da coluna. A amostra é introduzida na coluna como um pulso e os componentes individuais são separados devido a sua distribuição diferencial entre as fases (Zuñiga et al., 2003). As separações com altas resoluções podem ser adquiridas através da mudança na fase móvel, mediante o uso de eluição em gradiente (Héchard et al., 1992). Cromatografia de troca iônica, por interação hidrofóbica, de fase reversa, por exclusão molecular, dentre outras, são técnicas de separação cromatográficas utilizadas para purificação de bacteriocinas.

A cromatografia de troca iônica é a técnica de purificação de proteínas mais utilizada, tendo muita efetividade nas indústrias farmacêutica e de bioquímica para o escalonamento dos processos de isolamento de proteínas. Nesta técnica, a separação ocorre de acordo com a carga das biomoléculas (Gerberding and Byers, 1998). Existem no mercado diversos tipos de colunas e adsorventes utilizados na cromatografia de troca iônica.

A cromatografia por interação hidrofóbica foi desenvolvida com o intuito de separar proteínas, por meio do uso de colunas empacotadas com resina de agarose modificada. Essa técnica é muito empregada para separar proteínas com hidrofobicidade diferentes, sendo ideal na etapa de captura de biomoléculas ou no estágio de purificação intermediária. Sais de sulfato ou fosfato de amônio são utilizados para promover interações hidrofóbicas. A combinação desta técnica com sistemas aquosos bifásicos tem mostrado bons resultados na extração de proteínas a partir de um meio de células não clarificado (Brocklebank, 1987).

Na cromatografia de fase reversa, as separações podem ser obtidas com elevada rapidez (Maa and Horváth, 1988). Este tempo rápido na separação ajuda no monitoramento de bioprocessos, que necessita de análises periódicas dos compostos através do HPLC. Essa é uma técnica muito utilizada em laboratórios de análise de alimentos e no controle de qualidade. Porém, pode ser utilizada em grande escala em processos de purificação de compostos biofarmacêuticos. As fases estacionárias empregadas nesta técnica são estáveis em uma ampla faixa de pH (1 a 13) e são adequadas para a realização da separação de proteínas e peptídeos. Já as fases móveis são representadas pelas misturas orgânicas-aquosas, contendo acetonitrila, isopropanol, metanol, dentre outros como modificadores orgânicos (Tweeten and Tweeten, 1986).

Na cromatografia por exclusão molecular ocorre a separação de acordo com o tamanho das moléculas. A coluna é cheia de material inerte cujo tamanho dos poros é controlado. As moléculas maiores são excluídas de todos os poros e as moléculas menores podem penetrar na maioria dos poros apresentando maior tempo de retenção. Com isso, as moléculas maiores movem-se rapidamente através da coluna e, as menores são eluídas lentamente pela fase móvel (Irvine, 1997). A existência de colunas comerciais empacotadas e o desenvolvimento de diferentes tipos de géis contribuíram para melhorar a resolução das separações e para reduzir o tempo de análise. Esse fato causou um rápido aumento no emprego desta técnica como um método de separação e purificação de macromoléculas (Ricker and Sandoval, 1996).

A purificação do sobrenadante precipitado através de cromatografia de troca iônica tem permitido boa recuperação em casos de nisina, diplocina e lactocina A. Porém, esta metodologia não serve para todas as bactericionas produzidas por BAL, uma vez que afeta sensivelmente a recuperação da atividade inibitória (Barefoot,

1993). Alguns autores observaram que a cromatografia de interação hidrofóbica pode ser usada com êxito na purificação de alguns lantibióticos, como a nisina, lactocina A e lactocina S; entretanto, esse método não serve para purificar bacteriocinas de grande hidrofobicidade, como a plantaricina e lactocina 481, que na ausência de sal reagem espontaneamente com a matriz do gel (Janes et al., 1998; Uteng et al., 2002; Venema et al., 1997). Em casos específicos pode ser utilizada a cromatografia de fase reversa na purificação de bacteriocinas, entretanto a cromatografia de troca iônica é um método mais adaptado às moléculas hidrofóbicas. Essa técnica foi utilizada por Abriouel et al. (2003) associado à adsorção da bacteriocina em Carboxymethyl Sephadex CM-25, para a enterocina AS-48, bacteriocina produzida por *E. faecalis* subsp. *liquefaciens* AS-48. De acordo com propriedades semelhantes em diversas bacteriocinas, tais como resistência aos solventes orgânicos, carga positiva e pH ácido, esses autores conseguiram através dessa metodologia uma recuperação em larga escala da bacteriocina purificada em duas etapas de purificação.

Uma propriedade das bacteriocinas que pode ser utilizada como forma de purificação é a capacidade de serem adsorvidas pelas células produtoras. Isto é possível quando o sobrenadante livre de células é submetido a um tratamento térmico com o intuito de inviabilizar as formas vegetativas, precedidas do ajuste de pH, posterior incubação overnight a 4 ºC e agitação constante, por fim ressuspensão e centrifugação após a separação das células. As bacteriocinas que possuem adsorção dependente do pH serão adsorvidas pelas células mortas e liberadas pela força iônica do tampão de suspensão e separadas através da centrifugação, resultando em uma maior recuperação e com baixo custo de produção (Atrih et al., 2001).

5. Aplicabilidade de BAL bacteriocinogênicas e suas bacteriocinas

5.1. Potencial benéfico

Segundo a WHO/FAO (2002), probióticos são definidos como micro-organismos vivos que quando ingerido em concentrações adequadas conferem benefícios à saúde do consumidor. Esses micro-organismos podem ser bactérias, fungos e leveduras; entretanto, apenas BAL são consideradas de importância em relação à alimentação e nutrição (Holzapfel et al., 2001).

Vários são os benefícios à saúde associado ao consumo de produtos probióticos, devidamente comprovados cientificamente, incluindo: propriedades antimutagênicas (Le Leu et al., 2005), anticarcinogênicas (Wollowski et al., 2001), antioxidativas (Ahire et al., 2013), antidiarreicas (Clancy, 2003), estimulação do sistema imunológico (Hai et al., 2009), prevenção de eczema e atopia (Isolauri, 2004), redução da pressão arterial (Hlivak et al., 2005), redução na concentração de colesterol (Hlivak et al., 2005), maior resistência a doenças infecciosas (Li et al., 2008), estimulação do crescimento (Ohashi and Ushida, 2009), melhoria nas doenças gastrointestinais inflamatórias (Ryan et al., 2009), manutenção da microbiota equilibrada e melhora no metabolismo de lactose (Minelli et al., 2004).

O modo como os probióticos podem influenciar a microbiota do intestino do hospedeiro ainda não é totalmente conhecido, e assim os mecanismos para os efeitos benéficos são difíceis de determinar (Aguirre-Ezkauriatza et al., 2010). Apesar dos mecanismos de ação desses micro-organismos ainda não serem totalmente elucidados, estudos mostram a crescente evidência científica a respeito dos benefícios à saúde e sua segurança para o consumo humano (Aguirre-Ezkauriatza et al., 2010; Ahire et al.,

2013; Holzapfel et al., 1998). Pode-se presumir que a resistência a um baixo pH do estômago, ácidos biliares, bem como a capacidade para competir de forma eficaz no ecossistema intestinal faz com que esses micro-organismos sejam capazes de promover os efeitos benéficos no organismo do hospedeiro (Gibson and Fuller, 2000). O modo de ação dessas bactérias envolve a supressão de bactérias indesejáveis: produção de substância inibitórias, bloqueio dos sítios de adesão, competição por nutrientes, estimulação do sistema imune, degradação dos receptores de toxinas, supressão na produção de toxinas, redução do pH intestinal e atenuação da virulência (Cotter et al., 2005; Lebeer et al., 2008).

5.2. Potencial tecnológico

BAL apresentam várias características tecnológicas, dentre elas as mais importantes são: possuem propriedades sensoriais agradáveis, possuem resistência aos bacteriófagos; apresentam viabilidade durante o processamento e estabilidade no produto e durante sua estocagem (Mattila-Sandholm et al., 2002).

Em relação ao sabor dos produtos fermentados, é importante destacar que o processo de fermentação de BAL ocorre de duas formas, homofermentação ou heterofermentação. O processo homofermentativo ocorre principalmente em espécies do gênero *Streptococcus* e *Lactococcus*, sendo o ácido lático o principal produto gerado. Já o processo heterofermentativo é caracterizado pela produção adicional de etanol e dióxido de carbono, ocorrendo principalmente nos gêneros *Lactobacillus* e *Leuconostoc* (Axelsson, 2004). Além disso, BAL probióticas se multiplicam lentamente no leite, devido à baixa atividade proteolítica, gerando pouca quantidade de ácido lático, o que causa aumento no tempo de fermentação. Com isso, as BAL

probióticas não são utilizadas sozinhas, e sim em conjunto com a cultura *starter* do produto (Ordóñez, 2005). A combinação de BAL probiótica e culturas *starter* permite adquirir um produto final com características ideais de aroma, sabor, pH e textura (Shah, 2000).

De acordo com Shah (2000), *L. delbrueckii* subsp. *bulgaricus*, utilizada na produção de iogurte, continua produzindo ácido lático durante a estocagem sob refrigeração. Esse ácido produzido causa uma diminuição na viabilidade de BAL probiótica, por isso, recomenda-se o uso de *Streptococcus thermophilus*, pois este micro-organismo se torna o principal responsável pela fermentação. A viabilidade de BAL probióticas também depende da linhagem usada, da acidez final do produto, da interação entre as espécies presentes no produto e das concentrações de ácido acético e lático. Além disso, outros fatores interferem na viabilidade das culturas láticas probióticas, como a concentração de açúcares, o teor de oxigênio dissolvido e a permeabilidade do oxigênio através da embalagem, a disponibilidade de nutrientes e de promotores ou inibidores de crescimento, a quantidade de inóculo, a temperatura de incubação, o tempo de fermentação e a temperatura de estocagem. Porém, os principais fatores que causam a perda de viabilidade são a acumulação de ácido orgânicos, como resultado da multiplicação e da fermentação, com consequente redução do pH do meio. Elevado teor de oxigênio, por exemplo, pode afetar a multiplicação e a viabilidade de BAL anaeróbias.

5.3. Toxicidade de bacteriocinas

As principais pesquisas toxicológicas sobre as bacteriocinas produzidas por BAL referem-se aos testes feitos para a aprovação da utilização da nisina como

bioconservantes em alimentos. Segundo a Food and Drug Administration, estudos sobre a toxicidade crônica, sub-crônica, aguda, de sensibilidade cruzada e de resistência cruzada mostraram que a nisina é segura para o consumo humano com uma dose diária aceitável de 2,9 mg/pessoa/dia (Cleveland et al., 2001).

Os efeitos da nisina sobre a microbiota oral foram avaliados, pois a nisina é ingerida por essa via. A partir desses estudos, foi observado que um minuto após o consumo de leite achocolatado contendo nisina, foi possível detectar apenas 40% de sua atividade quando comparada a um controle na saliva (Claypool et al., 1966). Outro estudo demonstrou o efeito das enzimas gástricas sobre a nisina, e foi possível observar que a bacteriocina é inativada pela tripsina e, com isso, conclui-se que a ingestão da nisina não interfere sobre a microbiota gastrointestinal (Hara et al., 1962).

Outros estudos toxicológicos feitos para avaliar a nisina demonstraram que sua ingestão por humanos não causa efeitos tóxicos ao organismo nas doses em que a população é exposta, sendo observada uma DL50 de 6950 mg/Kg de peso corpóreo. A nisina administrada por via oral é rapidamente inativada pela quimiotripsina e é sensível à ptialina, não sendo detectada na saliva após 10 min do consumo de líquidos que possua essa bacteriocina (Chandrapati and O'Sullivan, 1998).

A nisina é a bacteriocina com maior uso comercial, porém a segurança de outras bacteriocinas utilizadas em alimentos também tem sido avaliada. Bhunia et al. (1990) injetaram pediocina PA-1(AcH) em ratos e camundongos e demonstraram que a bacterioncina não foi imunogênica para os animais testados; essa bacteriocina também é susceptível à proteólise por quimiotripsina e tripsina.

A natureza química das bacteriocinas produzidas por BAL faz com que essas substâncias sejam facilmente degradadas no trato gastrointestinal dos animais e do homem, na maioria das vezes, perdendo sua toxicidade (Mokoena, 2017). De acordo

com Ogaki et al. (2015), pesquisas feitas com diversas bacteriocinas indicaram que esses peptídeos não são tóxicos e nem causam reações imunológicas e, com isso, possuem grande potencial como bioconservantes em alimentos.

Pariza and Foster (1983) observaram alguns critérios de segurança para o uso de enzimas em indústrias de processamento de alimentos e os seguintes pontos foram analisados: segurança do micro-organismo com atenção especial para seu potencial patogênico e toxigênico, irritações primárias e alergias; antibióticos; carcinogênese; mutagênese; teratogênese e efeitos reprodutivos; produtos de reações enzimáticas; interações entre enzimas e outros componentes alimentares e efeitos direto das enzimas alimentares sobre o consumidor. De acordo com esse estudo, a avaliação de segurança de enzimas alimentares começa através do teste oral de toxicidade aguda em ratos com dose única, onde essa deve ser equivalente a no mínimo 100 vezes a exposição humana média estimada ou no mínimo 2.000 mg/Kg de peso corporal, segundo diretrizes estabelecidas pela OECD (1987) para testes em animais. O outro teste de toxicidade proposto é um estudo da dose-oral repetida (14-91 dias), de preferência em ratos, onde o material teste pode ser administrado via sonda esofágica ou na ração, sendo a dose mínima de pelo menos 100 vezes a exposição média humana estimada. Além disso, os autores recomendam que o material teste seja avaliado quanto à presença de micotoxinas, como por exemplo, as aflatoxinas, zearalenona, ocratoxina A, esterigmatocistina e toxina T-2.

5.4. Potencial de virulência de cepas produtoras

A seleção de BAL para utilização em alimentos é de extrema importância e deve ser muito criteriosa, pois essas bactérias podem ter genes associados à virulência.

Mesmo que a maioria desses micro-organismos não seja capaz de expressá-los e com isso, não sendo potencialmente patogênicos, esses micro-organismos podem agir como reservatórios e transferi-los para outras bactérias (Barbosa et al., 2010).

Algumas linhagens BAL da microbiota intestinal humana e animal são utilizados como suplementos alimentares probióticos, incluindo *E. faecium*, *L. plantarum*, *L. acidophilus* e *L. casei* subsp. *rhamnosus* e várias espécies de *Bifidobacterium* e *Propionibacterium.* BAL amplamente utilizadas como probióticos ou culturas *starter* têm o potencial de servir como um hospedeiro de genes de resistência a antibióticos com o risco de transferir os genes para outras BAL e outras bactérias patogênicas (Tannock, 1999).

A resistência das bactérias aos antibióticos ocorre de duas formas: mutação em um loci do cromossoma ou através da transferência horizontal de genes (Džidić et al., 2008). Geralmente, os genes presentes em plasmídeos responsáveis pela resistência codificam enzimas que inativam os antibióticos ou, então, reduzem a permeabilidade das células. Por outro lado, a resistência através de mutações cromossomais envolve a modificação do alvo (Neihardt, 2004).

A resistência aos antibióticos pode estar naturalmente presente em determinadas espécies de bactérias (resistência intrínseca), resultando na capacidade da bactéria em se multiplicar na presença do agente antimicrobiano. A resistência intrínseca é herdada pelos micro-organismos da mesma espécie através da divisão celular e não pode ser transferida. Esse tipo de resistência acontece devido a fatores bioquímicos e fisiológicos específicos da bactéria, por exemplo, *Pseudomonas aeruginosa* é naturalmente resistente à penicilina G (Kümmerer, 2009). Por outro lado, a resistência adquirida está presente em algumas linhagens dentro de espécies geralmente susceptíveis a determinado antibiótico e pode ser horizontalmente transferida entre as

bactérias. Esse tipo de resistência pode começar através de mutações no genoma do micro-organismo ou através da aquisição de gene que codificam para o mecanismo de resistência (Mathur and Singh, 2005).

Um foco maior tem sido dado aos alimentos como veículos de genes de resistência a antibióticos (Franz et al., 1999; Klein et al., 2000; Perreten et al., 1997). No entanto, ainda existem poucos estudos com o intuito de investigar a resistência adquirida a antibióticos em BAL presente em alimentos. A maioria dos dados existente é sobre *Enterococcus* patogênicos, enquanto o número de relatos sobre *Lactococcus* e *Lactobacillus* é limitado. Geralmente, BAL são resistentes a alguns antibióticos das famílias aminoglicosídeos, cefalosporinas e β-lactâmicos (Herreros et al., 2005).

Aminas biogênicas são compostos que possuem bases orgânicas (putrescina, cadaverina, espermidina e espermina), heterocíclicas (histamina e triptamina) e aromáticas (feniletilamina e tiramina) gerados principalmente através da descarboxilação de seu aminoácido precursor correspondente. Em relação à via biossintética, as aminas podem ser classificadas em biogênicas e naturais. As aminas biogênicas são formadas pela descarboxilação de aminoácidos por enzimas microbianas e nas aminas naturais, a formação ocorre *in situ* nas células à medida que são requeridas (Bardócz et al., 1995). Os aminoácidos livres também podem ocorrer em alimentos ou serem liberados através de proteólise. Micro-organismos com alta atividade proteolítica aumentam as chances de formação de aminas biogênicas em alimentos, devido ao aumento da disponibilidade de aminoácidos livres (Karovičová and Kohajdova, 2005). Estes compostos são termoestáveis e o cozimento e a exposição prolongada ao calor não atuam sobre a toxina (Duflos, 2009; Shalaby, 1996; Tapingkae et al., 2010).

O processo de fermentação de produtos cárneos é capaz de contribuir para a formação de aminas biogênicas, uma vez que necessitam de micro-organismos produtores de descarboxilase, concentrações adequadas de precursores de aminoácidos livres associados a fatores ambientais que estimulam a multiplicação microbiana e a síntese da descarboxilase (Curiel et al., 2011). A atividade proteolítica que ocorre nesses produtos e a grande quantidade de proteína promovem os precursores para a ação da descarboxilase das culturas *starter* e micro-organismos autóctones (Suzzi and Gardini, 2003). Em produtos cárneos fermentados, a tiramina é a mais encontrada e em maior concentração, entretanto, a cadaverina, putrescina, triptamina e feniletilamina também já foram detectadas (Galgano et al., 2009; Hernández-Jover et al., 1997; Suzzi and Gardini, 2003). Existem poucos estudos sobre aminas biogênicas em produtos cárneos brasileiros, na qual a maioria dos dados é sobre os teores de histamina em amostras de linguiça, presunto e salame (Caccioppoli et al., 2006; Leitão et al., 1983) e sobre os teores de aminas em carne e em produtos como, mortadela, linguiça frescal, almôndega, salsicha, hambúrguer e empanado (Silva and Glória, 2002).

A determinação destes compostos em alimentos não é simples e, em produtos cárneos as dificuldades são ainda maiores, pois são produtos que apresentam uma complexidade da matriz cárnea, como por exemplo, rica em proteínas, possui grande quantidade de gordura e a presença de vários ingredientes não cárneos de origem animal e/ou vegetal, assim como diferentes processos a que são submetidas (Silva and Glória, 2002). Os métodos moleculares (hibridização e PCR) são os mais utilizados na rotina de identificação compostos aminados (Björnsdóttir-Butler et al., 2010; Landete et al., 2007). A detecção de aminas biogênicas em alimentos é de extrema importância, pois estes compostos podem funcionar como indicadores químicos da presença de

contaminação microbiana indesejada, além de causarem riscos à saúde dos consumidores.

A histamina age como vasodilatador e neurotransmissor no sistema cardiovascular e no sistema nervoso central, sendo uma amina biogênica responsável por alguns episódios de envenenamento alimentar que se manifestam por reações alérgicas caracterizados por prurido, erupção, vômito, febre, dificuldade de respirar e hipertensão (Hernández-Jover et al., 1997; Naila et al., 2010). A triptamina causa aumento da pressão arterial, porém, não existe uma quantidade máxima de consumo dessa amina biogênica relacionada ao efeito tóxico em humanos. A putrescina, cadaverina, espermina e espermidina não foram associadas a efeitos tóxicos no organismo humano, entretanto são capazes de reagir com nitrito e formar as nitrosaminas carcinogênicas, atuando como um possível precursor mutagênico (Kalac, 2009; Kim et al., 2009; Shalaby, 1996).

Além da produção de aminas biogênicas e da presença de genes de resistência a antibióticos, outros fatores de virulência também são de extrema importância, como por exemplo: produção de proteínas de superfície, que são proteínas capazes de ancorar na parede celular e participam na formação de biofilmes (Hendrickx et al., 2009); produção de substâncias de agregação, que permitem o contato entre as células para conjugação e transmissão de plasmídeos de virulência (Hendrickx et al., 2009); produção de hialuronidase, que facilita a dispersão da bactéria e suas toxinas, causando degradação e danos aos tecidos do hospedeiro (Franz and Holzapfel, 2004); produção de gelatinase, responsável pela hidrólise de colágenos e podendo estar envolvida no começo e propagação de processos inflamatórios (Lopes et al., 2006); produção de citolisina, exotoxina de efeito hemolítico e bacteriocinogênica, possui capacidade de

invadir o sistema imune do hospedeiro e pode causar lise de eritrócitos (Franz and Holzapfel, 2004); dentre outros fatores.

6. Ensaios *in situ* de bacteriocinas

Listeria monocytogenes é um micro-organismo patogênico que já foi isolado de vários tipos de alimentos. Essa bactéria é onipresente no meio ambiente e a Commission on Microbiological Specifications for Foods (1996) considera que quando o nível de contaminação por *L. monocytogenes* for inferior a 100 UFC/g, o consumo é aceitável sem risco. Assim, o controle adequado da multiplicação de *L. monocytogenes* pode minimizar o risco do aumento da listeriose (Jay, 1996). Em produtos cárneos, *L. monocytogenes* pode representar grave perigo para a saúde devido à sobrevivência e proliferação ativa em temperaturas de refrigeração. Além disso, esse micro-organismo possui a capacidade de se multiplicar em alimentos embalados a vácuo e é relativamente tolerante ao sal e baixo pH, por isso é difícil controlar sua multiplicação em alimentos. Obstáculos adicionais devem ser utilizados em combinação com o armazenamento ao frio, a fim de aumentar a segurança de produtos cárneos refrigerados (Farber and Peterkin, 1991). Sistemas de biopreservação de alimentos, tais como BAL bacteriocinogênicas e/ou suas bacteriocinas tem tido maior atenção e novas abordagens têm sido desenvolvidas para o controle de micro-organismos patogênicos e deteriorantes. Alguns estudos demonstraram o antagonismo de bacteriocinas contra *Listeria* spp. em carnes e produtos cárneos.

Liserre et al. (2002) isolaram uma cepa bacteriocinogênica de *L. sakei* de linguiça italiana frescal e avaliaram o feito combinado da embalagem com atmosfera modificada e adição de *L. sakei* em relação à inibição de *L. monocytogenes* em linguiça

armazenada a 6 ºC. Após uma semana, esses autores observaram que em produtos que possuem apenas a embalagem com atmosfera modificada a inibição de *L. monocytogenes* foi significativa, enquanto a linguiça que possuía apenas *L. sakei* não influenciou significativamente na contagem de *L. monocytogenes*. Após duas semanas, uma redução de 1,3 a 1,4 log UFC/g nas contagens de *L. monocytogenes* foi observada em amostras contendo ou a embalagem com atmosfera modificada ou *L. sakei*, e houve uma redução de 3,5 log UFC/g naquelas amostras que foram submetidas a ambos os tratamentos, mostrando que a inibição de *L. monocytogenes* em linguiça frescal foi potencializada quando se utilizou a combinação de BAL bacteriocinogênica e embalagem com atmosfera modificada.

Martinis and Franco (1998) avaliaram a inibição de *L. monocytogenes* em um produto suíno com *L. sakei* bacteriocinogênica e observaram que, após 4 semanas a 8 ºC, as contagens de *L. monocytogenes* no produto suíno picado co-inoculado com essa cultura foram aproximadamente 6 log UFC/g menores do que em amostras controle contendo apenas *L. monocytogenes*. Estes resultados sugerem que a cepa de *L. sakei* produz uma bacteriocina que pode ser usada para inibir o desenvolvimento de *L. monocytogenes* em salsichas brasileiras.

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OBJETIVOS

Objetivo Geral

Isolar e caracterizar bactérias ácido láticas (BAL) presentes em embutidos cárneos fermentados artesanais através de técnicas fenotípicas e genotípicas, buscando a seleção de isolados com potencial bacteriocinogênico.

Objetivos Específicos

- ✓ Isolar BAL de embutidos cárneos fermentados artesanais
- ✓ Caracterizar o potencial bacteriocinogênico da coleção de BAL
- ✓ Identificar coleção de culturas bacteriocinogênicas através de técnicas de biologia molecular
- ✓ Caracterizar o potencial virulento, probiótico e resistência a antibióticos da coleção de culturas bacteriocinogênicas
- ✓ Purificar as bacteriocinas produzidas por cepas bacteriocinogênicas
- ✓ Sequenciar e identificar as bacteriocinas
- ✓ Avaliar as interações entre uma cultura lática bacteriocinogênica selecionada (*Lactobacillus curvatus* 12) e suas bacteriocinas com *Listeria monocytogenes* em um estudo *in situ* em linguiça frescal.

CAPÍTULO 1 - *Lactobacillus curvatus* strain and other lactic acid bacteria isolated from *calabresa*, a fermented meat product, present high bacteriocinogenic activity against *Listeria monocytogenes*

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Manuscript prepared and submitted to Frontiers in Microbiology (IF 4.076)

Title page

Lactobacillus curvatus strain and other lactic acid bacteria isolated from *calabresa*, a fermented meat product, present high bacteriocinogenic activity against *Listeria monocytogenes*

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Abstract

Lactic acid bacteria (LAB) isolates were obtained from four artisanal meat products and characterized by their bacteriocinogenic activity. After isolation, fingerprinting (rep-PCR and RAPD), detection of bacteriocin related genes, inhibitory spectrum and effect of chemical substances and temperature on inhibitory activity, three strains (all from *calabresa*) were selected for further studies, named as *Lactobacillus curvatus* 12, *L. curvatus* 36 and *Weissella viridescens* 23. The selected strains presented distinct growth and bacteriocin production was more evident and consistent for *L. curvatus* 12 and *W. viridescens* 23, reaching levels of 25,000 AU/mL after incubation at 25, 30 and 37 °C and 6, 9 and 12 h. The produced bacteriocins were adsorbed by the producer strains at different levels. *L. monocytogenes* 72 was inhibited by the produced bacteriocins, indicating their antimicrobial potential. Bacteriocins produced by the selected strains adsorbed to *L. monocytogenes* 72 at levels ranging from 87.5 to 100%. Partially purified bacteriocins of the selected strains kept their inhibitory activity after elution with isopropanol at 60% (v/v). Cell lysis patterns were similar for all tested strains, being increased after 24 h of interaction by these substances at concentrations presenting 50 to 1,600 AU/mL. The detection of β-galactosidase in cultures of the target strain *L. monocytogenes* 72 associated to the partially purified bacteriocins indicated destabilization of the cell membrane permeability. Bacteriocin produced by *L. curvatus* 12 was purified by HPLC and sequenced, resulting in four peptides with 3,102.79, 2,631.40, 1,967.06 and 2,588.31 Da, without homology of known bacteriocins. The selected isolates from *calabresa*, specially *L. curvatus* 12, were able to produce bacteriocins with high inhibitory activity against *L. monocytogenes*, indicating their potential application in the food industry as biopreservatives.

Keywords: *calabresa*, bacteriocin, lactic acid bacteria, *Listeria monocytogenes*, *Lactobacillus curvatus*

1. Introduction

Consumers interest in natural products, without chemicals and associated to health benefits, has been increasing in the last decades. Meat products fermented by lactic acid bacteria (LAB) can be one of these functional products, despite the possibility of carrying pathogenic agents (Leroy and De Vuyst, 2016; Zgomba Maksimovic et al., 2018). Fermented meat products provide favorable growth conditions not only for the beneficial LAB, but also to spoilage and pathogenic microorganisms, demanding alternative strategies to control the development of undesirable bacteria (Oliveira et al., 2018).

LAB can be considerate as a major component of fermented meats microbiota, playing an important role to develop specific flavor and texture, being associated to the preservative effect due to production of antimicrobial compounds, such as organic acids and bacteriocins (Toldrá, 2010; Oliveira et al., 2018). Fresh meat contains an autochthonous LAB microbiota that determines a mild fermentation process, without deep changing in the sensorial characteristics due to the strong buffer capacity of this food matrix; however, LAB are able to growth due to the adding of sugar in fermented meats, determining a deep decrease of pH and protein denaturation, resulting in specific sensorial characteristics and a microbial stabilization (Toldrá, 2010).

Bacteriocins produced by LAB are protein compounds that present a variable spectrum of antimicrobial activity, usually against closely related species to the producer strain (Cotter et al., 2005). Numerous bacteriocins produced by LAB species have been already described and they are well known by their activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium* sp., *Bacillus* sp. and other foodborne pathogens (Cotter et al., 2005). Also, some bacteriocins present

antimicrobial activity against some Gram negative bacteria, such as *Escherichia coli* and *Salmonella* Typhimurium (Gong et al., 2010), and even against *Campylobacter jejuni*, the major cause of gastroenteritis worldwide (Kaakoush et al., 2015).

Nowadays, several studies have been reporting on isolation of bacteriocinogenic LAB from different sources, in order to investigate their potential as natural biopreservatives in different products. Thus, the present study aimed the isolation of bacteriocinogenic strains of LAB in artisanal meats produced in Minas Gerais state, Brazil, and to characterize their produced bacteriocins towards their antimicrobial features and structure.

2. Material and Methods

2.1. Screening of bacteriocinogenic lactic acid bacteria

2.1.1. Isolation from meat products, antimicrobial activity and storage

Artisanal meat products (*lombo defumado*, *calabresa*, bacon and *chouriço*) were purchased at the Central Market of Belo Horizonte (Minas Gerais, Brazil) and portions of 25 g were aseptically transferred to 225 mL of sterile peptone water 0.1% (w/v) (SPW, Oxoid Ltd., Basingstoke, England), homogenized and ten-fold diluted with SPW. Aliquots of 100 µL from selected dilutions were surface plated in duplicates on de Man, Rogosa and Sharpe agar plates (MRS, Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA), overlaid with agar-agar 1% (w/v) (BD), and incubated at 37 °C for 48h. After incubation, colonies were enumerated and results were expressed as colony forming units per g (CFU/g).

Plates containing individual colonies were selected and overlaid with 5 mL of brain heart infusion (BHI, BD) supplemented with 0.75% (w/v) agar (BD) inoculated with *Listeria monocytogenes* 72, previously isolated from beef carcasses by Camargo et al. (2014), at approximately 10^6 CFU/mL. Plates were incubated at 37 °C for 24 h and colonies with clear and evident inhibition zones were transferred to MRS broth (BD) and incubated at 37 °C for 24 h (Todorov and Dicks, 2005a). The obtained isolates were subjected to Gram staining and tested for catalase production using hydrogen peroxide at 3% (v/v). Gram positive and catalase negative isolates were transferred to MRS broth (BD), incubated at 37 °C overnight, and the obtained cultures were stored at -20ºC with glycerol at 20% (v/v).

2.1.2. Bacteriocinogenic potential and identification

Aliquots of the isolates stock cultures (n = 94) were transferred to MRS broth (BD) and incubated at 37 °C for 24h. The obtained cultures were centrifuged (8,000 × *g*, 4 °C, 15 min) and the cell free supernatants (CFS) were adjusted to pH 6.0 with 1 M NaOH and heated at 80 °C for 10 min. The treated CFS were subjected to the spot-on-the lawn assay to identify the inhibitory activity of strains: aliquots of 10 µL of CFS were spotted on the surface of plates containing BHI agar (BD) or MRS agar (BD) previously inoculated with *L. monocytogenes* 72 or *Enterococcus faecalis* ATCC 19443, or *Lactobacillus sakei* ATCC 15521 (target strains at 10^6 CFU/mL), respectively. Plates were incubated at 37 °C for 24-48 h, and inhibition halos larger than 2 mm were indicative of bacteriocinogenic activity of the CFS producer isolate.

The isolates that presented bacteriocinogenic activity to at least one of the tested targets were selected (n = 17) and subjected to DNA extraction by using ZR

Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA). DNA concentrations were determined by using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), and used for fingerprinting by rep-PCR using primer GTG_5, according to Dal Bello et al. (2010), and RAPD-PCR using primers OPL01, OPL02, OPL04, OPL05, OPL14 and OPL20, according to Todorov et al. (2010). Based on rep-PCR and RAPD profiles, five strains were selected and their DNA were subjected to PCR to amplify a region of 16S rRNA (Felske et al., 1997), which were sequenced in the Center for Human Genome Studies (Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil). The obtained sequences were subjected to analysis by using the Basic Local Alignment Search Tool (BLAST, GenBank, National Center for Biotechnology Information, Bethesda, MD, USA) for identification.

2.2. Bacteriocinogenic activity

2.2.1. Bacteriocin related genes

DNA from the selected strains were obtained as described above and subjected to PCR reactions to detect genes related to the production of the following bacteriocins: enterocins A, P, B and L50B, pediocin PA-1, nisin, plantaricins W, NC8 and S, and sakacins GA-1, GA-2, X, A, Q, P, Tα and Tβ. The amplification PCR reactions were adopted based on the described by Du Toit et al. (2000), Todorov et al. (2016), Kruger et al. (2013), Holo et al. (2001), Maldonado et al. (2003), Stephens et al. (1998), Todorov et al. (2011), Macwana and Muriana (2012) and Dortu et al. (2008). Primer sequences and PCR conditions are described in the Supplementary Table.

2.2.2. Inhibitory spectrum

CFS of the selected strains was obtained and treated as described above, and tested for antimicrobial activity against a panel of 64 strains, composed by Gram positive (*Listeria* spp. = 27, *Enterococcus* spp. = 9, *Staphylococcus aureus* = 2, *Lactococcus lactis*: 1, *Lactobacillus* spp. = 13, *Pediococcus* spp. = 2, *Weissella paramesenteroides* = 2, *Corynebacterium vitaeruminis* = 1) and Gram negative (*Pseudomonas* spp. = 2, *Escherichia coli* = 2, *Salmonella* spp. = 3) strains obtained from reference culture collections and previously conducted studies. The spot-on-the-lawn method, as described above, was used for this characterization.

2.2.3. Effect of enzymes, pH, temperature and chemicals on stability of bacteriocins

CFS of selected strains were obtained and treated as described above. Sensibility to enzymes was assessed by adding enzymes (at 0.1 mg/mL: trypsin, proteinase K, papain, pepsin; at 1.0 mg/mL: protease, α-amylase, lipase, catalase, Sigma-Aldrich, St. Louis, MI, USA) to 1 mL aliquots of the CFS, followed by incubation at 30 °C for 30 min, heating at 90 °C for 5 min, and cooling, as described by van Reenen et al. (1998). pH was assessed by adding 1M HCl or 1M NaOH to CFS in order to reach pH values of 3.0, 5.0, 7.0, 8.0 and 10.0, and incubated at 30 °C for 1 h. Temperature was assessed by incubating CFS aliquots at 7, 25, 37, 40, 60 and 80 °C for 30 min. Also, obtained CFSs were supplemented with NaCl, EDTA and Tween 80 (all at end concentrations of 10 mg/mL, Sigma-Aldrich) and incubated at 30 °C for 30 min.

CFS subjected to treatments with enzymes and pH were tested for inhibitory activity against *L. monocytogenes* 72 by the spot-on-the lawn assay, as described above; CFS aliquots were also tested as control, without any of the described treatments. In addition, CFS subjected to treatments of temperature and chemicals were subjected to a quantitative assay to verify the inhibitory activity of the produced bacteriocins (van Reenen et al., 1998), being subjected to two-fold serial dilution with phosphate buffer (100 mM, pH 6.5), spotted (10 µL) on the surface of plates containing BHI agar (BD) inoculated with *L. monocytogenes* 72, and 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. Bacteriocin production

2.3.1. Effect of temperature, pH and MRS composition on bacteriocin production and growth by the bacteriocinogenic strains

Studied strains were inoculated in MRS broth (BD), and incubated at 25, 30 and 37 °C for 24 h. In each 1 h, aliquots of cultures were obtained and subjected to spectrophotometry at 600 nm (UV-M51, Bell Photonics do Brasil, São Paulo, SP, Brazil) and pH measuring (W3B, Bell). In each 3 h, CFS of the cultures were obtained and treated as described above, and subjected to a quantitative assay to verify the inhibitory activity against *L. monocytogenes* 72 of the produced bacteriocins, as described above and based on van Reenen et al. (1998)

Also, the selected strains were transferred to 10 mL of MRS broth (BD), incubated at 37 °C for 24 h, and their cells were obtained by centrifugation (5,000 × *g* for 10 min) and washed two times with sterile 0.85% NaCl (w/v). Then, cells were suspended with 10 mL of 0.85% NaCl (w/v), and aliquots de 100 µL were used for inoculation in MRS broth with modified characteristics, as described by Furtado et al. (2014), based on pH (MRS broth [BD] adjusted to 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0, by using 1M NaOH or 1M HCl), carbohydrate source (lactose, sacarose, dextrose, D-manitol, frutose, maltose or raffinose, all at 20.0 g/L [w/v] and from Sigma-Aldrich, instead of glucose), organic nitrogen source (tryptone at 20 mg/mL, meat extract at 20 mg/mL, yeast extract at 20 mg/mL, tryptone at 12.5 mg/mL and meat extract at 7.5 mg/mL, tryptone at 12.5 mg/mL and yeast extract at 7.5 mg/mL, meat extract at 10 mg/mL and yeast extract at 10 mg/mL, and tryptone at 10 mg/mL and meat extract at 5 mg/mL and yeast extract at 5 mg/mL, all from Sigma-Aldrich), and other chemicals (K_2HPO_4 at 0, 5.0 and 10.0 mg/mL; $MgSO_4$ at 0, 0.1 and 0.5 mg/mL; $MnSO_4$ at 0, 0.05 and 0.2 mg/mL; sodium acetate at 0, 5.0 and 10.0 mg/mL; tri-ammonium citrate at 0, 2.0 and 5.0 mg/mL; Tween 80 at 0, 1.0, 2.0 and 5.0 mg/mL; glycerol at 0, 0.5, 1.0, 2.0, 5.0 and 10.0 mg/mL). Cultures were incubated at 37 °C for 24 h, when the bacteriocin activity was determined against *L. monocytogenes* 72 by using a quantitative assay, as described above.

2.3.2. Adsorption of produced bacteriocins to the producer strains

Adsorption of produced bacteriocins to the cell surface of the bacteriocinogenic strains was assessed according Yang et al. (1992), with some modifications. Bacteriocinogenic strains were cultured in 10 mL of MRS broth (BD) at 37 °C for 24

h, when the pH was adjusted to 6.0 with 1 M NaOH. Then, cells were obtained by centrifugation (10,000 × *g*, 15 min, 4 °C) and washed twice with phosphate buffer (100 mM, pH 6.5); CFS was obtained and treated as described above. The washed cells were re-suspended in 10 mL NaCl 0.85% (w/v) (pH 2.0), stirred for 1 h at 4 ºC, centrifuged (12,000 × *g*, 15 min, 4 ºC); CFS was obtained and treated as described. CFS obtained before and after acid treatment were subjected to a quantitative assay to measure the antimicrobial activity, as described above, as compared to estimate the amount of bacteriocins attached to the bacteriocinogenic strains.

2.3.3. Inhibitory effects of produced bacteriocins on L. monocytogenes 72

L. monocytogenes 72 was selected as target strains to evaluate the inhibitory effect of the CFS produced by the selected LAB strains. *L. monocytogenes* 72 was cultured in BHI (BD) for 18 h at 37 °C, when the cells were obtained after centrifugation (5,000 × *g* for 5 min at 4 °C), washed twice with NaCl 0.85% (w/v) and resuspended in 10 mL of NaCl 0.85% (w/v). Equal volumes of *L. monocytogenes* 72 suspensions and CFS of the bacteriocinogenic strains (obtained as described above) were mixed and incubated at 37 °C for 1 h. Then, *L. monocytogenes* 72 cultures were ten-fold diluted (NaCl 0.85%, w/v) and pour plated in BHI agar (BD), followed by incubation at 37 °C for 24, when colonies were enumerated and results were expressed as CFU/mL. Target cell suspensions mixed with NaCl 0.85% (w/v) were considered as controls.

Also, the target strain was inoculated in BHI (BD) at approximately 10^6 CFU/mL and divided in four flasks each containing 100 mL. Cultures were incubated at 37 °C for 12 h; after the initial 3 h of incubation, 20 mL of the CFS of the bacteriocinogenic

strains (prepared as described above) were individually inoculated to the target cultures. Target culture without CFS inoculation was considered as growth control. In each hour, aliquots of the cultures were obtained and subjected to spectrophotometry at 600 nm (UV-M51, Bell) (Todorov and Dicks, 2006).

2.3.4. Adsorption of the produced bacteriocins to L. monocytogenes 72

Adsorption of bacteriocins to *L. monocytogenes* 72 was assessed as described by Yıldırım et al. (2002), with some modifications. CFS of bacteriocinogenic strains were obtained as described above, diluted 1:2 with NaCl 0.85 % (w/v) and their antimicrobial activity were measured by a quantitative assay as described above. *L. monocytogenes* 72 was cultured in BHI (BD) at 37 °C for 24 h, centrifuged (8,000 × *g*, 15 min., 4 °C) and washed twice with NaCl 0.85% (w/v). Then, washed cells were re-suspended in 500 µL of NaCl 0.85 % (w/v) and mixed with 500 µL of CFS, and subjected to variations of pH (4, 6, 8, 10, all at 37 °C for 1 h), temperatures (4, 25, 30 and 37 °C for 1 h) and chemicals (NaCl, Tween 80 and glycerol, all from Sigma-Aldrich, 1 %, w or v/v, at 37 °C for 1 h). After treatments, CFS were obtained, treated and tested for the antimicrobial activity by a quantitative assay, as described above. CFS obtained after incubation at 37 °C was considered as the control. The adsorption percentages were calculated based on the equation proposed by Furtado et al. (2014): Adsorption (%) = 100 - [(AU/mL after treatment / AU/mL control) × 100].

2.4. Inhibitory activity of partially purified bacteriocins

2.4.1. Partial purification of bacteriocins

Bacteriocinogenic strains were cultured in 1,000 mL of MRS (BD), at 37 °C for 24 h. CFS was obtained as described above. Proteins were precipitated by adding ammonium sulfate to 300 mL of the CFS, in order to obtain 40, 60 and 80% of saturation. The mixtures were stirred for 4h in orbital shaker at 4ºC, centrifuged (1 h, 20,000 × *g*, 4ºC), and the obtained pellet was re-suspended in 30 mL of phosphate buffer (PB, 100 mM, pH 6.5). Partial separation of proteins was performed by SepPakC_{18} hydrophobic column (Merck Millipore, Burlington, MA, USA), with a gradient of different concentrations of isopropanol (20, 40, 60, 80%) in PB (100 mM, pH 6.5). All obtained fractions were tested for bacteriocin activity by the spot-on-the-lawn method against *L. monocytogenes* 72, as described above. Fractions that presented bacteriocinogenic activity were selected and freeze dried. Before use, the dried material was resuspended in ultrapure water (MilliQ water, Merck Millipore).

2.4.2. Cell lysis of L. monocytogenes 72 by the partially purified bacteriocins

Cell lysis of target microorganisms was assessed based on the protocol proposed by Todorov et al. (2010). A cultures of *L. monocytogenes* 72 was obtained as described above and the cells were obtained by centrifugation (10,000 × *g*, 4ºC, 10 min), washed and resuspended with 5 mL of PB (100 mM, pH 6.5), and aliquots of 100 µL were distributed in wells of sterile flat-bottom 96-well microtiter plates (TPP, Trasadingen, Switzerland). Partially-purified bacteriocins (60% isopropanol) were sterile filtered

(0.22 µm, Merck Millipore) and subjected a quantitative assay to determine their inhibitory activity against *L. monocytogenes* 72, as described above. Then, partially-purified bacteriocins were subjected to serial two-fold dilution in PB (100 mM, pH 6.5), and aliquots of 50 µL of different dilutions were added to wells with the *L. monocytogenes* 72 cells suspensions. Wells with only cell suspensions and only diluted partially-purified bacteriocins were considered as controls. Plates were incubated at 37ºC for 24 h and subjected to spectrophotometry at 600 nm at times 0, 6, 9, 12 and 24 h (Biolisa Reader, Bioclin, Belo Horizonte, MG, Brazil). Cell lysis percentages values were calculated based on the equation proposed by Todorov et al. (2010): Cell lysis = [100 - ($OD_t/OD_0 \times 100$)], where OD_0 was the absorbance measured at time 0 and OD_t the absorbance measured at different times of incubation. Experiments were performed in duplicates.

2.4.3. Cell lysis of L. monocytogenes 72 by measuring the extracellular levels of β-galactosidase by the partially purified bacteriocins

Cell lysis of the target microorganisms were assessed by an alternative method, by measuring the levels of β-galactosidase, as proposed by Todorov et al. (2010). A culture of *L. monocytogenes* 72 and the partially purified bacteriocins (isopropanol 60%) were obtained and treated as described above. Aliquots of 2 mL of the cell suspensions were added to aliquots of 2 mL of the partially purified bacteriocins, incubated at 25 °C for 5 min, and added with 0.2 mL of 0.1M O-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma-Aldrich) diluted in 0.3M PB (100 mM, pH 6.5). The systems were incubated at 37 °C for 10 min and enzymatic reaction stopped by adding 2.0 mL of 0.1 M sodium carbonate. Cultures were then centrifuged (8,000 × *g*, 25 °C,

15 min) and the obtained supernatants were subjected to spectrophotometry at 420 nm (UV-M51, Bell). The assays were performed in duplicate in 2 independent assays.

2.5. Purification and sequencing of bacteriocins produced by L. curvatus 12

L. curvatus 12 was inoculated in MRS broth (BD) and incubated at 37 ºC for 24 h. Cells were removed by centrifugation (8,000 × g, 4 ºC, 15 min) and the CFS was obtained as described above. The CFS was precipitated by adding ammonium sulfate 40%, incubated at 4 ºC for 2 h, centrifuged (8,000 × g, 4 ºC, 20 min) and the obtained pellet was re-suspended in MiliQ water (Merck Millipore). The obtained extract was tested for its inhibitory activity against *L. monocytogenes* 72 using the spot-on-the-lawn assay, as described above.

The obtained extract was subjected to a HPLC using a C4 column equilibrated with solvent A (H_2O added to formic acid at 0.1%, v/v) and solvent B (acetonitrile added to formic acid at 0.1%, v/v). The system was washed with solvent A and B and the absorbed substances were eluted with a linear gradient from 5 to 80% solvent B. The fractions were collected and dried in SpeedVac (Thermo Fisher) for 1 h, and the pellet was re-suspended in MiliQ water (Merck Millipore) and tested for its inhibitory activity against *L. monocytogenes* 72, as described above.

Peptides with inhibitory activity against *L. monocytogenes* 72 purified by HPLC were dried and desalinated. Then, peptides were solubilized in 400 µL of 0.1% formic acid, packed in tubes suitable for application in the LC-MS/MS system. Aliquots of 1 µL of sample were analyzed by nano LC-MS using the nanoAcquity UPLC system (Waters, Milford, MA, USA), containing a nanoActity UPLC® 2G-V/MTrap 5 µm Symmetry® C18 180 µm x 20 mm trap column in a flow rate of 7 µL/min, for 3

minutes. The peptides were separated by a nanoAcquity UPLC® 1.7 7µm BEH130 100 µm x 100 mm column, operating at a flow rate of 0.3µL/min. The mobile phase of the chromatographic process was water acidified with solvents A and B. The chromatographic separation occurred according to the following schedule: 2% solvent B for 1 min; gradient from 2 to 30% solvent B for 59 min; gradient from 30 to 85% solvent B for 5 min; and maintenance at 2% solvent B for 10 min, totaling 85 min of chromatographic analysis.

The eluted peptides were automatically injected into a mass spectrometer model MAXIS 3G (Bruker Daltonics, Billerica, MA, USA), acting in online mode with a CaptiveSpray ionization source. Peptide analysis was performed using an appropriate method (IE_GCF_01-02-2017), with the drying gas flow rate of 3 L/min, temperature of ionization source of 150 ºC and the transmission voltage of 2kV. The raw data were converted to a mass list in the mzXML extension using CompassXport software version 3.0.13 (Bruker Daltonics, Billerica, MA, USA).

The mass list was compared against the *Lactobacillus* protein bank, deposited in the Uniprot Consortium using the PEAKS application version 7.0 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) (Ma et al., 2003). The parameters used for the research were: the analyzed peptides not originating from enzymatic cleavage; error tolerance for the 20 ppm parental ion and for the 0.6 Da fragments; carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification; for the identification to be accepted, the result should contain at least one unique peptide and the false discovery rate (FDR) less than one.

3. Results

3.1. Screening for bacteriocinogenic lactic acid bacteria

The LAB counts in the examined samples were 5.3×10^6 CFU/g for *chorição*, 6.4 $\times 10^6$ CFU/g for bacon and *calabresa* and 5.6 x 10^7 CFU/g for *lombo defumado*. A total of 94 LAB isolates was obtained from the tested samples and selected due to the presence of inhibition halo against *L. monocytogenes* 72; after confirmatory assays, 17 of them presented bacteriocinogenic activity against at least one of the tested microorganisms; *L. monocytogenes* 72 was the target strain that presented higher frequency of inhibition by the tested LAB isolates. These isolates were obtained from *calabresa*, being confirmed as LAB (Gram positive, catalase negative).

rep-PCR and RAPD allowed the determination of five genetic profiles, leading to the selection of five representative LAB strains. Based on BLAST analysis, the sequencing of 16S rRNA allowed the identification of *Lactococcus garvieae* (one strain, named as *L. garvieae* 32, with 100% of identity with other *L. garvieae* strains in GenBank), *Lactobacillus curvatus* (two strains, named as *L. curvatus* 12 and *L. curvatus* 36, with 99% and 75%, respectively, of identity with other *L. curvatus* strains in GenBank) and *Weissella viridescens* (two strains, named as *W. viridescens* 23 and *W. viridescens* 31, both with 99% of identity with other *W. viridescens* strains in GenBank).

3.2. Bacteriocinogenic activity

Results for amplification of bacteriocin related genes in the tested LAB strains are presented in Table 1. *L. curvatus* strains presented similar genetic profiles regarding bacteriocin related genes: both presented amplified products for *sakTA* and *sakTB*, and *L. curvatus* 36 also presented positive results for *plaW* and *plaS*. *W. viridescens* strains also presented similar genetic profiles, with positive results for *ped*, *pla*W, and *sakTA* and *sakTB*; *plaS* was also recorded in *W. viridescens* 31. *L. garvieae* 32 presented positive results only for *entP* and *sakTA*.

The inhibitory spectrum of the selected bacteriocinogenic LAB is presented in Table 2. Three of the tested strains (*L. curvatus* 12, *L. curvatus* 36 and *W. viridescens* 23) presented a high potential in inhibiting the Gram positive targets usually associated with human infections and usual focus of bacteriocin studies, like *L. monocytogenes*, *Enterococcus* spp. and *Staphylococcus*; low frequencies of inhibition were observed for LAB (except *Enterococcus* spp.), and no inhibitory activity was recorded against the tested Gram negative bacteria. *L. garvieae* 32 and *W. viridescens* 31 presented inhibitory activity against only one *L. monocytogenes* strain (Table 2). Considering the results observed until this step, *L. curvatus* 12, *L. curvatus* 36 and *W. viridescens* 23 were selected for further analyses regarding their bacteriocinogenic activity and *L. monocytogenes* 72 was selected as the target organism.

Treatment of the CFS with different enzymes resulted in complete inactivation of the inhibitory activity (Table 3). None of the tested CFS lost their antimicrobial activity after treatment with α-amylase, lipase or catalase, confirming that the studied antimicrobial peptides have not carbohydrate or lipid moiety in their structures, neither antimicrobial activity is result of production of H_2O_2 (Table 3). pH, temperature and

chemicals promoted different patterns of interference on the inhibitory activity of the CFS from the tested LAB strains (Table 3). Temperature did not affect substantially the inhibitory activity of the CFS of the studied strains: only for *L. curvatus* 36 was observed a decrease on the inhibitory activity after treatments at 40, 60 and 80 °C (Table 3). Regarding tested chemicals, CFS of *L. curvatus* 36 lost its inhibitory activity after treatment with NaCl and Tween 80, while the CFS of the other strains kept their inhibitory activity at different levels (Table 3).

3.3. Bacteriocin production and mode of action

Growth and bacteriocin production by the bacteriocinogenic strains cultured in MRS at 25, 30 and 37 °C are presented in Figure 1. All strains presented similar growth pattern, independently of the incubation temperature. Also, all strains presented a similar pattern of acidification, monitored by changes of pH in the tested temperatures, ranging from 6.0 to 4.0 along the incubation (data not shown). Independently of the level of bacteriocin production, inhibitory activity was stable in all tested conditions and no decrease of it was recorded in the end of stationary phase (Figure 1).

Table 4 shows the interference of variations on MRS composition on the inhibitory activity of the tested bacteriocinogenic strains. Based on the observed data, *L. curvatus* 12 was the strains that presented less influence of the tested variation on its inhibitory activity, being decreased only at some pH variations (2, 8 and 10), some carbohydrates sources (lactose, sacarose, mannitol and raffinose) and absence of $MnSO_4$ and Tween 80. A similar behavior was observed for *W. viridescens* 23, in which the inhibitory activity was totally inhibited when the strain was cultured at pH 10 and 12, and in absence of $MnSO_4$. *L. curvatus* 36 was highly affected by the MRS

composition: only the variations on MgSO4 did not result in a loss or decrease of the inhibitory activity.

The bacteriocins produced by the selected LAB strains were able to adsorb to their cell surfaces: CFS of *L. curvatus* 12 presented inhibitory activity before adsorption of 25,600 AU/mL, and after adsorption decreased to 3,200 AU/mL; the inhibitory activity of *L. curvatus* 36 CFS ranged from 6,400 AU/mL (before adsorption) to 200 AU/mL; CFS produced by *W. viridescens* 23 presented 25,600 AU/mL of inhibitory activity before adsorption, and 3,200 AU/mL after adsorption.

The inhibitory effects of the produced bacteriocins on *L. monocytogenes* 72 were assessed in two steps: first, in target cells at stationary phases, and second, in the beginning of their log growth phase (after 3 h). Considering the first approach, CFS of *L. curvatus* 12 determined complete inhibition of *L. monocytogenes* 72. CFS from *L. curvatus* 36 and *W. viridescens* 23 were able to reduce *L. monocytogenes* 72 populations to approximately 10^2 CFU/mL. Based on the second approach, Figure 2 shows the effects of the CFS when added to the target cultures of *L. monocytogenes* 72 after 3 h of incubation. CFS produced by the bacteriocinogenic strains were able to reduce substantially the growth of the target strains, specially *L. monocytogenes* 72 and the CFS produced by *L. curvatus* 12 presented a better performance in controlling both target strains when compared to the CFS produced by the other bacteriocinogenic strains.

Adsorption levels of bacteriocins produced by the studied strains to *L. monocytogenes* 72 are presented in Table 5. The target strain was able to adsorb the produced bacteriocins at high percentages, even after treatments of the CFS at different temperatures, chemicals and pH values.

3.4. Inhibitory activity of partially purified bacteriocins

After the partial purification procedures from the CFS produced by the bacteriocinogenics strains, inhibitory activity was observed after elution with 60% isopropanol. However, inhibitory activity was also observed with 40 % and 80% isopropanol, at lower levels when compared to 60% isopropanol (data not shown).

Lysis percentages of *L. monocytogenes* 72 cells after association with the partially purified bacteriocins from the LAB strains are presented in Table 6. The pattern of cell lysis was similar for all tested partially purified bacteriocins, being increased after 24 h of interaction by these substances at concentrations presenting 50 to 1,600 AU/mL.

3.5. Purification and sequencing of bacteriocins produced by L. curvatus 12

After precipitation with ammonium sulfate, the purified proteins present inhibitory activity against *L. monocytogenes* 72. The extract submitted to a HPLC using a C4 column also presented inhibitory activity against *L. monocytogenes* 72 and resulted in one isolated peak (Figure 3). The sequencing results indicated four different peptides: GFAIPSNEVVKIINQLVANGKVVRPALGIS (3,102.79 Da), TLGPASNNVETIAKLIEAGANVFRF (2,631.40 Da), IMNAIAYADAIYRLTR (1,967.06 Da) and KSYTPQEVSAMILQYIKKFAED (2,588.31 Da).

4. Discussion

4.1. Screening for bacteriocinogenic lactic acid bacteria

LAB counts in analyzed samples were in coherence with Prado et al. (2000), who reported that these counts in *linguiça* ranged from 10^7 to 10^8 CFU/g. Despite the current demand for artisanal products from animal origin by consumers, they can offer some microbiological hazards due to natural presence of pathogens, demanding food good sanitary practices during production, ripening and storage. Several LAB isolated from meat products presented ability to produce inhibitory substances against different microorganisms, mainly *L. monocytogenes* and other foodborne pathogens and spoilage bacteria (Todorov et al., 2010).

LAB constitute a relevant part of the initial microbiota in meats and their populations easily grow during cold storage, even when these products are packed under modified atmosphere or vacuum, and after processing of fermented products (Toldrá, 2010). The autochthonous LAB usually found in meats are *Carnobacterium piscicola*, *C. divergens*, *L. sakei*, *L. curvatus*, *L. plantarum*, *Leuconostoc mesenteroides*, *L. gelidum* and *L. carnosum* (Toldrá, 2010); *W. halotolerans*, *W. hellenica* and *W. viridescens* have been commonly associated with meat or meat products (Collins et al., 1993). As observed in the present study, Barbosa et al. (2015) isolated *L. curvatus* with anti-*Listeria* activity from Italian type salami produced in Brazil, and Dortu et al. (2008) isolated bacteriocinogenic strains of *L. sakei* from raw poultry and *L. curvatus* from raw beef. Chen et al. (2014) isolated a strain of *W. hellenica* from a Chinese artisanal fermented meat and characterized its bacteriocin as possessing a wide antimicrobial activity range.

4.2. Bacteriocinogenic activity

The variable pattern of results for bacteriocin related genes observed in the tested LAB isolates (Table 1) was already observed for other bacteriocingenic strains. Macwana and Muriana (2012) described that both sakacin Tα and Tβ are part of a two-bacteriocin complex and the presence of both peptides is required for the activity of a mature peptide system, indicating that *Lactobacillus* and *Weissella* strains would be able to produce this bacteriocin. Also, Holo et al. (2001) observed that plantaricin W, produced by *L. plantarum* LMG2379, is a bacteriocin composed by two-antimicrobial peptides that present synergistic effect. Barbosa et al. (2016) observed that *L. plantarum* MBS4 presented a fragment in its DNA homologous to *plaW*, indicating its ability to produce this bacteriocin. Stephens et al. (1998) also observed that plantaricin S produced by *L. plantarum* LPCO10 is a two peptide bacteriocin. Todorov et al. (2017) reported that some LAB isolated from Lukanka, a fermented meat product, presented bacteriocinogenic activity and were positive for *sakTA* and *sakTB*.

Cintas et al. (1998) observed that LAB strains that present several enterocin related genes do not necessarily express all these bacteriocins simultaneously; the efficiency of horizontal gene transfer mechanisms can explain the variety of genes and the production of multiple bacteriocins by the same culture, as well as high frequency of *Enterococcus* spp. with several genes of these bacteriocins (Franz et al., 2007). *Enterococcus* strains have mechanisms of conjugative genetic exchange (conjugative transposon and plasmids, with high frequency transfer) and not conjugative, as by plasmids, explaining the variable presence of enterocins related genes in wild strains (Franz et al., 2007).

Expression of pediocin PA-1 is associated to the presence of *pedA*, *pedB*, *pedC* and *pedD* in the bacteriocinogenic strain, most probably located in a plasmid (Todorov and Dicks, 2009). The obtained results for *ped* operon related genes indicate the presence of the full machinery for pediocin PA-1 production in the *Weissella* strains (Table 1), suggesting their ability to produce this bacteriocin.

The tested LAB isolates presented a wide inhibitory spectrum (Table 2). Assessing the inhibitory spectrum of bacteriocinogenic strains is required to evaluate the potential application of the producer strains as biopreservatives and probiotics, due to the natural biodiversity of target strains in food and gastrointestinal tract (Cotter et al., 2005). As observed in the present study, Barbosa et al. (2015) also demonstrated the inhibitory activity of two bacteriocins against 23 strains of *L. monocytogenes*, and no inhibition against the tested Gram negative bacteria. The low frequencies of LAB inhibition (Table 2) demonstrate the potential usage of the bacteriocinogenic strains as biopreservatives in fermented foods.

Confirmation of proteinaceous nature of bacteriocins is essential step in characterization of new antimicrobial peptides. CFS of selected LAB isolates added to enzymes resulted in no inhibition, confirming their proteinaceous natures (Table 3). Barbosa et al. (2014) also observed the inactivation of the inhibitory activity of the bacteriocin produced by *L. sakei* isolated from Brazilian salami after treatment with enzymes, an essential characteristic to determine the chemical nature of the inhibitory substance and to classify them as bacteriocins, antimicrobial peptides. α-amylase, lipase or catalase did not influence the inhibitory activity of LAB isolates (Table 3); testing such substances are important to identify possible components of bacteriocins structure, as well as to identify inhibitory activity due to the production of other antimicrobial substances produced by the producer strains. Todorov (2010) reported

that bacteriocin ST63BZ lost its inhibitory activity after treatment with α-amylase, as observed by Keppler et al. (1994) for leuconocin S, indicating that their activity was associated with glycosylation of the active peptide.

Inhibitory activity of LAB isolates were variable after pH, temperature and chemicals treatment (Table 3). pH played an important role on the inhibitory activity of the studied bacteriocins: *L. curvatus* 12 CFS kept its antimicrobial activity at pH values higher than 7.0, *L. curvatus* 36 CFS presented inhibitory activity only after pH 7.0, and *W. viridescens* 23 CFS kept its inhibitory activity after all tested pH values. Some studies reported a higher inhibitory activity of bacteriocins when subjected to acids when compared to alkaline: nisin is known by its sensitivity to alkaline pH values (Yang et al., 1992) and leucocin F10 kept its inhibitory activity after acid treatments, but it was sensitive to pH 7.0 and 9.0 (Parente et al., 1996). Regarding temperature, once bacteriocins are small peptides, they are usually thermostable, as previously described (Cotter et al., 2005; Favaro et al., 2015) and observed in the present study (Table 3). However, lactocin NK24 lost around 90% of its inhibitory activity after treatment at 121 °C for 15 min (Lee and Paik, 2001). Also, resistance to different chemicals is important to lead the technological application of bacteriocins, as well as to guide the adoption of different laboratorial procedures for further characterization of them. Von Mollendorff et al. (2006) demonstrated different levels of interference by urea, Tween 20, Tween 80 and EDTA on the inhibitory activity of four bacteriocins produced by LAB isolated from *boza*, as well as Todorov and Dicks (2005b) for pediocin ST18 and Gálvez et al. (1998) for enterocin EJ97.

4.3. Bacteriocin production and mode of action

When compared to other tested LAB isolates, *L. curvatus* 12 reached higher levels of growth (Figure 1). Also, this isolate and *W. viridescens* 23 presented high levels of production of bacteriocin after 6 or 9 h of incubation, independently of the temperature; *L. curvatus* 36 presented higher production of bacteriocin when cultured at 25 °C, compared to other incubation temperatures, and only after 21 h of incubation (Figure 1). Similar profile of bacteriocin production was already reported for several antimicrobial peptides expressed by different strains of *Lactobacillus* spp. (Todorov et al., 2011; Barbosa et al., 2014; Barbosa et al., 2015; Barbosa et al., 2016).

Based on variations on MRS composition, tested LAB isolates presented a variable pattern of inhibitory activity (Table 4). pH plays an important role in bacteriocin stability (Table 3) and production (Table 4) and many studies describe the relevance of its initial value in the culture media considered for bacteriocin production by bacteriocinogenic strains (Daeschel et al., 1990; Kelly et al., 1996; Todorov et al., 2010). *L. curvatus* 12 was able to produce bacteriocins at low pH (Table 4), despite the produced substances being susceptible to these conditions (Table 3); *L. curvatus* 36 produced bacteriocins at high levels only in neutral pH (Table 4), which corresponds to pH for its bacteriocins stability (Table 3); despite *W. viridescens* 23 CFS were stable at all tested pH (Table 3), the production of bacteriocins occurred only and acid and neutral pH values (Table 4).

Production of bacteriocins can be related to the metabolism of growth medium and it did not need to occur in coherence with the microbial growth. Todorov et al. (2010) and Kelly et al. (1996) demonstrated a different patterns of production of some bacteriocins associated to variations of different carbohydrate and organic nitrogen

sources. Plantaricin 423 production was enhanced by the presence of peptone in growth broth of the bacteriocinogenic strain (Verellen et al., 1998), as well observed for pediocin PA-1/AcH in culture media with meat extract (Bhunia et al., 1988).

Little is known about the influence of potassium ions on the bacteriocins production. Todorov et al. (2010) reported that high concentrations of K_2HPO_4 decreased the production of bacteriocins, and plantaricin UG1 production was enhanced by the presence of this substance at 7.0 g/L (Enan et al., 1996). Tri-ammonium citrate was described as enhancer of bacteriocin ST8KF production (Powell et al., 2007), while the absence of $MgSO_4$ and $MnSO_4$ determined a decrease on bacteriocin ST8KF production (Powell et al., 2007). Tween 80 was also described as responsible to enhance the bacteriocin production at specific concentrations (Todorov et al., 2010).

Different patterns of bacteriocins absorbance to the cells of the bacteriocinogenic LAB isolate were recorded, as already described for some bacteriocins (Todorov and Dicks, 2005b; Albano et al., 2007), being a relevant feature to be assessed in order to choose adequate protocols to optimize their purification from growth cultures (Cintas et al., 2001).

L. monocytogenes 72 was totally inhibited by the CFS from *L. curvatus* 12, while CFS from *L. curvatus* 36 and *W. viridescens* 23 reduced its populations at 10^2 CFU/mL. Considering a similar approach, bacteriocin HA-6111-2 produced by *Pediococcus acidilactici* was able to determine a complete inhibition of *E. faecium* HKLHS populations (Albano et al., 2007). Similarly, when the CFS from *L. curvatus* 12 was added to the log growth of *L. monocytogenes* 72, the recorded inhibition was higher when compared to the effects of the CFS from the other tested LAB isolates (Figure 1). Furtado et al. (2014) observed that adding the bacteriocin DF04Mi at 3,200

AU/mL to a 3 h-old culture of *L. monocytogenes* resulted in growth inhibition for at least 12 h. Other bacteriocins, such as the ones produced by *E. faecium* ST5Ha and *P. acidilactici* HA- 6111 - 2, presented a similar behavior (Albano et al., 2007; Todorov, 2010).

The results for adsorption of bacteriocins to *L. monocytogenes* 72, indicate high interaction in distinct conditions (Table 5), as already observed by Cintas et al. (2001). Barbosa et al. (2016) identified that bacteriocin MBSa4 presented a strong interaction with the *L. monocytogenes* (100%), differently from pediocin 31-1 (Liu et al., 2008) and AMA-K (Todorov, 2008), that presented adsorption to target cells of 50 % and 75%, respectively. Adsorption of the bacteriocin to the cell surface of the target strains is an important first step in the characterization of their inhibitory effect. Modulation of the levels of adsorption by different environmental conditions (pH, temperature, presence of chemicals) is an important tool that can predict the inhibitory activity in specific conditions when applied in some fermented food products.

4.4. Inhibitory activity of partially purified bacteriocins

After partial purification of bacteriocins from CFS of the tested LAB isolates, the fraction of 60% isopropanol presented higher inhibitory activity when compared to other fractions. Todorov and Dicks (2009) observed that after hydrophobic chromatography on SepPakC18, the level of the inhibitory activity of bacteriocin ST44AM was recorded in 60% isopropanol, and Todorov et al. (2004) reported that the active fraction of plantaricin ST31 was eluted with 40% isopropanol.

The results of cell lysis recorded for all partially purified bacteriocins (Table 6), indicate a potential application of the bacteriocins produced by *L. curvatus* 12, *L.*

curvatus 36 and *W. viridescens* 23 in controlling food contamination by *L. monocytogenes*, even at low concentrations (Table 6). Todorov and Dicks (2009) described a similar pattern of inhibition of *L. monocytogenes* by bacteriocins produced by *P. pentosaceus*. As an alternative to assess the cell lysis, the detection of β-galactosidase in cultures of the target strain *L. monocytogenes* 72 associated to the partially purified bacteriocins confirmed the previous results (Table 6), indicating destabilization of the cell membrane permeability. This approach was also considered in other bacteriocin studies to assess the lysis of target strains, and similar results were observed (Yıldırım et al., 2002; Todorov and Dicks, 2006).

4.5. Purification and sequencing of bacteriocins produced by L. curvatus 12

Considering the obtained results, the bacteriocins produced by *L. curvatus* 12 were purified and the peak with inhibitory activity (Figure 3) revealed four peptides. Zhu et al. (2014) used a four-step purification method, including XAD-2, cation-exchange chromatography, gel chromatography and HPLC and successfully obtained a pure peptide; these authors observed that the purified bacteriocin was resistant to N-terminal sequencing and the sequencing showed no homology with other known bacteriocins. Barbosa et al. (2015) purified two bacteriocins achieve by the three-step procedure, such as cation-exchange followed by sequential hydrophobic-interaction and reversed-phase chromatography, and they observed two peaks in the final chromatogram of each bacteriocin tested; these procedures resulted in successful purification of both bacteriocins.

5. Conclusions

Based on the obtained results, LAB isolates obtained from an artisanal meat product (*calabresa*) presented high inhibitory potential, and the strains *L. curvatus* 12, *L. curvatus* 36 and *W. viridescens* 23 were able to produce bacteriocins with potential application in the food industry as alternative substances to control foodborne pathogens, specially *L. monocytogenes*. Among these isolates, bacteriocins produced by *L. curvatus* 12 presented a better inhibitory performance and the purification procedures revealed four peptides with sequences not described for bacteriocins to date.

Author Contributions

NPAC, LLO, SDT, and LAN conceived and designed the experiments. NPAC, MC, and SDT collected data. NPAC, LLO, SDT, and LAN analyzed the data. All authors contributed to draft writing and revision. All authors read and approved the final manuscript.

Funding

This research was funded by CNPq, CAPES, and FAPEMIG.

Acknowledgments

We sincerely thank the Núcleo de Análise de Biomoléculas from UFV for the support on the peptides sequencing and analysis.

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Table 1. Results for bacteriocin related genes in five bacteriocinogenic strains of lactic acid bacteria isolated from *calabresa*, obtained by PCR (positive: +; negative: -).

Target genes	Bacteriocinogenic strain				
	L. garvieae 32	*L. curvatus* 12	*L. curvatus* 36	*W. viridescens* 23	*W. viridescens* 31
entA	-	-	-	-	-
entP	+	-	-	+	-
entB	-	-	-	-	-
entL50B	-	-	-	-	-
*ped*pro	-	-	-	+	+
nis	-	-	-	-	-
plaW	-	-	+	+	+
plaNC8	-	-	-	-	-
plaS	-	-	+	-	+
sakGA1	-	-	-	-	-
sakGA2	-	-	-	-	-
sakX	-	-	-	-	-
sakA	-	-	-	-	-
sakQ	-	-	-	-	-
sakP	-	-	-	-	-
sakTA	-	+	+	+	+
sakTB	+	+	+	+	+

Obs.: primers and PCR conditions are detailed in Supplementary Table.

Table 2. Frequencies of inhibitory activity of the cell free supernatant of bacteriocinogenic lactic acid bacteria isolated from *calabresa* against different targets, assessed by the spot-on-the-lawn methodology.

Genus	Species/Serotype	n	Producer strain				
			L. garvieae 32	*L. curvatus* 12	*L. curvatus* 36	*W. viridescens* 23	*W. viridescens* 31
Listeria	*L. monocytogenes*	27	1	24	24	24	1
Enterococcus	*E. faecium*	3	0	3	3	3	0
	E. faecalis	4	0	3	3	3	0
	E. durans	1	0	1	1	1	0
	E. hirae	1	0	1	1	1	0
Staphylococcus	*S. aureus*	2	0	1	1	1	0
Lactococcus	*L. lactis* subsp. *cremoris*	1	0	1	1	1	0
Lactobacillus	*L. sakei*	1	0	1	1	1	0
	L. casei	4	0	1	1	0	0
	L. acidophilus	1	0	0	0	0	0
	L. nagelli	1	0	0	0	0	0
	L. harbinensis	2	0	0	0	0	0
	L. fermentum	1	0	0	0	0	0
	L. plantarum	3	0	1	1	0	0
Pediococcus	*P. pentosaceus*	1	0	0	0	0	0
	P. acidilactici	1	0	0	0	0	0
Weissella	*W. paramesenteroides*	2	0	0	0	0	0
Corynebacterium	*C. vitaeruminis*	1	0	1	1	0	0
Pseudomonas	*P. aeruginosa*	1	0	0	0	0	0
	P. fluorescens	1	0	0	0	0	0
Escherichia	*E. coli*	2	0	0	0	0	0
Salmonella	*S.* Thyphimurium	2	0	0	0	0	0
	S. Typhi	1	0	0	0	0	0

Table 3. Effects of different substances and incubation conditions on the inhibitory activity of the cell free supernatant of bacteriocinogenic lactic acid bacteria isolated from *calabresa* against *L. monocytogenes* 72.

effect	substance/condition	concentration	producer strain		
			L. curvatus 12	*L. curvatus* 36	*W. viridescens* 23
Enzymes[1]	trypsin	0.1 mg/mL	AI	AI	AI
	proteinase K	0.1 mg/mL	AI	AI	AI
	papain	0.1 mg/mL	AI	AI	AI
	pepsin	0.1 mg/mL	AI	AI	AI
	protease	1 mg/mL	AI	AI	AI
	α-amilase	1 mg/mL	I	I	I
	lipase	1 mg/mL	I	I	I
	catalase	1 mg/mL	I	I	I
pH[1]	3		AI	AI	I
	5		AI	AI	I
	7		I	I	I
	8		I	AI	I
	10		I	AI	I
Temperature[2]	7 °C for 30 min		3,200	12,800	25,600
	25 °C for 30 min		3,200	25,600	25,600
	37 °C for 30 min		25,600	25,600	25,600
	40 °C for 30 min		25,600	12,800	25,600
	60 °C for 30 min		25,600	12,800	25,600
	80 °C for 30 min		25,600	6,400	25,600
Chemicals[2]	NaCl	10 mg/mL	3,200	0	200
	EDTA	10 mg/mL	200	800	800
	Tween 80	10 mg/mL	200	0	400

[1] effect of enzymes was assessed by the spot-on-the-lawn method. “AI” indicates absence of inhibition, “I” indicates inhibition; [2] effects of pH, temperature and chemicals were assessed by a quantitative assay, and results expressed as arbitrary units per mL

Table 4. Effects of variations on MRS broth on inhibitory activity of bacteriocinogenic lactic acid bacteria isolated from *calabresa* against *L. monocytogenes* 72, assessed by a quantitative assay.

Effect	MRS variant	Concentration (mg/mL)	Bacteriocinogenic strain		
			L. curvatus 12	*L. curvatus* 36	*W. viridescens* 23
Control	MRS	-	25,600	25,600	25,600
pH	2	-	200	0	1,600
	4	-	25,600	200	1,600
	6	-	25,600	25,600	25,600
	8	-	1,600	0	1,600
	10	-	12,800	0	0
	12	-	25,600	0	0
Carbo-hydrate	lactose	20	1,600	0	25,600
	sacarose	20	800	0	25,600
	manitol	20	3,200	0	800
	fructose	20	25,600	0	25,600
	dextrose	20	25,600	25,600	25,600
	maltose	20	25,600	0	25,600
	raffinose	20	12,800	100	25,600
Organic nitrogen	peptone	25	25,600	0	25,600
	meat extract	25	25,600	0	25,600
	yeast extract	25	25,600	100	25,600
	peptone + meat extract	12,5 + 12,5	25,600	100	12,800
	peptone + yeast extract	15 + 7,5	25,600	200	25,600
	meat extract + yeast extract	15 + 7,5	25,600	100	25,600
	peptone + meat extract + yeast extract	10 + 10 + 5	25,600	25,600	25,600
Chemicals	KH_2PO_4	0	25,600	100	12,800
		2	25,600	25,600	25,600
		5	25,600	100	25,600
		10	25,600	0	25,600
	$MgSO_4$	0	25,600	25,600	100
		0,1	25,600	25,600	25,600
		0,5	25,600	25,600	100
	$MnSO_4$	0	1,600	25,600	0
		0,05	25,600	25,600	25,600
		0,2	25,600	0	100
	Sodium acetate	0	25,600	0	25,600
		5	25,600	25,600	25,600
		10	25,600	100	25,600
	Tri-ammonium citrate	0	25,600	0	25,600
		2	25,600	25,600	25,600
		5	25,600	100	25,600
	Tween 80	0	12,800	0	12,800
		1	25,600	25,600	25,600
		2	25,600	100	25,600
		5	25,600	200	25,600

Table 5. Percentages of adsorption to *L. monocytogenes* 72 target strain by the bacteriocins presented in the cell-free-supernatant produced by bacteriocinogenic lactic acid bacteria isolated from *calabresa*.

Target strain	CFS	Control	CFS treatment										
		37 °C, pH 6.5	Temperature (°C)				NaCl	Tween 80	Glycerol	pH			
			4	25	30	37	1% (w/v)	1% (w/v)	1% (w/v)	4	6	8	10
L. monocytogenes 72	*L. curvatus* 12	96.87	99.6	99.2	99.2	99.6	87.5	93.8	87.5	96.9	99.2	93.8	96.9
	L. curvatus 36	99.21	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.61	100.0	99.61	100.0
	W. viridescens 23	96.87	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	98.4

Table 6. Lysis of *Listeria monocytogenes* 72 cells (%) after treatment with partially purified bacteriocins from *Lactobacillus curvatus* 12, *L. curvatus* 36 and *Weissella viridescens* 23, at difference concentrations (indicated by the inhibitory activity, AU/mL) and times of interaction (h).

bacteriocinogenic strain	time (h)	Inhibitory activity of the partially purified bacteriocin (AU/mL)*										
		25	50	100	200	400	800	1,600	3,200	6,400	12,800	25,600
L. curvatus 12	6	36.4	38.0	42.0	44.0	41.3	48.6	47.1	46.4	47.3	60.6	59.4
	9	52.7	52.2	60.8	61.0	57.5	55.0	56.2	45.9	51.0	56.6	54.8
	12	62.5	62.9	68.4	70.3	68.2	64.3	64.1	51.7	52.2	59.2	59.4
	24	78.0	79.9	85.2	88.2	87.8	84.8	84.8	78.1	79.2	79.6	77.1
L. curvatus 36	6	31.6	28.7	32.6	34.5	34.0	41.8	38.9	39.1	38.4	43.4	48.5
	9	54.3	50.7	57.3	60.3	60.7	54.7	52.4	44.5	44.2	43.9	45.1
	12	64.2	65.5	66.9	70.3	70.6	64.0	59.7	54.5	50.6	53.8	54.9
	24	65.4	74.3	77.6	84.4	84.1	80.9	80.2	74.7	69.3	73.7	70.6
W. viridescens 23	6	37.3	44.9	45.7	45.5	41.7	52.3	45.8	44.1	44.7	52.1	51.3
	9	52.1	60.5	64.7	64.9	59.9	58.9	53.8	41.8	48.0	51.3	44.5
	12	62.5	69.8	70.0	73.4	69.0	66.4	61.9	52.3	57.2	57.7	52.9
	24	78.2	80.8	81.7	85.0	85.9	84.6	85.4	78.2	73.3	73.7	71.6

* Color intensity of results indicates the degree of inhibitory activity.

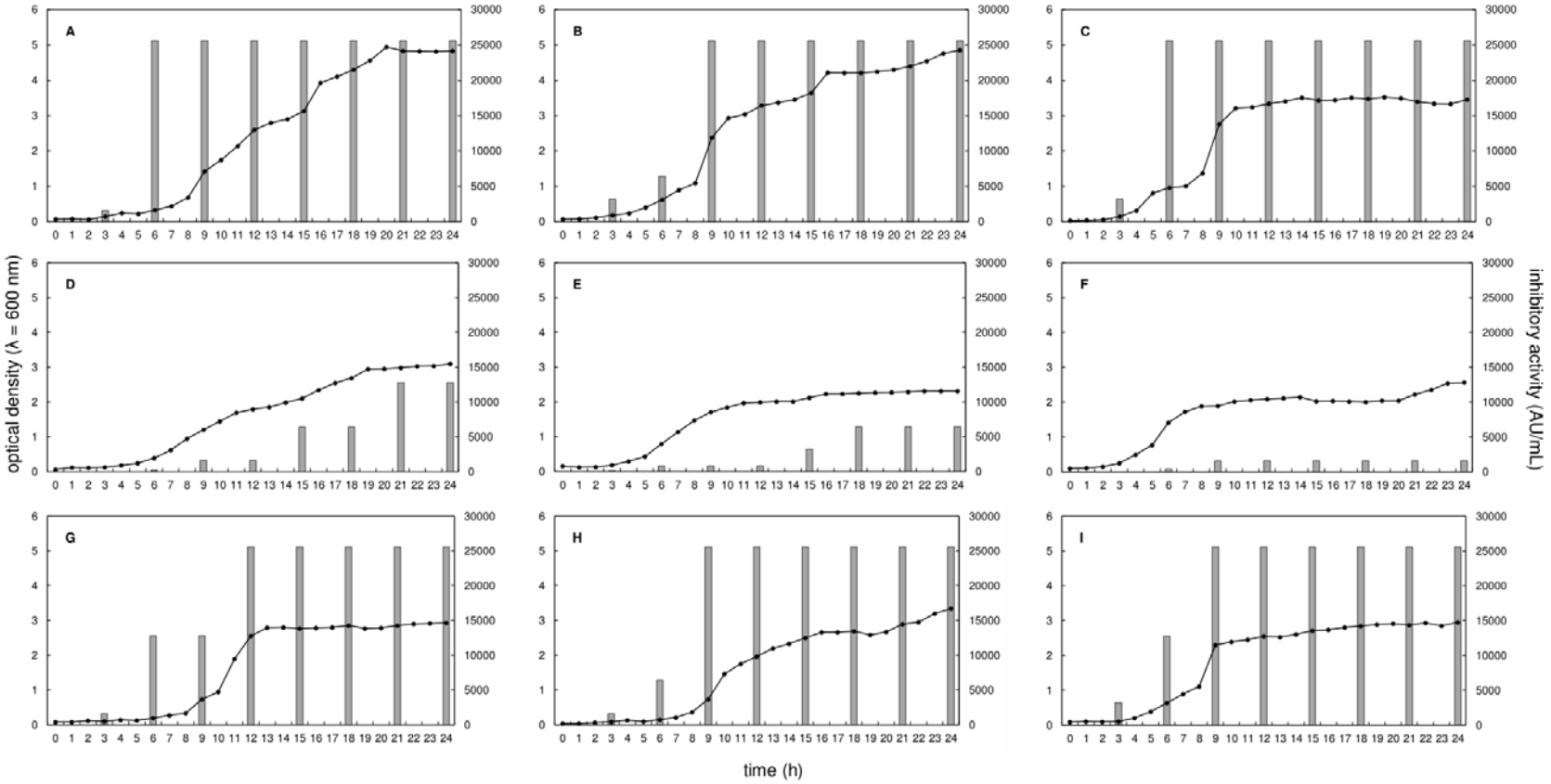

Figure 1. Growth (optical density at λ = 600 nm) and inhibitory activity (arbitrary units per mL) of *Lactobacillus curvatus* 12 (A, B, C), *L. curvatus* 36 (D, E, F) and *Weissella viridescens* 23 (G, H, I) cultivated in MRS broth and incubated at 25 (A, D, G), 30 (B, E, H) and 37 °C (C, F, I).

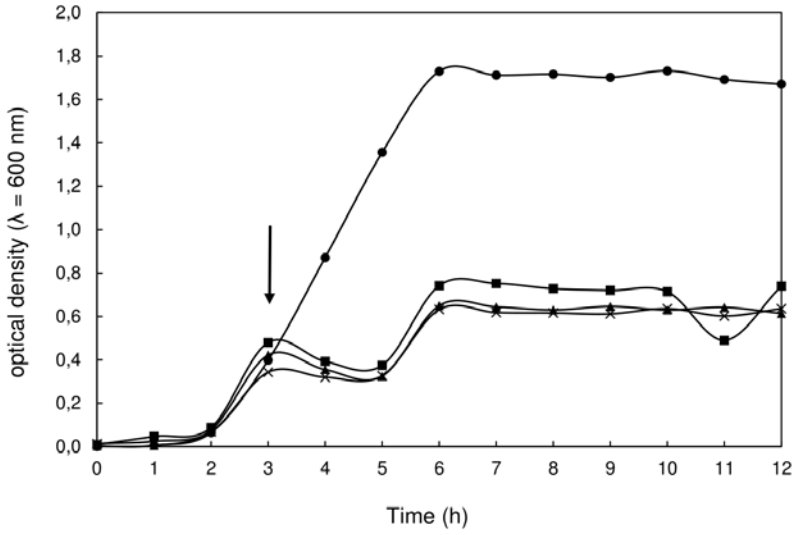

Figure 2. Effect of adding cell free supernatant of the bacteriocionogenic strains of *Lactobacillus curvatus* 12 (▲), *L. curvatus* 36 (×) and *Weissella viridescens* 23 (■) at 3 h (arrow) of growth of *Listeria monocytogenes* 72 (●).

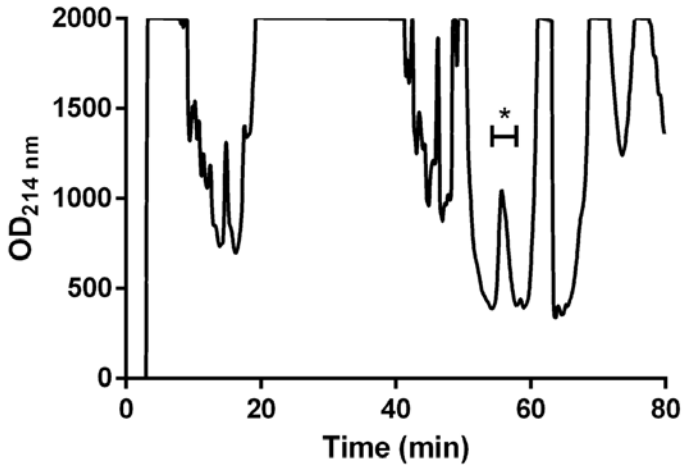

Figure 3. Chromatogram of the purified bacteriocins produced by *Lactobacillus curvatus* 12 (C4 reversed-phase HPLC). Asterisk indicates the peak with inhibitory activity against *L. monocytogenes* 72.

Supplementary Table. Primers and PCR conditions for detection of bacteriocin related genes in five bacteriocinogenic lactic acid bacteria isolated from *calabresa.*

Bacteriocin	Target gene	Primer	Sequence (5'-3')	Annealing (°C)	Size (bp)	Reference
Enterocin A	*entA*	EntA - F	AAATATTATGGAAATGGAGTGTAT	34	452	du Toit et al., 2000
		EntA - R	GCACTTCCCTGGAATTGCTC			
Enterocin P	*entP*	EntP- F	TATGGTAATGGTGTTTATTGTAAT	41	216	du Toit et al., 2000
		EntP- R	ATGTCCCATACCTGCCAAAC			
Enterocin B	*entB*	EntB- F	GAAAATGATCACAGAATGCCTA	41	159	du Toit et al., 2000
		EntB- R	GTTGCATTTAGAGTATACATTTG			
Enterocin L50B	*entL50*	EntL50B- F	STGGGAGCAATCGCAAAATTAG	44	135	du Toit et al., 2000
		EntL50B- R	ATTGCCCATCCTTCTCCAAT			
Pediocin PA-1	*ped*pro	PedA - F	CAAGATCGTTAACCAGTTT	44	1238	Todorov et al., 2016
		PedA- R	CCGTTGTTCCCATAGTCTAA			
Nisin	*nis*	NisF	ATGAGTACAAAAGATTTCAACTT	48	203	Kruger et al., 2013
		NisR	TTATTTGCTTACGTGAACGC			
Plantaricin W	*plaW*	PlanW- F	TCACACGAAATATTCCA	41	165	Holo et al., 2010
		PlanW- R	GGCAAGCGTAAGAAATAAATGAG			
Plantaricin NC8	*plaNC8*	PlanNC8- F	GGTCTGCGTATAAGCATCGC	35	207	Maldonado et al., 2003
		PlanNC8- R	AAATTGAACATATGGGTGCTTTAAATTCC			
Plantaricin S	*plaS*	PlanS - F	GCCTTACCAGAGTAATGCCC	45	450	Stephens et al., 1998
		PlanS - R	CTGGTGATGCAATCGTTAGTTT			
Sakacin GA-1	*sakGA1*	SakGA1- F	TTAGAACTACACTGCTCGTG	38	259	Todorov et al., 2011
		SakGA1- R	TGGAAGAATGAGTACTTGTT			
Sakacin GA-2	*sakGA2*	SakGA2- F	CGTTACAACAGAACTTCAAG	38	259	Todorov et al., 2011
		SakGA2- R	TGGAAGAATGAGTACTTGTT			
Sakacin X	*sakX*	SakX - F	AGCTATGAAAGGTATTGTCGGG	62	156	Macwana and Muriana, 2012
		SakX - R	TAAGATTTCCAGCCAGCAGC			
Sakacin A	*sakA*	SakA- F	GAAWTRMMANCAATTAYMGGTGG	55	150	Dortu et al., 2008
		SakA- R	CAGCCGCTAATCATACCACC			
Sakacin Q	*sakQ*	SakQ- F	GAARTWSYANCAATTADNGGTGG	53	130	Dortu et al., 2008
		SakQ- R	TACCACCAGCAGCCATTCCC			
Sakacin P	*sakP*	SakP- F	ATGGAAAAGTTTATTGAATTA	40	186	Reminger et al., 1996
		SakP- R	TTATTTATTCCAGCCAGCGTT			
Sakacin Tα	*sakTA*	SakT-α - F	TCGGTGGCTATACTGTCTAAACA	58	160	Macwana and Muriana, 2012
		SakT-α - R	TGTCCTAAAAATCCACCAATGC			
Sakacin Tβ	*sakTB*	SakT-β - F	AAGAAATGATAGAAATTTTTGGAGG	56	151	Macwana and Muriana, 2012
		SakT-β - R	TGTGAAATCCAATCTTGTCCTG			

CAPÍTULO 2 - Beneficial and safety features of bacteriocinogenic lactic acid bacteria isolated from *calabresa*, an artisanal sausage

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Manuscript prepared for submission to *Letters in Applied Microbiology* (IF 1.575)

Title page

Beneficial and safety features of bacteriocinogenic lactic acid bacteria isolated from *calabresa*, and artisanal sausage

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Running headline: LAB beneficial and safety aspects

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Significance and Impact of the Study

The food industry has a particular interest in using bacteriocinogenic lactic acid bacteria (LAB) as biopreservatives in end products, and the characterization of additional beneficial features is important to identify potential probiotic strains. However, these strains can only be added in end products after being properly characterized according their negative aspects, such as virulence and antibiotic resistance genes. So, a wide characterization of beneficial and safety aspects of bacteriocinogenic LAB is determinant to guide the proper utilization of these strains, or their purified bacteriocins, by the food industry.

Abstract

Despite of the beneficial relevance of lactic acid bacteria (LAB) in the food industry, microorganisms belonging to this group can determine spoilage in foods and carry a number of virulence and antibiotic resistance related genes. This study aimed the characterization of beneficial and safety aspects of five bacteriocinogenic LAB strains (*Lactobacillus curvatus* 12, *L. curvatus* 36, *Weissela viridescens* 23, *W. viridescens* 31 and *Lactococcus garvieae* 36) isolated from an artisanal *calabresa*. Regarding their safety aspects, all tested isolates were positive for *mub*, while EF226-cbp, EF1249-fbp and EF2380-maz were detected in at least one tested strain; none isolates presented *map*, *EFTu* or *prgB*. The tested strains presented a variable pattern of virulence related genes and none strain presented *gelE*, *cylA*, *efsA*, *cpd*, *int-Tn* or *sprE*, and other virulence-related genes were detected at different frequencies. Antibiotic resistance genes were also detected at different patterns. Despite presenting some beneficial aspects, the presence of virulence and antibiotic resistance genes jeopardize their utilization as starter or biopreservatives cultures in food. Considering the inhibitory potential of these strains, an alternative would be the use of their bacteriocins after semi-purification or purification procedures.

Keywords: lactic acid bacteria, virulence, beneficial aspects, antibiotic resistance, *calabresa*

Introduction

Lactic acid bacteria (LAB) are of great importance for the food industry (Arqués et al. 2015). Microorganisms belonging to this group are widely used as *starter* cultures, causing changes in raw material due to the production of acids and conferring specific flavors and aromas (Woraprayote et al. 2016; Mokoena 2017). LAB are also usually known by their beneficial features: many strains present probiotic characteristics, being able to colonize the gastrointestinal tract and to improve the beneficial balance of the autochthonous microbiota in this environment (Quinto et al. 2014; Alvarez-Sieiro et al. 2016), and many strains are able to produce different antimicrobial substances, specially bacteriocins, being able to inhibit the growth and contamination by spoilage and foodborne pathogens (Alvarez-Sieiro et al. 2016).

However, LAB may also determine early spoilage in food through the production of proteolytic substances and organic acids (Quinto et al. 2014), and they can also carry and express a number of genes associated to virulence and antibiotic resistance (Huddleston 2014; Fraqueza 2015; Porto et al. 2017). So, the proper characterization of beneficial and virulence features in LAB strains is mandatory before their usage as beneficial, bio-preservatives and *starter* cultures in food (Alvarez-Sieiro et al. 2016; Porto et al. 2017). This study aimed the characterization of beneficial and virulence potential of five bacteriocinogenic LAB strains previously isolated from an artisanal *calabresa*: *Lactobacillus curvatus* 12, *L. curvatus* 36, *Weissela viridescens* 23, *W. viridescens* 31 and *Lactococcus garvieae* 36.

Results and Discussion

All tested isolates were positive for *mub*, while EF226-cbp, EF1249-fbp and EF2380-maz were detected in at least one tested strain; none isolate presented *map*, *EFTu* or *prgB* (Table 1). *mub* encodes extracellular mucus-binding proteins and it is usually found in intestinal *Lactobacillus* sp., being highly associated with adhesion ability (Ramiah et al. 2007; Colombo et al. 2017). *map*, *EFTu* and *prgB* are also associated with adhesion (Todorov et al. 2017a), despite not being detected in any of the tested strain (Table 1). EF226-cbp, EF1249-fbp and EF2380-maz are related to adsorption of nutrients and defense, characteristics that enhance the beneficial potential of isolates (Souza et al. 2018). The presence of beneficial related genes in potential probiotic candidates stains is highly recommended, but not necessarily mandatory: the studied genes are relevant for the adhesion properties of the cultures in the intestinal cells, indicating an advantage in colonization and expression of other beneficial features (Archer et al. 2018). However, the expression of such genes is a complex process, widely influenced by a number of factors such as available nutrients, microbial competition and communication, and presence of specific receptors in the host cells (Miyoshi et al. 2006; Ramiah et al. 2007; Todorov and Dicks 2008). Based on obtained results, the tested strains have some beneficial potential, due to their possible ability of colonize the intestinal cells (Table 1).

Despite their beneficial potential, the tested strains presented a variable pattern of virulence related genes: none strain presented *gelE*, *cylA*, *efaA*, *cpd*, *int-Tn* or *sprE*, and other virulence-related genes were detected at different frequencies (Table 1). According to Lopes et al. (2006), the presence of *gel*E gene is probably not enough for gelatinase activity, since the complete *fsr* operon seems to be essential for the

expression of this virulence marker. Though, *fsr* operon seems to be easily damaged and lost mainly during the freezing in the laboratory conditions. According to Cotter et al. (2005), cytolisin is a substance considered as an antibacterial, but it is also virulent due to its hemolytic potential. The genes *asa*1, *esp, efa*A and *ace* are related to the production of various substances enrolled in the microbial colonization and adhesion at microorganism surfaces, and they are also associated to evasion of the host immune system (Valenzuela et al. 2009). The gene *hyl* is enrolled in the dispersion of the producing bacteria or their toxins in the host tissue, and maybe this gene can interact with lymphocyte receptors inducing immune diseases (Girish and Kemparaju 2007). *ccf, cob* and *cpd* genes are responsible for encoding sexual pheromones; although these substances are not considered as virulence factors, they are important to conjugation process and their production can support the transfer of resistance to antibiotics and virulence genes, either horizontally or vertically, which would cause an increase in the pathogenicity of some bacteria (Eaton and Gasson 2001). *int* and *int*-Tn are genes related to a transposon integrase and *spr*E is related to serine protease (Fortina et al. 2008).

Antibiotic resistance genes were also detected at different patterns (Table 1). Except for *L. garvieae* 32, all tested isolates presented positive results for one gene related to resistance to erythromycin. Only the two *L. curvatus* strains presented positive results for two genes related to tetracycline resistance (*telK* or *tetL*), and only *L. curvatus* 12 presented positive results for *aac(6')-Ie-aph(2")-Ia*, related to resistance to gentamycin. Aminoglycosides resistance genes were detected in all tested LAB isolates, being detected in frequencies varying from 2 to 3 out of 6 tested genes; resistance to aminoglycosides is considered as an intrinsic characteristic of LAB, being usually attributed to the absence of cytochrome-mediated electron transport, that

mediates drug uptake (Danielsen and Wind 2003). Only two LAB isolates presented genes associated to resistance to chloramphenicol, and only *L. curvatus* 12 presented one out of three tested genes linked to resistance to bacitracin (*bcrB*). As for aminoglycosides, all tested LAB isolates presented positive results for at least two genes related to resistance to vancomycin. *vanA* and *vanB* are located on a plasmid, while *vanC* is located in bacterial chromosome (Martín-Platero et al. 2009; Perin et al. 2014). The presence of resistance related genes in plasmids represents a major concern for safety, once these elements are easily transferred among microorganisms through horizontal transfer, enhancing the widespread of antibiotic resistance (Huddleston 2014).

The presence of such genes in the genome of the bacteriocinogenic LAB does not necessarily indicate their virulence or antibiotic resistance, once their expression should be assessed. However, these genes can be easily transferred to other LAB since they are usually located in conjugative plasmids, once they are usually associated with *Enterococcus* spp. (Eaton and Gasson 2001). Horizontal gene transfer is a common occurrence in mixed populations, such as the intestinal tract in which the probiotic LAB strains develop their beneficial features (Huddleston 2014; Todorov et al. 2017b). This is a special concern related to antibiotic resistance genes, a current concern worldwide; even the LAB strains not being able to express the resistance related to these genes, they could act as spreaders of this characteristic to pathogenic and common microorganisms present in the gastrointestinal tract (Huddleston 2014).

The LAB strains presented some beneficial aspects, besides the bacteriocins production, but the presence of virulence and antibiotic resistance genes is a concern in using them as *starter* or biopreservatives cultures in food. Considering the inhibitory potential of these strains, an alternative would be the use of their bacteriocins after

purification or semi-purification procedures, demanding additional assays to evaluate their resistance to different conditions of foods and gastrointestinal tract, and their cytotoxicity.

Materials and methods

Lactic acid bacteria strains and storage

Lactobacillus curvatus 12, *L. curvatus* 36, *Weissela viridescens* 23, *W. viridescens* 31 and *Lactococcus garvieae* 36 were isolated from *calabresa*, an artisanal sausage, and characterized as bacteriocinogenic (Castilho et al., Capítulo 1). Stock cultures were keep in de Man, Rogosa and Sharpe broth (MRS, Becton, Dickinson and Company - BD, Franklin Lakes, NJ, USA) added to glycerol at 20% (v/v) at -20 ºC.

Beneficial, virulence and antibiotic resistance related genes detection by PCR

Aliquots of the stock cultures were transferred to MRS (BD) and incubated at 37 °C for 24h; total DNA from LAB cultures were obtained using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) and DNA concentrations were determined by using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA).

DNA were used in PCR assays to detect the beneficial related genes *mub*, *map*, *EFTu*, *prgB*, *EF2662*-cbp, *EF1249*-fbp and *EF2380*-maz, as described by Kao et al. (1991), Fortina et al. (2008) and Todorov et al. (2012). Also, DNA were used to in PCR assays to detect virulence and antibiotic resistance related genes (*gelE*, *hyl*, *asa1*, *esp*, *cylA*, *efaA*, *ace*, *vanA*, *vanB*, *vanC1*, *vanC2*, *ermA*, *ermB*, *ermC*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *aac*(6′)-Ie-aph(2″)-Ia, *aph*(3′)-IIIa, *ant*(4′)-Ia, *aph*(2″)-Id, *aph*(2″)-Ic,

aph(2″)-Ib, *ant*(6)-Ia, *catA*, *bcrB*, *bcrD*, *bcrR*, *ccf*, *cob*, *cpd*, *sprE*, *int* and *int*-Tn), according protocols described by Fortina et al. (2008) and Moraes et al. (2012).

Acknowledgments

CAPES, CNPq and FAPEMIG.

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Table 1. Results for beneficial, virulence and antibiotic resistance related genes in five bacteriocinogenic strains of lactic acid bacteria isolates from *calabresa*, an artisanal sausage.

aspect	target gene	function/resistance to	bacteriocinogenic strains of lactic acid bacteria				
			L. curvatus 12	*W. viridescens* 23	*W. viridescens* 31	*L. garvieae* 32	*L. curvatus* 36
beneficial	*mub*	adhesion	+	+	+	+	+
	map	adhesion	-	-	-	-	-
	EFTu	adhesion	-	-	-	-	-
	*prg*B	surface protein	-	-	-	-	-
	EF2662-cbp	choline-binding protein	-	+	-	-	-
	EF1249-fbp	fibrinogen-binding protein	-	+	+	-	-
	EF2380-maz	membrane-associated zinc metalloprotease	-	-	+	-	+
virulence	*gelE*	gelatinase	-	-	-	-	-
	hyl	hyaluronidase	-	-	+	-	+
	asa1	aggregation substance	-	-	-	+	+
	esp	enterococcal suface protein	-	+	+	-	+
	cylA	cytolisin	-	-	-	-	-
	efaA	endocarditis antigen	-	-	-	-	-
	ace	adhesion of collagen	+	-	-	-	-
	ccf	related to sex pheromones	+	-	-	-	-
	cob	related to sex pheromones	-	+	-	-	-
	cpd	related to sex pheromones	-	-	-	-	-
	int	transposom	+	-	-	-	+
	int-Tn	transposom	-	-	-	-	-
	sprE	serine protease	-	-	-	-	-

Table 1, cont.

aspect	target gene	function/resistance to	bacteriocinogenic strains of lactic acid bacteria				
			L. curvatus 12	*W. viridescens* 23	*W. viridescens* 31	*L. garvieae* 32	*L. curvatus* 36
antibiotic resistance	*ermA*	erythromycin	-	-	-	-	-
	ermB	erythromycin	-	+	-	-	-
	ermC	erythromycin	+	-	+	-	+
	tetK	tetracycline	-	-	-	-	+
	tetL	tetracycline	+	-	-	-	-
	tetM	tetracycline	-	-	-	-	-
	tetO	tetracycline	-	-	-	-	-
	tetS	tetracycline	-	-	-	-	-
	aac(6')-Ie-aph(2")-Ia	gentamycin	+	-	-	-	-
	aph(3')-IIIa	aminoglycosides	-	+	+	-	+
	ant(4')-Ia	aminoglycosides	-	-	-	+	-
	aph(2")-Ib	aminoglycosides	+	-	+	+	-
	aph(2")-Ic	aminoglycosides	+	+	+	-	+
	aph(2")-Id	aminoglycosides	-	-	-	-	-
	ant(6)-Ia	aminoglycosides	-	-	-	-	-
	catA	chloramphenicol	-	-	+	+	-
	bcrB	bacitracin	+	-	-	-	-
	bcrD	bacitracin	-	-	-	-	-
	bcrR	bacitracin	-	-	-	-	-
	vanA	vacomycin	-	+	+	+	+
	vanB	vacomycin	+	+	+	-	-

Table 1, cont.

aspect	target gene	function/resistance to	bacteriocinogenic strains of lactic acid bacteria				
			L. curvatus 12	*W. viridescens* 23	*W. viridescens* 31	*L. garvieae* 32	*L. curvatus* 36
antibiotic resistance	*vanC1*	vacomycin	-	-	-	+	-
	vanC2	vacomycin	+	+	-	-	+

+: positive, -: negative

CAPÍTULO 3 – Inhibition of *Listeria monocytogenes* in fresh sausage by bacteriocinogenic *Lactobacillus curvatus* 12 and its semi-purified bacteriocin

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Manuscript prepared for submission to *Food Microbiology* (IF 3.759)

Title page

Inhibition of *Listeria monocytogenes* in fresh sausage by bacteriocinogenic *Lactobacillus curvatus* 12 and its semi-purified bacteriocin

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Abstract

The aim of the study was to evaluate the inhibitory activity of *Lactobacillus curvatus* 12 and its bacteriocin on *Listeria monocytogenes* in controlled food matrix. Fresh sausages were prepared and inoculated in different combinations with *L. curvatus* 12 (bacteriocingeonic strain, 10^6 CFU/g), *L. sakei* ATCC 15521 (non-bacteriocinogenic strain, 10^6 CFU/g), *L. monocytogenes* (target, 10^3 CFU/g), nisin (12.5 mg/g) and partially purified bacteriocin from *L. curvatus* 12 (12.5 mg/g and 6.25 mg/g), being stored at 7 °C for 10 days. Microbial populations and bacteriocin production was assessed on days 1, 4, 7 and 10. In general, LAB counts did not present significant differences amongst treatments and storage periods ($p > 0.05$). *L. monocytogenes* counts of sausages inoculated with the pathogen and the bacteriocinogenic strain ranged from 1.0 to 2.0 log CFU/g, being significantly different from the pathogen control ($p < 0.05$). Nisin and partially purified bacteriocin also determined a decrease of *L. monocytogenes* counts when compared with the pathogen control, ranging from 1.0 to 3.0 log CFU/g ($p > 0.05$). These results indicate that bacteriocinogenic LAB was able to determine a significant decrease of *L. monocytogenes* counts in fresh sausage stored at 7 °C.

Keywords: fresh sausage, bacteriocin, *L. monocytogenes*, bacteriocinogenic *L. curvatus*

1. Introduction

Listeria monocytogenes is a foodborne pathogen often associated with meat products and described as resistant to a variety of adverse conditions. This pathogen can remain viable and multiply in microaerophilic environments, low temperatures and in the presence of salts and low pH, in addition to persist in industrial environments due to its ability of adhering in surfaces and forming biofilms (Buchanan et al., 2017; Ferreira et al., 2014). Due to these characteristics, the control of contamination and elimination of *L. monocytogenes* in foods is a constant challenge for the industry, especially in ready-to-eat meat products that are produced for direct human consumption, without additional heat treatments (Bersot et al., 2001; Camargo et al., 2017; Samelis et al., 2005).

Sausages are meat products particularly susceptible to contamination and consequent development of *L. monocytogenes*. Fresh sausages, particularly those produced with pork, present high water activity, low pH and different types of ingredients, besides intense handling during production, increasing the chances of contamination by different microorganisms, including *L. monocytogenes* (Giello et al., 2018; Liserre et al., 2002; Samelis et al., 2005). Thus, appropriate hygiene practices are necessary to minimize possible contamination during the production of sausages; but even these practices may not be enough to assure the safety of these products.

In this sense, the food industry has been adopting alternatives to minimize contamination and control the multiplication of microorganisms that may contaminate food during the production chain, such as bacteriocins produced by lactic acid bacteria (LAB). In recent years, studies on the use of bacteriocins indicate that they can bring a number of benefits in food, such as reducing the risk of dissemination of foodborne

pathogens, increasing the shelf life and safety of the end products, less use of chemical preservatives and the possibility of using milder technological treatments during production (Castellano et al., 2017; Gálvez et al., 2007; Mokoena, 2017). Nowadays, nisin is the only bacteriocin that can be used as a food additive for the control microorganisms in food, because of its GRAS (Generally Recognized As Safe) status; the use of nisin in food production is authorized in approximately 50 countries (Gharsallaoui et al., 2016; Guinane et al., 2005; Punyauppa-path et al., 2015).

These limitations of alternatives have stimulated several research groups to characterize new bacteriocinogenic isolates and their bacteriocins, aiming at potential application in food as biopreservatives. Based on this information, the aim of the study was to evaluate the interactions between bacteriocinogenic *Lactobacillus curvatus* 12 and its bacteriocins with *Listeria monocytogenes* in fresh sausage, a food system potentially subject to contamination by this pathogen.

2. Material and Methods

2.1. Bacterial strains, partial purification of bacteriocin produced by Lactobacillus curvatus 12 and growth conditions

Lactobacillus curvatus 12 was isolated from *calabresa* and characterized as a bacteriocinogenic strain with high inhibitory activity against *L. monocytogenes* (Castilho et al., Capítulo 1). *Lactobacillus sakei* ATCC 15521 was used in the present study as negative control, once it is a non-bacteriocinogenic strain. *Listeria monocytogenes* 72 was isolated from beef carcasses (Camargo et al., 2014), and considered in the presented study as the target organism. LAB and *L. monocytogenes* strains were innoculated in de Mann-Rogosa-Sharpe (MRS, Becton, Dickinson and

Company - BD, Franklin Lakes, NJ, USA) and trypticase soya broth (TSB, BD), respectively, incubated at 35 °C for 24 h, added with glycerol at 20 % (v/v) and stored at -20 °C. For use, aliquots of the cultures were transferred to MRS (BD, for LAB) and TSB (BD, for *L. monocytogenes*), incubated overnight at 35 °C and diluted in NaCl 0.85% (w/v) until a turbidity similar to tube 1 of MacFarland scale, corresponding to approximately 3 x 10^8 colony forming units per mL (CFU/mL). Then, cultures were ten-fild diluted with NaCl 0.85% (w/v) until adequate populations for sausage inoculation.

Also, bacteriocin produced by *L. curvatus* 12 was subjected to partial purification. An aliquot of the stock culture of *L. curvatus* 12 was transferred to MRS (BD) and incubated at 35 ºC for 24 h. Then, the culture was centrifuged (8,000 × *g*, 4 °C, 15 min) and the cell free supernatants (CFS) were treated with NaOH 1M to pH 6.0 and heated at 80 ºC for 10 min. Then, proteins were precipitated with trichloroacetic acid (TCA) at 4% (w/v) and incubated at 4 ºC for 1 h, centrifuged (8,000 × *g*, 4 °C, 20 min) and pellet was re-suspended in TRIS. The concentration of the partially purified bacteriocin was determined by using the BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer instructions and using bovine serum albumin as standard. Semi purified bacteriocin was used in two different concentrations: 12.5 and 6.25 mg/g. The obtained extract was evaluated for bacteriocin activity against *L. monocytogenes* 72 by the spot-on-the-lawn method (Todorov and Dicks, 2005a).

2.2. Fresh sausage production

Fresh sausage batches were produced in experimental conditions, using equipment and utensils previously cleaned and sanitized. Fresh sausage was produced

by using raw pork (shoulder and fat) cut in small pieces (2-3 cm^3) and ground by using a meat grinder with a mincer screen of 0.5 cm (Arbel, São José do Rio Preto, SP, Brazil). Then, the obtained grounded pork was mixed for three minutes in order to obtain a mass of 6.0 kg, composed by 5.4 kg of shoulder and 0.6 kg of fat. The obtained pork mixture was mixed for 3 min with ingredients and spices, previously diluted in 36.5 mL of sterile distilled water: sugar (6 g), garlic paste (12 g), black pepper (3 g), chilli pepper (5.5 mL), nutmeg (3 g) and erythorbate (7.5 g). Then, curing salt (150 mg) and NaCl (100 g) were diluted in 50 mL of sterile distilled water and added to the pork mixture, followed by manual homogenization for 3 min. The obtained pork mixture was stored at 2 °C for 24 h.

The pork mixture was incubated at 25 °C for 4 h and divided in 12 portions of 0.5 kg that were inoculated with the LAB, *L. monocytogenes* strains, nisin and partially purified bacteriocin produced by *L. curvatus* 12 according to the treatments described in Table 1. Then, the inoculated mixtures were incubated at 25 °C for 2 h and casing was conducted using funnels, resulting in pork sausages with approximately 100 g. Sausages were packed in sterile styrofoam boxes, identified and stored at 7 °C for 10 days. Sausages and treatments were produced in 3 independent repetitions.

2.3. Physicochemical analysis

Samples of fresh sausages (only from control treatment, Table 1) were obtained after 1 and 10 day of storage and subjected to physicochemical analyzes.

2.3.1. Water activity

The obtained samples were submitted to determination of the water activity (a_w), using the AquaLab Lite (Meter Group, Pullman, WA, USA). Each sample unit was

analyzed in duplicate, with the instrument previously calibrated, following the manufacturer's operating manual.

2.3.2. Moisture

Crucibles were previously kept at 105 ºC for at least 1 h and after being removed from the oven were kept in the desiccator for at least 30 min to reach the thermal equilibrium with the environment. Then, the crucibles were weighed and portions of 5 g of the samples were added and incubated at 105 ºC for 4 h. After the incubation time, the crucibles were again kept in the desiccator for 30 min, and then weighed. Based on recorded weights, the percentage of moisture was calculated using the following equation: Moisture = $[(Weight_{crucibles} + Weight_{sample}) - Weight_{final\ of\ crucibles}] \times 100 / Weight_{sample}$.

2.3.3. pH

The pH was measured by using a pHmeter. Portions of 50 g of the samples were diluted in 10 mL of distilled water (neutral pH), to allow adequate electrode contact from a previously calibrated pH meter with the sample.

2.4. Microbiological analysis

Samples of sausages from all experiments were obtained on days 1, 4, 7 and 10. Portions of 25 g from sausages were transferred to sterile bags with 225 mL of NaCl 0.85% (w/v), homogenized and ten-fold diluted in NaCl 0.85% (w/v).

2.4.1. Enumeration of hygiene indicator micro-organisms

Selected dilutions were plated in Petrifilm™ AC and Petrifilm™ EC for enumeration of mesophilic aerobes, total coliforms and *Escherichia coli*, and incubated at 35 °C for 24 to 48 h. The final results were expressed in CFU/g.

2.4.2. Enumeration of lactic acid bacteria

LAB populations were monitored by pour plate of selected dilutions on MRS agar (BD) and incubated at 35 °C for 48 h. After incubation time, the colonies were enumerated and the results were expressed in CFU/g.

To estimate the population of the bacteriocinogenic LAB added, plates containing individual colonies were selected and overlaid with 5 mL of brain heart infusion (BHI, BD) supplemented with agar 0.75% (w/v) inoculated with *L. monocytogenes* 72 (approximately 10^6 CFU/mL) and incubated at 35 ºC for 24 h (Todorov and Dicks, 2005b). Colonies that presented inhibition halo were considered as the bacteriocinogenic LAB added

2.4.3. Detection and enumeration of L. monocytogenes

Samples were subjected to *L. monocytogenes* detection and enumeration according to ISO 11290-1/Amd 1 (ISO, 1996, 2004), with some modifications. Aliquots of 40 mL of the sample homogenates were centrifuged (4 °C, 15 min, 1,000 × *g*), the supernatant discarded and the obtained pellet suspended with 10 mL of Half-Fraser (Oxoid Ltd., Basingstoke, UK), followed by incubation at 30 °C for 24 h. Then, aliquots of 0,1 mL were transferred to Fraser broth (Oxoid), incubated at 35 °C for 24 h, and streaked onto plates containing Chromogenic Listeria agar (Oxoid), incubated

at 35 °C for 24 h. The presence of typical *L. monocytogenes* colonies (blue greenish, with white opaque halo) was indicative of positive result.

Aliquots of 1 mL from the Half-Fraser cultures, after 1 h of incubation, were plated onto the surfaces of agar plates containing Chromogenic Listeria agar (Oxoid) and incubated at 35 °C for 24 h. Then, typical colonies of *L. monocytogenes* were enumerated and the results were expressed as CFU/g.

2.4.4. Bacteriocin production in situ

The production of bacteriocin by *L. curvatus* 12 in the inoculated fresh sausages was assessed according to Ávila et al. (2006), with modifications. Portions of fresh sausages (5 g) were homogenized with 5 mL of 0.02 N HCl in stomacher and centrifuged (12,000 × g, 20 min, 4 °C). The pH of the obtained supernatant was adjusted to 6.0 (1N NaOH) and lyophilized. Then, the lyophilized samples were diluted in 200 μL of Ringer and 50 μL were transferred to 5 mm wells prepared on BHI (BD) supplemented with agar 0.75% (w/v) inoculated with *L. monocytogenes* 72 (approximately 10^6 CFU/mL) and incubated at 35 °C for 24 h. The presence of inhibition halos was the indicative of bacteriocin production in the tested samples.

2.5. Statistical analysis

All treatments were prepared in 3 independent repetitions and the results of the obtained microbial counts were converted to log10 and compared to verify significant differences between the treatments and the storage periods of the sausages by Analysis of Variance and Tukey test ($p < 0.05$), by using the software XLStat 19.01 (Adinsoft, New York, NY, USA).

3. Results and Discussion

The mean values of a_w of the samples evaluated on days 1 and 10 were 0.97 e 0.98, respectively. The development of microorganisms is favored by intrinsic characteristics of the meat, particularly its chemical composition, high water availability and pH close to neutrality (Contreras et al., 2002; Pothakos et al., 2015). The a_w of a food is the most accurate measure to determine the possibility of microbial growth (Lenovich, 2017; Troller, 2017), as it influences microbial stability and for many foods this growth is prevented with a value between 0.6 to 0.7 (Bourne, 2017).

According to the Brazilian Technical Guidelines of Identity and Quality of Sausage (Brasil, 2000), the acceptable moisture content for fresh sausage is at most 70%. The mean moisture found in the samples on days 1 and 10 was 61.36 and 59.41%, respectively, indicating that this parameter followed the current Brazilian legislation.

The mean pH values of the fresh sausage samples on storage days 1 and 10 were 6.36 and 6.29, respectively. Buchanan et al. (2017) observed that *L. monocytogenes* is able to grow well at pH as low as 4.1. Some studies have shown that the inhibition of *Listeria* spp. in meat products containing bacteriocinogenic LAB was greater than in products containing non bacteriocininogenic LAB, for the same range of pH, indicating that the inhibition is more likely to be due to the bacteriocin than to the presence of lactic acid (Campos et al., 1997; Hugas et al., 1998).

Based on mesophilic aerobes and coliforms counts, no significant differences were observed amongst treatments and storage periods ($p > 0.05$, data not shown). Also, *Escherichia coli* was not detected in any sample (data not shown). These results indicate that the added LAB did not influence the autochthonous microbiota of the fresh sausages. LAB counts, in general, did not present significant differences amongst

treatments and storage periods ($p > 0.05$, Table 2); significant differences of LAB counts were observed only in sausages inoculated with only the non-bacteriocinogenic strain and only nisin (after 7 days of storage) when compared to the sausages control, inoculated with only *L. monocytogenes* and inoculated with *L. monocytogenes* and nisin (after 1 day of storage) ($p < 0.05$, Table 2). Sausages that were inoculated with LAB presented higher counts (around 6 log CFU/g) when compared to sausages that were not inoculated with LAB (3 to 4 log CFU/g). Also, only the sausages that were inoculated with *L. curvatus* 12 presented colonies in MRS agar with inhibition halos, confirming the non-bacteriocinogenic nature of the *L. sakei* ATCC 15521 and absence of an autochthonous LAB microbiota with bacteriocinogenic activity.

LAB constitute a part of the initial microbiota which develops easily after the meat is processed to fermented sausages, stored or packed under vacuum or modified atmosphere. These microorganisms have been playing an important role in food fermentations leading to flavor and texture changes together with a preservative effect, resulting in an increase in the shelf life of the transformed product (Mokoena, 2017). LAB in fresh meat bring about a mild fermentation process without producing variations in the sensorial characteristics because of the low carbohydrate content and the strong buffering capacity of meat (Toldrá, 2010). In a similar process, LAB growth in fermented meats is enhanced by the adding of sugar, resulting to the production of lactic acid that decreases pH and denatures proteins, leading to a microbial stabilization in the end products (Beraquet, 2005; Toldrá, 2010; Woraprayote et al., 2016). Dias et al. (2015) described a LAB count range of 3.0 to 8.9 log CFU/g in pork sausage, while Prado et al. (2000) observed a range of 7 to 8 log CFU/g in fresh sausage.

All sausages that were inoculated with *L. monocytogenes* presented positive results for this pathogen in the detection procedure, independently of the storage period, except for the sausages inoculated with the pathogen and nisin after 1 and 4 days of storage. Sausages that were not inoculated with the pathogen did not present positive results in the detection assay, indicating absence of natural contamination of ingredients and cross contamination during sausage production. *Listeria monocytogenes* counts of sausages inoculated with the pathogen and the non-bacteriocinogenic strain ranged from 2.0 to 3.5 log CFU/g through the storage period, without significant differences when compared to the pathogen control ($p > 0.05$, Figure 1), while these counts in the sausages inoculated with the pathogen and the bacteriocinogenic strain ranged from 1.0 to 2.0 log CFU/g, being significantly different from the pathogen control ($p < 0.05$, Figure 1). Nisin and partially purified bacteriocin (at both concentrations) also determined a decrease of *L. monocytogenes* counts when compared with the pathogen control, ranging from 1.0 to 3.0 log CFU/g ($p > 0.05$, Figure 2). These results indicate that bacteriocinogenic LAB was able to determine a significant decrease of *L. monocytogenes* counts, while its partially purified bacteriocins, at the tested concentrations, determined only a non-significant decrease of the pathogen count. Cleveland et al. (2001) described that nisin activity may be affected in meat products by combination with phospholipids or inactivation by γ-glutamyl transferase, what might explain the detection and enumeration of *L. monocytogenes* in sausages inoculated with the pathogen and nisin after 7 days of storage (Figure 2).

Sant'Anna et al. (2013) evaluated the antimicrobial activity of the bacteriocin BLS P34 against *L. monocytogenes* in chicken sausage; this bacteriocin was able to inhibit the pathogen *in situ* in all incubation times for up to 10 days at 5 ºC, and its

effectiveness was increased by associate it with nisin. Giello et al. (2018) evaluated the effectiveness of the bacteriocin produced by *L. curvatus* 54M16 to inhibit *L. monocytogenes* in fermented sausage and they observed that inhibitory activity was lower during the production steps and total after fermentation. Martinis and Franco (1998) evaluated the inhibition of *L. monocytogenes* in a pork product by a bacteriocinogenic *L. sakei* strain and observed that after 4 weeks at 8 ºC the pathogen counts were 6 log CFU/g lower when compared to the pathogen control. Liserre et al. (2002) evaluated the inhibition potential of the same *L. sakei* strain against *L. monocytogenes* in sausages stored under modified atmosphere-package, demonstrating that this condition enhanced the inhibitory activity of the produced bacteriocin. Also, Ghalfi et al. (2006) demonstrated the inhibitory activity of the bacteriocin produced by *L. curvatus* CWBI-B28 against *L. monocytogenes* in cold-smoked salmon at 4 ºC storage, considering different approaches of bacteriocin-target interactions.

All sausages that the bacteriocinogenic strain was inoculated presented bacteriocin production *in situ*, independently of the storage period. Also, sausages that were inoculated with the partially purified bacteriocins presented positive results for the detection of the bacteriocin *in situ*. These results indicate that the bacteriocin was produced in the inoculated sausages by the bacteriocinogenic strain, and that it was not inhibited by the sausages compounds. During the fermentation process, LAB can produce various organic compounds, including several organic acids (lactic acid, acetic acid, propionic acid, formic acid), diacetyl, acetaldehyde, hydrogen peroxide, bacteriocins and other compounds with antimicrobial properties (Toldrá, 2010). Bacteriocins produced by LAB are antimicrobial protein compounds synthesized via the ribosomes, presenting variable spectrum of activity (Cotter et al., 2005). Numerous

bacteriocins produced by LAB species have been already described and it is well known for their activity against *L. monocytogenes*, *Staphylococcus aureus*, *Clostridium* sp., *Bacillus* sp. and other foodborne pathogens (Caly et al., 2017; Cotter et al., 2005; Du et al., 2017; Zhao et al., 2016; Zhu et al., 2014). Several LAB isolated from meat products presented ability to produce inhibitory substances against many tested microorganisms, mainly *L. monocytogenes* (Barbosa et al., 2014; Djadouni and Kihal, 2012; Du et al., 2017; Todorov et al., 2010).

The results obtained in this study indicated that *L. curvatus* 12 presented better inhibition against *L. monocytogenes* when compared to its partially purified bacteriocin at both tested concentrations (Figures 1 and 2). Despite being more practical, the use of starter cultures in fermented foods must be considered with care, once these microorganisms can carry a number of virulence and antibiotic resistance related genes, posing as potential hazards for the consumers. Besides the presence of such genetic elements, it is important to evaluate the expression of such virulent characteristics. Once these hazards are identified, one alternative would be the usage of the purified or partially purified bacteriocins of these strains. These issues lead to further studies to assess the safety of the bacteriocinogenic strains, expression of virulence traits and/or purification of bacteriocins, followed by characterization of tis cytotoxicity. Todorov et al. (2017) observed that genes related to the virulence factors, vancomycin resistance and production of biogenic amines were presented in low frequency in LAB isolated from “lukanka”, and artisanal sausage. Perin et al. (2014) studied the antibiotic resistance, virulence and biogenic amine production in bacteriocinogenic *Lactococcus* spp. and *Enterococcus* spp., and they observed the presence of different combinations of virulence related genes, but not necessarily the expression of such factors.

Based on the obtained results, *L. curvatus* 12 is capable to inhibit the growth of *L. monocytogenes* in a fresh sausage system during storage at 7 °C due to the production of a bacteriocin. However, the partially purified bacteriocin was not able to determine a significant decrease of *L. monocytogenes* populations in the fresh sausage in the tested concentrations, but partially purified bacteriocins reduced 1.0 to 3.0 log CFU/g. Further studies must be conducted to assess the safety aspects of *L. curvatus* 12 and the inhibitory activity of its bacteriocin at different concentrations and after additional purification steps.

Acknowledgments

CAPES, CNPq, FAPEMIG.

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Table 1. Treatments of fresh sausages prepared with different associations of *Lactobacillus curvatus* 12 (bacteriocinogenic strain), *L. sakei* ATCC 15521 (non-bacteriocinogenic strain), *Listeria monocytogenes* (target), nisin and partially purified bacteriocin from *L. curvatus* 12.

Treatment	Strains/bacteriocin	Specifications
1	control	-
2	*L. monocytogenes* 72 (target)	10^3 UFC/g
3	*L. curvatus* 12 (bacteriocinogenic)	10^6 UFC/g
4	*L. sakei* ATCC 15521 (non-bacteriocinogenic)	10^6 UFC/g
5	*L. monocytogenes* 72 & *L. curvatus* 12	10^3 & 10^6 UFC/g
6	*L. monocytogenes* 72 & *L. sakei* ATCC 15521	10^3 & 10^6 UFC/g
7	nisin	12.5 mg/g
8	*L. curvatus* 12 bacteriocin [I]	12.5 mg/g
9	*L. curvatus* 12 bacteriocin [1/2]	6.25 mg/g
10	*L. monocytogenes* 72 & nisin	10^3 UFC/g & 12.5 mg/g
11	*L. monocytogenes* 72 & *L. curvatus* 12 bacteriocin [I]	10^3 UFC/g & 12.5 mg/g
12	*L. monocytogenes* 72 & *L. curvatus* 12 bacteriocin [1/2]	10^3 UFC/g & 6.25 mg/g

Table 2. Mean counts (± standard deviation) of lactic acid bacteria in fresh sausages inoculated with different associations of *Lactobacillus curvatus* 12 (bacteriocinogenic strain), *L. sakei* ATCC 15521 (non-bacteriocinogenic strain), *Listeria monocytogenes* (target), nisin and partially purified bacteriocin from *L. curvatus* 12 and stored at 7 °C for 10 days.

Treatment	Strains/bacteriocin	Time (days)*			
		1	4	7	10
1	control	3.86 ± 0.19c	4.92 ± 1.52bc	8.56 ± 0.29ab	7.34 ± 1.72abc
2	*L. monocytogenes* 72 (target)	4.32 ± 1.56c	5.36 ± 2.01abc	8.36 ± 0.40ab	7.43 ± 1.76abc
3	*L. curvatus* 12 (bacteriocinogenic)	6.22 ± 1.31abc	7.50 ± 1.15abc	8.50 ± 0.16ab	8.32 ± 0.14ab
4	*L. sakei* ATCC 15521 (non-bacteriocinogenic)	6.47 ± 1.22abc	6.66 ± 1.24abc	9.12 ± 0.44a	6.99 ± 1.26abc
5	*L. monocytogenes* 72 & *L. curvatus* 12	5.79 ± 1.78abc	8.07 ± 0.41abc	8.38 ± 0.35ab	8.54 ± 0.18ab
6	*L. monocytogenes* 72 & *L. sakei* ATCC 15521	6.44 ± 1.14abc	7.21 ± 1.95abc	7.73 ± 0.71abc	7.31 ± 1.18abc
7	nisin	3.56 ± 0.39abc	4.50 ± 1.23abc	8.64 ± 0.77a	6.12 ± 2.19abc
8	*L. curvatus* 12 bacteriocin [I]	4.93 ± 1.74abc	5.57 ± 1.71abc	6.85 ± 2.04abc	6.90 ± 2.42abc
9	*L. curvatus* 12 bacteriocin [1/2]	4.52 ± 1.45abc	5.66 ± 1.86abc	6.64 ± 2.31abc	7.04 ± 2.47abc
10	*L. monocytogenes* 72 & nisin	2.88 ± 0.16c	4.02 ± 1.50abc	5.80 ± 1.39abc	7.86 ± 1.56abc
11	*L. monocytogenes* 72 & *L. curvatus* 12 bacteriocin [I]	3.66 ± 0.36abc	6.34 ± 1.97abc	6.38 ± 1.78abc	6.97 ± 1.56abc
12	*L. monocytogenes* 72 & *L. curvatus* 12 bacteriocin [1/2]	3.33 ± 0.31bc	4.64 ± 1.69abc	5.92 ± 1.97abc	6.69 ± 2.22abc

* Mean counts followed by different letters are significantly different by ANOVA and Tukey test ($p < 0.05$).

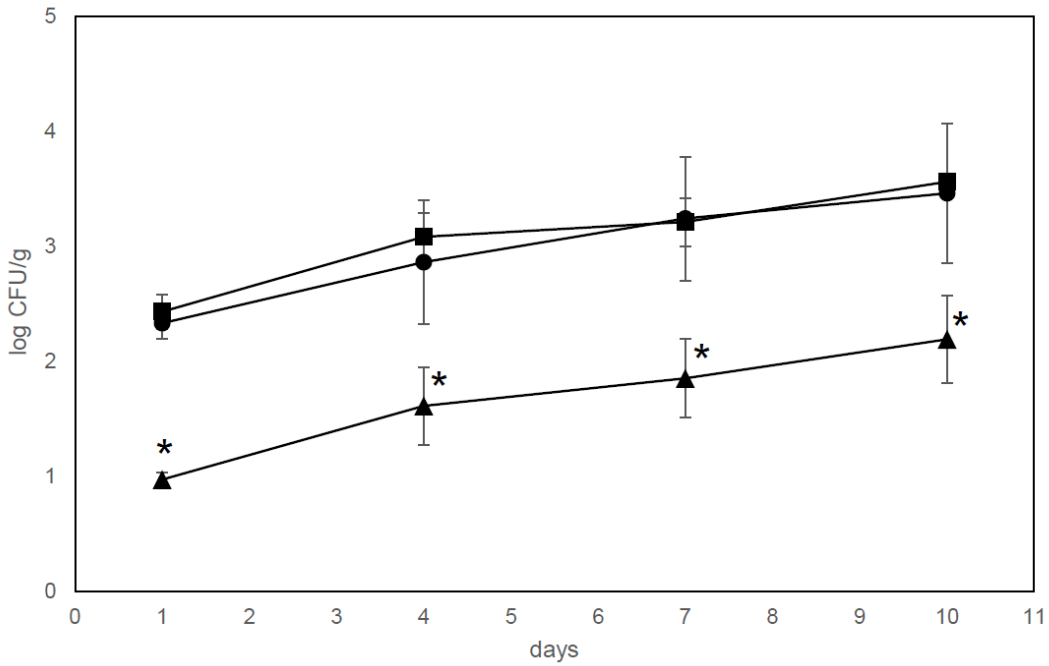

Figure 1. Mean counts (± standard deviation) of *Listeria monocytogenes* 72 inoculated in fresh sausages (●: alone; ■: with *Lactobacillus sakei* ATCC 15521, non-bacteriocinogenic; ▲: with *L. curvatus* 12, bacteriocinogenic strain) and stored at 7 °C for 10 days. Asterisks indicate significant differences by analysis of variance and Tukey ($p < 0.05$)

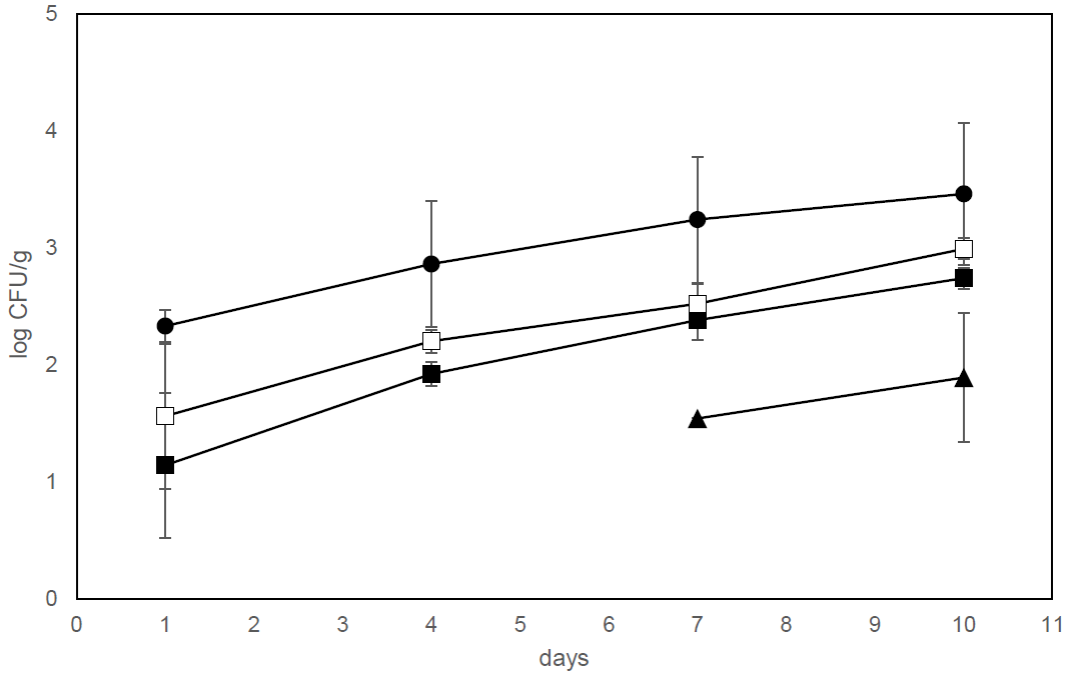

Figure 2. Mean counts (± standard deviation) of *Listeria monocytogenes* 72 inoculated in fresh sausages (●: alone; ▲: with nisin 12.5 mg/g; ■: with *L. curvatus* 12 partially purified bacteriocin at 12.5 mg/g; □: with *L. curvatus* 12 partially purified bacteriocin at 6.25 mg/g) and stored at 7 °C for 10 days.

CONCLUSÕES

- ✓ Linguiça calabresa artesanal é uma importante fonte de cepas de BAL bacteriocinogênicas;
- ✓ BAL isoladas de linguiça calabresa artesanal apresentaram alto potencial inibitório contra *L. monocytogenes*, sendo *L. curvatus* 12 e 36 e *W. viridescens* 23 capazes de produzir bacteriocinas com potencial aplicação na indústria de alimentos;
- ✓ Cepas de BAL bacteriocinogênicas isoladas de linguiça calabresa podem apresentar genes relacionados à virulência, resistência a antibióticos e probióticos;
- ✓ Bacteriocinas produzidas por BAL podem ser purificadas, sequenciadas e identificadas a partir de uma série de métodos cromatográficos;
- ✓ Quatro bacteriocinas produzidas por *L. curvatus* 12 foram purificadas, e apresentaram sequencias sem similaridade com outras bacteriocinas;
- ✓ *L. curvatus* 12 produtor de bacteriocina foi capaz de reduzir a multiplicação da população de *L. monocytogenes* em linguiça frescal potencialmente sujeita à contaminação pelo patógeno durante a estocagem a 7 °C por 10 dias devido a produção de bacteriocina;
- ✓ A bacteriocina semi purificada, em diferentes concentrações, produzida por *L. curvatus* 12 não foi capaz de determinar uma redução significativa de *L. monocytogenes*.