UNIVERSIDADE DE SÃO PAULO – USP
FACULDADE DE CIÊNCIAS FARMACÊUTICAS – FCF
PROGRAMA DE PÓS-GRADUAÇÃO DE CIÊNCIA DOS ALIMENTOS
ÁREA DE BROMATOLOGIA

**Bactérias láticas produtoras de bacteriocinas em salame: isolamento,
caracterização, encapsulação e aplicação no controle de *Listeria
monocytogenes* em salame experimentalmente contaminado**

Matheus de Souza Barbosa

Tese para obtenção do grau de
DOUTOR

Orientadora:
Prof^ª Dr^a Bernadette D. G. M. Franco

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Comissão Julgadora da
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São Paulo, _____ de _____.

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BARBOSA, M. S. **Bactérias lácticas produtoras de bacteriocinas em salame: isolamento, caracterização, encapsulação e aplicação no controle de *Listeria monocytogenes* em salame experimentalmente contaminado.** São Paulo, 2013. [Tese de Doutorado- Faculdade de Ciências Farmacêuticas, Universidade de São Paulo].

RESUMO

A tecnologia da microencapsulação apresenta várias aplicações na indústria de alimentos. Sabendo-se que diferentes fatores intrínsecos e extrínsecos dos alimentos podem influenciar a produção e atividade antimicrobiana das bacteriocinas produzidas pelas bactérias lácticas, este estudo teve como principal objetivo avaliar a funcionalidade da encapsulação de bactérias lácticas (BAL) bacteriocinogênicas em alginato de cálcio no controle de *Listeria monocytogenes* em salame experimentalmente contaminado. Para atingir este objetivo, foram isoladas novas cepas de BAL a partir de salame, que foram identificadas e caracterizadas quanto às propriedades das bacteriocinas produzidas, avaliando-se a influência do processo de encapsulação na produção de bacteriocinas. Foram isoladas quatro cepas produtoras de bacteriocinas, identificadas como *Lactobacillus sakei* (uma cepa), *Lactobacillus curvatus* (duas cepas) e *Lactobacillus plantarum* (uma cepa), nomeadas MBSa1, MBSa2, MBSa3 e MBSa4, respectivamente. As bacteriocinas produzidas pelas quatro cepas foram termoestáveis e com exceção da cepa MBSa2, sensíveis a pH acima de 8. Todas inibiram todas as cepas de *Listeria monocytogenes* testadas e várias espécies de BAL, mas foram inativas contra bactérias Gram negativas. As bacteriocinas foram purificadas por cromatografia de troca iônica seguida de cromatografia de interação hidrofóbica sequencial e cromatografia de fase reversa, observando-se que *L. sakei* MBSa1 produz um peptídeo de 4303 Da, com uma sequência parcial de aminoácidos idêntica à sequência presente em sakacina A. As cepas MBSa2 e MBSa3 produzem dois peptídeos ativos cada, idênticos nas duas cepas, um de 4457 Da e outro de 4360 Da, que apresentam sequências parciais idênticas às presentes na sakacina P e na sakacina X, respectivamente. Aparentemente, a cepa *L. plantarum* MBSa4 produz uma bacteriocina composta por duas sub-unidades. O DNA genômico da cepa *L. sakei* MBSa1 contém os genes da sakacina A e curvacina A, enquanto o DNA da cepa *L. plantarum* MBSa4 foi positivo para o gene da plantaricina W. A cepa *L. curvatus* MBSa2 foi encapsulada em alginato de cálcio e testada quanto à produção de bacteriocinas *in vitro*, observando-se que o processo de encapsulação não influenciou a produção de bacteriocina. Quando testada *in situ*, ou seja, no salame experimentalmente contaminado com *Listeria monocytogenes*, não foi observada ação anti-*Listeria* por *L. curvatus* MBSa2 encapsulado e não encapsulado, durante o 30 dias de fabricação do salame.

Palavras-chave: Bacteriocina, Bactéria láctica, Encapsulação, Salame, *Listeria monocytogenes*.

BARBOSA, M. S. **Bacteriocin-producing Lactic Acid Bacteria in Salami: Isolation, Characterization, Encapsulation and Application for the Control of *Listeria monocytogenes* in Experimentally Contaminated Salami.** São Paulo, 2013. [Thesis (Doctorate Degree)- Faculdade de Ciências Farmacêuticas, Universidade de São Paulo].

ABSTRACT

The microencapsulation technology has several applications in the food industry. Knowing that different intrinsic and extrinsic factors can influence production and antimicrobial activity of bacteriocins produced by lactic acid bacteria in foods, this study aimed at evaluating the functionality of the encapsulation of bacteriocinogenic lactic acid bacteria (LAB) in calcium alginate in the control of *Listeria monocytogenes* in experimentally contaminated salami. To achieve this goal, new strains of LAB were isolated from salami, identified and characterized for the properties of the produced bacteriocins, evaluating the influence of the encapsulation process in the bacteriocins production. Four bacteriocin producing strains were isolated and identified as *Lactobacillus sakei* (one strain), *Lactobacillus curvatus* (two strains) and *Lactobacillus plantarum* (one strain), named MBSa1, MBSa2, MBSa3 and MBSa4 respectively. The bacteriocins produced by the four strains were thermostable and with the exception of strain MBSa2, sensitive to pH above 8. All inhibited all tested *Listeria monocytogenes* strains and various species of LAB but were inactive against Gram-negative bacteria. The bacteriocins were purified by cation-exchange followed by sequential hydrophobic-interaction and reversed-phase chromatography, indicating that *L. sakei* MBSa1 produces a peptide of 4303 Da, with a partial amino acid sequence identical to the sequence present in sakacin A. *L. curvatus* MBSa2 and MBSa3 produce two active peptides, identical in the two strains, one of 4457 Da and the other of 4360 Da, with partial aminoacid sequences identical to those present in sakacin X and sakacin P, respectively. Apparently, *L. plantarum* MBSa4 produces a bacteriocin composed of two subunits. Genomic DNA of *L. sakei* MBSa1 indicated that this strain contains genes for sakacin A and curvacin A, while the DNA of *L. plantarum* MBSa4 was positive for the plantaricin W gene. The strain *L. curvatus* MBSa2 was encapsulated in calcium alginate and tested for bacteriocin production *in vitro*, observing that the encapsulation process did not affect the production of bacteriocin. When tested *in situ*, i.e. in the salami experimentally contaminated with *L. monocytogenes* was not observed anti-*Listeria* action by *L. curvatus* MBSa2 encapsulated and non-encapsulated during the 30 day manufacture of salami.

Key-words: Bacteriocin, Lactic Acid Bacteria, Entrapment, Salami and *Listeria monocytogenes*.

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1. INTRODUÇÃO

A encapsulação pode ser definida como um processo para isolar ou “blindar” uma substância (líquido, sólido ou gás) ou partícula dentro de outra substância, que irá constituir a parede da cápsula (NEDOVIC et al., 2011).

A técnica da encapsulação pode ser aplicada para diversos fins, como por exemplo para proteger substâncias (aromas, antioxidantes, óleos poli-insaturados, vitaminas, fármacos, etc.) ou microrganismos do ambiente que as envolve, liberar as substâncias de forma controlada, diminuir o gosto e odor desagradáveis das substâncias, entre outras aplicações (NEDOVIC et al., 2011; NESTERENKO et al., 2013).

Dependendo do tamanho das cápsulas, a encapsulação pode ser de dois tipos: macroencapsulação e microencapsulação. A macroencapsulação é caracterizada pela formação de cápsulas poliméricas de tamanho variando de alguns milímetros a centímetros. Por outro lado, a microencapsulação produz cápsulas de tamanho variando de 1 a 1000 μm . Como na macroencapsulação há mais dificuldade dos nutrientes difundirem até o centro das cápsulas e também acúmulo de metabolitos tóxicos no interior das cápsulas afetando a viabilidade microbiana, a microencapsulação em cápsulas de tamanhos inferiores a 1000 μm tem sido escolhida para a encapsulação de microrganismos vivos (RATHORE et al., 2013).

A microencapsulação pode ser realizada por vários processos, como por exemplo, “spray-drying”, evaporação do solvente, polimerização em emulsão, extrusão, etc. (NESTERENKO et al., 2013). Muitas substâncias podem ser utilizadas para compor a parede das cápsulas, no entanto, para a aplicação em alimentos estas substâncias devem ser certificadas como “geralmente reconhecida como seguras” (generally

recognized as safe - GRAS) (NEDOVIC et al., 2011). A escolha do processo e da substância encapsuladora para a realização da técnica de microencapsulação dependerá do tamanho das cápsulas que se objetiva, da biocompatibilidade e biodegradabilidade das cápsulas (características físico-químicas) no ambiente ao qual serão expostas e dos custos do processo (NEDOVIC et al., 2011; NESTERENKO et al., 2013).

A tecnologia da microencapsulação apresenta várias aplicações na indústria de alimentos e farmacêutica (NEDOVIC et al., 2011; NESTERENKO et al., 2013). Uma das muitas aplicações é a proteção de bactérias probióticas, visando o aumento da viabilidade das células no trato intestinal e nos alimentos fermentados como iogurtes, queijos, cremes fermentados e doces lácteos (KRASAEKOOPT et al., 2003; ISLAM et al., 2010). Em particular, a encapsulação de probióticos em alginato vem sendo bastante utilizada, pois se trata de um material não tóxico, e, portanto, seguro para utilização em alimentos (DING e SHAH, 2008; COOK et al., 2012). As cápsulas em gel de alginato formam uma barreira entre a célula bacteriana e o ambiente, protegendo-a contra o ambiente desfavorável. A estrutura formada pela encapsulação age ao redor da célula bacteriana como uma parede semipermeável, esférica e fina, que os nutrientes e os metabólitos atravessam facilmente (KAILASAPATHY, 2002; ANAL E SINGH, 2007).

Além do efeito protetor da cápsula de alginato para as bactérias probióticas, alguns estudos mostram que a encapsulação de bactérias lácticas (BAL) neste material também influencia na produção de ácido láctico (ABDEL-RAHMAN et al., 2013). Garbayo et al., (2004) observaram que a produção de ácido láctico por *Streptococcus thermophilus* e *Lactobacillus bulgaricus* co-encapsulados em alginato de cálcio foi influenciada pela concentração de alginato (1–2% p/v) e cloreto de cálcio (0,1–1,5 M), sendo a melhor condição para a produção de ácido láctico por estas cepas, a concentração de 1% (p/v) de alginato em 0.1 M de CaCl₂. Idris e Suzana (2005) reportaram que a

produção de ácido láctico por *L. delbrueckii* subsp. *delbrueckii* ATCC 9646 foi máxima quando a cepa foi encapsulada em alginato de cálcio com uma concentração de 2% de alginato. Resultado semelhante foi relatado por Rao et al. (2008) para *L. delbrueckii* NCIM 2365.

A encapsulação de BAL bacteriocinogênicas em cápsulas de alginato de cálcio com o objetivo de aumentar a produção de bacteriocina tem sido pouco estudada e parece ser dependente das cepas produtoras de bacteriocina. Scannell et al. (2000) observaram que a encapsulação de *Lactococcus lactis* subsp. *lactis* DPC 3147 produtora de lacticina 3147 e *L. lactis* DPC 496 produtora de nisina não aumentou a quantidade de bacteriocinas produzidas, mas aumentou sua estabilidade, quando comparadas com as bacteriocinas produzidas pelas cepas não encapsuladas. O mesmo foi observado por Sarika et al. (2012) para *L. plantarum* MTCC B1746 produtora de plantaricina e *L. lactis* MTCCB440 produtora de nisina. Contudo, Ivanova et al. (2000-2002) reportaram que a produção de bacteriocina por *Enterococcus faecium* A2000 encapsulado foi aproximadamente 50% superior à produzida pela cepa não encapsulada.

Bacteriocinas produzidas por BAL são peptídeos catiônicos, hidrofóbicos, com 20 a 60 resíduos de aminoácidos, ponto isoeletrico elevado, características anfipáticas, sendo sintetizadas nos ribossomos e secretadas pelas bactérias produtoras. As bacteriocinas variam em relação ao espectro de atividade antimicrobiana (estreito ou amplo), modo de ação, massa molecular, origem genética e propriedades bioquímicas. As bacteriocinas podem ser produzidas espontaneamente ou induzidas, sendo as bactérias produtoras imunes a elas devido à produção de proteínas de imunidade específica. A produção de bacteriocinas por bactérias Gram-positivas geralmente ocorre durante o final da fase exponencial, na transição para a fase estacionária-(COTTER et al., 2005; GALVEZ et al., 2008; MILLS et al., 2011; DOBSON et al., 2012; NISHIE et

al., 2012). Atualmente, um grande número de espécies de BAL produtoras de bacteriocina tem sido caracterizadas e descritas na literatura (BALCIUNAS et al., 2013).

As bacteriocinas podem ser utilizadas em alimentos de três formas: (1) adição das BAL produtoras de bacteriocinas diretamente ao alimento; (2) adição das bacteriocinas purificadas ou semi-purificadas e (3) adição de um ingrediente fermentado por cepas bacteriocinogênicas (CHEN e HOOVER, 2003; COTTER et al., 2005; DEEGAN et al., 2006). Um aspecto importante a ser considerado é que para a utilização de bacteriocinas purificadas ou semi-purificadas pelas indústrias de alimentos é necessária a aprovação dos órgãos regulamentadores. Como as BAL são oriundas dos alimentos, e por isso tem status GRAS, a utilização de BAL produtoras de bacteriocinas desperta mais interesse do que a adição de bacteriocinas (VATANYOOPASARN et al., 2011).

Até o momento, as bacteriocinas comerciais de aplicação em alimentos são a nisina, produzida por *Lactococcus lactis* subsp. *lactis* e a pediocina PA-1, produzida por *Pediococcus acidilactici*, comercializadas como Nisaplin™ e ALTA™ 2431, respectivamente (DEEGAN et al., 2006).

A aplicação de nisina em carnes é um assunto bastante controverso. A efetividade da aplicação de nisina na superfície de salsichas, conforme preconizado pelo Ministério da Agricultura do Brasil foi avaliado por CASTRO (2002), que demonstrou que esse procedimento é pouco efetivo no controle de *L. monocytogenes* ou de microrganismos deteriorantes, incluindo psicotróficos e BAL. Por outro lado, Hampikyan e Ugur (2007) observaram que a nisina adicionada à linguiça fermentada nas concentrações de 100 µg.g⁻¹ e 50 µg.g⁻¹ foi capaz de inibir a multiplicação *L. monocytogenes* por 20 e 25 dias, respectivamente.

Listeria monocytogenes é o agente etiológico da listeriose, doença importante para indivíduos imunocomprometidos, mulheres grávidas, idosos, neonatos e pacientes com HIV, podendo causar infecção do Sistema Nervoso Central, bacteremia, endocardite, aborto, parto pré-maturo e septicemia neonatal. Os principais vetores de *L. monocytogenes* são os alimentos, com destaque para a tolerância deste patógeno às altas concentrações de sal e a capacidade de multiplicação em temperaturas de refrigeração, podendo assim proliferar em alimentos mantidos nestas condições (CARPENTIER e CERF, 2011; TODD e NOTTERMANS, 2011; MILILLO et al., 2012).

Listeria monocytogenes possui elevada resistência fisiológica, sendo difícil controlar ou prevenir sua presença em alimentos, principalmente naqueles que não sofrem tratamento térmico. Esta resistência, aliada à capacidade de formar biofilmes nos equipamentos de plantas processadoras de alimentos, torna este microrganismo uma ameaça à indústria (TODD e NOTTERMANS, 2011). A contaminação de linhas processadoras de alimento por *L. monocytogenes* pode acontecer de diferentes maneiras e as boas práticas de higiene e planos de APPCC podem ser insuficientes para o controle ou eliminação do patógeno (TOMPKIN et al., 1999; TOMPKIN, 2002). Além disso, sabe-se que este patógeno pode sobreviver às barreiras tecnológicas encontradas na fabricação de salame, tal como a diminuição do pH e a adição de sal e nitrito. (VORGEL et al., 2010).

Pesquisas realizadas no Brasil com salames comercializados no varejo indicam que *L. monocytogenes* é comum nestes alimentos. Em estudo realizado no estado do Rio de Janeiro, detectou-se que 13,3% das 81 amostras adquiridas no comércio foram positivas. No estado de São Paulo, o patógeno foi detectado em 6,7% (SAKATE et al., 2003) e 6,2% das amostras estudadas (MARTINS & GERMANO, 2011).

O controle de *L. monocytogenes* em alimentos depende da combinação de vários fatores tais como atividade de água, temperatura, pH, e presença de sais, compostos químicos e antimicrobianos naturais. A combinação adequada destes fatores permite criar um ambiente adverso para o patógeno resultando na redução da sua taxa de multiplicação (BOZIARIS et al, 2007). No entanto, segundo Rodgers (2001), a utilização de compostos químicos para a conservação de alimentos não é compatível com a imagem de produtos “frescos”. Além disso, conservantes químicos como nitritos adicionados em alimentos cárneos visando o aumento da segurança e da vida útil, podem levar à formação de nitrosaminas carcinogênicas (CHEN e HOOVER, 2003). Dessa forma, a utilização de BAL produtoras de bacteriocinas tem sido estudada como tecnologia alternativa para o aumento da segurança e da qualidade alimentar (DEEGAN et al., 2006; GALVEZ et al., 2008; JUNEJA et al., 2012).

Dicks et al. (2004) observaram que as cepas *Lactobacillus plantarum* 423, produtora de plantaricina, e *Lactobacillus curvatus* DF126, produtora de curvacina, inibiram a multiplicação de *L. monocytogenes* durante a fermentação de salame de carne de avestruz por 9 dias à uma temperatura entre 16 e 18 °C. Contudo, observou-se que após o décimo dia de incubação, o patógeno voltou a multiplicar-se, atingindo no vigésimo segundo dia as mesmas contagens das amostras não adicionadas das cepas bacteriocinogênicas.

Sabendo-se que fatores intrínsecos e extrínsecos dos alimentos podem influenciar a produção e atividade antimicrobiana das bacteriocinas, a encapsulação de BAL bacteriocinogênicas surge como uma alternativa tecnológica interessante a ser explorada com o objetivo de melhorar o controle de *Listeria monocytogenes* em produtos cárneos.

2. OBJETIVOS

Face ao exposto em relação ao potencial da encapsulação de bactérias lácticas produtoras de bacteriocinas como alternativa tecnológica para melhorar a segurança de produtos cárneos quanto à contaminação por *Listeria monocytogenes*, o presente trabalho teve os seguintes objetivos:

1. Isolar e identificar bactérias lácticas produtoras de bacteriocinas a partir de salame tipo italiano disponível no mercado de São Paulo;
2. Caracterizar as bacteriocinas produzidas pelas bactérias lácticas isoladas;
3. Avaliar o efeito da adição de bacteriocinas semi-purificadas à massa de produção de salame no controle de *Listeria monocytogenes* durante a fabricação do produto;
4. Avaliar a influência da encapsulação de uma cepa selecionada de bactéria láctica bacteriocinogênica em alginato de cálcio na sua sobrevivência e produção de bacteriocinas em condições *in vitro* que simulam as condições ambientais (pH, Aw e temperatura) encontradas durante a fabricação de salame;
5. Avaliar a funcionalidade da cepa bacteriocinogênica selecionada, encapsulada em alginato de cálcio e adicionada à massa de produção de salame, no controle de *Listeria monocytogenes* durante a fabricação do produto.

3. ORGANIZAÇÃO DA TESE DE DOUTORADO

A apresentação desta tese de Doutorado foi dividida em quatro capítulos, preparados na forma de artigos científicos. O **capítulo 1** corresponde a caracterização, purificação e identificação da bacteriocina produzida pela cepa *Lactobacillus sakei* MBSa1 isolada de salame. O **capítulo 2** relata a caracterização inicial e purificação da bacteriocina produzida pela cepa *Lactobacillus plantarum* MBSa4 isolada de salame. No **capítulo 3** descrevem-se os resultados do estudo de caracterização, purificação e identificação de duas bacteriocinas produzidas pelas cepas *Lactobacillus curvatus* MBSa2 e *Lactobacillus curvatus* MBSa3, bem como a aplicação das bacteriocinas produzidas pela cepa MBSa2 para o controle de *Listeria monocytogenes*, durante o processo de fabricação de salame. No **capítulo 4**, são apresentados os resultados da produção de bacteriocinas pela cepa *Lactobacillus curvatus* MBSa2 *in vitro* e durante o processo de fermentação e maturação de salame, quando encapsulada em alginato de cálcio.

4. RESUMO DOS RESULTADOS

4.1 Isolamento e identificação de bactérias láticas produtoras de bacteriocinas a partir de salame tipo italiano disponível no mercado de São Paulo

Das colônias isoladas a partir de salame em agar MRS, foram selecionadas quatro que apresentaram características de BAL, ou seja, eram Gram-positivas e negativas para os testes de KOH 3%, catalase e oxidase, e foram produtoras de substâncias inibidoras de *L. monocytogenes* Scott A. Através da PCR e sequenciamento do gene 16S rDNA, esses isolados foram identificados como *Lactobacillus sakei* (cepa MBSa1), *Lactobacillus curvatus* (cepas MBSa2, MBSa3) e *Lactobacillus plantarum* (MBSa4). As quatro cepas foram igualmente sensíveis ao tratamento com enzimas proteolíticas, comprovando que as substâncias inibidoras produzidas eram de natureza protéica, podendo ser consideradas bacteriocinas.

Estes resultados estão descritos nos artigos referentes aos capítulos 1, 2 e 3.

4.2 Caracterização das bacteriocinas produzidas pelas bactérias láticas isoladas

4.2.1 Avaliação do efeito do pH na atividade antimicrobiana das bacteriocinas

Os resultados da avaliação do efeito do pH na atividade das bacteriocinas presentes nos sobrenadantes livres de células (CFS – *cell free supernatant*) das culturas das quatro cepas isoladas de salame estão apresentados na Tabela 1. O CFS da cepa MBSa 2 foi o único não afetado pelo pH. Os CFS das culturas MBSa1 e MBSa4 apresentaram atividade mais elevada em pH 2,0, 4,0 e 6,0, enquanto o CFS da cultura MBSa 3 apresentou a mesma atividade em pH 2 até 8, mas foi bem mais reduzida em pH 10.

Tabela 1. Atividade dos sobrenadantes das culturas MBSa1, MBSa2, MBSa3 e MBSa4 após exposição a diferentes valores de pH por 1 h a 25° C

pH	Atividade das bacteriocinas (UA.mL ⁻¹)			
	MBSa1	MBSa2	MBSa3	MBSa4
2	400	12800	12800	400
4	400	12800	12800	400
6	400	12800	12800	400
8	100	12800	12800	100
10	100	12800	400	0

4.2.2 Avaliação do efeito da temperatura na atividade antimicrobiana das bacteriocinas

As bacteriocinas produzidas pelas quatro cepas foram afetadas de forma idêntica pelo tratamento térmico, ou seja, mantiveram a mesma atividade (UA.mL⁻¹) após 1 hora a 4° C, 25° C, 30° C, 37° C, 45° C, 60° C e 80° C e 15 min a 121°C.

Estes resultados estão descritos nos artigos referentes aos capítulos 1, 2 e 3.

4.2.3 Avaliação do espectro de ação das bacteriocinas produzidas pelas BAL

4. Resumo dos Resultados

A Tabela 4 apresenta os resultados da inibição de diferentes microrganismos pelas bacteriocinas produzidas pelas cepas isoladas de salame. Todas as cepas de *L. monocytogenes* foram inibidas pelas quatro cepas bacteriocinogênicas isoladas de salame. As bacteriocinas, de uma forma geral, não apresentaram atividade contra cepas comerciais de aplicação tecnológica em alimentos, como por exemplo, *Lactobacillus acidophilus* La5, *Lactobacillus acidophilus* Lac4 e *Lactobacillus acidophilus* La14.

Estes resultados estão descritos nos artigos referentes aos capítulos 1, 2 e 3.

Tabela 2. Espectro de ação das bacteriocinas produzidas pelas cepas *Lactobacillus sakei* MBSa1, *L. curvatus* MBSa2, *L. curvatus* MBSa3 e *L. plantarum* MBSa4 isoladas de salame

Microrganismo alvo*		Diâmetro do halo de inibição (mm)			
		MBSa1	MBSa2	MBSa3	MBSa4
<i>Bacillus cereus</i>	ATCC 1178	0	0	0	0
<i>Staphylococcus aureus</i>	ATCC 29213	0	0	0	7
<i>Staphylococcus aureus</i>	ATCC 25923	0	0	0	0
<i>Staphylococcus aureus</i>	ATCC 6538	0	0	0	0
<i>Listeria welshimeri</i>	USP ¹	9	0	0	7
<i>Listeria seeligeri</i>	USP	0	0	0	0
<i>Listeria ivanovii</i> subsp. <i>ivanovii</i>	ATCC 19119	12	15	16	8
<i>Listeria innocua</i>	ATCC 33090	13	18	21	7
<i>Listeria innocua</i> 225/07 sorovar 6a	FIOCRUZ ²	9	15	16	7
<i>Listeria innocua</i> 224/07 sorovar 6a	FIOCRUZ	8	11	15	8
<i>Listeria innocua</i> 047/07 sorovar 6a	FIOCRUZ	8	15	14	7
<i>Listeria innocua</i> 588/08 sorovar 6a	FIOCRUZ	9	14	11	8
<i>Listeria monocytogenes</i> Scott A	FCF/USP	8	13	13	9
<i>Listeria monocytogenes</i> 602/08 sorovar 1/2a	FIOCRUZ	7	13	13	6
<i>Listeria monocytogenes</i> 046/07 sorovar 1/2c	FIOCRUZ	8	11	14	6
<i>Listeria monocytogenes</i> 103 sorovar 1/2a	USP	9	0	15	6
<i>Listeria monocytogenes</i> 106 sorovar 1/2a	USP	9	13	14	6
<i>Listeria monocytogenes</i> 104 sorovar 1/2a	USP	7	14	15	10
<i>Listeria monocytogenes</i> 409 sorovar 1/2a	USP	8	12	14	9
<i>Listeria monocytogenes</i> 506 sorovar 1/2a	USP	9	14	14	7
<i>Listeria monocytogenes</i> 709 sorovar 1/2a	USP	9	11	12	9
<i>Listeria monocytogenes</i> 607 sorovar 1/2b	USP	6	18	17	8
<i>Listeria monocytogenes</i> 603 sorovar 1/2b	USP	9	10	20	8
<i>Listeria monocytogenes</i> 426 sorovar 1/2b	USP	8	10	14	6
<i>Listeria monocytogenes</i> 637 sorovar 1/2c	USP	9	10	14	6
<i>Listeria monocytogenes</i> 422 sorovar 1/2c	USP	8	12	15	5
<i>Listeria monocytogenes</i> 712 sorovar 1/2c	USP	10	13	15	9
<i>Listeria monocytogenes</i> 408 sorovar 1/2c	USP	9	14	15	7
<i>Listeria monocytogenes</i> 211 sorovar 4b	USP	9	15	16	9

4. Resumo dos Resultados

<i>Listeria monocytogenes</i> 724 sorovar 4b	USP	9	19	16	8
<i>Listeria monocytogenes</i> 101 sorovar 4b	USP	10	18	18	9
<i>Listeria monocytogenes</i> 703 sorovar 4b	USP	9	18	20	8
<i>Listeria monocytogenes</i> 620 sorovar 4b	USP	10	20	20	8
<i>Listeria monocytogenes</i> 302 sorovar 4b	USP	7	15	14	5
<i>Escherichia coli</i>	ATCC 8739	0	0	0	0
<i>Escherichia coli</i> O157:H7	ATCC 35150	0	0	0	0
<i>Enterobacter aerogenes</i>	ATCC 13048	0	0	0	0
<i>Salmonella</i> Typhimurium	ATCC 14028	0	0	0	0
<i>Salmonella</i> Enteritidis	ATCC 13076	0	0	0	0
<i>Enterococcus faecalis</i>	ATCC 12755	10	10	13	11
<i>Enterococcus hirae</i> D105	FCF	8	10	15	12
<i>Enterococcus faecium</i> S5	AGRIS ³	0	0	11	0
<i>Enterococcus faecium</i> S154	AGRIS	0	0	0	0
<i>Enterococcus faecium</i> S100	AGRIS	8	10	10	8
<i>Enterococcus faecium</i> ST62	AGRIS	0	0	0	0
<i>Enterococcus faecium</i> ST211	AGRIS	0	0	0	0
<i>Enterococcus faecium</i> ET 12	UCV ⁴	0	0	0	0
<i>Enterococcus faecium</i> ET 88	UCV	0	0	0	0
<i>Enterococcus faecium</i> ET 05	UCV	0	0	0	0
<i>Lactococcus lactis</i> V94	USP	0	10	0	0
<i>Lactobacillus fermentum</i> ET35	UCV	0	0	0	0
<i>Pediococcus pentosaceus</i> ET 34	UCV	0	0	0	0
<i>Lactobacillus curvatus</i> ET 06	UCV	0	0	9	0
<i>Lactobacillus curvatus</i> ET 31	UCV	0	0	0	0
<i>Lactobacillus curvatus</i> ET 30	UCV	0	0	0	0
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> 2a	USP	0	0	0	0
<i>Lactobacillus sakei</i>	ATCC 15521	9	10	11	8
<i>Lactobacillus plantarum</i> V69	USP	0	0	0	0
<i>Lactobacillus delbrueckii</i> B5	USP	0	0	0	0
<i>Lactobacillus delbrueckii</i> ET32	UCV	0	0	0	0
<i>Lactobacillus acidophilus</i> La14	Rhodia	0	0	0	0
<i>Lactobacillus acidophilus</i> Lac4	Rhodia	0	0	0	0
<i>Lactobacillus acidophilus</i> La5	Rhodia	0	0	0	0
<i>Lactococcus lactis</i> B16	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> MK02R	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D2	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B1	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D4	USP	0	0	10	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B2	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B15	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D3	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D5	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B17	USP	0	0	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> R704	Chr. Hansen	0	0	0	0

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4.2.4 Purificação das bacteriocinas

Os cromatogramas apresentados na Figura 1 indicam que a metodologia utilizada para a purificação da bacteriocina produzida pela cepa *L. sakei* MBSa1, isto é, cromatografia de troca iônica seguida de cromatografia de interação hidrofóbica sequencial e cromatografia de fase reversa, foi eficaz para a obtenção de um produto puro, com a formação de somente um pico durante a eluição das frações aderidas à coluna utilizada, correspondentes ao gradiente em que bacteriocinas são eluídas. No caso das cepas *L. curvatus* MBSa2 e *L. curvatus* MBSa3 (Figuras 1b e 1c), foram detectados vários picos, sendo que dois, denominados P1 e P2, apresentaram atividade antimicrobiana. O cromatograma referente à cepa *L. plantarum* MBSa4 é mostrado na Figura 1d, com formação de 14 picos. A atividade do material de cada um destes picos contra *L. ivanovii* está apresentado na Figura 2a, onde pode ser observado que somente o material correspondente ao pico P9 apresentou uma clara atividade antimicrobiana. Observou-se também que o material correspondente ao pico 10 apresentou inibição parcial quando testado próximo do P9, sugerindo um efeito sinérgico entre esses dois materiais. Para confirmar este fato, o material do pico P9 foi misturado na proporção 1;1 com os materiais de todos os demais picos presentes e testado quanto à atividade antimicrobiana. Os resultados deste teste são apresentados na Figura 2b, onde pode ser observado que os materiais referentes aos picos P10, P11 e P12 passaram a ter atividade.

4. Resumo dos Resultados

Figura 1. Cromatogramas referentes à terceira etapa de purificação (C_{18} HPLC fase reversa) das bacteriocinas produzidas por *Lactobacillus sakei* MBSa1 (a), *L. curvatus* MBSa2 (b), *L. curvatus* MBSa3 (c) e *L. plantarum* MBSa4 (d).

Figura 2. Atividade anti-*Listeria* das frações após a última etapa da purificação (C_{18} HPLC fase reversa) da bacteriocina produzida por *Lactobacillus plantarum* MBSa4 (a) e quando as frações foram combinadas (1:1) com a fração 9 (b).

4. Resumo dos Resultados

A eficácia de cada etapa da purificação (rendimento, atividade específica e fator de purificação) das bacteriocinas produzidas pelas cepas MBSa1, MBSa2 e MBSa3 está resumida na Tabela 3. A purificação da bacteriocina produzida pela cepa *L. plantarum* MBSa4 deu resultados muito baixos, devido à pouca quantidade de bacteriocina produzida e à rápida perda de atividade, não sendo possível calcular a atividade específica.

Tabela 3. Purificação das bacteriocinas produzidas por *Lactobacillus sakei* MBSa1, *Lactobacillus curvatus* MBSa2 e *Lactobacillus curvatus* MBSa3.

Etapa da Purificação	Volume (mL)	Atividade (UA/mL)	Proteína (mg/mL)	Atividade específica (UA/mg)	Fator de Purificação	Rendimento (%)
MBSa1						
Sobrenadante	400	6400	3,42	1871,34	1,00	100
Troca catiônica	190	3200	1,86	1720,43	0,92	23,75
Fase reversa	70	12800	1,96	6530,61	3,49	35
C ₁₈ HPLC-FR	1	819200	10,93	74949,67	40,05	32
MBSa2						
Sobrenadante	200	800	3,10	257,65	1,00	100
Troca catiônica	700	200	2,46	81,20	0,31	87,5
Fase reversa	20	6400	2,54	2519,56	9,78	80
C ₁₈ HPLC- P1	2	16000	2,18	7353,19	28,54	20
FR P2	2	8000	1,89	4242,23	16,46	10
MBSa3						
Sobrenadante	200	800	4,41	181,26	1,00	100
Troca catiônica	700	200	1,93	103,85	0,57	87,5
Fase reversa	20	6400	2,32	2753,78	15,19	80
C ₁₈ HPLC- P1	2	16000	2,14	7491,33	41,33	20
FR P2	2	8000	1,88	4263,16	23,52	10

Os resultados do sequenciamento de aminoácidos e da identificação e determinação do peso molecular das bacteriocinas produzidas pelas cepas *L. sakei* MBSa1, *L. curvatus* MBSa2 e *L. curvatus* MBSa3 são apresentados na Tabela 4. A cepa *L. sakei* MBSa1 produz uma bacteriocina com peso molecular de 4303 Da, com uma

sequencia de aminoácidos em sua região C-terminal idêntica a uma parte da região C-terminal da sakacina A descrita por Holck et al.,1992.

Tabela 4. Sequencia dos aminoácidos e peso molecular das bacteriocinas produzidas pelas cepas de *Lactobacillus sakei* MBSa1, *Lactobacillus curvatus* MBSa2 e *Lactobacillus curvatus* MBSa3.

Cepa	Sequencia dos aminoácidos	Peso molecular (Da)	Bacteriocina
MBSa1	SIIGGMISGWASGLAG	4303	Sakacina A
MBSa2	P1 AAANWATGGNAG	4457	Sakacin P
	AGNSSNFLHKLQQLFT	2228	Proteína sinal
	P2 AVANLTTGGAGG	4360	Sakacin X
MBSa3	P1 AAANWATGGNAG	4457	Sakacin P
	AGNSSNFLHKLQQLFT	2228	Proteína sinal
	P2 AVANLTTGGAGG	4360	Sakacin X

As cepas MBSa2 e MBSa3 produzem dois compostos ativos (P1 e P2), com tempo de retenção distintos (Figura 1b e 1c). A espectrometria de massa do pico P1 das cepas MBSa2 e MBSa3 indicou tratar-se de dois peptídeos diferentes, sendo um peptídeo de 4457 Da com atividade anti-*Listeria* e um segundo peptídeo de 2228 Da não ativo. O sequenciamento dos aminoácidos destes peptídeos indicou que o peptídeo de 4457 Da corresponde à sakacina P e que o peptídeo de 2228 Da corresponde à proteína sinal que age como um fator de indução de bacteriocina na célula produtora.

A espectrometria de massa e sequenciamento dos aminoácidos revelaram que os peptídeos P2 produzidos pelas cepas MBSa2 e MBSa3 são idênticos, com 4360 Da e a sequencia AVANLTTGGAGG, também presente na sakacina X (Tabela 4).

Estes resultados estão descritos nos artigos referentes aos capítulos 1, 2 e 3.

4.2.5 Pesquisa de genes das bacteriocinas

Os resultados da amplificação dos genes de bacteriocinas investigados no DNA genômico das quatro cepas de BAL bacteriocinogênicas estão apresentados na Figura 3. Verificou-se que ao empregar os primers específicos para os genes de curvacina A (CurA-F/CurA-R) e sakacina A (SakA-F/SakA-R), houve amplificação de um fragmento de aproximadamente 171 pb e 150 pb no DNA genômico da cepa MBSa1, respectivamente (Figura 3a e 3b). A Figura 3c mostra que, ao utilizar o primer específico para sakacina P (SakP-F/SakP-R), houve amplificação de fragmento de 186 pb nos DNA genômicos das cepas MBSa2 e MBSa3. Empregando-se os primers PlanW-F e PlanW-R, específicos para plantaricina W, houve a amplificação de um fragmento de aproximadamente 165 pb no DNA genômico da cepa MBSa4 (Figura 3d).

Estes resultados estão descritos nos artigos referentes aos capítulos 1, 2 e 3.

Figura 3. Produtos da amplificação do DNA genômico de *Lactobacillus sakei* MBSa1, *Lactobacillus curvatus* MBSa2, *Lactobacillus curvatus* MBSa3 e *Lactobacillus plantarum* MBSa4 por PCR com primers para os genes de curvacina A (a), sakacina A (b), sakacina P (c) e plantaricina W (d). Linha M, Marcador de peso molecular (100 pb); linha A, controle negativo (água ultra purificada).

4.3 Avaliação do efeito da adição de bacteriocinas semi-purificadas à massa de produção de salame no controle de *Listeria monocytogenes* durante a fabricação do produto

Os resultados da ação anti-*Listeria* da bacteriocina MBSa2 semi-purificada, quando aplicada na massa de produção de salame são apresentados na Figura 4. Uma redução de aproximadamente 0,5 Log UFC.g⁻¹ na população do patógeno no tempo zero da produção do salame foi observado para a amostra adicionada da bacteriocina. Ao longo dos 30 dias de produção do salame, um menor número populacional da *L. monocytogenes* nas amostras contendo bacteriocina foi observado quando comparado com as amostras sem a adição de bacteriocina.

Estes resultados estão descritos no artigo referente ao capítulo 2.

Figura 4. Contagem de *Listeria monocytogenes* em salame contendo bacteriocina produzida por *Lactobacillus curvatus* MBSa 2(●-), em salame contendo água esterilizada (-■-) e em salame contendo somente *Listeria monocytogenes* (-▲-).

4.4. Avaliação da influência da encapsulação da cepa *Lactobacillus curvatus* MBSa2 em alginato de cálcio na sua sobrevivência e produção de bacteriocinas em condições *in vitro* que simulam as condições ambientais (pH, Aw e temperatura) encontradas durante a fabricação de salame

A Figura 5 apresenta os resultados da enumeração ($\log.UFC.mL^{-1}$) e produção de bacteriocina ($UA.mL^{-1}$) pela cepa *Lactobacillus curvatus* MBSa2, antes e após a encapsulação em alginato de cálcio. Os resultados mostram que o processo de encapsulação pode gerar uma perda de aproximadamente $2 \log.UFC.mL^{-1}$ na população de *L. curvatus* MBSa2, contudo o fato da BAL estar imobilizada em cápsulas de alginato não interferiu na produção de bacteriocina.

Figura 5. Sobrevivência (barra cinza) e produção de bacteriocina (barra preta) por *Lactobacillus curvatus* MBSa2, antes (livre) e depois (encapsulado) do processo de encapsulação.

Os resultados da sobrevivência e produção de bacteriocina por *Lactobacillus curvatus* MBSa2 livre e encapsulado com cápsula de tamanho de 266 μm e 473 μm de diâmetro, ao longo de 14 dias de incubação a 24° C e 18° C em caldo MRS, em diferentes valores pH e atividades de água são apresentados na Figura 6, Figura 7 e Figura 8, respectivamente. Não foram observadas melhoras na sobrevivência ou na produção de bacteriocina por *L. curvatus*, quando encapsulada em alginato de cálcio nas diferentes condições estudadas, com exceção da BAL em caldo MRS com valor de atividade de água ajustada para 0,97.

Estes resultados estão descritos no artigo referente ao capítulo 4.

4. Resumo dos Resultados

Figura 6. Sobrevivência e produção de bacteriocina por *Lactobacillus curvatus* MBSa2 livre e encapsulado em alginato de cálcio, durante armazenamento a 24 °C e 18 °C por 14 dias em caldo MRS.

4. Resumo dos Resultados

Figura 7. Sobrevivência e produção de bacteriocina por *Lactobacillus curvatus* MBSa2 livre e encapsulado em alginato de cálcio, durante armazenamento a 30 °C por 14 dias em caldo MRS com pH ajustado para 6, 5,5 e 5.

4. Resumo dos Resultados

Figura 8. Sobrevivência e produção de bacteriocina por *Lactobacillus curvatus* MBSa2 livre e encapsulado em alginato de cálcio, durante armazenamento a 30 °C por 14 dias em caldo MRS com valores de atividade de água ajustado para 0,97, 0,90 e 0,85.

4.5 Avaliação da funcionalidade da cepa *Lactobacillus curvatus* MBSa2, encapsulada em alginato de calcio e adicionada à massa de produção de salame, no controle de *Listeria monocytogenes* durante a fabricação do produto.

Na Figura 9 estão apresentados os resultados da enumeração de *Lactobacillus curvatus* MBSa2 ($\log \text{UFC.mL}^{-1}$) (livre e encapsulado) durante a fabricação de salame no controle de *Listeria monocytogenes*. Os resultados indicam que as contagens mantiveram-se praticamente as mesmas em todo o tempo estudado, indicando que a cepa em estudo sobrevive bem no salame ao longo de sua fabricação.

Figura 9. Enumeração de *Lactobacillus curvatus* MBSa2 livre (MBSa2 L) e encapsulado (MBSa2 E) em salame com e sem *L. monocytogenes* (LM), durante 30 dias de fabricação do produto.

Na Figura 10 estão apresentados os resultados das contagens de *Listeria monocytogenes* (log UFC.mL⁻¹) no salame contendo a cepa *Lactobacillus curvatus* MBSa2 (livre e encapsulado) bacteriocinogênica, durante 30 dias de fabricação do salame. Verificou-se que as contagens do patógenos foram as mesmas ($p>0,05$) quando em presença de *Lactobacillus curvatus* MBSa2 livre ou encapsulado ao longo do tempo estudado.

Figura 10. Enumeração de *Listeria monocytogenes* (LM) em salame adicionado de *Lactobacillus curvatus* MBSa2 livre (MBSa2 L) e encapsulado (MBSa2 E) durante 30 dias de fabricação do produto.

Quanto ao pH dos salames estudados ao longo dos 30 dias de fabricação, verificou-se que todos os valores mensurados foram independentes das culturas microbianas presentes. Do valor médio de 5,95 na mistura inicial de ingredientes, o pH

no 4º dia de fabricação abaixou para o valor médio de 5,19, subindo novamente em seguida, atingindo o valor médio de 5,44 no 30º dia. Em relação à A_w , verificou-se que houve uma gradativa queda no valor, independentemente das culturas microbianas presentes, passando de uma média de 0,98 na mistura de ingredientes, para uma média de 0,89 no 30º dia de fabricação.

Estes resultados estão descritos no artigo referente ao capítulo 4.

5. CONCLUSÃO

Por meio dos resultados obtidos pelo presente trabalho, é possível concluir que as quatro cepas BAL isoladas do salame, identificadas como *Lactobacillus sakei* MBSa1, *Lactobacillus curvatus* MBSa2, *Lactobacillus curvatus* MBSa3 e *Lactobacillus plantarum* MBSa4, são produtoras de bacteriocinas, sendo que a cepa MBSa1 produz sakacina A, as cepas MBSa2 e MBSa3 produzem sakacina P e sakacina X e a cepa MBSa4 produz uma bacteriocina composta por duas sub-unidades e apresenta em seu DNA genômico a sequência da bacteriocina plantaricina W. O processo de encapsulação em alginato de cálcio não influenciou negativamente na produção de bacteriocina pela cepa *L. curvatus* MBSa2 em meio de cultura. Em salame, o encapsulamento da cepa *L. curvatus* MBSa2 em alginato de cálcio não melhorou seu desempenho em relação ao controle de *Listeria monocytogenes* no produto durante a sua fabricação.

Capítulo 01

“Purification and characterization of the bacteriocin produced by *Lactobacillus sakei*
MBSa1 isolated from Brazilian salami”

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Purification and characterization of the bacteriocin produced by *Lactobacillus sakei* MBSa1 isolated from Brazilian salami

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Abstract

Aims: The study aimed at determining the biochemical characteristics of the bacteriocin produced by *Lactobacillus sakei* MBSa1, isolated from salami, correlating the results with the genetic features of the producer strain.

Methods and Results: Identification of strain MBSa1 was done by 16S rDNA sequencing. The bacteriocin was tested for spectrum of activity, heat and pH stability, mechanism of action, and molecular mass and amino acid sequence when purified by cation-exchange and reversed phase HPLC. Genomic DNA was tested for bacteriocin genes commonly present in *L. sakei*. Bacteriocin MBSa1 was heat-stable, unaffected by pH 2.0 to 6.0 and active against all tested *Listeria monocytogenes* strains. Maximal production of bacteriocin MBSa1 (1600 AU ml⁻¹) in MRS broth occurred after 20 h at 25 °C. The molecular mass of produced bacteriocin was 4303.3 Da and the molecule contained the SIIGGMISGWAASGLAG sequence, also present in sakacin A. The studied strain carried the genes for sakacin A and curvacin A.

Conclusions: Under studied conditions, *L. sakei* MBSa1 produced sakacin A, a class II bacteriocin, with remarkable anti-*Listeria* activity.

Significance and Impact of Study: The study covers essential aspects of the characterization of bacteriocins: purification, determination of molecular mass, amino acid sequencing and identification of the gene(s) involved in the production.

Key-words: *Lactobacillus sakei*, bacteriocin, *Listeria monocytogenes*, salami.

Introduction

Several preservation technologies can be used to ensure that foods maintain an acceptable level of quality from manufacture until consumption (Zhou *et al.* 2010). Fermentation is a millennial process used to extend the shelf-life of easily perishable products such as raw meat (Rantsiou and Cocolin 2006). The manufacturing process of many meat products includes a fermentation step, performed under conditions that inhibit the growth of several spoilage and pathogenic bacteria. However, few pathogens, such as *Listeria monocytogenes*, can survive in fermented products and become a health hazard (Thévenot *et al.* 2005).

The use of natural antimicrobials as food preservatives is receiving increased attention, since they are a promising tool for improvement of food safety and may replace or reduce the use of chemical additives (Deegan *et al.* 2006; Gálvez *et al.* 2007; Juneja *et al.*, 2012). Among these antimicrobial compounds, bacteriocins produced by lactic acid bacteria (LAB) that target pathogenic bacteria without toxic or other adverse effects for consumers are under intensive investigation (de Vuyst and Leroy 2007; Mills *et al.* 2011; Dobson *et al.* 2012; O'Shea *et al.* 2013). Many bacteriocins produced by LAB were already described and they vary in spectrum of their activities (narrow or broad), modes of action, molecular masses and genetic and biochemical properties (Mills *et al.* 2011; Dobson *et al.* 2012; Nishie *et al.*, 2012).

Fermented sausages contain many species of LAB and several studies have shown that some of them may produce bacteriocins (Table 1). However, to the best of our knowledge there is no report on the occurrence of such type of LAB in similar fermented meat products in Brazil. This survey aimed at isolating bacteriocin-producing LAB strains in salami samples collected on the Brazilian market, and determining the

biochemical characteristics of the bacteriocin produced by the isolate *Lactobacillus sakei* MBSa1, correlating the results with the genetic features of the producer strain.

Material and Methods

Search for LAB with anti-Listeria activity in salami

Salami samples (50 g), collected on local markets in the city of Sao Paulo (Brazil), were homogenized in a stomacher (Seward 400, London, UK) with 450 ml of 0.1% sterile peptone water (Difco, Detroit, MI, USA) and submitted to subsequent decimal dilutions in 0.1% sterile peptone water (Difco). Each dilution was plated on MRS agar (Oxoid) in duplicates and incubated 48 h at 30 °C. Growing colonies were randomly selected and tested for inhibitory activity against *Listeria monocytogenes* Scott A by the triple-layer method (Todorov and Dicks, 2005). In this method, plates of MRS agar presenting isolated colonies are overlaid with approximately 5 ml of semi-solid BHI medium [BHI broth (Oxoid) supplemented with 0.75% bacteriologic agar (Oxoid)] containing *L. monocytogenes* Scott A (10^5 - 10^6 CFU ml⁻¹) and incubated for 24 h at 37 °C. Colonies presenting growth inhibition zones around them were transferred to MRS broth (Difco), incubated for 24 h at 30 °C and then plated on MRS agar (Oxoid) and incubated for 24 h at 30 °C. Isolated colonies were submitted to Gram staining, and tested for catalase production using 3% hydrogen peroxide (v/v). Gram-positive and catalase-negative cultures presenting anti-*Listeria* activity were freeze-dried and stored at -20 °C.

Strains presenting anti-*Listeria* activity were grown in MRS broth (Difco) for 24 h at 30 °C and submitted to centrifugation at 4000 x g for 15 min at 4 °C (Hettich Zentrifugen, model Mikro 22R, Tuttlingen, Germany). The pH of the obtained cell-free supernatant (CFS) was adjusted to 6.0-6.5 with 1 mol l⁻¹ NaOH (Synth, Sao Paulo, Brazil), heated 30 min at 70 °C and sterilized by filtration (Millex GV 0.22 µm

[Millipore, Billerica, MA, USA]). Anti-*Listeria* activity of the CFS was tested by the spot-on-the-lawn method (van Reenen *et al.* 1998) with modifications. An aliquot of 10 μ l of CFS was spotted onto the surface of a plate containing 10-12 ml of 1.5% bacteriologic agar (Difco), overlaid with 5 ml of BHI semi-solid agar (BHI broth [Oxoid] added of 0.85% [w/v] bacteriological agar [Oxoid]) containing *L. monocytogenes* Scott A (10^5 - 10^6 CFU ml^{-1}). The plates were incubated at 37 °C for 12 h and observed for the formation of clear zones of inhibition around the spotted CFS. Bacteriocin production was confirmed by testing the proteinaceous nature of the antimicrobial compound. For this test, the CFS was treated (1 h at 37 °C) with the following proteolytic enzymes (0.1 mg ml^{-1}): α -chymotrypsin from bovine pancreas type II, *Streptomyces griseus* protease type XIV, trypsin and proteinase K (all from Sigma-Aldrich, St. Louis, MO, USA) solubilized in 20 mmol l^{-1} phosphate buffer pH 7 (Noonpakdee *et al.* 2003). After treatment, CFS was heated at 90 °C for 5 min for enzyme inactivation and tested for residual antimicrobial activity by the spot-on-the-lawn method (van Reenen *et al.* 1998). Absence of zone of inhibition after enzymatic treatment indicated the presence of bacteriocin(s). Control tests with non-treated CFS were also performed.

Identification of bacteriocin-producing LAB isolates

Bacteriocin-producing LAB isolated from the salami samples were submitted to 16S rDNA sequence analysis, by amplification of genomic DNA with primers 8f (5'-CAC GGA TCC AGA CTT TGA T(C/T)(A/C) TGG CTC AG-3') and 1512r (5'- GTG AAG CTT ACG G(C/T)T AGC TTG TTA CGA CTT-3') as described by Felske *et al.* (1997). The 20 μ l reaction volume contained 100 pmol l^{-1} each primer, 1x PCR buffer (New England BioLabs, Ipswich, MA, USA), 24 μ mol l^{-1} dNTP (Fermentas, Hanover,

MD, USA), 2 mmol l⁻¹ MgCl₂ (Fermentas) and 0.0125 U *Taq* DNA polymerase (New England BioLabs). Amplification was carried out in a DNA MasterCycler® (Eppendorf Scientific, Hamburg, Germany). PCR conditions included denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 10 s, primer annealing at 61 °C for 20 s, polymerization at 68 °C for 2 min and then at 72 °C for 7 min. PCR-amplified DNA fragments were separated by 0.8% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide (0.1 mg ml⁻¹). Fluorescent bands of approximately 831 bp were made visible using an UVP BioImaging System (DIGIDOC-IT System, Upland, CA, USA). The bands were purified with QIAquick® PCR Purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and submitted to amino acid sequencing at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil. The sequences were compared to those deposited in GenBank, using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>). The identifications of species were confirmed by species-specific PCR amplification assays as described by Berthier and Ehrlich (1998), using primers Ls-F (ATG AAA CTA TTA AAT TGG TA) and Ls-R (GCT GGA TCA CCT CCT TTC C). The PCR reactions were performed with 1x PCR buffer (New England BioLabs), 25 µmol l⁻¹ dNTP (Fermentas), 100 µmol l⁻¹ MgCl₂ (Fermentas) and 0.025 U *Taq* DNA polymerase (New England BioLabs). PCR conditions were: denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 30 s, polymerization at 72 °C for 1 min and a final polymerization at 72 °C for 5 min. PCR-amplified DNA fragments were separated by 2% (w/v) agarose gel electrophoresis and visualized by treatment with ethidium bromide (0.1 mg ml⁻¹) and made visible by using an UVP BioImaging System (DIGIDOC-IT System). Strain MBSa1, identified as *Lactobacillus sakei*, presented a

good anti-*Listeria* activity and therefore was selected for genetic and biochemical characterization of the bacteriocin.

Titration of the bacteriocin produced by strain MBSa1

The amount of bacteriocin produced by strain MBSa1 was determined using two-fold dilutions and the spot-on-the-lawn method described by van Reenen *et al.* (1998). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that resulted in production of a clear zone of inhibition of *L. monocytogenes* Scott A. Results were expressed in AU ml⁻¹ (Kaiser and Montville, 1996; van Reenen *et al.* 1998).

Effect of pH and temperature on activity of bacteriocin MBSa1

The effect of pH and temperature on activity of bacteriocin MBSa1 was determined as described by Albano *et al.* (2007). The pH of the CFS was adjusted to 2.0, 4.0, 6.0, 8.0 and 10.0 with concentrated phosphoric acid (Synth) or 1 mol l⁻¹ NaOH (Synth) and tested for activity against *L. monocytogenes* Scott A after 1 h at 25 °C. For the antilisterial tests, the pH of the CFS was adjusted to 6.0-6.5 with 1 mol l⁻¹ NaOH (Synth) or concentrated phosphoric acid (Synth). The effect of temperature on the activity of the bacteriocin was evaluated by keeping the CFS at 4, 25, 30, 37, 45, 60, 80 and 100 °C for 60 min and at 121 °C for 15 min and then testing for activity against *L. monocytogenes* Scott A.

Spectrum of activity of bacteriocin MBSa1

The antimicrobial activity of the CFS containing the bacteriocin produced by strain MBSa1 was determined against a variety of Gram-negative and Gram-positive bacteria isolated from foods, listed in Table 2. For testing, lactobacilli and enterococci were

grown in MRS broth (Difco) at 30 °C for 24 h and the other strains were grown in BHI broth (Oxoid) at 37 °C for 24 h. The spot-on-the lawn test (van Reenen *et al.* 1998) was used in this determination.

Effect of temperature on growth and bacteriocin production by strain MBSa1

Growth and production of bacteriocin by strain MBSa1 in MRS Broth (Difco) were evaluated at 25 °C, 30 °C and 37 °C. Growth was monitored at every 2 h up to 24 h, measuring absorbance at 600 nm (Ultrospec 2000; Pharmacia Biotech, Little Chalfont, UK). The anti-*Listeria* activity in the CFS was monitored by the spot-on-the-lawn method, using *L. monocytogenes* Scott A as indicator of activity (van Reenen *et al.* 1998).

Search for bacteriocin genes

The MBSa1 strain was investigated for the presence of known sakacin and curvacin A genes using PCR and the primers listed in Table 3. Total DNA was extracted and submitted to amplification in a reaction mixture (20 µl) containing approximately 25 ng µl⁻¹ of extracted DNA, 1x PCR buffer (New England BioLabs), 100 µmol l⁻¹ MgCl₂ (Fermentas), 200 µmol l⁻¹ dNTPs (Fermentas), 0.025 U *Taq* polymerase (New England BioLabs) and 1 pmol l⁻¹ each primer. Amplification was achieved in 35 cycles using a DNA thermocycler MasterCycler[®] PCR (Eppendorf Scientific). PCR conditions are show in Table 3. PCR-amplified DNA fragments were separated by 2% (w/v) agarose gel electrophoresis, stained with ethidium bromide (0.1 mg ml⁻¹) and observed using the UVP BioImaging System (DIGIDOC-IT System). For each primer, the corresponding bands (sizes described in Table 3) were purified with QIAquick[®] PCR Purification kit (Qiagen) according to the manufacturer's instructions and submitted to sequencing at the

Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil. The sequences were compared to those deposited in GenBank, using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>).

Purification of bacteriocin MBSa1

Bacteriocin MBSa1 was purified according to Batdorj *et al.* (2006), with modifications. MRS broth (Biokar, Beauvais, France) was inoculated with a 1% (v/v) overnight culture of MBSa1 strain and after 18 h at 25 °C, cells were removed by centrifugation at 6000 x g for 15 min at 4 °C (Centrifuge GR 2022, Jouan, France). The pH of the CFS was adjusted to 6.8 with 10 mol l⁻¹ NaOH (Euromedex, Souffelweyersheim, France) and loaded into a SP-Sepharose Fast Flow cation-exchange column (GE Healthcare, Amersham, Uppsala, Sweden) equilibrated with 20 mmol l⁻¹ phosphate (Sigma-Aldrich) buffer pH 6.8 (buffer A). The column was washed with buffer A and the absorbed substances were eluted with a linear gradient from 0 to 100% buffer B (20 mmol l⁻¹ sodium phosphate + 1 mol l⁻¹ NaCl [Euromedex] pH 6.8). The fractions were collected and tested for anti-*Listeria* activity using the spot-on-the-lawn test, and *L. ivanovii* subsp. *ivanovii* ATCC 19119 as indicator of activity.

Active fractions were pooled and loaded into a reversed phase (RP) column (SOURCE™15RPC 10 ml; GE Healthcare) equilibrated with solvent A [0.05% trifluoroacetic acid (TFA) (Sigma-Aldrich), 95% H₂O and 5% solvent B (80% acetonitrile (Biosolve, Valkenswaard, Netherlands), 10% isopropanol (Sigma-Aldrich), 10% H₂O, 0.03% TFA)]. Elution was performed with solvent B with a linear gradient from 0 to 100% for 25 min, at a flow rate of 5 ml min⁻¹. After drying under reduced pressure (Speed-Vac, SC110A, Savant, Holbrook, NY, USA), each fraction was tested for anti-*Listeria* activity using the spot-on-the lawn test, using *L. ivanovii* subsp.

invanovii ATCC 19119 as indicator strain. Fractions presenting activity were pooled and submitted to another purification step by RP-high performance liquid chromatography (RP-HPLC) using Unicorn 3.21 software (Amersham Pharmacia Biotech). The pool was loaded into a preparative C₁₈ column (Symmetry 300™ C₁₈, 5 µm 4·6 x 50 mm Waters, Hertfordshire, UK) equilibrated with solvent C (0·05% TFA, 5% solvent D [80% acetonitrile, 20% H₂O, 0·03 % TFA], 95% H₂O). Elution was performed with solvent D using a linear gradient from 25% to 60% in 35 min, at a flow rate of 6 ml min⁻¹. Peaks were detected by monitoring absorbance at 220 nm. Fractions were collected, dried under vacuum, dissolved in sterile ultra-pure water (Milli-Q, Millipore, Billerica, MA, USA) and tested for anti-*Listeria* activity. The protein concentration in this material, corresponding to purified bacteriocin MBSa1, was measured in microtiter plates using Pierce® BCA protein assay kit (Thermo Fisher Scientific, Schwerte, Germany), with albumin (Sigma-Aldrich) as standard.

The molecular mass of the purified bacteriocin MBSa1 was determined in a quadrupole-time-of-flight hybrid mass spectrometer (Q-TOF Global, Waters), equipped with an electrospray ionization (ESI) source and operated in the positive ion mode. Fractions collected from the HPLC chromatography were diluted in a mixture of water and acetonitrile (1:1, v/v) acidified with 0·1% formic acid, and infused into the mass spectrometer at a continuous flow rate of 5 µl min⁻¹. Following parent mass determination, ions were fragmented in the collision cell of the mass spectrometer and the obtained MS/MS spectra were interpreted to reconstruct the sequence tag of the peptide. This tag was further searched against NCBI databank using the BLAST software.

Test for disulfide bonds in bacteriocin MBSa1 activity

The presence of disulfide bonds in bacteriocin MBSa1 was checked according to Joerger and Klaenhammer (1986) with modifications. The dried purified bacteriocin MBSa1 was resuspended in 50 mmol l⁻¹ Tris-HCl buffer pH 8.0 and divided in four portions of 100 ml: to the first an aqueous solution of 100 mmol l⁻¹ dithiothreitol (DTT) (Sigma-Aldrich) was added, trypsin (0.1 mg ml⁻¹) was added to the second, proteinase K (0.1 mg ml⁻¹) (controls of proteic character of the studied substance) was added to the third, and the last portion was used as positive control. The mixtures were incubated 1 h at 37 °C and checked for anti-*Listeria* activity by the agar diffusion method.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Killing Concentration (MKC) of the purified bacteriocin MBSa1

MIC was determined as described by Nielsen *et al.* (1990) with modifications. The dried purified bacteriocin MBSa1 was re suspended in 50 mmol l⁻¹ Tris-HCl buffer pH 8.0 and submitted to serial two-fold dilutions in 96-well microtiter-plates (TPP, Trasadingen, Switzerland) containing 100 µl of BHI broth (Oxoid) in each well. In the next step, 20 µl of an overnight culture of *L. monocytogenes* Scott A obtained in BHI broth at 37 °C were added to each well, achieving 10²-10³ CFU ml⁻¹ in the wells. For determination of MIC, the microtiter-plates were incubated 24 h at 37 °C and observed for turbidity in the wells. For determination of MKC, the content of each well was plated on TSA-YE agar plates and checked for growth of colonies. MIC was recorded as the lowest concentration of bacteriocin that resulted in absence of turbidity in the well and MKC was recorded as the lowest concentration of bacteriocin that resulted in absence of growth of *L. monocytogenes* Scott A in the TSA-YE agar plates in 24 h.

In vitro anti-Listeria activity of the purified bacteriocin MBSa1

The anti-*Listeria* activity of the purified bacteriocin MBSa1 was tested according to Todorov and Dicks (2004). A 24 h culture of *L. monocytogenes* Scott A in BHI broth was transferred to fresh BHI broth and purified bacteriocin MBSa1 at concentration corresponding to the MIC was added to the culture at times 0 h, 6 h (early exponential phase) and 8 h (late exponential phase), and incubated at 37 °C. Absorbance measurements (*Thermo Fisher Scientific Multiskan®FC*) were done at 595 nm every hour up to 24 h. A culture of *L. monocytogenes* Scott A without addition of the bacteriocin MBSa1 was used as control.

Results

Several LAB isolated from the studied salami samples presented anti-*Listeria* activity, indicating that this meat product is a good source for new strains with potential application in the control of undesired microorganisms in foods. One isolate (MBSa1) was especially active against most tested *Listeria* strains, mainly *L. monocytogenes* belonging to different serotypes and isolated from a variety of foods (Table 2). However, this strain was inactive against the tested Gram-negative bacteria (*Salmonella*, *Escherichia coli* and *Enterobacter*), *Bacillus cereus* and *Staphylococcus aureus*. Three out of ten tested strains of *Enterococcus* spp. were inhibited by strain MBSa1. When tested against other species of LAB, a limited antimicrobial activity was observed: only one (*Lactobacillus sakei* ATCC 15521) out of 25 strains was inhibited.

As shown in Table 4, the bacteriocin produced by MBSa1 strain was heat resistant. Full residual activity was observed even after autoclaving during 15 min at 121 °C. Frozen storage did not affect its activity as well (data not shown). As for the effect of pH, the bacteriocin remained stable at pH 2.0 to 6.0, but lost part of the activity at pH 8.0 and 10.0, with residual activity of 41.6% and 33.6%, respectively.

Treatment with proteinase K, trypsin, pepsin, α -chymotrypsin and protease type XIV resulted in total loss of activity (Table 4).

Identification based on 16S rDNA sequencing, confirmed by amplification with the species-specific primers, indicated that MBSa1 strain is *Lactobacillus sakei* (GenBank access number is AB593361.1).

Bacteriocin production (AU ml^{-1}) and pH reduction during growth of *Lactobacillus sakei* MBSa1 in MRS broth at 25 °C, 30 °C and 37 °C are shown in Figure 1. *L. sakei* MBSa1 grew well in MRS broth in the three tested temperatures, causing similar decrease of pH of the medium. For all tested temperatures, bacteriocin production started in the early exponential growth phase (4 h of incubation). The optimum condition for bacteriocin production (1600 AU ml^{-1}) was 25 °C and 20 h of incubation time (Figure 1).

When the DNA extracted from *L. sakei* MBSa1 was tested for bacteriocin genes using primers CurA-F/CurA-R, flanking the curvacin A structural gene (*curA*) and primers SakA-F/SakA-R, flanking the sakacin A structural gene (*sakA*), only DNA fragments of 171 bp and 150 bp length were obtained, respectively (Figure 2). No other structural sakacin genes (Table 3) were detected.

The effectiveness of each purification step (yield, specific activity and purification factor) of bacteriocin MBSa1 is summarized in Table 5. The chromatogram of the bacteriocin at the final step of purification (C_{18} RP-HPLC) presented only one peak at 13 min retention time (Figure 3). The purification sequence, i.e. cation-exchange followed by sequential hydrophobic-interaction and reversed-phase chromatography, resulted in a stepwise increase of the specific activity. When tested against *L. ivanovii*, the purified bacteriocin presented a high specific activity ($74\,949.6 \text{ AU mg}^{-1}$).

The molecular mass of bacteriocin MBSa1, determined by Q-TOF-MS, was 4303.3 Da. The amino acid sequencing by MS/MS indicated that the molecule contained the SIIGGMISGWASGLAG sequence (Table 6) also present in the C-terminal region of sakacin A (Holck *et al.* 1992), sakacin K (Aymerich *et al.* 2000) and curvacin A (Tichaczek *et al.* 1992).

Treatment with DTT resulted in mild change in antimicrobial activity, indicating that disulfide bonds are not essential for the antimicrobial activity of bacteriocin MBSa1 (Figure 4).

Growth of *L. monocytogenes* Scott A in BHI broth at 37 °C after addition of purified bacteriocin MBSa1 at the determined MIC/MKC values (3497 AU mg⁻¹ for both MIC and MKC) is shown in Figure 5. Addition of the bacteriocin at times 0 h and 8 h inhibited completely the growth of *L. monocytogenes*, indicating a bacteriostatic effect. However, when the bacteriocin was added after 6 h (early exponential phase), an inhibitory effect was observed only until 20 h of incubation.

Discussion

Bacteria belonging to *Lactobacillus* species are common in fermented and non-fermented foods such as dairy (Zago *et al.* 2011; Morales *et al.* 2011) meat products (Castro *et al.* 2011; Aquilanti *et al.* 2007) and vegetables (Chen *et al.* 2010). They are also common in animal (Yin and Zheng 2005) and human isolates (Dubos *et al.* 2011). *Lactobacillus sakei* was initially described in saké, an alcoholic beverage made by fermenting rice (Katagiri *et al.* 1934), thereby its name. The species has been considered a transient member of the human GI tract (Chiaramonte *et al.* 2009) and mutant strains were recently reported to colonize the GI tract of axenic mice (Chiaramonte *et al.* 2009; Chiaramonte *et al.* 2010), a finding which could lead to increased interest for this

species. *L. sakei* is specially adapted to the meat environment and has been widely used as a starter culture for the manufacture of a variety of meat products (Hugas and Monfort 1997; Carr *et al.* 2002). Chaillou *et al.* (2005) determined the complete genome sequence of the French sausage isolate *L. sakei* 23K, showing that this strain has a specialized metabolic repertoire that may contribute to its competitive ability in these foods.

Due to production of antimicrobial compounds, such as lactic and acetic acids, diacetyl, hydrogen peroxide and bacteriocins, some *L. sakei* strains possess interesting biotechnological potential application for food biopreservation (Carr *et al.* 2002). Several bacteriocins produced by *L. sakei* have been identified, such as sakacin A (Schillinger and Lucke 1989; Holck *et al.* 1992), sakacin M (Sobrino *et al.* 1992), bavaricin A (Larsen *et al.* 1993; Messens and de Vuyst 2002), sakacin P (Holck *et al.* 1994; Tichaczek *et al.* 1994; Vaughan *et al.* 2001; Urso *et al.* 2006; de Carvalho *et al.* 2010), sakacin K (Hugas *et al.* 1995), bavaricin MN (Kaiser and Montville 1996), sakacins 5T and 5X (Vaughan *et al.* 2001), sakacin G (Simon *et al.* 2002), sakacin Q (Mathiesen *et al.* 2005), sakacin C2 (Gao *et al.* 2010) and sakacin LSJ618 (Jiang *et al.*, 2012). In this study, it was observed that the bacteriocin produced by the *L. sakei* MBSa1 strain isolated from salami shares several properties with several bacteriocins produced by *L. sakei*. Bacteriocin MBSa1 presents the same heat stability as sakacin M (Sobrino *et al.* 1992), sakacin C2 (Gao *et al.* 2010), sakacin P (de Carvalho *et al.* 2010) and sakacin LSJ618 (Jiang *et al.* 2012). The resistance to pH in the range 2.0-6.0 is similar to that of sakacin LSJ618 (Jiang *et al.* 2012). However, sakacin C2 (Gao *et al.* 2010) and sakacin P (de Carvalho *et al.* 2010) are stable at high pH (pH>8.0), which was not observed for bacteriocin MBSa1.

The maximum production of bacteriocin MBSa1 in lactobacilli MRS broth occurred in the late logarithmic phase of growth (20 h at 25 °C). Bacteriocin activity was first detected after 4 h of incubation at 25 °C (late lag phase), which is similar to that found for sakacin A produced by *L. sakei* Lb796 (Schillinger and Lucke 1989) and sakacin P produced by *L. sakei* (Urso *et al.* 2006). However, maximum production of sakacin P by another *L. sakei* strain (*L. sakei* CCUG 42687) was reported at 20 °C (Aasen *et al.* 2000).

Like other bacteriocins produced by *L. sakei*, bacteriocin MBSa1 was inactive against Gram-negative bacteria. Until now, only two sakacins (C2 and LSJ618) are known for this activity: sakacin C2 inhibits *Escherichia coli* ATCC 25922, *Salmonella typhimurium* CMCC 47729 and *Shigella flexneri* CMCC 51606 (Gao *et al.* 2010); and sakacin LSJ618 inhibits *Escherichia coli* ECX4 and *Proteus* sp. (Jiang *et al.* 2012). However, the capability of bacteriocin MBSa1 to inhibit all tested food borne strains of *L. monocytogenes*, besides *L. monocytogenes* Scott A, is remarkable. *L. monocytogenes* is a foodborne pathogen able to survive during manufacture of dry sausages and its control is of great importance for the food industry. Bacteriocin MBSa1 did not inhibit the tested commercial probiotic strains (*Lactobacillus acidophilus* La14, *Lactobacillus acidophilus* Lac4 and *Lactobacillus acidophilus* La5), suggesting an interesting potential for anti-*Listeria* technological application in fermented foods.

Since most bacteriocins produced by LAB contain positively charged amino acid residues and present hydrophobic characteristics (Carolissen-Mackay *et al.* 1997; Nishie *et al.* 2012), most bacteriocin purification strategies have used ion-exchange and hydrophobic-interaction chromatographies. The bacteriocin produced by *L. sakei* MBSa1 strain was successfully purified by cation-exchange, sequential hydrophobic-interaction and reversed-phase chromatography. Similar procedure was used for

purification of sakacin A (Holck *et al.* 1992), bavaricin A (Larsen *et al.* 1993) and sakacin P, sakacin 5X and sakacin 5T (Vaughan *et al.* 2001).

The C-terminal partial amino acid sequence and molecular mass of the purified bacteriocin MBSa1 were identical to those of sakacin A (Table 6). The amplification of DNA of *L. sakei* MBSa1 with specific primers targeting six different sakacin genes (sakacin T α , T β , Q, X, P and G) generated negative results, but when PCR was performed with primers for sakacin A (SakA-F/SakA-R) and curvacin A (CurA-F/CurA-R), homologous fragments for the two bacteriocin genes were obtained (GenBank accession numbers AB292465.1 and MSUXNA4Z015, respectively). This is not surprising, since many similarities between different bacteriocins have been already reported. Sakacin A produced by *L. sakei* Lb706 (Axelsson and Holck 1995) and curvacin A produced by *L. curvatus* LTH1174 (Tichaczek *et al.* 1992) contain identical genetic background for bacteriocin production and regulation (Eijsink *et al.* 1998; Aymerich *et al.* 2000), sakacin K produced by *L. sakei* CTC494 (Aymerich *et al.* 2000) is also identical to curvacin A and sakacin A, as are leucocin A and leucocin B (Felix *et al.* 1994), carnobacteriocin BM1 and piscicocin V1b (Bhugaloo-Vial *et al.* 1996) and pediocin PA-1 and pediocin SJ-1 (Schved *et al.* 1994).

The similarity among bacteriocins produced by different strains generates some confusion, suggesting that their nomenclature needs to be revised. Knowing that the *L. sakei* and *L. curvatus* species are phylogenetically closely related (Collins *et al.* 1991; Berthier and Ehrlich 1999) and that sakacin A produced by *L. sakei* Lb706 (Axelsson and Holck 1995), curvacin A produced by *L. curvatus* LTH1174 (Tichaczek *et al.* 1993) and sakacin K produced by *L. sakei* CTC494 (Aymerich *et al.* 2000) were isolated from meat products, a future change in the nomenclature may solve the misunderstandings about their identity. A new nomenclature should take into consideration the source of

the bacteriocin-producing strains, the amino acid sequence and genetic characterization of the bacteriocins.

Class II bacteriocins are known for having at least one disulfide bridge in the molecule. These bridges influence the antimicrobial activity (Ennahar *et al.* 2000), and bacteriocins with more than one disulfide bridge have higher activity than those with only one (Rihakova *et al.* 2009). Holck *et al.* (1992) and Tichaczek *et al.* (1992) have shown that sakacins A and P contain one single disulfide bond, and when treated with dithiothreitol (DTT), only part of the activity is lost, indicating that this bond is important but not essential for antimicrobial activity. Similarly, the antimicrobial activity of bacteriocin MBSa1 was only moderately reduced when treated with DTT (Figure 4).

When bacteriocin MBSa1 was added to a culture of *L. monocytogenes* Scott A to achieve the concentration corresponding to the MIC/MKC values, the growth of the pathogen was inhibited regardless the growth phase (lag-phase or exponential phase), indicating a bacteriostatic activity. Sakacins produced by other *L. sakei* presented similar activities against *Listeria* spp. (Sobrino *et al.* 1991, 1992; Trinetta *et al.* 2008).

The control of *L. monocytogenes* in meat products is essential, as this pathogen causes outbreaks with high fatality rates (20% to 30%), especially among high risk groups, such as pregnant women, neonates, elderly and immuno-compromised persons (Zunabovic *et al.* 2011). *L. monocytogenes* is a ubiquitous pathogen and may persist in the food industry environment due to its capability to produce resistant biofilms on equipment surfaces and premises (Carpentier and Cerf 2011). The entrance or recontamination of *L. monocytogenes* in the processing plants can have multiple sources, mainly raw ingredients, and Good Hygiene Practices and HACCP systems may be inefficient to avoid persistence in the processing environment and presence of

Listeria in the final product (Tompkin *et al.* 1999; Tompkin 2002). Therefore, application of antimicrobial compounds may be necessary to inhibit the growth of pathogen. In this context, bacteriocins and bacteriocinogenic LAB can be explored as technological alternatives or ingredients for increasing the safety of the products manufactured in such conditions.

To conclude, sakacin A produced by the strain *L. sakei* MBSa1 isolated from salami produced in Brazil is a heat-resistant and pH-stable class II bacteriocin, with remarkable anti-*Listeria* activity and bacteriostatic action when applied in the concentration corresponding to the MIC value. Further *in situ* work in food systems will evaluate the potential application of this strain and its bacteriocin for control of *L. monocytogenes* in foods.

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References

Aasen, I.M., Moretro, T., Katla, T., Axelsson, L. and Storro, I. (2000) Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. *Applied Microbiology Biotechnology* **53**, 159–166.

Abrams, D., Barbosa, J., Albano, H., Silva, J., Gibbs, P.A. and Teixeira, P. (2011) Characterization of bacPPK34 a bacteriocin produced by *Pediococcus pentosaceus* strain K34 isolated from “Alheira”. *Food Control* **22**, 940–946.

Albano, H., Todorov, S.D., van Reenen, C.A., Hogg, T., Dicks, L.M.T. and Teixeira, P. (2007) Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from “Alheira”, a fermented sausage traditionally produced in Portugal. *International Journal of Food Microbiology* **116**, 239–247.

Aquilanti, L., Santarelli, S., Silvestri, G., Osimani, A., Petruzzelli, A. and Clementi, F. (2007) The microbial ecology of a typical Italian salami during its natural fermentation. *International Journal of Food Microbiology* **120**, 136–145.

Aymerich, M.T., Garriga, M., Monfort, J.M., Nes, I. and Hugas, M. (2000) Bacteriocin-producing lactobacilli in Spanish-style fermented sausages: characterization of bacteriocins. *Food Microbiology* **17**, 33–45.

Axelsson, L. and Holck, A. (1995) The genes involved in production of and immunity to Sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. *Journal of Bacteriology* **177**, 2125–2137

Batdorj, B., Dalgarrondo, M., Choiset, Y., Pedroche, J., Métro, F., Prévost, H., Chobert, J.-M. and Haertlé, T. (2006) Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. *Journal of Applied Microbiology* **101**, 1364–5072.

Belgacem, Z.B., Ferchichi, M., Prévost, H., Dousset, X. and Manai, M. (2008) Screening for anti-*Listeria* bacteriocin-producing lactic acid bacteria from “Gueddid” a traditionally Tunisian fermented meat. *Meat Science* **78**, 513–521.

Berthier, F and Ehrlich, S.D. (1998) Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA. *FEMS Microbiology Letters* **161**, 97–106.

Bhugaloo-Vial, P., Dousset, X., Metivier, A., Sorokine, O., Anglade, P., Boyaval, P. and Marion, D. (1996) Purification and amino acid sequences of Piscicocins V1a and V1b, two Class IIa bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. *Applied and Environmental Microbiology* **62**, 4410–4416.

Carolissen-Mackay, V., Arendse, G. and Hastings, J.W. (1997) Purification of bacteriocins of lactic acid bacteria: problems and pointers. *International Journal of Food Microbiology* **34**, 1–16.

Carpentier, B. and Cerf, O. (2011) Review — Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology* **145**, 1–8.

Carr, F.J., Chill, D. and Maida, N. (2002) The lactic acid bacteria: A literature survey. *Critical Reviews in Microbiology* **28**, 281–370.

Castro, M.P., Palavecino, N.Z., Herman, C., Garro, O.A. and Campos, C.A. (2011) Lactic acid bacteria isolated from artisanal dry sausages: characterization of antibacterial compounds and study of the factors affecting bacteriocin production. *Meat Science* **87**, 321–329.

Chaillou, S., Champomier-Vergès, M.C., Cornet, M., Crutz-Le Coq, A.M., Dudez, A.M., Martin, V., Beaufils, S., Darbon-Rongère, E., Bossy, R., Loux, V. and Zagorec, M. (2005) The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nature Biotechnology* **23**, 1527–1533.

Chen, Y.S., Wu, H.C., Liu, C.H., Chen, H.C. and Yanagida, F. (2010) Isolation and characterization of lactic acid bacteria from jiang-sun (fermented bamboo shoots), a traditional fermented food in Taiwan. *Journal of the Science of Food and Agriculture* **90**, 1977–1982.

Chiaromonte, F., Blugeon, S., Chaillou, S., Langella, P., Zagorec, M. (2009) Behavior of the meat-borne bacterium *Lactobacillus sakei* during its transit through the gastrointestinal tracts of axenic and conventional mice. *Applied Environmental Microbiology* **75**, 4498–4505.

Chiaromonte, F., Anglade, P., Baraige, F., Gratadoux, J.J., Langella, P., Champomier-Verges, M.C., Zagorec, M. (2010) Analysis of *Lactobacillus sakei* mutants selected after adaptation to the gastrointestinal tract of axenic mice. *Applied Environmental Microbiology* **76**, 2932–2939.

Collins, M.D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M. and Wallbanks, S. (1991) Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiology Letters* **77**, 5–12.

de Carvalho, K.G., Bambirra, F.H.S., Kruger, M.F., Barbosa, M.S., Oliveira, J.S., Santos, A.M.C., Nicoli, J.R., Bemquerer, M.P., Miranda, A., Salvucci, E.J., Sesma, F.J.M. and Franco, B.D.G.M. (2010) Antimicrobial compounds produced by *Lactobacillus sakei* subsp. *sakei* 2a, a bacteriocinogenic strain isolated from a Brazilian meat product. *Journal of Industrial Microbiology and Biotechnology* **37**, 381–390.

Deegan, L.H., Cotter, P.D., Hill, C. and Ross, P. (2006) Bacteriocins: biological tools for bio-preservation and shelf-life extension. *International Dairy Journal* **16**, 1058–1071.

de Vuyst, L. and Leroy, F. (2007) Bacteriocins from lactic acid bacteria: Production, purification, and food applications. *Journal of Molecular Microbiology and Biotechnology* **13**, 194–199.

Dobson, A., Cotter, P.D., Ross, R.P. and Hill, C. (2012) Bacteriocin production: a probiotic trait? *Applied Environmental Microbiology* **78**, 1–6.

Dortu, C., Huch, M., Holzapfel, W.H., Franz, C.M.A.P. and Thonart, P. (2008) Antilisterial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. *Letters in Applied Microbiology* **47**, 581–586.

Dubos, C., Vega, N., Carvallo, C., Navarrete, P., Cerda, C., Brunser, O. and Gotteland, M. (2011) Identification of *Lactobacillus* spp. in colostrum from Chilean mothers. *Archive Latinoamerican Nutricion* **61**, 66–68.

Eijsink, V.G.H., Skeie, M., Middlhoven, P.H., Brurberg, M.B. and Nes, I.F. (1998) Comparative studies of class IIa bacteriocins of lactic acid bacteria. *Applied and Environmental Microbiology* **64**, 3275–3281.

Ennahar, S., Sashihara, T., Sonomoto, K. and Ishizaki, A. (2000) Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiology Reviews* **24**, 85–106.

Felix, J.V., Papathanasopoulos, M.A., Smith, A.A., von Holy, A. Hastings, J.W. (1994) Characterization of Leucocin B-Talla: a bacteriocin from *Leuconostoc carnosum* Talla isolated from meat. *Current Microbiology* **29**, 207–212.

Felske, A., Rheims, H., Wokerink, A., Stackebrandt, E. and Akkermans, A.D.L. (1997) Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology* **143**, 2983–2989.

Gálvez, A., Abriouel, H., Lucas López, R. and Ben Omar, N. (2007) Bacteriocin based strategies for food biopreservation. *International Journal of Food Microbiology* **120**, 51–70.

Gao, Y., Jia, S., Gao, Q. and Tan, Z. (2010) A novel bacteriocin with a broad inhibitory spectrum produced by *Lactobacillus sake* C2, isolated from traditional Chinese fermented cabbage. *Food Control* **21**, 76–81.

Holck, A, Axelsson, L, Birkeland, S, Aukrust, T. and Blom, H. (1992) Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. *Journal of General Microbiology* **138**, 2715–2720.

Holck, A.L., Axelsson, L., Hühne, K. and Kröckel, L. (1994) Purification and cloning of sakacin 674, a bacteriocin from *Lactobacillus sake* Lb674. *FEMS Microbiology Letters* **115**, 143–149.

Hugas, M., Garriga, M., Aymerich, M.T. and Monfort, J.M. (1995) Inhibition of *Listeria* in dry fermented sausages by the bacteriocinogenic *Lactobacillus sake* CTC494. *Journal of Applied Bacteriology* **79**, 322–330.

Hugas, M. and Monfort, J.M. (1997) Bacterial starter cultures for meat fermentation. *Food Chemistry* **59**, 547–554.

Jiang, J., Shi, B., Zhu, D., Cai, Q., Chen, Y., Li, J., Qi, K. and Zhang, M. (2012) Characterization of a novel bacteriocin produced by *Lactobacillus sakei* LSJ618 isolated from traditional Chinese fermented radish. *Food Control* **23**, 338–344.

Joerger, M.C. and Klaenhammer, T.R. (1986) Characterization and purification of Helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. *Journal of Bacteriology* **167**, 439–446.

Juneja, V.K., Dwivedi, H.P. and Yan, X. (2012) Novel natural food antimicrobials. *Annual Review of Food Science and Technology* **3**, 381–403.

Kaiser, A.L. and Montville, T.J. (1996) Purification of the bacteriocin bavaricin MN and characterization of its mode of action against *Listeria monocytogenes* Scott A cells and lipid vesicles. *Applied and Environmental Microbiology* **62**, 4529–4535.

Katagiri, H., Kitahara, K., Fukami, K. (1934) The characteristics of the lactic acid bacteria isolated from moto, yeast mashes for sake manufacture. Part IV. Classification of the lactic acid bacteria. *Bulletin of the Agricultural Chemical Society of Japan* **10**, 156–157.

Larsen, A.G., Vogensen, F.K. and Josephsen, J. (1993) Antimicrobial activity of lactic acid bacteria isolated from sourdoughs: purification and characterization of bavaricin A, a bacteriocin produced by *Lactobacillus bavaricus* MI401. *Journal of Applied Bacteriology* **75**, 113–122.

Macwana, S. and Muriana, P.M. (2012) Spontaneous bacteriocin resistance in *Listeria monocytogenes* as a susceptibility screen for identifying different mechanisms of resistance and modes of action by bacteriocins of lactic acid bacteria. *Journal of Microbiological Methods* **88**, 7–13.

Mandal, V., Mandal, N.C. and Sen, S.K. (2011) Isolation and characterization of pediocin NV 5 producing *Pediococcus acidilactici* LAB 5 from vacuum-packed fermented meat product. *Indian Journal of Microbiology* **51**, 22–29.

Mathiesen, G., Huehne, K., Kroeckel, L., Axelsson, L. and Eijsink, V.G.H. (2005) Characterization of a new bacteriocin operon in sakacin P-producing *Lactobacillus*

sakei, showing strong translational coupling between the bacteriocin and immunity genes. *Applied and Environmental Microbiology* **71**, 3565–3574.

Messens, W. and de Vuyst, L. (2002) Inhibitory substances produced by Lactobacilli isolated from sourdoughs-a review. *International Journal of Food Microbiology* **72**, 31–43.

Mills, S., Stanton, C., Hill, C. and Ross, R.P. (2011) New developments and applications of bacteriocins and peptides in foods. *Annual Review of Food Science and Technology* **2**, 299–329.

Morales, F., Morales, J.I., Hernández, C.H. and Hernández-Sánchez, H. (2011) Isolation and partial characterization of halotolerant lactic acid bacteria from two Mexican cheeses. *Applied Biochemistry and Biotechnology* **164**, 889–905.

Müller, D.M., Carrasco, M.S., Tonarelli, G.G. and Simonetta, A.C. (2009) Characterization and purification of a new bacteriocin with a broad inhibitory spectrum produced by *Lactobacillus plantarum* lp 31 strain isolated from dry-fermented sausage. *Journal of Applied Microbiology* **106**, 2031–2040.

Nielsen, J.W., Dickson, J.S. and Crouse, J.D. (1990) Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Applied and Environmental Microbiology* **56**, 2142–2145.

Nishie, M. Nagao, J.-I. and Sonomoto, K. (2012) Antibacterial peptides “Bacteriocins”: an overview of their diverse characteristics and applications. *Biocontrol Science* **17**, 1–16.

Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K. and Panyim, S. (2003) Isolation of nisin-producing *Lactococcus lactis* WNC20 strain from nham, a traditional Thai fermented sausage. *International Journal of Food Microbiology* **81**, 137–145.

O'Shea, E.F., Cotter, P.D., Ross, R.P. and Hill, C. (2013) Strategies to improve the bacteriocin protection provided by lactic acid bacteria. *Current Opinion in Biotechnology* **24**, 130–134.

Phumkhachorn P., Rattanachaikunsopon P. and Khunsook S. (2007) Use of the *gfp* gene in monitoring bacteriocin-producing *Lactobacillus plantarum* N014, a potential starter culture in nham fermentation. *Journal of Food Protection* **70**, 419–424.

Rantsiou, K. and Cocolin, L. (2006) New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: A review. *International Journal of Food Microbiology* **108**, 255–267.

Reminger, A., Ehrmann, M.A. and Vogel, R.F. (1996) Identification of bacteriocin-encoding genes in Lactobacilli by Polymerase Chain Reaction (PCR). *Systematic Applied Microbiology* **19**, 28–34.

Rihakova, J., Petit, V.W., Demnerova, K., Prévost, H., Rebuffat, S. and Drider, D. (2009) Insights into structure-activity relationships in the C-terminal region of divercin V41, a class IIa bacteriocin with high-level antilisterial activity. *Applied and Environmental Microbiology* **75**, 1811–1819.

Sabia, C., Manicardi, G., Messi, P., de Niederhäusern, S. and Bondi, M. (2002) Enterocin 416K1, an antilisterial bacteriocin produced by *Enterococcus casseliflavus* IM 416K1 isolated from Italian sausages. *International Journal of Food Microbiology* **75**, 163–170.

Samelis, J., Maurogenakis, F. and Metaxopoulos J. (1994) Characterization of lactic acid bacteria isolated from naturally fermented Greek dry salami. *International Journal of Food Microbiology* **23**, 179–196.

Schillinger, U. and Lucke, F.-K. (1989) Antibacterial activity of *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology* **55**, 1901–1906.

Schved, F., Lalazar, A., Lindner, P., and Juven, B.J. (1994) Interaction of the bacteriocin produced by *Pediococcus acidilactici* SJ-1 with the cell envelope of *Lactobacillus* spp. *Letters in Applied Microbiology* **19**, 281–283.

Simon, L., Fremaux, C., Cenatiempo, Y. and Berjeaud, J.-M. (2002) Sakacin G, a new type of antilisterial bacteriocin. *Applied and Environmental Microbiology* **68**, 6416–6420.

Sobrinho, J., Rodriguez, J.M., Moreira, W.L., Fernandez, M.F., Sanz, B. and Hernandez, P.E. (1991) Antibacterial activity of *Lactobacillus sake* isolated from dry fermented sausages. *International Journal of Food Microbiology* **13**, 1–10.

Sobrinho, J., Rodriguez, J.M., Moreira, W.L., Cintas, L.M., Fernandez, M.F., Sanz, B. and Hernandez, P.E. (1992) Sakacin M, a bacteriocin-like substance from *Lactobacillus sake* 148. *International Journal of Food Microbiology* **16**, 215–225.

Thévenot, D., Delignette-Muller, M.L., Christieans, S. and Vernozy-Rozand, C. (2005) Fate of *Listeria monocytogenes* in experimentally contaminated French sausages. *International Journal of Food Microbiology* **101**, 189–200.

Tichaczek, P.S., Nissen-Meyer, J., Nes, I.F., Vogel, R.F. and Hammes, W.P. (1992) Characterization of the bacteriocins curvacin A from *Lactobacillus curvatus* LTH1174 and sakacin from *L. sake* LTH673. *Systematic and Applied Microbiology* **15**, 460–468.

Tichaczek, P. S., Vogel, R. F. and Hammes, W. P. (1994) Cloning and sequencing of sakP encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH673. *Microbiology* **140**, 361–367.

Todorov, S.D. and Dicks, L.M.T. (2004) Characterization of mesentericin ST99, a bacteriocin produced by *Leuconostoc mesenteroides* subsp. *dextranicum* ST99 isolated from boza. *Journal of Industrial Microbiology and Biotechnology* **31**, 323–329.

Todorov, S.D. and Dicks, L.M.T. (2005) *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria. *Enzyme and Microbial Technology* **36**, 318–326.

Todorov, S.D., Ho, P., Vaz-Velho, M. and Dicks, L.M.T. (2010) Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Science* **84**, 334–343.

Todorov, S.D., Rachman, C., Fourrier, A., Dicks, L.M.T., van Reenen, C.A., Prévost, H. and Dousset, X. (2011) Characterization of a bacteriocin produced by *Lactobacillus sakei* R1333 isolated from smoked salmon. *Anaerobe* **17**, 23–31.

Todorov, S.D., Favaro, L., Gibbs, P. and Vaz-Velho, M. (2012) *Enterococcus faecium* isolated from Lombo, a Portuguese traditional meat product: characterisation of antibacterial compounds and factors affecting bacteriocin production. *Beneficial Microbes* **3**, 319–330.

Todorov, S.D., Vaz-Velho, M., Franco, B.D.G.M. and Holzapfel, W.H. (2013) Partial characterization of bacteriocins produced by three strains of *Lactobacillus sakei*, isolated from salpicao, a fermented meat product from North-West of Portugal. *Food Control* **30**, 111–121.

Tompkin, R.B., Scott, V.N., Bernard, D.T., Sveum, W.H. and Gombas, K.S. (1999) Guidelines to prevent post-processing contamination from *Listeria monocytogenes*. *Dairy Food Environmental* **19**, 551–562.

Tompkin, R.B. (2002) Control of *Listeria monocytogenes* in the food-processing environment. *Journal of Food Protection* **65**, 709–725.

Trinetta, V., Rollini, M., Limbo, S. and Manzoni, M. (2008) Influence of temperature and sakacin A concentration on survival of *Listeria innocua* cultures. *Annals of Microbiology* **58**, 633–639.

Urso, R., Rantsiou, K., Cantoni, C., Comi, G. and Cocolin, L. (2006) Sequencing and expression analysis of the sakacin P bacteriocin produced by a *Lactobacillus sakei* strain isolated from naturally fermented sausages. *Applied Microbiology and Biotechnology* **71**, 480–485.

van Reenen, C.A., Dicks L.M.T. and Chikindas, M.L. (1998) Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology* **84**, 1131–1137.

Vaughan, A., Eijssink, V.G.H., van Sinderen, D., O'Sullivan, T.F. and O'Hanlon, K. (2001) An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *Journal of Applied Microbiology* **91**, 131–138.

Xiraphi, N., Georgalaki, M., van Driessche, G., Devreese, B., van Beeumen, J., Tsakalidou, E., Metaxopoulos, J. and Drosinos, E.H. (2006) Purification and characterization of curvaticin L442, a bacteriocin produced by *Lactobacillus curvatus* L442. *Antonie van Leeuwenhoek* **89**, 19–26.

Yin, Q. and Zheng, Q. (2005) Isolation and identification of the dominant *Lactobacillus* in gut and faeces of pigs using carbohydrate fermentation and 16S rDNA analysis. *Journal of Bioscience and Bioengineering* **99**, 68–71.

Zago, M., Fornasari, M.E., Carminati, D., Burns, P., Suárez, V., Vinderola, G., Reinheimer, J. and Giraffa, G. (2011) Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. *Food Microbiology* **28**, 1033–1040.

Zhou, G.H., Xu, X.L. and Liu, Y. (2010) Preservation technologies for fresh meat – A review. *Meat Science* **86**, 119–128.

Zunabovic, M., Domig, K.J. and Kneifel, W. (2011) Practical relevance of methodologies for detecting and tracing of *Listeria monocytogenes* in ready-to-eat foods and manufacture environments - A review. *LWT - Food Science and Technology* **44**, 351–362.

Table 1. Bacteriocinogenic lactic acid bacteria isolated from fermented meat products.

Strain	Source	Reference
<i>Lactobacillus sakei</i> ST22Ch	Salpicao	Todorov <i>et al.</i> 2013
<i>Lactobacillus sakei</i> ST153Ch		
<i>Lactobacillus sakei</i> ST154Ch		
<i>Enterococcus faecium</i> ST211CH	Lombo	Todorov <i>et al.</i> 2012
<i>Pediococcus pentasaceus</i> K34	Fermented sausage “alheira”	Abrams <i>et al.</i> 2011
<i>Pediococcus acidilactici</i> LAB 5	Vacuum-packed fermented meat product	Mandal <i>et al.</i> 2011
<i>Lactobacillus plantarum</i> bacST202Ch	Chouriço	Todorov <i>et al.</i> 2010
<i>Lactobacillus plantarum</i> bacST216Ch	Beloura	Todorov <i>et al.</i> 2010
<i>Lactobacillus plantarum</i> LP 31	Argentinian dry-fermented sausage	Müller <i>et al.</i> 2009
<i>Enterococcus faecium</i> MMZ17	Tunisian fermented meat	Belgacem <i>et al.</i> 2008
<i>Pediococcus acidilactici</i> HA-6111-2 <i>Pediococcus acidilactici</i> HA-5692-3	Portuguese fermented sausage	Albano <i>et al.</i> 2007
<i>Lactobacillus plantarum</i> N014	Thai fermented pork	Phumkhachorn <i>et al.</i> 2007
<i>Lactobacillus curvatus</i> L442	Greek fermented sausage	Xiraphi <i>et al.</i> 2006
<i>Lactobacillus sakei</i> I151	Fermented sausages	Urso <i>et al.</i> 2006
<i>Lactococcus lactis</i> WNC 20	Thai fermented sausage	Noonpakdee <i>et al.</i> 2003
<i>Enterococcus casseliflavus</i> IM416K1	Italian sausages	Sabia <i>et al.</i> 2002
<i>Lactobacillus sakei</i> CTC494	Fermented sausage	Aymerich <i>et al.</i> 2000
<i>Lactobacillus sakei</i> 251	Greek dry sausage	Samelis <i>et al.</i> 1994
<i>Lactobacillus curvatus</i> LTH 1174 <i>Lactobacillus sakei</i> LTH 673	Fermented sausage	Tichaczek <i>et al.</i> 1992
<i>Lactobacillus sakei</i> 148	Spanish dry fermented sausage	Sobrino <i>et al.</i> 1991

Table 2. Spectrum of activity of the bacteriocin produced by *Lactobacillus sakei* MBSa1.

Indicator microorganism	Source	Activity ^a
<i>Bacillus cereus</i>	ATCC 1178	-
<i>Staphylococcus aureus</i>	ATCC 29213	-
<i>Staphylococcus aureus</i>	ATCC 25923	-
<i>Staphylococcus aureus</i>	ATCC 6538	-
<i>Listeria welshimeri</i>	USP ^b	+
<i>Listeria seeligeri</i>	USP	-
<i>Listeria ivanovii</i> subsp. <i>ivanovii</i>	ATCC 19119	++
<i>Listeria innocua</i>	ATCC 33090	++
<i>Listeria innocua</i> 225/07 sorovar 6a	FIOCRUZ ^c	+
<i>Listeria innocua</i> 224/07 sorovar 6a	FIOCRUZ	+
<i>Listeria innocua</i> 047/07 sorovar 6a	FIOCRUZ	+
<i>Listeria innocua</i> 588/08 sorovar 6a	FIOCRUZ	+
<i>Listeria monocytogenes</i> Scott A	USP	+
<i>Listeria monocytogenes</i> 602/08 sorovar 1/2a	FIOCRUZ	+
<i>Listeria monocytogenes</i> 046/07 sorovar 1/2c	FIOCRUZ	+
<i>Listeria monocytogenes</i> 103 sorovar 1/2a	USP	+
<i>Listeria monocytogenes</i> 106 sorovar 1/2a	USP	+
<i>Listeria monocytogenes</i> 104 sorovar 1/2a	USP	+
<i>Listeria monocytogenes</i> 409 sorovar 1/2a	USP	+
<i>Listeria monocytogenes</i> 506 sorovar 1/2a	USP	+
<i>Listeria monocytogenes</i> 709 sorovar 1/2a	USP	+
<i>Listeria monocytogenes</i> 607 sorovar 1/2b	USP	+
<i>Listeria monocytogenes</i> 603 sorovar 1/2b	USP	+
<i>Listeria monocytogenes</i> 426 sorovar 1/2b	USP	+
<i>Listeria monocytogenes</i> 637 sorovar 1/2c	USP	+
<i>Listeria monocytogenes</i> 422 sorovar 1/2c	USP	+
<i>Listeria monocytogenes</i> 712 sorovar 1/2c	USP	+
<i>Listeria monocytogenes</i> 408 sorovar 1/2c	USP	+
<i>Listeria monocytogenes</i> 211 sorovar 4b	USP	+
<i>Listeria monocytogenes</i> 724 sorovar 4b	USP	+
<i>Listeria monocytogenes</i> 101 sorovar 4b	USP	+
<i>Listeria monocytogenes</i> 703 sorovar 4b	USP	+
<i>Listeria monocytogenes</i> 620 sorovar 4b	USP	+
<i>Listeria monocytogenes</i> 302 sorovar 4b	USP	+
<i>Escherichia coli</i>	ATCC 8739	-
<i>Escherichia coli</i> O157:H7	ATCC 35150	-
<i>Enterobacter aerogenes</i>	ATCC 13048	-
<i>Salmonella</i> Typhimurium	ATCC 14028	-
<i>Salmonella</i> Enteritidis	ATCC 13076	-
<i>Enterococcus faecalis</i>	ATCC 12755	+
<i>Enterococcus hirae</i> D105	AGRIS ^d	+
<i>Enterococcus faecium</i> S5	AGRIS	-
<i>Enterococcus faecium</i> S154	AGRIS	-

<i>Enterococcus faecium</i> S100	AGRIS	+
<i>Enterococcus faecium</i> ST62	AGRIS	-
<i>Enterococcus faecium</i> ST211	AGRIS	-
<i>Enterococcus faecium</i> ET 12	UCV ^e	-
<i>Enterococcus faecium</i> ET 88	UCV	-
<i>Enterococcus faecium</i> ET 05	UCV	-
<i>Lactobacillus</i> sp. V94	USP	-
<i>Lactobacillus fermentum</i> ET35	UCV	-
<i>Pediococcus pentosaceus</i> ET 34	UCV	-
<i>Lactobacillus curvatus</i> ET 06	UCV	-
<i>Lactobacillus curvatus</i> ET 31	UCV	-
<i>Lactobacillus curvatus</i> ET 30	UCV	-
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> 2a	USP	-
<i>Lactobacillus sakei</i>	ATCC 15521	+
<i>Lactobacillus plantarum</i> V69	USP	-
<i>Lactobacillus delbrueckii</i> B5	USP	-
<i>Lactobacillus delbrueckii</i> ET32	UCV	-
<i>Lactobacillus acidophilus</i> La14	Rhodia	-
<i>Lactobacillus acidophilus</i> Lac4	Rhodia	-
<i>Lactobacillus acidophilus</i> La5	Rhodia	-
<i>Lactococcus lactis</i> B16	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> MK02R	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D2	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B1	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D4	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B2	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B15	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D3	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D5	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B17	USP	-
<i>Lactococcus lactis</i> subsp. <i>lactis</i> R704	Chr. Hansen	-

^a - no inhibitory activity; + inhibition halo diameter 1-10 mm; ++ inhibition halo diameter 11-15 mm; +++ inhibition halo diameter >20 mm.

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Table 3. Targeted bacteriocin genes and primers used in the study.

Target bacteriocin	Primer*	Sequence (5'-3')**	Size (bp)	Reference	Initial denaturation	Denaturation	Annealing	Elongation
Sakacin A	SakA-F SakA-R	GAATWTRMMANCAATTAYMGTTGG CAGCCGCTAATCATACCACC	150	Dortu <i>et al.</i> , 2008	95°C, 15 min	95°C, 30 s	55°C, 1 min	72°C, 1 min
Sakacin T-α	SakT-α-F SakT-α-R	TCGGTGGCTATACGTCTAAACAA TGTCCTAAAAATCCACCAATGC	160	Macwana and Muriana (2012)	95°C, 15 min	95°C, 30 s	58°C, 1 min	72°C, 1 min
Sakacin T-β	SakT-β-F SakT-β-R	AAGAAATGATAGAAAATTTTGGAGG TGTGAAATCCCAATCTGTCTCTG	151	Macwana and Muriana (2012)	95°C, 15 min	95°C, 30 s	56°C, 1 min	72°C, 1 min
Sakacin Q	SakQ-F SakQ-R	GAARTW SYA NCA ATT ADN GGT GG TAC CAC CAG CAG CCA TTC CC	130	Dortu <i>et al.</i> (2008)	95°C, 15 min	95°C, 30 s	53°C, 1 min	72°C, 1 min
Sakacin X	SakX-F SakX-R	AGCTATGAAAGGTATTGTCGGG TAAAGATTTCCAGCCAGCAGC	156	Macwana and Muriana (2012)	95°C, 15 min	95°C, 30 s	58°C, 1 min	72°C, 1 min
Sakacin P	SakP-F SakP-R	ATG GAA AAG TTT ATT GAA TTA TTA T TT ATT CCA GCC AGC GTT	186	Reminger <i>et al.</i> (1996)	94°C, 3 min	94°C, 30 s	40°C, 1 min	72°C, 1 min
Sakacin G	SakGA1-F SakGA1-R	TTA GAA CTA CAC TGA TCG TG TGG AAG AAT GAG TAC TTG TT		Todorov <i>et al.</i> (2011)	94°C, 4 min	94°C, 30 s	38°C, 30 s	72°C, 30 s
Sakacin G	SakGA2-F SakGA2-R	CGT TAC AAC AGA ACT TCA AG TGG AAG AAT GAG TAC TTG TT		Todorov <i>et al.</i> (2011)	94°C, 4 min	94°C, 30 s	38°C, 30 s	72°C, 30 s
Curvacin A	CurA-F CurA-R	GTA AAA GAA TTA AGT ATG ACA TTA CAT TCC AGC TAA ACC ACT	171	Reminger <i>et al.</i> (1996)	94°C, 3 min	94°C, 30 s	40°C, 1 min	72°C, 1 min

*F- Forward; R- Reverse

**deoxyninosine; R, T/A; W, A/G; S, C/A; Y, A/C; D, C/T; N, A/C.

Table 4. Effect of proteolytic enzymes, temperature and pH on activity of the bacteriocin produced by *Lactobacillus sakei* MBSa1.

Treatment		Residual bacteriocin activity (%)
Enzyme		
	Proteinase K	0
	Trypsin	0
	Pepsin	0
	α -chymotrypsin	0
	Protease Type XIV	0
Temperature		
	4 – 100 °C (60 min)	100
	121° C (15 min)	100
pH		
	2.0 – 6.0	100
	8.0	41.6
	10.0	33.3

Table 5. Purification of bacteriocin produced by *Lactobacillus sakei* MBSa1.

Purification step	Volume (ml)	Activity (AU ml ⁻¹)	Total Activity (AU)	Yield (%)	Protein (mg ml ⁻¹)	Specific activity (AU mg ⁻¹)	Purification factor
Supernatant	400	6400	2.56×10^6	100	3.42	1871.34	1.00
Cation exchange	190	3200	6.08×10^5	23.75	1.86	1720.43	0.92
(SOURCE TM 15RPC)	70	12800	8.96×10^5	35	1.96	6530.61	3.49
C ₁₈ RP-HPLC	1	819200	8.19×10^5	32	10.93	74949.6	40.05

Table 6. Amino acid sequence and molecular mass of bacteriocin MBsa1 and other bacteriocins produced by *Lactobacillus sakei*.

Bacteriocin*	Sequence	Molecular Mass
Sakacin 5T ¹	KTNWGSVVGSCVAGGLVGALGGTPIWIGAGPLVVGAGQDAISQ	4083
Sakacin 5X ¹	KYYGNGLSCNKGSCVDWSKAISIIGNNAVANLTITGGAAGWKS	4365
Sakacin P ^{2,3}	KYYGNGVHCGKHSTVDWGTAGNIGNNAAANWATGAGWVK	4437
Sakacin G ⁴	KYYGNGVSCNSHGCNVNWQAWTGCVNHLANGGHGVC	3834
Sakacin Q ⁵	MQNTKELSVVELQQLLGKR ASFGKCVVGAAGAGAGAGVSG	4486
Bavaricin MN ⁶	TKYYGNGVYXNSKKXWVDWGAAGGIGQTVVXGWLGAIPGK	4769
Bavaricin A ⁷	KYYGNGVHGXGKHSXTVDWGTAGNIGNNAAANXATGXNAGG	4002
Sakacin K ⁸	ARSYGNGVVCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	4308
Curvacin A ⁹	ARSYGNGVVCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	4308
Sakacin A ¹⁰	ARSYGNGVVCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	4308
MBsa1	SIIGGMISGWASGLAG	4303

*¹Vaughan *et al.* 2001; ²Tichaczek *et al.* 1994; ³Holck *et al.* 1994; ⁴Simon *et al.* 2002; ⁵Mathiesen *et al.* 2005; ⁶Kaiser and Montville 1996; ⁷Larsen *et al.* 1993; ⁸Aymenich *et al.* 2000; ⁹Tichaczek *et al.* 1992; ¹⁰Holck *et al.* 1992.

Figure 1. Growth (OD 600 nm), bacteriocin-production (AU ml⁻¹) and pH reduction of *Lactobacillus sakei* MBSa1 in MRS broth at 25 °C, 30 °C and 37 °C.

Figure 2. DNA fragments obtained after PCR with genomic DNA from *Lactobacillus sakei* MBSa1 using curvacin A specific primers (CurA-F/CurA-R) (a) and sakacin A specific primers (SakA-F/SakA-R) (b). Lane 1, molecular weight marker (100 bp); lane 2, amplicon obtained using genomic DNA; lane 3, amplicon obtained using sterile water (control).

Figure 3. Chromatogram of the purified bacteriocin produced by *Lactobacillus sakei* MBSa1 (C₁₈ reversed-phase HPLC).

Figure 4. Activity of the purified bacteriocin MBSa1 against *L. monocytogenes* Scott A, after treatment with Tris-HCl (50 mmol l^{-1}) at pH 8.0, proteinase K (1 mg ml^{-1}), trypsin (1 mg ml^{-1}), and dithiothreitol (100 mmol l^{-1}).

Figure 5. Growth of *Listeria monocytogenes* Scott A in BHI broth at 37°C after addition of the purified bacteriocin MBSa1, added at time 0 h (■), 6 h (▲) and 8 h (●). Control curve, without addition of bacteriocin (♦).

Capítulo 02

**“Preliminary characterization of the two-peptides bacteriocin produced by
Lactobacillus plantarum MBSa4 isolated from salami”**

*Artigo em preparação para submissão para publicação em
Food Research International*

**Preliminary characterization of the two-peptides bacteriocin produced by
Lactobacillus plantarum MBSa4 isolated from salami**

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Abstract:

The objective this work was to characterize the bacteriocin produced by *Lactobacillus plantarum* MBSa4 isolated from salami produced in the Brazil. Bacteriocin produced by *L. plantarum* MBSa4 was not affected by range of temperature from 4°C to 100°C, even at 121°C by 15 min. The bacteriocin is stable only in acid conditions (pH 2.0 to 6.0). Bacteriocin produced by MBSa4 strains was active against *Listeria monocytogenes*, *Enterococcus* spp and *Lactobacillus sakei* ATCC 15521. Bacteriocin MBSa4 was not active against the tested gram-negative bacteria. MBSa4 strain showed antagonistic activities against fungi, but antifungal activity was not observed by antimicrobial compounds produced by LAB. The high level of bacteriocin was detected at temperature of 25°C (1600 AU/mL) and bacteriocin MBSa4 had bacteriostatic effect against *L. monocytogenes* Scott A. The molecular weight of the bacteriocin produced by *L. plantarum* was around of 2500 daltons. After of the last step for partial purification of the bacteriocin produced by *L. plantarum* MBSa4, only one peak showed anti-*Listeria* activity, however when this active peak was combined with others peaks non-active (ratio 1:1), an activity synergistically between active peak and non-active peak was observed. These characteristics are in accordance with bacteriocins classified as two-peptides bacteriocins. The results of investigation of bacteriocin genes for bacteriocin-producing *L. plantarum* MBSa4 was positive for plantaricin W.

Key-words: *Lactobacillus plantarum*, salami and class IIb bacteriocin.

Introduction

The term bacteriocin is applied to ribosomally synthesized antimicrobial peptides, which are known to be active against closely related bacteria and activity against unrelated strains, especially those that are pathogenic and responsible for food spoilage (De Vuyst and Leroy 2007; Mills et al., 2011; Dobson et al., 2012). Although a variety of gram-positive bacteria have been reported to produce bacteriocins, those produced by lactic acid bacteria (LAB) have been more widely investigated because of their potential use as biopreservatives for food (Cotter et al., 2005; De Vuyst & Leroy, 2007; Mills et al., 2011; Dobson et al., 2012).

Klaenhammer (1993) classified the bacteriocins produced by LAB into four classes. The bacteriocins of class I and II are the best known. Small (molecular weight $\leq$ 5 kDa) and thermo-stable peptides containing thioether amino acids are classified to Class I (lantibiotics). Small (molecular weight $\leq$ 10 kDa) and thermo-stable peptides non-lanthionine containing peptides are classified to Class II. Class III and IV are labile and can be hydrophilic proteins or protein complexes consisting of phospholipids and/or carbohydrates. Kemperman et al. (2003) suggested a new classification including the circular bacteriocins into a new class, class V. Cotter et al. (2005) proposed a new classification, dividing the bacteriocins in three classes: the lantibiotics (Class I) divided into 11 groups (Nisin, Epidermin, Streptin, Pep5, Lacticin 481, Mersacidin, LtnA, Cytolysin, Lactocin S, Cinnamycin and Sublancin group), the non-lantibiotics (Class II) divided in four groups (classes IIa, IIb, IIc [formerly class V from Kemperman's classification] and IId) and bacteriolysins (Class III), which are high molecular weight and thermo-labile proteins. Due to the limited availability of data about Class IV bacteriocins (Klaenhammer's classification), this class was not included in the current classification. Deegan et al. (2006) defined Class I bacteriocins as small (<5 kDa) and

thermostable peptides, with residues of lanthionine and methyl lanthionine amino acids. This class was divided into two subclasses: subclass Ia composed of long, flexible and positively charged peptides, which act on the cytoplasmic membrane through pore formation and the subclass Ib was characterized by spherical, rigid, and neutral or negatively charged peptides. The class II bacteriocins are small (<10 kDa), thermostable and residues non-lanthionine and non-methyl lanthionine peptides. However, only two types are common to all classification systems and were retained in this proposed classification scheme: the class IIa, which contains the N-terminal consensus YGNGVXCXXXXCXV and the class IIb which is composed of two-peptides bacteriocins for their antimicrobial activity. Recently, Nishie et al (2012) revised the bacteriocin's classification. The division for class II was follows the classification proposed by Cotter et al. (2005). However, for class I was proposed a new classification in class I lantibiotics and class II lantibiotics, taking as base the pathway by which maturation of the peptide occurs. The class I lantibiotics consist in the bacteriocins that their prepeptides are modified by enzymes LanB and LanC and class II lantibiotics are modified by enzyme LanM.

Several different groupings and classification for bacteriocins have been suggested, but their heterogeneous nature makes rational classification difficult. In this context, studies of isolation of new bacteriocin-producing LAB and characterization of its bacteriocin are of great importance for increase the knowledge about these antimicrobials peptides and hereafter help to define the bacteriocin's classification. Moreover, the characterization of the LAB and bacteriocin produced has to be considered for an optimal selection of strains of interest for application in food. Therefore, the objective this work was to characterize the bacteriocin produced by *Lactobacillus plantarum* isolated from salami produced in the Brazil.

Material and Methods

Isolation of lactic acid bacteria (LAB) with anti-listerial activity in salami

Samples salami were homogenized in a stomacher (Seward 400, London, UK) with 0.1% sterile peptone water (Difco, USA), submitted to serial decimal dilutions and each dilution was plated on MRS agar (Oxoid, UK) in duplicate. After 48 h incubated at 30°C, colonies on MRS agar plates were overlaid with soft-agar BHI (BHI [Oxoid, UK] supplemented with 0.75% bacteriologic agar [Difco, USA]) inoculated with *L. monocytogenes* Scott A (10^5 - 10^6 CFU/mL) and incubated for 24 h at 37°C for test of inhibitory activity against *Listeria monocytogenes* (Todorov and Dicks 2005). Colonies presenting growth inhibition zones were transferred to MRS broth (Difco, USA) and incubated at 30°C for 24 h. Colonies isolated were submitted to Gram staining and tested for catalase production using 3% hydrogen peroxide (v/v). Cultures with anti-*Listeria* activity were freeze-dried and stored at -20°C.

Confirmation of Bacteriocin production

The antimicrobial activities of isolated were assayed using spot-on-the-lawn method, described by van Reenen et al. (1998) with modifications. Isolates were grown in MRS broth (Difco, USA) for 24 h at 30°C and removed by centrifugation at 4000 xg for 15 min at 4°C (Hettich Zentrifugen, model Mikro 22R, Germany). The pH of cell free supernatant (CFS) was adjusted to 6.0-6.5 with 1 N NaOH (Synth, Brazil), after heated at 70°C for 30 min and then sterilized by filtration (Millex GV 0,22 µm [Millipore, USA]). Indicator microorganism, *L. monocytogenes* Scott A (10^5 - 10^6 CFU/mL), was added in 5 ml of BHI soft-agar and overlaid in plate containing 10-12 ml of 1.5% bacteriologic agar (Difco, USA). An aliquot of 10 µL of CFS was spotted onto the surface of plate with medium and after complete absorption of the CFS, the plates

were incubated at 37°C for 12 h and observed for the formation of a clear zone of inhibition around of the CFS spotted. Bacteriocin production was confirmed trough treated with proteolytic enzymes (0.1 mg/mL) showed in the Table 1, as described by Noonpakdee et al. 2003. After incubation at 37°C for 1 h, CFS treated with proteolytic enzymes were heated at 80°C for 5 min for enzyme inactivation, and then tested for residual antimicrobial activity using the spot-on-the-lawn method, as described before. Absence of zone of inhibition after enzymatic treatment indicated the presence of bacteriocin(s). Control tests, with non-treated CFS whit proteolytic enzymes were also performed.

Titration of bacteriocin

The titration of the bacteriocin was arbitrarily assayed using serial dilutions two-fold and spot-on-the-lawn methods (van Reenen et al., 1998). One arbitraty unit (AU) was defined as the reciprocal of the highest dilution that showed a distinct clear zone of inhibition, expressed in AU/mL (Kaiser and Montville, 1996).

Strain Identification

The bacteriocin-producing LAB isolated were submitted to 16S rDNA sequence analysis, by amplification of genomic DNA with primers 8f (5'-CAC GGA TCC AGA CTT TGA T(C/T)(A/C) TGG CTC AG-3') and 1512r (5'- GTG AAG CTT ACG G(C/T)T AGC TTG TTA CGA CTT-3') (Felske et al., 1997). The 20 µL reaction volume contained 100 pM each primer, 1x PCR buffer (Fermentas, Lithuania), 24 µM dNTP, 2 mM MgCl₂ (Fermentas, Lithuania) and 0.0125 U *Taq* DNA polymerase (Fermentas, Lithuania) was used. Amplification was carried out in a DNA thermocycler MasterCycler®PCR (Eppendorf Scientific, Germany). PCR conditions included

denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 10 s, primer annealing at 61°C for 20 s, polymerization at 68°C for 2 min and then at 72°C for 7 min. PCR-amplified DNA were analyzed on 0.8% (w/v) agarose gel electrophoresis and visualized by ethidium bromide (0.1 mg/mL) fluorescence using an UVP BioImaging System (DIGIDOC-IT System, USA). Band with approximately 831 bp was cut from the gel and purified with QIAquick®PCR Purification kit (Qiagen, Germany) according to the manufacturer's instructions and submitted to sequencing at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of São Paulo, Brazil. The sequences were compared to those deposited in GenBank, using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>).

Effect of pH and temperature on activity of bacteriocin MBSa4

The effect of pH and temperature on activity of bacteriocin MBSa4 was determined as described by Albano et al. (2007). The pH of the CFS was adjusted from 2.0 to 10.0 with 1N NaOH (Synth, Brazil) or concentrated phosphoric acid (Synth, Brazil), and incubated for 1 h at 25°C. Before test, the pH of the CFS was adjusted to 6.0-6.5 with 1N NaOH (Synth, Brazil) or concentrated phosphoric acid (Synth, Brazil). For test of the effect of temperature on the anti-*Listeria* activity of the bacteriocin, the CFS was kept in different combinations binominal of time/temperatures (Table 1) and then testing for activity against indicator microorganism. All samples were tested for anti-*Listeria* activity by using spot-on-the-lawn method as described before.

Spectrum of Activity of Bacteriocin MBSa4

The antimicrobial spectrum of the bacteriocin MBSa4 strain was determined against a variety of gram-negative and gram-positive bacteria food isolates (Table 2)

using spot-on-the lawn method (van Reenen et al. 1998). For testing, lactobacilli and enterococci were grown in MRS broth (Difco, USA) at 30°C for 24 h, while the other strains were grown in BHI broth (Oxoid, UK) at 37°C for 24 h.

Antifungi assay

Antagonism of MBSa4 strain against moulds and yeast were tested using a dual-culture overlay methodology, as described by Magnusson et al. (2003) with some modifications. The fungi used are listed in Table3 and all were grown on Potato Dextrose Agar (PDA) medium (AES, Bruz, France) at 30°C for 48 to 96 h. Yeast cells and moulds spores were collected and resuspended in saline buffer (0.8% NaCl) and enumerated on counting cells plate. Then, these suspensions were standardized at a final concentration of 10^4 - 10^5 cells or spores per ml.

One overnight culture of the *L. plantarum* MBSa4 incubated at 30°C in MRS broth (Biokar, France) was inoculated in modified MRS (without sodium acetate) soft-agar (MRS broth plus 0.85% [w/v] of bacteriological agar [Biokar, France]), placed into 12-well plates and incubated at 30°C for 48 h. After period incubation, 100 µl of solution with yeast cells or moulds spores was dropped on surface of the MRS soft-agar previously inoculated with MBSa4 strain and observed for fungal inhibition after 72 h of incubation at 30°C.

Determination of antifungi activity of the compounds produced by MBSa4 strain was performed adapting agar well diffusion method. Population of each yeast cells or moulds spores (10^4 - 10^5 cells per milliliter) that showed positives results for antagonism test was “spread-inoculated” onto the surface of MRS soft-agar (MRS broth (Biokar, France) plus 0.85% [w/v] of bacteriological agar [Biokar, France]). The wells with approximately 8 mm of diameter were cut from the MRS soft-agar and CFS of MBSa4

strain was delivered into them. After incubation for 72 h at 30°C, all plates were examined for clear zones inhibition.

Bacteriocin production during MBSa4 strain growth

The bacteriocin production by MBSa4 strain in MRS Broth (Difco, USA) was evaluated at 25°C, 30°C and 37°C. Growth was monitored at every 2 h, up to 24 h, by spectrophotometric measurements (Ultrospec 2000 spectrophotometer; Pharmacia Biotech, UK) at 600 nm. At the same time, anti-*Listeria* activity in CFS was determined using by spot-on-the-lawn method described by van Reenen et al. (1998).

Determination of Minimal Inhibitory Concentration (MIC) and in vitro anti-Listeria activity of the bacteriocin MBSa4

Bacteriocin extraction

Bacteriocin produced by MBSa4 strain was precipitated by saturation with 60% of ammonium sulfate added in CFS. After stirring at 4°C for 4 h, supernatants were centrifuged at 10,000 xg (4°C) for 1 h and the sediments were resuspended with ammonium acetate buffer (25 mM) pH 6.5. The solution was applied on Sep-Pak C18 columns (Waters), and eluted with ammonium acetate buffer (25 mM) pH 6.5 containing increasing concentrations of i-propanol (20%, 40%, 60% and 80%). Anti-*Listeria* activity of the bacteriocin in each fraction was tested using spot-on-the-lawn (van Reenen et al., 1998) and fractions which showed activity were pooled and dehydrated under reduced pressure (Speed-Vac) and stored at -20°C.

Determination of Minimal Inhibitory Concentration (MIC)

The MIC was determined as described by Nielsen et al. (1990) with modifications. The bacteriocin extracted was resuspended in sterile distilled water and submitted to serial two-fold dilutions in 96-well microtiter-plates (TPP, Switzerland) containing 100 µl BHI broth (Oxoid, UK) in each well. In the next step, an overnight culture of *L. monocytogenes* Scott A obtained in BHI broth (Oxoid, UK) at 37°C were added to each well (10^5 - 10^6 CFU/mL) and the microplates were incubated at 37°C for 24 h. The MIC was determined as the lowest concentration of bacteriocin that resulted in absence of visible bacterial growth in 24 h.

In vitro anti-Listeria activity of the bacteriocin MBSa4

The bacteriostatic or bactericidal effect of the bacteriocin produced by *L. plantarum* MBSa4 against *Listeria monocytogenes* Scott A was tested according to Todorov and Dicks (2011). A 24 h culture of *L. monocytogenes* Scott A (10^5 - 10^6 CFU/mL) in BHI broth (Oxoid, UK) was transferred to fresh BHI broth (Oxoid, UK) and bacteriocin MBSa4 (value of MIC) was added to the culture at times 0 h and 6 h of incubation at 37°C. Spectrophotometric measurements (*Thermo Fisher Scientific Multiskan®FC*, Germany) at 595 nm were done each two hour during 24 h, the culture of *L. monocytogenes* Scott A without the addition of the bacteriocin was used as control.

Bacteriocin MBSa4 adsorption to Listeria monocytogenes cells

Bacteriocin MBSa4 adsorption at *L. monocytogenes* Scott A was tested as described by Yildirim et al (2002) with modifications. The culture of *L. monocytogenes* Scott A obtained from BHI broth (Oxoid) for 24 h at 37°C was centrifuged at 4000 xg

(4°C) for 15 min and washed twice in sterile phosphate buffer 5 mM pH 6.5. Cells were suspended using the same buffer in order to obtain a suspension with optical density at 600 nm equal to 1.0. Cell suspension was added an equal volume of CFS containing the bacteriocin, prepared as described above, and incubated at 37°C for one hour. After this period, the suspensions were centrifuged and the supernatant was tested for anti-*Listeria* activity of bacteriocin unbound by the test spot-on-the-lawn and adsorption percentage was calculated using the following equation:

$$\text{Adsorption\%} = 100 - (\text{AU/mL after treatment} / \text{AU/mL original} \times 100)$$

Estimative of the molecular weight of the bacteriocin MBSa4

The migration of the bacteriocin MBSa4 in Tris-Tricine Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed in continuous gradient gel designed to low molecular weight proteins, as described by Schagger and von Jagow (1987). Bacteriocin MBSa4 obtained (item 2.9.1) was injected into to two well of the same gel containing three layers: 1. stacking gel 10% polyacrylamide; 2. “spacer” gel 10% polyacrylamide; 3. and separating gel of 16.5% polyacrylamide. As standard low molecular weight marker was used ranging from 26,600 Da to 1,060 Da (Sigma). After electrophoresis in an apparatus (BioRad) at 90 V for 4 h, the gel was cut into two vertical parts. One part of the gel (with marker) was fixed with 5% formaldehyde solution for 20 min, rinsed with sterile ultra-purified water (Milli-Q®, Millipore) and stained with Coomassie Brilliant Blue R250. Then, kept at 4°C with agitation (80 rpm) for 18-24 h, destained in solution decolorizing and observed for formation of the bands by the peptide (s). The other part of the gel was used for detection of the anti-*Listeria* activity, as described by Bhunia et al. (1987). Bacteriocin

in the gel was fixed for 2 h in a solution of 20% i-propanol and 10% acetic acid, followed by rinsing with ultra purified water in Milli-Q® (Millipore) sterilized. The gel was kept at 4°C with agitation (80 rpm). After 24 h, the gel was added in an plate dish and overlaid with BHI soft-agar previously inoculated with *L. monocytogenes* Scott A (10^5 - 10^6 CFU/mL), incubated for 18 h at 37°C and examined for zone inhibition.

Partial Purification of bacteriocin MBSa4

Bacteriocin produced by *Lactobacillus plantarum* MBSa4 was partially purified according to Batdorj et al. (2006), with modifications. MRS broth (Biokar, France) was inoculated with 1% (v/v) overnight culture of MBSa4 and after 18 h at 25°C, cells were removed by centrifugation (6000 xg for 15 min at 4°C) (Centrifuge GR 2022, Jouan, France). The pH of the CFS was adjusted to 6.8 with 10 N NaOH (Euromedex, France). CFS was injected into a SP-Sepharose Fast Flow cation exchange column (GE Healthcare, Amersham, Sweden) equilibrated with 20 mM/L phosphate (Sigma-Aldrich, USA) buffer pH 6.8 (buffer A). The column was washed with buffer A and subsequently the absorbed substances were eluted in a linear gradient from 0 to 100% buffer B (20 mM/L sodium phosphate [Sigma-Aldrich, USA] + 1 M/L NaCl [Euromedex, France] pH 6.8). The fractions were collected and activity was tested against *L. ivanovii* subsp. *ivanovii* ATCC 19119.

Active fractions were pooled (Fraction 1) and applied into a reverse phase (RP) column (SOURCE™15RPC 10 mL;GE Healthcare, Amersham, Sweden) equilibrated with solvent A (0.05% trifluoroacetic acid [TFA] [Sigma-Aldrich, USA], 95% H₂O and 5% solvent B [80% acetonitrile {Biosolve, Netherlands} , 10% isopropanol {Sigma-Aldrich, USA}, 10% H₂O, 0.03% TFA {Sigma-Aldrich, USA}]). Elution was performed with solvent B in a linear gradient from 0-100% for 25 min, at a flow rate of

5 mL/min. After drying under reduced pressure (Speed-Vac, SC110A, Savant, USA), each fraction was tested for anti-*Listeria* activity.

The active fractions were pooled (Fraction 2) and submitted to another purification step, by reverse phase high performance liquid chromatography (RP-HPLC) using Unicorn 3.21 software (Amersham Pharmacia Biotech, Sweden). Fraction 2 was injected into a preparative C₁₈ column (Symmetry 300™ C₁₈, 5 µm 4,6x50 mm Waters, UK) equilibrated with solvent C (0.05% TFA [Sigma-Aldrich, USA], 5% solvent D [80% acetonitrile { Biosolve, Netherlands}, 20% H₂O, 0.03% TFA {Sigma-Aldrich, USA}]). Elution was performed with solvent D using a linear gradient from 25% to 60% in 35 min, at a flow rate of 6 mL/min. Peaks were detected by monitoring absorbance at 220 nm. Fractions were collected, dried under vacuum, dissolved in sterile ultra pure water (Milli-Q, Millipore, USA) and tested for anti-listerial activity. Then, activity fraction was combined with non activity fraction (1/1), tested again for anti-listerial activity and the activity fraction combined or not, was stored at -20°C.

Identification of genes encoding bacteriocin production

Isolate *L. plantarum* MBSa4 was investigated for the presence of known bacteriocin genes, using PCR and appropriate primers (Table 4). Total DNA was extracted using kit ZR Fungal/Bacterial DNA MiniPrep (Zymo Research) and submitted to amplification in a reaction mixture (20 µL) containing approximately 25 ng/µL of extracted DNA, 1x PCR buffer (Fermentas, Lithuania), 100 µM MgCl₂ (Fermentas, Lithuania), 200 µM dNTPs (Fermentas, Lithuania), 0.025 U *Taq* polymerase (Fermentas, Lithuania) and 1 pM each primer. Amplification was achieved in 35 cycles using a DNA thermocycler MasterCycler®PCR (Eppendorf Scientific, Germany) and PCR conditions for each primer are show on Table 5. PCR-amplified DNA were

separated on 2% (w/v) agarose gel electrophoresis and visualized by ethidium bromide (0.1 mg/mL) fluorescence using an UVP BioImaging System (DIGIDOC-IT System, USA). For each bacteriocin primer, a band corresponding to the correct size (Table 2) was purified from the gel using QIAquick® PCR Purification kit (Qiagen, Germany) according the manufacturer's instructions and submitted to sequencing at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of São Paulo, Brazil. The sequences were compared to those deposited in GenBank, using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>).

Results

Identification based on 16S rRNA sequencing indicated that one LAB isolated from salami is *Lactobacillus plantarum*. Further, *L. plantarum* strain was called MBSa4 by our researcher team of the FCF-USP and as showed in the Table 3, MBSa4 strain is a bacteriocin-producing strain, due lost antimicrobial activity when its CFS was treated with protease K, trypsin, pepsin, α -Chymotrypsin and Protease Type XIV (Table 1).

Bacteriocin produced by *L. plantarum* MBSa4 was not affected by range of temperature from refrigeration (4°C) to cooking (100°C), even temperature of autoclaving at 121°C by 15 min (Table 1). Full residual activity was observed at pH 2.0 to 6.0, but lost part of its activity at pH 8.0 (20.8%) and complete inactivation was observed at pH 10.0 (Table 1).

As shown in the Table 2, bacteriocin produced by *L. plantarum* MBSa4 was active against all *Listeria* spp. belonging to different serotypes tested, with exception for *Listeria seeligeri*. When tested against three strains of *Staphylococcus aureus*, antimicrobial activity was observed only for one (*Staphylococcus aureus* ATCC 29213). Bacteriocin produced by this strain was active for three out of ten strains of

Enterococcus spp and one (*Lactobacillus sakei* ATCC 15521) out of 25 strains of LAB. Bacteriocin MBSa4 was not active against the tested gram-negative bacteria (*Salmonella*, *Escherichia coli* and *Enterobacter*), nor against *Bacillus cereus*. Notably, MBSa4 strain showed antagonistic activities against all fungal tested, with exception of *Geotrichum candidum* (Table 3). However, antifungal activity was not observed by antimicrobial compounds production (data not shown).

L. plantarum MBSa4 growth (OD 600 nm), its bacteriocin-production (AU/mL) and pH reduction in MRS broth (Difco) when incubated at 25°C, 30°C and 37°C are shown in the Fig. 1. MBSa4 strain grew well in MRS broth (Difco) in the three temperatures tested, but caused fastest decrease of pH of the medium in the temperatures of 30°C and 37°C. The high level of bacteriocin was detected at 25°C for 22 h of incubation (1600 AU/mL) and bacteriocin production started in 14 h of incubation in this temperature (100 AU/mL). However, early bacteriocin production was detected at 30°C in the stationary growth phase (12 h of incubation), beginning with 200 AU/mL.

The determined value of the Minimal Inhibitory Concentration (MIC) for the extracted bacteriocin MBSa4 against *L. monocytogenes* Scott A was 1600 AU/mL. To test for bactericidal or bacteriostatic effect of the bacteriocin produced by *L. plantarum* MBSa4 (MIC value) was assayed using *L. monocytogenes* Scott A as indicator (Fig. 2). Bacteriocin MBSa4 had bacteriostatic effect on culture of pathogen, when added at times early lag phase (0 h) and early exponential phase (6 h). A low number of cells survived and were able to grow in the presence of bacteriocin, not inhibiting completely the growth of *L. monocytogenes*. One hundred percent of the bacteriocin MBSa4 added in the medium (100 AU/mL) was adsorbed to *L. monocytogenes* Scott A cells after one hour of incubation at 37°C.

The molecular weight of the bacteriocin produced by *L. plantarum* was determined by SDS-PAGE to be around of 2500 daltons (Fig. 3). The last step for partial purification (C₁₈ RP-HPLC) of the bacteriocin produced by *L. plantarum* MBSa4 is shown in the Fig.4. The chromatogram presents many peak and the results for anti-*Listeria* activity for each peak isolated and in combination is show in the Fig.5. When anti-*Listeria* activity of the peaks was tested separately, only peak 9 showed inhibitory zones (Fig. 5a). However, when the peak 9 was combined with others peaks (ratio 1:1) was observed a weak inhibition zone from the peak 1 at peak 8, and a strong inhibition zone was observed from the peak 10 at peak 12 (Fig. 5b).

The results of investigation of bacteriocin genes for bacteriocin-producing *L. plantarum* strain isolated from salami are listed in Table 6. The primers PlanW-F and PlanW-R specific for plantaricin W were able of to generate a DNA-fragment of approximately 165 bp with its DNA and the nucleotides sequencing of this amplicon corresponded to plantaricin W.

Discussion

This work describes the isolation, characterization and partial purification of an antimicrobial compound produced by a strain of *L. plantarum* (MBSa4) isolated from salami. The active compound has a proteinaceous nature because its activity was lost after treatment with proteases. According the definition of bacteriocins of Gram-positive bacteria given by Klaenhammer (1988), the antimicrobial compound produced by MBSa4 strain was confirmed to be bacteriocin. It known that the species of lactobacilli most commonly present in meat and meat products are *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* (Hugas and Monfort, 1997; Santos et al. 1998; Aymerich et al. 2006) and bacteriocin-producing *L. plantarum* strains have

been isolated from meat products (Messi et al., 2001; Rattanachaikunsopon and Phumkhachorn, 2006; Müller et al., 2009; Smaoui et al., 2010; Todorov et al., 2010).

The bacteriocin MBSa4 has interesting technological properties. A primary property is its thermostability, which the bacteriocin should keep its antimicrobial activity after heat treatment usually applied in food processing, similar results were observed for other bacteriocins as lactacin F (Muriana and Klaenhammer, 1991), plantaricins S (Jiménez-Díaz et al., 1993), plantaricin C (González et al., 1994), enterocin 1071 (Balla et al., 2000), plantaricin ASM1 (Hata et al., 2010) and plantaricin MG (Gong et al., 2010) and plantaricin C (Pei et al., 2013). Second its good stability at acid pH, also observed for nisin (Liu and Hansen, 1990), plantaricin C from *L. plantarum* LL441 (González et al., 1994), plantaricin MG produced by *L. plantarum* KLDS1.0391 (Gong et al., 2010) and bacteriocin produced by *L. plantarum* ST71KS (Martínez et al., 2013) which is required in the case of application in acidified products with a long shelf-life.

From the standpoint of its inhibitory spectra, bacteriocin MBSa4 appears to take a position more close to the IIa bacteriocins, which are very effective against *Listeria monocytogenes* (Nes and Holo, 2000; Cotter et al., 2005; Nishie et al., 2012), than class IIb bacteriocins, seems to be more active against closely related microorganisms, as example bacteriocins produced by *L. plantarum* C-11 (Daeschel et al., 1990), plantaricin S and plantaricin T from *L. plantarum* LPCO10 (Jiménez-Díaz et al., 1993), plantaricin C from *L. plantarum* LL441 (González et al., 1994), enterocin 1071 from *E. faecalis* BFE 1071 (Balla et al., 2000), Lactococcin Q from *Lactococcus lactis* QU4 (Zendo et al., 2006) and plantaricin ASM1 produced by *L. plantarum* A-1 (Hata et al., 2010). No antimicrobial activity of the bacteriocin MBSa4 was observed against Gram-negative bacteria. Stevens et al. (1991) theorized that bacteriocins of lactic acid bacteria

are inefficient to inhibit Gram-negative bacteria because the outer membrane hinders the site for bacteriocin action. More recently, some bacteriocins produced by *L. plantarum* strain with action against Gram-negative bacteria have been reported, such as bacteriocin produced by *L. plantarum* ST26MS and *L. plantarum* ST28MS can inhibit *Acinetobacter*, *Escherichia coli* and *Pseudomonas* (Todorov and Dicks, 2005), bacteriocin produced by *L. plantarum* AMA-K can inhibit *E. coli* (Todorov et al., 2007), plantaricin MG produced by *L. plantarum* KLDS1.0391 in that it can inhibit *E. coli*, *P. fluorescens*, *P. putida* and *S. typhimurium* (Gong et al., 2010) and bacteriocin produced by *L. plantarum* TN635 can inhibit *S. enterica*, *P. aeruginosa*, *Hafnia* sp. and *Serratia* sp. (Smaoui et al., 2010). However, its mechanism of action remains unclear.

Other interest technological properties shown by MBSa4 strain was the antagonist action against fungal. Filamentous moulds and yeast are common spoilage organism of food product and some moulds may also produce health damaging mycotoxins. However, antifungal activity not was observed by compounds action produced by MBSa4 strain. In the literature, some antifungal compounds producing BAL have been reported (Magnusson and Schnürer, 2001; De Muynck et al., 2004; Gerez et al., 2009; Stoyanova et al., 2010; Belgesmia et al., 2013).

Bacteriocin production by *L. plantarum* MBSa4 started at the late exponential phase and reached its maximum at the medium of the stationary phase (22 h at 25°C), suggesting that the antimicrobial peptide was a secondary metabolite, as is nisin (Hurst, 1981). However, high biomass values occurred at temperatures of 30°C and 37°C. Our results showed that the decrease of temperature below the optimum for growth improved the bacteriocin production. Many reports has been showed that unfavourable growth conditions such as low temperature, nutrient limitation, osmotic stress, etc. should stimulate bacteriocin production (De Vuyst et al. 1996; Kim et al., 1997; Aasen

et al., 2000; Leal-Sánchez et al., 2002; Mataragas et al., 2003; Delgado et al., 2005; Delgado et al., 2007). The positive effect of low temperatures in the bacteriocin production may be due increased availability of amino acids and energy at low growth rates and enzymatic reactions (Aasen et al., 2000).

The mode of action of bacteriocin of *L. plantarum* MBSa4, when added in the MIC value (1600 AU/mL), studied here may be supposed as bacteriostatic against *L. monocytogenes*, even when added in different times of growth of this pathogen, similar results was observed for plantaricin D produced by *L. plantarum* BFE 905 (Franz et al., 1998), plantaricin C19 produced by *L. plantarum* C19 (Atrih et al., 2001), bacteriocin produced by *L. plantarum* lp 31 (Müller et al., 2009). Todorov (2009) reported that the class II bacteriocins demonstrate a bactericidal mode of action against other closely related organisms. Bactericidal action of some bacteriocins has been described in the literature, such as bacteriocin produced by *L. plantarum* KLDS1.0391 show antimicrobial activity against *Salmonella typhimurium* ATCC14028 (Gong et al., 2010), bacteriocin BacTN635 produced by *L. plantarum* TN635 against *L. ivanovii* BUG 496 (Smaoui et al., 2010), bacteriocin ST71KS produced by *L. plantarum* ST71KS against *L. monocytogenes* (Martinez et al., 2013) and plantaricin C produced by *L. paracasei* CICC 20241 against *A. acidoterrestris* and *L. helveticus* (Pei et al., 2013).

It is common knowledge that electrostatic interactions with cytoplasmatic membrane bacterial are responsible for the initial binding some bacteriocins (Dridet et al., 2006). The bacteriocin MBSa4 showed a strong interaction with *Listeria monocytogenes* cells (100% adsorbed), differently of bacteriocin AMA-K (Todorov et al., 2007) and Pentocin 31-1 (Liu et al., 2008) that shown adsorption of 75% and 50%, respectively.

In SDS-PAGE gel, fraction active of bacteriocin produced by *L. plantarum* MBSa4 migrated as a peptide of approximately 2.5 kDa, similar to plantaricin S (Jimenez-Diaz et al., 1993) a two-peptide bacteriocin nonlantibiotic (Table 7).

The study of amino acid sequence not was possible due either the obtainment of little amount of bacteriocin or appearing unstable of the peptides in this last purification step (C₁₈ RP-HPLC). Similar phenomenon was observed by Nissen-Meyer et al. (1993), which reported that much of the purified plantaricin A activity was lost during reverse-phase chromatography. According to the criteria reported by Cotter et al. (2005) for classification of the bacteriocin, the anti-*Listeria* compound produced by *L. plantarum* MBSa4 could be characterized as two-peptides bacteriocin, whose antimicrobial activity of some fractions after last step of the purification was dependent upon the complementation of the fraction active. Moreover, fragment homologous to plantaricin W gene was obtained using DNA of *L. plantarum* MBS4 with specific primers for PlanW-F/PlanW-R. Holo et al., (2001) reported that plantaricin W from *Lactobacillus plantarum* LMG 2379 is a two-peptides bacteriocin lantibiotics. Number of other bacteriocin two-peptides lantibiotic and nonlantibiotic that works at a 1:1 ratio have been isolated from different sources (Table 7).

In 2000, Leistner have been defined that an intelligent application of combined preservative factors (hurdles) ensures the microbial safety and stability as well as the sensory and nutritional quality of the foods. The most common hurdles used in food preservation are temperature, water activity, acidity, redox potential, competitive microorganisms and chemical preservatives (e.g., nitrite, sorbate). However, with the increasing demand for more natural and microbiologically safe food products, there is a need for new preservation techniques. Among the emerging preservative technologies, the bacteriocins of LAB have been highlighted, e.g. with the use of the nisin, currently

approved in more than 48 countries and by the US Food and Drug Administration (de Arauz et al., 2009), and also others bacteriocins with potential application for control of pathogens have been reported in the literature (Nishie et al., 2012).

The emergence of nisin-resistant *L. monocytogenes* mutants has already been reported (Gravesen et al., 2002; Martinez et al., 2005; Saá Ibusquiza et al., 2011). Kaur et al. (2011) reviewed possible mechanisms involved in the development of resistance to nisin and Class IIa bacteriocins for some foodborne pathogens. Therefore, studies of characterization and application of other bacteriocin classes actually are important for use more effective of this antimicrobial like biopreservative in food. Macwana and Muriana, 2012, reported that a possible use of the mixtures of bacteriocins of different modes of action could provide greater inhibition than mixtures of bacteriocins of the same mode of action.

Some two-peptides bacteriocin have been applied as food preservation, such as Lactocin 705 for control of *L. monocytogenes* in ground beef (Vignolo et al., 1996), lacticin 3147 for control of *L. monocytogenes* in cottage cheese (McAuliffe et al., 1999), lacticin 3147 for the control of *L. monocytogenes* and *Bacillus cereus* in natural yogurt and cottage cheese (Morgan et al., 2001), lacticin 481 for control of *L. monocytogenes* during the manufacture and storage of cottage cheese (Dal Bello et al., 2012).

In conclusion, the properties of bacteriocin produced by *L. plantarum* MBSa4 described here appear quite promising for development of consistent salami of high quality. However, further study of application in food model and optimization of the purification process, repectively, will help to evaluate its effectiveness for the control of pathogens in this product and to classify this bacteriocin.

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References

Aasen, I.M., Moretro, T., Katla, T., Axelsson, L. and Storro, I. (2000) Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. *Applied Microbiology Biotechnology* **53**, 159-166.

Albano, H., Todorov, S.D., van Reenen, C.A., Hogg, T., Dicks, L.M.T. and Teixeira, P. (2007) Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from “Alheira”, a fermented sausage traditionally produced in Portugal. *International Journal of Food Microbiology* **116**, 239–247.

Atrih, A., Rekhif, N., Moir, A.J., Lebrihi, A. and Lefebvre, G. (2001) Mode of action, purification and amino acid sequence of plantaricin C19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C19. *Int J Food Microbiol.* **68**, 93-104.

Aymerich, T., Martin, B., Garriga, M., Vidal-Carou, M.C., Bover-Cid, S. and Hugas, M. (2006) Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *Journal of Applied Microbiology* **100** 40–49.

Balla, E., Dicks, L.M.T., Du Toit, M., Van Der Merwe, M.J. and Holzapfel, W.H. (2000) Characterization and Cloning of the Genes Encoding Enterocin 1071A and Enterocin 1071B, Two Antimicrobial Peptides Produced by *Enterococcus faecalis* BFE 1071. *Applied and Environmental Microbiology*, 1298–1304

Batdorj, B., Dalgarrondo, M., Choiset, Y., Pedroche, J., Métro, F., Prévost, H., Chobert, J.-M. and Haertlé, T. (2006) Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. *Journal of Applied Microbiology* **101**, 1364-5072.

Belguesmia, Y., Choiset, Y., Rabesona, H., Baudy-Floc'h, M., Le Blay, G., Haertlé, T., Chobert, J.M. (2013) Antifungal properties of durancins isolated from *Enterococcus durans* A5-11 and of its synthetic fragments. *Lett Appl Microbiol.* 56, 237-44.

Cotter, P. D., Hill, C. & Ross R. P. (2005) Bacteriocins: developing innate immunity for food. *Nature Review Microbiology*, 3, 777-788.

Daeschel, M.A., McKenney, M.C. and McDonald, L.C. (1990) Bacteriocidal activity of *Lactobacillus plantarum* C-11. *Food Microbiology*, 7, 91-98.

Dal Bello, B., Cocolin, L., Zeppa, G., Field, D., Cotter, P.D. and Hill, C. (2012) Technological characterization of bacteriocin producing *Lactococcus lactis* strains employed to control *Listeria monocytogenes* in Cottage cheese. *International Journal of Food Microbiology* 153 58–65

de Arauz, L.J., Jozala, A.F., Mazzola, P.G. and Penna, T.C.V. (2009) Nisin biotechnological production and application: a review. *Trends in Food Science & Technology* 20 146-154.

De Muynck, C., Leroy, A.I., De Maeseneire, S., Arnaut, F., Soetaert, W., Vandamme, E.J. (2004) Potential of selected lactic acid bacteria to produce food compatible antifungal metabolites. *Microbiol Res.* 159, 339-46.

De Vuyst, L. and Leroy, F. (2007) Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications. *J. Mol. Microbiol. Biotechnol.* 13 (4), 194–199.

De Vuyst, L., Callewaert, R. and Crabbe, K. (1996) Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology* 142, 817-827.

Deegan, L. H., Cotter, P. D., Hill, C. & Ross, P. (2006) Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *International Dairy Journal*, 16, 1058–1071.

Delgado, A., Brito, D., Peres, C., Noé-Arroyoc, F. and Garrido-Fernández, A. (2005) Bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration. *Food Microbiology* 22 521–528

Delgado, A., López, F.N.A., Brito, D., Peres, C., Fevereiro, P., Garrido-Fernández, A. (2007) Optimum bacteriocin production by *Lactobacillus plantarum* 17.2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. *J Biotechnol.* 130 193-201.

Dobson, A., Cotter, P.D., Ross, R.P. and Hill, C. (2012) Bacteriocin production: a probiotic trait? *Applied Environmental Microbiology* **78**, 1-6.

Drider, D., Fimland, G., Héchard, Y., McMullen, L.M. and Prévost, H. (2006) The Continuing Story of Class IIa Bacteriocins. *MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS*, 70, 564–582.

Felske, A., Rheims, H., Wokerink, A., Stackebrandt, E. and Akkermans, A.D.L. (1997) Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology* **143**, 2983-2989.

Franz, C.M.A.P., Du Toit, M., Olasupo, N.A., Schillinger, U. and Holzapfel, W.H. (1998) Plantaricin D, a bacteriocin produced by *Lactobacillus plantarum* BFE 905 from ready-to-eat salad. *Letters in Applied Microbiology*, 26, 231–235.

Gerez, C.L., Torino, M.I., Rollán, G. and de Valdez, G.F. (2009) Prevention of bread mould spoilage by using lactic acid bacteria with antifungal properties. *Food Control* 20, 144–148.

Gong, H.S., Meng, X.C. and Wang, H. (2010) Plantaricin MG active against Gram-negative bacteria produced by *Lactobacillus plantarum* KLDS1.0391 isolated from “Jiaoke”, a traditional fermented cream from China. *Food Control* 21 89–96

Gonzalez, B., Arca, P., Mayo, B. and Suárez, J.E. (1994) Detection, Purification, and Partial Characterization of Plantaricin C, a Bacteriocin Produced by a *Lactobacillus*

plantarum Strain of Dairy Origin. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 2158-2163

Gravesen, A., Jydegaard Axelsen, A.M., Mendes da Silva, J., Hansen, T.B., Knøchel, S. (2002) Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. Appl Environ Microbiol. 68, 756-764.

Hata, T., Tanaka, R. and Ohmomo, S. (2010) Isolation and characterization of plantaricin ASM1: A new bacteriocin produced by *Lactobacillus plantarum* A-1. International Journal of Food Microbiology 137 94–99

Holo, H., Jeknic, Z., Daeschel, M., Stevanovic, S. and Nes, I.F. (2001) Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. *Microbiology* 147, 643–651.

Hugas, M. and Monfort, J. Ma. (1997) Bacterial starter cultures for meat fermentation. *Food Chemistry*, Vol. 59, No. 4, pp. 547-554,

Hurst, A., 1981. Nisin. Adv. Appl. Microbiol., 27: 85–122.

Jímenez-Díaz, R., Ríos-Sánchez, R.M., Desmazeaud, M., Ruiz-Barba, J.L. and PIARD, J.-C. (1993) Plantaricins S and T, Two New Bacteriocins Produced by *Lactobacillus plantarum* LPCO10 Isolated from a Green Olive Fermentation Applied and Environmental Microbiology, May 1993, p. 1416-1424

Kaiser, A. L. & Montville, T. J. (1996) Purification of the Bacteriocin Bavaricin MN and Characterization of Its Mode of Action against *Listeria monocytogenes* Scott A Cells and Lipid Vesicles. *Applied and Environmental Microbiology*, 4529–4535.

Kaur, G., Malik, R.K., Mishra, S.K., Singh, T.P., Bhardwaj, A., Singroha, G., Vij, S. and Kumar, N. (2011) Nisin and Class IIa Bacteriocin Resistance Among *Listeria* and Other Foodborne Pathogens and Spoilage Bacteria Microbial Drug Resistance, 17 197-205.

Kemperman, R., Kuipers, A., Karsens, H., Nauta, A., Kuipers, O. and Kok, J. (2003) Identification and Characterization of Two Novel Clostridial Bacteriocins, Circularin A and Closticin 574. *Applied and Environmental Microbiology* 69 (3), 1589–1597.

Kim, W.S., Hall, R.J. and Dunn, N.W. (1997) The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*. *Appl Microbiol Biotechnol* 48: 449±453.

Klaenhammer, T. R. (1988) Bacteriocins of lactic acid bacteria. *Biochimie* 70 337-349.

Klaenhammer, T.R. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews*, v.12, p.39-85, 1993.

Leal-Sánchez, M.V., Jiménez-Díaz, R., Maldonado-Barragán, A., Garrido-Fernández, A. and Ruiz-Barba, J.L. (2002) Optimization of Bacteriocin Production by Batch Fermentation of *Lactobacillus plantarum* LPCO10. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, 68, 4465–4471.

Leistner, L. (2000) Basic aspects of food preservation by hurdle technology. *International Journal of Food Microbiology* 55 181–186.

Liu, G., Lv, Y., Li, P., Zhou, K. and Zhang, J. (2008) Pentocin 31-1, an anti-*Listeria* bacteriocin produced by *Lactobacillus pentosus* 31-1 isolated from Xuan-Wei Ham, a traditional China fermented meat product. *Food Control* 19 353–359

Liu, W. and Hansen, J.N. (1990) Some Chemical and Physical Properties of Nisin, a Small-Protein Antibiotic Produced by *Lactococcus lactis* *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, Aug. 1990, p. 2551-2558

Macwana, S. and Muriana, P.M. (2012) Spontaneous bacteriocin resistance in *Listeria monocytogenes* as a susceptibility screen for identifying different mechanisms of resistance and modes of action by bacteriocins of lactic acid bacteria. *Journal of Microbiological Methods* 88 7–13.

Magnusson, J. and Schnürer, J. (2001) *Lactobacillus coryniformis* subsp. *coryniformis* Strain Si3 Produces a Broad-Spectrum Proteinaceous Antifungal Compound. *Appl Environ Microbiol.* 67, 1–5.

Magnusson, J., Ström, K., Roos, S., Sjögren, J. and Schnürer, J. (2003) Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiology Letters* 219 (1), 129–135.

Martínez, B., Bravo, D., Rodríguez, A. (2005) Consequences of the development of nisin-resistant *Listeria monocytogenes* in fermented dairy products. *J Food Prot.* 68, 2383-2388.

Martinez, R.C.R., Wachsman, M., Torres, N.I., LeBlanc, J.G., Todorov, S.D. and Franco, B.D.G.M. (2013) Biochemical, antimicrobial and molecular characterization of a noncytotoxic bacteriocin produced by *Lactobacillus plantarum* ST71KS. *Food Microbiology* Volume 34, Issue 2, June 2013, Pages 376–381

Mataragas, M., Metaxopoulos, J., Galiotou, M. and Drosinos, E.H. (2003) Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. *Meat Science* 64 265–271.

McAuliffe, O., Hill, C. and Ross, R.P. (1999) Inhibition of *Listeria monocytogenes* in cottage cheese manufactured with a lacticin 3147-producing starter culture. *Journal Applied Microbiology*, 86, 251-256.

Messi, P., Bondi, M., Sabia, C., Battini, R. and Manicardi, G. (2001) Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. *International Journal of Food Microbiology* 64 193–198

Mills, S.; Stanton, C., Hill, C. & Ross, R. P. (2011) New Developments and Applications of Bacteriocins and Peptides in Foods. *Annual Review of Food Science and Technology*, 2, 299–329.

BARBOSA, M. S.

Morgan, S.M., Galvin, M., Ross, R.P. and Hill, C. (2001) Evaluation of a spray-dried lactacin 3147 powder for the control of *Listeria monocytogenes* and *Bacillus cereus* in a range of food systems. *Lett Appl Microbiol.* 33, 387-391.

Müller, D.M., Carrasco, M.S., Tonarelli, G.G. and Simonetta, A.C. (2009) Characterization and purification of a new bacteriocin with a broad inhibitory spectrum produced by *Lactobacillus plantarum* lp 31 strain isolated from dry-fermented sausage. *Journal of Applied Microbiology* 106 2031–2040

Muriana, P. M. and Klaenhammer, T.R. (1991) Purification and Partial Characterization of Lactacin F, a Bacteriocin Produced by *Lactobacillus acidophilus* 11088. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, Jan., p. 114-121 Vol. 57, No. 1

Nes, I.F. and Holo, H. (2000) Class II Antimicrobial Peptides from Lactic Acid Bacteria. *Biopolymers* Vol. 55, 50–61

Nielsen, J.W., Dickson, J.S. and Crouse, J.D. (1990) Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Applied and Environmental Microbiology* **56**, 2142-2145.

Nishie, M. Nagao, J.-I. and Sonomoto, K. (2012) Antibacterial Peptides “Bacteriocins”: An Overview of Their Diverse Characteristics and Applications. *Biocontrol Science* **17**, 1-16.

Nissen-Meyer, J., Larsen, A.G., Sletten, K., Daeschel, M. and Nes, I.F. (1993) Purification and characterization of plantaricin A, a *Lactobacillus plantarum* bacteriocin whose activity depends on the action of two peptides. *Journal of General Microbiology* 139, 1973–1978.

Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K. & Panyim, S. (2003) Isolation of nisin-producing *Lactococcus lactis* WNC 20 strain from nham, a traditional Thai fermented sausage. *International Journal of Food Microbiology*, 81, 137–145.

Pei, J., Yuan, Y. and Yue, T. (2013) Primary characterization of bacteriocin paracin C – A novel bacteriocin produced by *Lactobacillus paracasei*. *Food Control* 34 168-176

Rattanachaikunsopon, P. and Phumhachorn, P. (2006) Isolation and Preliminary Characterization of a Bacteriocin Produced by *Lactobacillus plantarum* N014 Isolated from Nham, a Traditional Thai Fermented Pork. *Journal of Food Protection*, Vol. 69, No. 8, Pages 1937–1943

Saá I.P., Herrera, J.J.R. and Cabo, M.L. (2011) Comparison between the Resistance of Benzalkonium Chloride-Adapted and -Nonadapted Biofilms of *Listeria monocytogenes* to Modified Atmosphere Packaging and Nisin Once Transferred to Mussels. *J Food Prot.* Jul;74(7):1112-8.

Santos, E. M., González-Fernández, C., Jaime, I. and Rovira, J. (1998) Comparative study of lactic acid bacteria house flora isolated in different varieties of 'chorizo'. *International Journal of Food Microbiology* 39 123–128.

Schagger, H. and Von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 166, 368–379.

Smaoui, S., Elleuch, L., Bejar, W., Karray-Rebai, I., Ayadi, I., Jaouadi, B., Mathieu, F., Chouayekh, H., Bejar, S., Mellouli, L. (2010) Inhibition of Fungi and Gram-Negative Bacteria by Bacteriocin BacTN635 Produced by *Lactobacillus plantarum* sp. TN635. *Appl Biochem Biotechnol* 162:1132–1146

Stevens, K.A., Sheldon, B.W., Klapes, N.A., Klaenhammer, T.R. (1991) Nisin treatment for inactivation of *Salmonella* species and other Gram-negative bacteria. *Appl. Environ. Microbiol.*, 57, 3613-3615.

Stoyanova, L.G., Ustyugova, E.A., Sultimova, T.D., Bilanenko, E.N., Fedorova, G.B., Khatrukha, G.S. and Netrusov, A.I. (2010) New Antifungal Bacteriocin-Synthesizing Strains of *Lactococcus lactis* ssp. *lactis* as the Perspective Biopreservatives for Protection of Raw Smoked Sausages. *American Journal of Agricultural and Biological Sciences* 5 (4): 477-485.

Todorov, S.D. and Dicks, L.M.T. (2005) *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria. *Enzyme and Microbial Technology* **36**, 318–326.

Todorov, S.D. (2008) Bacteriocin production by *Lactobacillus plantarum* AMA-K isolated from Amasi, a Zimbabwean fermented milk product and study of the adsorption of bacteriocin AMA-K to *Listeria* sp. *Braz. J. Microbiol.*, 39, 178-187.

Todorov, S.D. (2009) Bacteriocins from *Lactobacillus plantarum* production, genetic organization and mode of action: produção, organização genética e modo de ação. *Braz. J. Microbiol.* 40, 209-221.

Todorov, S.D., Ho, P., Vaz-Velho, M. and Dicks, L.M.T. Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal (2010) *Meat Science* 84 334–343.

Todorov, S.D., Rachman, C., Fourrier, A., Dicks, L.M.T., van Reenen, C.A., Prévost, H. and Dousset, X. (2011) Characterization of a bacteriocin produced by *Lactobacillus sakei* R1333 isolated from smoked salmon. *Anaerobe* **17**, 23–31.

van Reenen, C.A., Dicks L.M.T. and Chikindas, M.L. (1998) Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology* **84**, 1131–1137.

Vignolo, G., Fadda, S., de Kairuz, M.N., de Ruiz Holgado, A.A.P. and Oliver, G. (1996) Control of *Listeria monocytogenes* in ground beef by 'Lactocin 705', a bacteriocin produced by *Lactobacillus casei* CRL 705. International Journal of Food Microbiology 29 397-402

Yıldırım, Z., Avşar, Y. K. **and** Yıldırım, M. (2002) Factors affecting the adsorption of buchnericin LB, a bacteriocin produced by *Lactocobacillus buchneri*. Microbiol. Res. 157 (2), 103–107.

Zendo, T., Koga, S., Shigeri, Y., Nakayama, J. and Sonomoto, K. (2006) Lactococcin Q, a Novel Two-Peptide Bacteriocin Produced by *Lactococcus lactis* QU 4. Applied and Environmental Microbiology, 3383–3389.

Table 1 Effects of different treatments on the bacteriocin activity produced by *Lactobacillus plantarum* MBSa4.

Treatment	Residual bacteriocin activity (%)
Enzymes	
Protease K	0
Trypsin	0
Pepsin	0
α -Chymotrypsin	0
Protease Type XIV	0
Temperature	
4° C (60 min)	100
25° C (60 min)	100
30° C (60 min)	100
37° C (60 min)	100
45° C (60 min)	100
60° C (60 min)	100
80° C (60 min)	100
100° C (60 min)	100
121° C (15 min)	100
pH	
2	100
4	100
6	100
8	20.8
10	0

Table 2 Spectrum of activity of the bacteriocin produced by *Lactobacillus plantarum* MBSa4.

Indicator microorganism	Source	Activity (mm)
<i>Bacillus cereus</i>	ATCC 1178	0
<i>Staphylococcus aureus</i>	ATCC 29213	7
<i>Staphylococcus aureus</i>	ATCC 25923	0
<i>Staphylococcus aureus</i>	ATCC 6538	0
<i>Listeria welshimeri</i>	USP ^a	7
<i>Listeria seeligeri</i>	USP	0
<i>Listeria ivanovii</i> subsp. <i>ivanovii</i>	ATCC 19119	8
<i>Listeria innocua</i>	ATCC 33090	7
<i>Listeria innocua</i> AL225/07 sorovar 6a	FIOCRUZ ^b	7
<i>Listeria innocua</i> AL224/07 sorovar 6a	FIOCRUZ	8
<i>Listeria innocua</i> AL047/07 sorovar 6a	FIOCRUZ	7
<i>Listeria innocua</i> AL588/08 sorovar 6a	FIOCRUZ	8
<i>Listeria monocytogenes</i> Scott A	USP	9
<i>Listeria monocytogenes</i> AL602/08 sorovar 1/2a	FIOCRUZ	6
<i>Listeria monocytogenes</i> AL046/07 sorovar 1/2c	FIOCRUZ	6
<i>Listeria monocytogenes</i> 103 sorovar 1/2a	USP	6
<i>Listeria monocytogenes</i> 106 sorovar 1/2a	USP	6
<i>Listeria monocytogenes</i> 104 sorovar 1/2a	USP	10
<i>Listeria monocytogenes</i> 409 sorovar 1/2a	USP	9
<i>Listeria monocytogenes</i> 506 sorovar 1/2a	USP	7
<i>Listeria monocytogenes</i> 709 sorovar 1/2a	USP	9
<i>Listeria monocytogenes</i> 607 sorovar 1/2b	USP	8
<i>Listeria monocytogenes</i> 603 sorovar 1/2b	USP	8
<i>Listeria monocytogenes</i> 426 sorovar 1/2b	USP	6
<i>Listeria monocytogenes</i> 637 sorovar 1/2c	USP	6
<i>Listeria monocytogenes</i> 422 sorovar 1/2c	USP	5
<i>Listeria monocytogenes</i> 712 sorovar 1/2c	USP	9
<i>Listeria monocytogenes</i> 408 sorovar 1/2c	USP	7
<i>Listeria monocytogenes</i> 211 sorovar 4b	USP	9
<i>Listeria monocytogenes</i> 724 sorovar 4b	USP	8
<i>Listeria monocytogenes</i> 101 sorovar 4b	USP	9
<i>Listeria monocytogenes</i> 703 sorovar 4b	USP	8
<i>Listeria monocytogenes</i> 620 sorovar 4b	USP	8
<i>Listeria monocytogenes</i> 302 sorovar 4b	USP	5
<i>Escherichia coli</i>	ATCC 8739	0
<i>Escherichia coli</i> O157:H7	ATCC 35150	0
<i>Enterobacter aerogenes</i>	ATCC 13048	0
<i>Salmonella</i> Typhimurium	ATCC 14028	0
<i>Salmonella</i> Enteritidis	ATCC 13076	0
<i>Enterococcus faecalis</i>	ATCC 12755	11
<i>Enterococcus hirae</i> D105	AGRIS ^c	12
<i>Enterococcus faecium</i> S5	AGRIS	0
<i>Enterococcus faecium</i> S154	AGRIS	0

<i>Enterococcus faecium</i> S100	AGRIS	8
<i>Enterococcus faecium</i> ST62	AGRIS	0
<i>Enterococcus faecium</i> ST211	AGRIS	0
<i>Enterococcus faecium</i> ET 12	UCV ^d	0
<i>Enterococcus faecium</i> ET 88	UCV	0
<i>Enterococcus faecium</i> ET 05	UCV	0
<i>Lactobacillus</i> sp. V94	USP	0
<i>Lactobacillus fermentum</i> ET35	UCV	0
<i>Pediococcus pentosaceus</i> ET 34	UCV	0
<i>Lactobacillus curvatus</i> ET 06	UCV	0
<i>Lactobacillus curvatus</i> ET 31	UCV	0
<i>Lactobacillus curvatus</i> ET 30	UCV	0
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> 2a	USP	0
<i>Lactobacillus sakei</i>	ATCC 15521	8
<i>Lactobacillus plantarum</i> V69	USP	0
<i>Lactobacillus delbrueckii</i> B5	USP	0
<i>Lactobacillus delbrueckii</i> ET32	UCV	0
<i>Lactobacillus acidophilus</i> La14	Rhodia	0
<i>Lactobacillus acidophilus</i> Lac4	Rhodia	0
<i>Lactobacillus acidophilus</i> La5	Rhodia	0
<i>Lactococcus lactis</i> B16	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> MK02R	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D2	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B1	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D4	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B2	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B15	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D3	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D5	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B17	USP	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> R704	Chr. Hansen	0

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Table 3 Antagonistic activities of the *Lactobacillus plantarum* MBSa4 against fungi

Indicator microorganism	Antifungal Activities*
<i>Penicillium roqueforti</i> LMSA1.12.138	+
<i>Penicillium expansum</i> LMSA1.08.102	+
<i>Fusarium</i> sp	+
<i>Geotrichum candidum</i>	-
<i>Mucor plumbeus</i> s LMSA1.03.032	+
<i>Cladosporium</i> sp LMSA1.12.139	+
<i>Debaromyces hansenii</i> LMSA2.11.003	+

*+ inhibited the strain; - not inhibited the strain

Table 4 Primers used in this study.

Specificity	Primers	Sequence (5' -3')	Expected size (bp)	Reference
Plantaricin S	PlanS-F	GCC TTA CCA GCG TAA TGC CC	450	Moghadam et al., 2010
	PlanS-R	CTG GTG ATG CAA TCG TTA GTT T		
Plantaricin NC8	PlanNC8-F	GGT CTG CGT ATA AGC ATC GC	207	Moghadam et al., 2010
	PlanNC8-R	AAA TTG AAC ATA TGG GTG CTT TAA ATT CC		
Plantaricin W	PlanW-F	TCA CAC GAA ATA TTC CA	165	Moghadam et al., 2010
	PlaW-R	GGC AAG CGT AAG AAA TAA ATG AG		
Sakacin T- α	SakT- α -F	TCGGTGGCTATACTGTCTAAACA	160	Macwana and Muriana (2012)
	SakT- α -R	TGTCCTAAAAATCCCAATGC		
Sakacin T- β	SakT- β -F	AAGAAATGATAGAAATTTTGGAGG	151	Macwana and Muriana (2012)
	SakT- β -R	TGTGAAATCCCAATCTTGCTCG		
Sakacin Q	SakQ-F	GAA (T/A)T(A/G)(C/A)(A/C)A NCA ATT A(C/T)(A/C) GGT GG	130	Dortu et al. (2008)
	SakQ-R	TAC CAC CAG CAG CCA TTC CC		
Sakacin X	SakX-F	AGCTATGAAAGGTATGTGCGG	156	Macwana and Muriana (2012)
	SakX-R	TAAAGATTCCAGCCAGCAGC		
Sakacin P	SakP-F	ATG GAA AAG TTT ATT GAA TTA	186	Reminger et al. (1996)
	SakP-R	TTA T TT ATT CCA GCC AGC GTT		
Sakacin G	SakGA1-F	TTA GAA CTA CAC TGA TCG TG		Todorov et al. (2011)
	SakGA1-R	TGG AAG AAT GAG TAC TTG TT		
Sakacin G	SakGA2-F	CGT TAC AAC AGA ACT TCA AG		Todorov et al. (2011)
	SakGA2-R	TGG AAG AAT GAG TAC TTG TT		
Curvacin A	CurA-F	GTA AAA GAA TTA AGT ATG ACA	171	Reminger et al. (1996)
	CurA-R	TTA CAT TCC AGC TAA ACC ACT		

Table 5 Optimized cycling conditions used for the amplification of bacteriocin genes.

Primers	Initial denaturation	Denaturation	Annealing	Elongation
PlanS-F	94 °C, 3 min	94 °C, 30 s	45 °C, 1 min	72 °C, 1 min
PlanNC8	94 °C, 3 min	94 °C, 1 min	51 °C, 1 min	72 °C, 30 s
PlanW	94 °C, 3 min	94 °C, 1 min	41 °C, 1 min	72 °C, 30 s
SakT- α (F/R)	95 °C, 15 min	95 °C, 30 sec	58 °C, 1 min	72 °C, 1 min
SakT- β (F/R)	95 °C, 15 min	95 °C, 30 sec	56 °C, 1 min	72 °C, 1 min
SakQ (F/R)	95 °C, 15 min	95 °C, 30 sec	53 °C, 1 min	72 °C, 1 min
SakX (F/R)	95 °C, 15 min	95 °C, 30 s	58 °C, 1 min	72 °C, 1 min
SakP (F/R)	94 °C, 3 min	94 °C, 30 s	40 °C, 1 min	72 °C, 1 min
SakG (F/R)	94 °C, 4 min	94 °C, 30 s	38 °C, 30 s	72 °C, 30 s
CurA (F/R)	94 °C, 3 min	94 °C, 30 s	40 °C, 1 min	72 °C, 1 min

Table 6 Translated gene of the bacteriocin genes amplified using specific primers for bacteriocins.

Specificity	Primers	Amplification	Translated gene
Plantaricin S	PlanS (F/R)	Negative	
Plantaricin NC8	PlanNC8 (F/R)	Negative	
Plantaricin W	PlanW (F/R)	Positive	MKISKIEAQAARKDFFKKIDTNSNLLNVNGAKCKWWNISCE
Sakacin T- α	SakT- α (F/R)	Negative	
Sakacin T- β	SakT- β (F/R)	Negative	
Sakacin Q	SakQ (F/R)	Negative	
Sakacin X	SakX(F/R)	Negative	
Sakacin P	SakP (F/R)	Negative	
Sakacin G	SakGA1 (F/R)	Negative	
Sakacin G	SakGA2 (F/R)	Negative	
Curvacin A	CurA (F/R)	Negative	
Enterocin A	EntA (F/R)	Negative	
Enterocin B	EntB (F/R)	Negative	
Enterocin P	EntP (F/R)	Negative	
Enterocin L50B	EntL50B (F/R)	Negative	
Nisin	Nis (F/R)	Negative	
Pediocin PA1	PedPA1 (F/R)	Negative	

Table 7 Summary of known two-peptide bacteriocins lantibiotic and nonlantibiotic.

Strain	Source	Bacteriocin	Classification	Reference
<i>Bacillus thuringiensis</i> DPC 6431	human fecal	Thuricin CD	nonlantibiotic	Rea et al., 2010
<i>Brochothrix campestris</i> ATCC 43754	Soil	Brochocin-C	nonlantibiotic	Talon et al., 1988 McCormick et al., 1998
<i>Enterococcus faecalis</i> C901	human colostrum	Enterocin C	nonlantibiotic	Maldonado-Barragán et al., 2009
<i>Enterococcus faecalis</i> NKR-4-1	Thai fermented fish	Enterocin W	lantibiotic	Sawa et al., 2012
<i>Enterococcus faecalis</i>	Clinical isolates	Cytolysin	lantibiotic	Booth et al., 1996
<i>Enterococcus faecalis</i> BFE 1071	feces of minipigs	Enterocin 1071	nonlantibiotic	Balla et al., 2000
<i>Enterococcus faecalis</i> FAIR-E 309	Argentinian cheese	Enterocin 1071	nonlantibiotic	Franz et al., 2002
<i>Enterococcus faecium</i> L50	Dry-fermented sausage	Enterocin L50	nonlantibiotic	Cintas et al., 1995 Cintas et al., 1998
<i>Enterococcus faecium</i> KU-B5	Sugar apples	Enterocin X	nonlantibiotic	Hu et al., 2010
<i>Lactobacillus casei</i> CRL 705	Fermented sausage	Lactocin 705	nonlantibiotic	Cuozzo et al., 2000
<i>Lactobacillus johnsonii</i> VPI11088	Human intestine	Lactacin F	nonlantibiotic	Fremaux et al., 1993 Allison et al., 1994
<i>Lactococcus lactis</i> LMG 2081		Lactococcin G	nonlantibiotic	Nisse-Meyer et al., 1992
<i>Lactococcus lactis</i> QU 4	corn	Lactococcin Q	nonlantibiotic	Zendo et al., 2006
<i>Lactococcus lactis</i> subsp. <i>lactis</i> DPC3147	Irish kefir grain	Lacticin 3147	lantibiotic	Ryan et al., 1999
<i>Lactobacillus salivarius</i> DPC6005	porcine intestinal	Salivaricin P	nonlantibiotic	Barret et al., 2007
<i>Lactobacillus salivarius</i> UCC118	Human gastrointestinal tract	ABP-118	nonlantibiotic	Flynn et al., 2002
<i>Lactobacillus plantarum</i> C11	cucumber fermentations	Plantaricin A Plantaricin	nonlantibiotic	Daeschel et al., 1990 Nissen-

		EF Plantaricin JK		Meyer et al., 1993 Andersen et al., 1998 Jiménez- Díaz et al., 1993 Jiménez- Díaz et al., 1995 Aukrust and Blom, 1992 Maldonado et al., 2003
<i>Lactobacillus plantarum</i> LPCO10	Green olive Fermentation	Plantaricin S	nonlantibiotic	
<i>Lactobacillus plantarum</i> NC8	Grass silage	plantaricin NC8	nonlantibiotic	
<i>Lactobacillus plantarum</i> LMG 2379	fermenting Pinot Noir wine	Plantaricin W	lantibiotic	Holo et al., 2001
<i>Leuconostoc</i> MF215B		Leucocin H	nonlantibiotic	Blom et al., 1999
<i>Staphylococcus aureus</i> C55	Human skin	Staphylococc in C55	lantibiotic	Dajani et al., 1968 Navaratna et al., 1998
<i>Streptococcus bovis</i> HJ50	Raw milk	Bovicin HJ50	lantibiotic	Xiau et al., 2004
<i>Streptococcus mutans</i> UA140	Caries-active dental patient	Mutacin IV	nonlantibiotic	Qi et al., 2001
<i>Streptococcus thermophilus</i> SF13	Nestle strain collection	Thermophili n 13	nonlantibiotic	Marciset et al., 1997

Figure 1 Bacteriocin production (bar) and pH reduction (dotted line) and growth (continue line) of *Lactobacillus plantarum* MBSa4 in MRS broth, when incubated at 25°C, 30°C and 37°C by 24 h.

Figura 2 Growth of *Listeria monocytogenes* Scott A in BHI broth at 37°C after addition of the bacteriocin produced by *Lactobacillus plantarum* MBSa4, added at time 0 h (■), 6 h (▲) and without bacteriocin (♦).

Figure 3 SDS-PAGE gel containing bacteriocin produced by *Lactobacillus plantarum* MBSa4. (a) gel stained with Coomassie Brilliant Blue R250 (b) gel overlaid with BHI soft-agar inoculated with *Listeria monocytogenes* Scott A after incubation at 37°C for 12 h.

Figure 4 Chromatogram of the bacteriocin produced by *Lactobacillus plantarum* MBSa4 (C₁₈ reverse-phase HPLC).

Figure 5 Anti-litteral activities of fractions after last step of purification (C₁₈ reverse-phase HPLC) of the bacteriocin produced by *Lactobacillus plantarum* MBSa4 isolated (a) and combined with fraction 9 (b).

Capítulo 03

**“Control of *Listeria monocytogenes* in Italian type salami by bacteriocins produced
by autochthonous *Lactobacillus curvatus*”**

Artigo em preparação para publicação no periódico Meat Science

**Control of *Listeria monocytogenes* in salami by bacteriocins produced by
autochthonous *Lactobacillus curvatus***

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Abstract:

The aims of this study were to isolate lactic acid bacteria with anti-*Listerial* activity from salami samples, characterize the bacteriocins produced by selected isolates, semi-purify the bacteriocin(s) produced by these strains and evaluate their effectiveness for the control of *Listeria monocytogenes* during manufacturing of experimentally contaminated salami. Two isolates, identified as *Lactobacillus curvatus* based on 16S rDNA sequencing (named MBSa2 and MBSa3), presented activity against all tested *L. monocytogenes* strains and several other Gram-positive bacteria. Temperature, pH and NaCl had little effect on antimicrobial activity. The three-step purification procedure indicated that *Lb. curvatus* MBSa2 and MBSa3 produced two active peptides each (4457.9 Da and 4360.1 Da, sharing homology to sakacins P and X), identical in the two isolates. Addition of the semi-purified bacteriocins produced by MBSa2 strain to experimentally contaminated batter for production of salami caused 1.98 log and 1.77 log reductions in the counts of *L. monocytogenes* in salami after 10 and 20 days respectively, evidencing the potential application of these bacteriocins to improve safety of salami during its manufacturing.

Key-words: Bacteriocin, salami, *Lactobacillus curvatus*, *Listeria monocytogenes*, biopreservation

1. Introduction

Lactic acid bacteria (LAB), especially *Lactobacillus sakei* and *Lactobacillus curvatus*, are part of the microbiota of many types of fermented meat products. These two species of LAB are well adapted to the meat environment, playing a key role for improved flavor and accelerated maturation of fermented meat products (Chaillou et al., 2005; Lahtinen et al., 2011). LAB are also essential agents for hygienic quality of foods, preventing growth of spoilage and pathogenic microorganisms by acidification and production of antimicrobial compounds, like bacteriocins contributing to improved safety and quality (Fadda, López, & Vignolo, 2010; Balciunas et al., 2013; Mangia et al., 2013).

Bacteriocins produced by LAB are antimicrobial proteinaceous compounds synthesized by the ribosomes, presenting variable spectrum of activity. Most bacteriocins are small molecules with amphipathic characteristics and high isoelectric point. The producer cells are immune to the bacteriocins they produce due to synthesis of specific immunity proteins (Deegan et al., 2006; Mills, Stanton, Hill, & Ross, 2011; Dobson et al., 2012; Nishie, Nagao, & Sonomoto, 2012). Currently, numerous bacteriocins produced by different LAB species have been described (Balciunas et al., 2013). According to Cotter, Hill, & Ross, 2005, between 30 and 99% of the prokaryotes (Bacteria and Archaea) produce at least one bacteriocin.

Bacteriocins produced by LAB are well known for their activity against *Listeria monocytogenes*, a ubiquitous Gram-positive pathogen that has caused several food related outbreaks in the last decades (Kumar, 2011; Scallan, et al., 2011). Hang et al. (2007) even dedicated a special class in their classification of bacteriocins to those with anti-Listerial activity. It is well known that *L. monocytogenes* can survive the

technological hurdles usually encountered during manufacture of dry fermented products, such as low pH, salt and presence of nitrites (Vogel et al., 2010). Due to this anti-*Listeria* activity, bacteriocinogenic LAB and their bacteriocins have a potential application as preservation agents in fermented products, and can be used as technological alternatives to chemical preservatives, fitting the increased demand for foods with less or no additives (Dickson-Spillmann, Siegrist, & Keller, 2011).

Surveys in Brazil indicate that *L. monocytogenes* is a frequent contaminant in fermented meat products, such as sausages and salami, detected in 6.2%- 6.7% (Martins & Germano, 2011; Sakate et al., 2003) to 13.3% (Borges et al., 1999) of the tested samples. In this study, we describe the isolation of LAB with anti-Listerial activity from Italian type salami produced in Brazil, characterization of the bacteriocins produced by two selected isolates, and evaluation of the effectiveness of the semi-purified bacteriocin produced by one of the isolates on the control of *L. monocytogenes* in experimentally contaminated salami during manufacturing.

2. Material and Methods

2.1 Isolation and identification of bacteriocinogenic LAB from salami

Italian type salami samples were purchased in retail markets in the city of Sao Paulo (Brazil), and 50 g of each sample were submitted to microbiological analysis aiming at isolating LAB capable to produce bacteriocins, using the methodology described in Todorov et al, 2010. Identification of the strains was done using recommended morphological, biochemical and genetic approaches, including 16S rDNA sequence analysis of genomic DNA, amplified with primers 8f (5'-CAC GGA TCC AGA CTT TGA T(C/T)(A/C) TGG CTC AG-3') and 1512r (5'- GTG AAG CTT ACG G(C/T)T

AGC TTG TTA CGA CTT-3'), as described by Felske et al, 1997. Purified amplified PCR products were sequenced at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil, and sequences were compared to known sequences in GenBank using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). The genetic similarity of the bacteriocinogenic isolates was tested by Random Amplification of Polymorphic DNA (RAPD), as described by Todorov et al. (2010).

2.2 Titration of the produced bacteriocins

The amount of bacteriocin produced by two selected bacteriocinogenic isolates (*Lb. curvatus* MBSa2 and MBSa3) was determined testing two-fold dilutions of cell free supernatants (CFS) for antimicrobial activity according to van Reenen, Dicks, & Chikindas, (1998), using *L. monocytogenes* Scott A as indicator strain. For preparation of the CFS, strains were grown in MRS broth (Difco, Detroit, MI, USA) for 24 h at 30 °C and cells were removed by centrifugation at 4000 x g for 15 min at 4 °C (Hettich Zentrifugen, model Mikro 22R, Tuttlingen, Germany). The pH of CFS was adjusted to 6.0-6.5 with 1 mol l⁻¹ NaOH (Synth, Sao Paulo, Brazil), heated 30 min at 70 °C and filter-sterilized (Millex GV 0.22 µm, Millipore, Billerica, MA, USA). One arbitrary unit (AU) was defined as the reciprocal of the highest dilution that resulted in production of a clear zone of inhibition of *L. monocytogenes*. Results were expressed in AU ml⁻¹ (van Reenen, Dicks, & Chikindas, 1998).

2.3 Characterization of the bacteriocinogenic strains

2.3.1 Growth and bacteriocin production in MRS broth

The bacteriocinogenic strains *Lb. curvatus* MBSa2 and MBSa3 were tested for growth and bacteriocin production in MRS broth (Difco) at 25 °C, 30 °C and 37 °C. Growth was monitored measuring absorbance at 600 nm (Ultrospec 2000; Pharmacia Biotech, Little Chalfont, UK) at every 2 h up to 24 h. Changes in pH of the cultures were recorded. Presence of bacteriocins in the CFS was monitored at every 2 h up to 24 h, using the spot-on-the-lawn method and *L. monocytogenes* Scott A as indicator of activity, as described before.

2.3.2 Influence of NaCl content and pH of MRS broth on growth

Bacteriocinogenic strains *Lb. curvatus* MBSa2 and MBSa3 were tested for growth in MRS broth containing increasing NaCl contents and acid pH, simulating conditions that occur during manufacturing of salami. Strains were inoculated (10^6 - 10^7 CFU/mL) in MRS broth containing from 1% up to 10% NaCl and pH adjusted to 4 or 6 with 1N lactic acid, and incubated at 30 °C. Growth was monitored at every 2 h up to 24 h, measuring changes in absorbance as described before.

2.4 Characterization of the bacteriocin produced by the strains

2.4.1 Effect of temperature, pH and salt content on activity

CFS of the strains *Lb. curvatus* MBSa2 and MBSa3, prepared as described before, were tested for antimicrobial activity after exposing them at 4 °C, 25 °C, 30 °C, 37 °C, 45 °C, 60 °C, 80 °C and 100 °C for 60 min, and at 121 °C for 15 min. The influence of pH on activity was tested after adjustment of the pH of the CFS to values ranging from 2 to 10, using 1N NaOH or 10 M phosphoric acid, and incubation for 1 h at 25 °C. Before

testing for antimicrobial activity, the pH of each CFS was neutralized to 6.0-6.5. The effect of salt on bacteriocin activity was tested adding 1% up to 10% NaCl to the CFS of the cultures, and incubating at 7 °C, 30 °C and 37 °C for 2 h. Sterile MRS broths containing the same amounts of NaCl were used as negative controls. For all tests, the residual antimicrobial activity of the treated CFS was measured using the spot-on-the-lawn method and *L. monocytogenes* Scott A as indicator of activity, as described before.

2.4.2 Spectrum of activity

The CFS of strains *Lb. curvatus* MBSa2 and MBSa3, prepared as described before, were tested for antimicrobial activity against several Gram-negative and Gram-positive bacteria, listed in Table 1. The activity was measured by the spot-on-the-lawn method, as described before.

2.4.3 Search for bacteriocin genes

Lb. curvatus MBSa2 and MBSa3 were investigated for the presence of known bacteriocin genes using PCR and the primers listed in Table 3. Total DNA was extracted and submitted to amplification in a reaction mixture (20 µl) containing 25 ng µl⁻¹ of extracted DNA, 1x PCR buffer (New England BioLabs), 100 µmol l⁻¹ MgCl₂ (Fermentas), 200 µmol l⁻¹ dNTPs (Fermentas), 0.025 U *Taq* polymerase (New England BioLabs) and 1 pmol l⁻¹ each primer. Amplification was achieved in 35 cycles using a DNA thermocycler MasterCycler[®] PCR (Eppendorf Scientific). PCR conditions are show in Table 3. PCR-amplified DNA fragments were separated by 2% (w/v) agarose gel electrophoresis, stained with ethidium bromide (0.1 mg ml⁻¹) and visualized using the UVP BioImaging System (DIGIDOC-IT System). For each primer, the

corresponding bands (sizes described in Table 3) were purified with QIAquick® PCR Purification kit (Qiagen) according to the manufacturer's instructions and submitted to sequencing at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil. The sequences were compared to those deposited in GenBank, using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST>).

2.5 Purification of bacteriocins

Bacteriocins produced by strains *Lb. curvatus* MBSa2 and MBSa3 were purified as described by Batdorj et al. (2006), with some modifications. MRS broth (Biokar, Beauvais, France) was inoculated with a 1% (v/v) overnight culture of the bacteriocinogenic strain and incubated for 18 h at 25 °C, then the cells were removed by centrifugation at 6000 x g for 15 min at 4 °C (Centrifuge GR 2022, Jouan, France). The pH of the CFS was adjusted to 6.8 with 10 N NaOH (Euromedex, Souffelweyersheim, France) and loaded into a SP-Sepharose Fast Flow cation-exchange column (GE Healthcare, Amersham, Uppsala, Sweden) equilibrated with 20 mmol l⁻¹ phosphate (Sigma-Aldrich) buffer pH 6.8 (buffer A). The column was washed with buffer A and the absorbed substances were eluted with a linear gradient from 0 to 100% buffer B (20 mmol l⁻¹ sodium phosphate + 1 mol l⁻¹ NaCl [Euromedex] pH 6.8). The fractions were collected and tested for antimicrobial activity using the spot-on-the-lawn method and *L. ivanovii* subsp. *ivanovii* ATCC 19119 as sensitive microorganism (van Reenen et al, 1998). Fractions presenting activity were pooled and submitted to RP-high performance liquid chromatography (RP-HPLC), using Unicorn 3.21 software (Amersham Pharmacia Biotech). The pools were loaded into a preparative C₁₈ column (Symmetry 300™ C₁₈, 5 µm 4.6 x 50 mm Waters, Hertfordshire, UK) equilibrated with solvent A

(0.05% TFA, 5% solvent B [80% acetonitrile, 20% H₂O, 0.03 % TFA], 95% H₂O). Elution was performed with solvent B using a linear gradient from 0 to 100% in 25 min, at a flow rate of 5 ml min⁻¹. Peaks were detected by monitoring absorbance at 220 nm. Fractions were collected, dried under vacuum, dissolved in sterile ultra-pure water (Milli-Q, Millipore, Billerica, MA, USA) and tested for anti-*Listeria* activity. The protein concentration in this material, corresponding to purified bacteriocins, was measured in microtiter plates using Pierce[®] BCA protein assay kit (Thermo Fisher Scientific, Schwerte, Germany), with albumin (Sigma-Aldrich) as standard. Molecular mass measurement was performed on a quadrupole-time-of-flight hybrid mass spectrometer (Q-TOF Global, Waters, Manchester UK), equipped with an electrospray ionization (ESI) source and operated in the positive ion mode. Fractions collected from the HPLC were diluted in a mixture of water and acetonitrile (1:1, v/v) acidified with 0.1% formic acid, and infused into the mass spectrometer at a continuous flow rate of 5 µl min⁻¹. Following parent mass determination, ions were fragmented in the collision cell of the mass spectrometer using an appropriate energy. The obtained MS/MS spectra were interpreted to reconstruct a sequence tag of the peptide. Results were searched against NCBI databank using the BLAST program.

2.6 Control of *L. monocytogenes* in salami by bacteriocin produced by *Lb. curvatus* MBSa2

2.6.1 Preparation of the bacteriocin for application in salami

The bacteriocinogenic strain *Lb. curvatus* MBSa2 was selected for the tests of control of *Listeria monocytogenes* in salami. The CFS obtained after culturing the strain in MRS broth for 24 h at 30 °C, centrifuged at 4000 x g for 15 min at 4 °C, was subjected to

ammonium sulphate precipitation (80%) at 4 °C for 4 h, and centrifuged at 10,000 x g at 4 °C for 1 h. The pellet was resuspended with 25 mM ammonium acetate buffer pH 6.5 and the suspension was applied to a Sep-Pak C18 column (Waters, Hertfordshire, UK). The proteins were separated by increasing concentrations of isopropanol (20%, 40%, 60% and 80%) in ammonium acetate buffer (25 mM) pH 6.5. The collected fractions were tested for antimicrobial activity using the spot-on-the-lawn method and *L. ivanovii* subsp. *ivanovii* ATCC 19119 as sensitive microorganism. Fractions presenting activity were pooled, dehydrated under reduced pressure (Speed-Vac) and stored at -20°C.

2.6.2 Determination of Minimal Inhibitory Concentration (MIC)

The Minimal Inhibitory Concentration (MIC) of the semi-purified bacteriocin MBSa2 was determined by the microdilution method described by Nielsen et al, 1990, using 96-well microplates containing 100 µL of BHI broth in the wells. A culture of *L. monocytogenes* Scott A (10^4 - 10^5 CFU ml⁻¹) was used as indicator of the antimicrobial activity.

2.6.3 Manufacture of salami and experimental contamination with *L. monocytogenes* Scott A

Salami was prepared in the pilot plant of a meat industry, located in Sao Paulo, SP, Brazil, following the manufacture procedure used in this industry. Salami was formulated with 10% bovine meat, ground through a 3 mm disc, 75% pork shoulder, ground through a 8 mm disc and 15% lard, chopped into cubes of appr. 125 mm³. The meats were added of 1.3% NaCl, 1% Compact Salami 160 (Kraki and Kienast Ltda, Brazil), correspondent to a preformulated mixture of maltodextrin, sugar, garlic powder,

onion powder, ground red pepper, ground white pepper, sodium nitrate, sodium erythorbate, garlic essential oil and nutmeg essential oil, and 0.02% Bactoferm™ T-SPX starter culture (*Pediococcus pentosaceus* and *Staphylococcus xylosus*) (CHR Hansen, Denmark). The ingredients were mixed in a stainless steel meat homogenizer (CAF HG 120/114S, Brazil) for 3 to 5 min and the resulting batter was kept under refrigeration until used. For experimental contamination, a culture of *L. monocytogenes* Scott A in BHI broth incubated at 37 °C for 24 h was centrifuged at 6000 x g for 15 min and the pellet was resuspended in sterile 0.1% peptone (w/v) water. This procedure was repeated three times in order to eliminate all components of the BHI medium. The salami batter was divided in four parts: one was added of the suspension of *L. monocytogenes* Scott A to achieve a contamination level of 10^4 - 10^5 CFU g⁻¹; the second was added of the same suspension of *L. monocytogenes* Scott A and the semi-purified bacteriocin MBSa2 at the concentration determined in the MIC test; the third was added of the same suspension of *L. monocytogenes* Scott A and sterile water (same volume as the semi-purified bacteriocin) and the last one served as control (received no additional cultures). The batters were transferred into caliber 60 collagen casings (Fibran S.A., Brazil), pre-hydrated in 15% saline solution for 30 min, using a small-scale stainless steel filling machine (Filizola, Brazil). Prior each use, the cylinder and the piston of the filling machine were autoclaved at 121°C for 15 min. The casings containing the batter (approx.. 20 cm long) were transferred to EL111 chambers (Eletrolab, Brazil) where the temperature and relative humidity (RH) were controlled as follows: 4 days at 20°C and 97% RH (fermentation step), 5 days at 18°C and RH from 95% to 87% and then for 20 days at 15 °C and RH from 87% to 75% (maturation step). These experiments were performed in triplicates.

2.6.4 Counts of *L. monocytogenes* in the experimentally inoculated salami

Counts of *L. monocytogenes* were performed in the batters (time 0) and at 4, 10, 20 and 30 days of manufacture of salami. For the tests, 25 g of the product were removed and homogenized with 225 ml sterile 0.1% peptone water in a stomacher. The mixtures were submitted to decimal serial dilutions in sterile 0.1% peptone water and surface plated on Oxford agar (Difco) in duplicates. Plates were incubated at 37 °C for 24h, when colonies were counted. Results were expressed in log CFU g⁻¹.

2.6.5 pH and water activity (a_w) measurements

The pH and the a_w of the samples at times 0, 4, 10, 20 and 30 days of manufacture of salami were measured using a HI1090B6 pH electrode (Hannah Instruments, USA) and Novasina AWC500 (Novasina AG, Switzerland), respectively. Both measurements were made in duplicates.

2.6.6 Statistic analyses

All experiments were repeated twice. Counts of *Listeria monocytogenes* were submitted to analysis of variance (ANOVA) and to Tukey's Test when applicable. The Statistica software version 7.0 was used in these tests and the adopted level of significance was 5% ($p < 0.05$)

3. Results and Discussion

3.1. Isolation of the bacteriocinogenic strains and characterization of the produced bacteriocins

Several LAB strains isolated from the salami samples presented capability to produce inhibitory substances against the tested microorganisms. However, when submitted to the appropriate tests for bacteriocin production (Todorov et al, 2010), only two of them were bacteriocinogenic, as demonstrated by the sensitivity of the inhibitory substances to proteolytic enzymes (α -chymotrypsin, *Streptomyces griseus* protease type XIV, trypsin, pepsin and proteinase K). The 16S rDNA sequencing indicated that the two strains were *Lactobacillus curvatus* (MBSa2 and MBSa3). The RAPD-PCR performed with primers OPL-01, OPL-02, OPL-04, OPL-14 and OPL-20 indicated that they were two distinct strains (Fig. 1).

Despite the common presence of LAB in meat and meat products, there are very few reports on strains with antimicrobial activity isolated from these products. Surdiman et al., (1993) isolated eight strains with antimicrobial activity among 56 isolates of *Lactobacillus* spp strains obtained from semidry sausages. Cintas et al., 1995 reported that only fifty-five among 500 LAB isolates from Spanish dry-fermented sausages presented antagonistic activity against *L. monocytogenes* Scott A. Aymerich et al. (2006) failed in the isolation of LAB presenting *in vitro* anti-Listerial activity from fuet, chorizo and salchichon. Belgacem et al. (2008) reported that 9% of the 48 LAB isolated from gueddid, a Tunisian fermented meat, were active against *L. monocytogenes*. Vermeiren et al. (2004) obtained better results, as 38% of strains originating from meat products inhibited *L. monocytogenes*, *Leuconostoc mesenteroides*, *Leuconostoc carnosum* and *Brochotrix thermosphacta*. Todorov et al. (2013) reported on *Lb. sakei* isolated from portuguese fermented meat products with activity against *L. monocytogenes*.

As shown in Fig 2, production of bacteriocins MBSa2 and MBSa3 in MRS broth started in the early exponential growth phase (4 h of incubation), regardless the temperature. However, when the incubation was performed at 37 °C, after 12 h the amount of produced bacteriocins started to decrease, for both strains. The maximum production of bacteriocin MBSa2 ($12\,800\text{ AU ml}^{-1}$) occurred at 8 h at 25 °C and 37 °C, and 6 h at 30 °C. Production of bacteriocin MBSa2 presented a similar profile, however the maximum production at 25 °C occurred at 10 h. These features indicating primary metabolite kinetics were also observed for other bacteriocins, such as sakacin K produced by *Lb. sakei* CTC 494 (Leroy & De Vuyst, 1999), sakacin P produced by *Lb. sakei* CCUG 42687 (Moreto et al., 2000), curvacin A produced by *Lb. curvatus* LTH 1174 (Messens et al., 2003), curvaticin L442 produced by *Lb. curvatus* L422 (Xiraphi et al., 2006) and the bacteriocin produced by *Lb. plantarum* ST16Pa (Todorov et al., 2011).

The spectra of activity of bacteriocins MBSa2 and MBSa3 can be seen in Table 1. The bacteriocin MBSa2 inhibited 22 out of 23 *L. monocytogenes* strains, while the bacteriocin MBSa3 inhibited all 23. The two bacteriocins inhibited some other Gram-positive bacteria in a similar pattern and none of them inhibited the tested Gram-negative bacteria. These results are not surprising, as bacteriocins are defined as compounds that are active against closely related species (Deegan et al., 2006). The spectra of activity of these strains can be considered similar to that reported for several other bacteriocins isolated from meat products (Surdiman et al., 1993; Belgacem et al., 2008; Vermeiren et al., 2004; Todorov et al., 2010, Todorov et al., 2013), reinforcing the potential application of bacteriocinogenic strains or their bacteriocins as additional hurdles for inhibition of undesirable microorganisms.

Presence of NaCl in MRS broth had a negative effect on the growth of both bacteriocinogenic strains only when the concentration was equal or higher than 6% (Fig 3), which is not surprising as lactobacilli do not grow well in presence of high levels of NaCl. However, the capability to grow and produce bacteriocins at 4-6% NaCl, even if lower than in the absence of salt, is an important feature of these strains, as they can be applied in salted meat products such as salami, without affecting their inhibitory potential. It should be noted that capability to grow and produce bacteriocins in the presence of salt seems to be a strain-dependent feature. Coppola et al., (1997) reported that all 183 strains of *Lactobacillus* spp isolated from fermented sausage during maturation were able to grow in MRS broth containing 8% NaCl and most of them at 10% NaCl. Papamanoli et al., (2003) observed that among 49 strains of *Lb. sakei*, 24 strains of *Lb. curvatus* and 7 strains of *Lb. plantarum*, 24%, 17% and 100% presented growth in the presence of 10% NaCl. Other studies have shown that salt may affect the activity of bacteriocins in different intensity. Garcia et al. (2004) observed that 2, 4 and 6% NaCl did not affect the activity of enterocin EJ97 against *L. monocytogenes* CECT 4032, while Bouttefroy et al. (2000) reported that 1% to 6% NaCl reduced the antimicrobial activity of curvacin 13.

For both strains, a better growth was detected at pH 6.0 than at pH 4 (Fig 3). As shown in Table 2, pH had similar effect on the activity of the two bacteriocins, except that for bacteriocin produced by MBSa3, when exposed to pH 10, the residual antimicrobial activity was reduced to 26%. As for stability at acidic pH, detected for the bacteriocins MBSa2 and MBSa3, several studies have shown that most bacteriocins are stable over a wide pH range, such as pediocin L50 (Cintas et al., 1995), piscicocin CS526 (Yamazaki et al., 2005), acidocin D20079 (Deraz et al., 2005) and pediocin NV

5 (Mandal et al., 2011). Less pH stability was described for plantaricin LP 31 (Müller et al., 2009). This tolerance to pH is a convenient characteristic of these strains because they may be used in acidic as well as non-acidic foods for biopreservation.

Effect of pH on growth of LAB in general of bacteriocinogenic strains in particular is another feature that is strain-dependent. Papamanoli et al. (2003) reported that none of the 49 *Lb. sakei* strains isolated from salami was capable to grow in MRS at pH 4, but 10 out of 24 *Lb. curvatus* strains and all 7 *Lb. plantarum* strains grew well in these conditions.

Results shown in Table 2 indicate that the bacteriocins MBSa2 and MBSa3 were heat stable molecules. Both maintained the same antimicrobial activity after autoclaving at 121°C for 15min. This property indicates that both can be used in foods that are submitted to different degrees of heat treatment, without affecting their biopreservative characteristics. Usually, low molecular weight bacteriocins are heat-stable as they are small polypeptides. Same properties have been already described for sakacin M (Sobrino et al., 1992), pediocin L50 (Cintas et al., 1995), piscicocin CS526 (Yamazaki et al., 2005), acidocin D20079 (Deraz et al., 2005), plantaricin LP31 (Müller et al., 2009), sakacin P (de Carvalho et al., 2010) and pediocin NV 5 (Mandal et al., 2011).

The purification of bacteriocins MBSa2 and MBSa3, achieved by the three-step procedure (cation-exchange, followed by sequential hydrophobic-interaction and reversed-phase chromatography), resulted in two peaks (P1 and P2) in the final chromatogram of each bacteriocin (Table 4), with a yield of purification of 20% and 10%, respectively. This three-step procedure resulted in successful purification of both bacteriocins MBSa2 and MBSa3. Other studies have used other purification methods, with different degrees of success. The direct injection of bacterial culture supernatants

into a cation-exchange chromatography was used for purification of pediocin PA-1 (Uteng et al., 2002), divergicin M35 (Tahiri et al., 2004) and enterocin A5-11 (Batdorj et al., 2006). Todorov et al. (2004) observed that purification with and without a previous precipitation with ammonium sulfate achieved the same results.

Mass spectrometry analysis conducted in the purified materials indicated that peak 1 (P1) contained two peptides, with molecular masses of 4457.9 Da and 2228.16 Da, and the partial aminoacid sequences AAANWATGGNAG and AGNSSNFLHKLQQLFT, respectively. Database screening indicated that first peptide is sakacin P, and the second corresponds to a bacteriocin-type signal sequence domain protein found in *Lb. curvatus* CRL 705. The peak 2 (P2) contained one peptide of 4360.1 Da and partial amino acid sequence AVANLTTGGAGG, also present in sakacin X. These results suggest that both *Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa3 produce two different bacteriocins.

When the DNA extracted from *L. curvatus* MBSa2 and MBSa3 were tested for bacteriocin genes using primers listed in Table 3, positive amplicons were obtained only with primers SakP-F/SakP-R, targeting sakacin P structural gene (*sakA*). The sequence of the amplified product of 186 bp presented homology to sakacin P structural gene and was detected in both strains (Fig 4).

The literature contains description of several LAB capable to produce two or more bacteriocins. *Carnobacterium piscicola* V1 produced piscicocin V1a with molecular mass 4416 Da and piscicocin V1b with molecular mass 4526 Da (Bhugallo-Vial et al., 1996). *Leuconostoc mesenteroides* TA33a produces three bacteriocins: leucocin A-TA33a (3933 Da), leucocin B-TA33a (3466 Da) and leucocin C-TA33a (4598 Da) (Papathanasopoulos et al., 1997). *Lb. sakei* 5 produced sakacin 5T, 5X and

5P, and *L. mesenteroids* 6 produced leucocin 6A and leucocin 6C (Vaughan et al., 2001). *Enterococcus durans* A5-11 is a strain that produces of two different bacteriocins with molecular mass 5206 Da (enterocin A5-11A) and 5218 Da (enterocin A5-11B) (Batdorj et al., 2006). *Lb. sakei* subsp. *sakei* 2a produces at least three compounds with antimicrobial activity: sakacin P (4.4 kDa), a ribosomal protein S21 (6.8 kDa) and a histone-like DNA-binding protein (9.5 kDa) produced by *Lb. sakei* subsp. *sakei* 23 K (Carvalho et al., 2010). *Enterococcus faecium* L50 produces four enterocins: L50A, L50B, Q and P (Criado et al., 2006) and *Enterococcus faecium* NKR-5-3 also produces four enterocins: NKR-5-3A (5242.3 Da), NKR-5-3B (6316.4 Da), NKR-5-3C (4512.8 Da) and NKR-5-3D (2843.5 Da) (Ishibashi et al., 2012). This ability to produce multiple bacteriocins may be advantageous for a strain, enhancing its ability to compete with other bacteria in the same environment (Vaughan et al., 2001).

3.2 Control of *Listeria monocytogenes* by bacteriocins produced by *Lactobacillus curvatus* MBSa2 in salami

The MIC value of the semi-purified bacteriocin produced by *Lb. curvatus* MBSa2 against *L. monocytogenes* was 200 AU ml⁻¹, which corresponded to the amount added to the salami batter (200 AU g⁻¹) for evaluation of the capability to control the growth of this pathogen.

Measurements of pH and a_w and counts of *L. monocytogenes* in the batter and salami during the manufacturing process are presented in Tables 5 and 6 and Fig. 5. The pH dropped from an average of 5.81 in the batter to 4.81 in the product at the 4th day of manufacturing (fermentation step), increasing again to 5.36 and 5.43 at the 20th and 30th day of manufacturing (maturation step). At the end of the fermentation period (4th day),

the average pH in the four types of salami was similar, and the same occurred in the maturation step (Table 5). The water activity (a_w) dropped gradually from 0.99 in the batter to 0.88 in the product at the 30th day of production (Table 6).

The semi-purified bacteriocin produced by *Lb. curvatus* MBSa2 caused a small reduction (0.5 log) in the counts of *L. monocytogenes* (Fig 5) immediately after its addition to the batter. The counts remained the same up to the 4th day of fermentation ($p>0.05$) and started to decrease afterwards. The decrease was more evident in the samples containing the bacteriocin, and on the 10th day, the counts of *L. monocytogenes* were almost 2 log lower than in samples without added bacteriocin. At the end of the maturation step (30th day), the detected difference in the CFU/g counts was 1.77 log.

The manufacturing process of Italian type salamis, such as the one used in this study, is expected to reduce the counts of pathogens present in these products. However, the reduction may be not enough to for effective control of pathogens that are common in such products and may cause disease, like *L. monocytogenes*. Nightingale et al, 2006, have shown that counts of *Salmonella* spp in experimentally contaminated Italian-style salami batter dropped from 7.4 log CFU to 4.5 log CFU/g when the moisture/protein ratio in the product was 1.4:1. However, *L. monocytogenes* populations in these products reduced less than 1 log CFU/g, indicating that supplemental measures are necessary to achieve the expected 5 log reduction determined by the regulatory agencies in the Unites States. In Brazil, *L. monocytogenes* is a frequent contaminant in salami (Sakate et al., 2003; Petruzzelli et al., 2009; Di Pinto et al., 2010; Okada et al., 2012), so that the application of bacteriocins produced by LAB can be a technological alternative to be considered to increase safety of these products.

Bacteriocins can be used in foods for biopreservation in three ways: 1) application of bacteriocin-producing LAB strain, alone or in combination with starter cultures in the fermentation process; 2) addition of the purified or semi-purified bacteriocins. Nisin is a good example for application in biopreservation as a commercial semi-purified preparation and 3) incorporation of an ingredient previously fermented with a bacteriocin-producing strain (Mills et al., 2011). All approaches offer advantages and disadvantages, but the use of the purified or semi-purified bacteriocins is the best option to promote safety, as they inhibit the proliferation of food-borne pathogenic and spoilage-causing bacteria without changing the taste or odor of the product (Nishie et al., 2012).

A number of studies have tested the effect of adding purified or semi-purified bacteriocins to foods for the control of pathogenic bacteria, with controversial results. The application of enterocin CCM 4231 (12 800 AU/g) in dry fermented Hornad salami reduced the counts of *L. monocytogenes* immediately after addition of the bacteriocin and maintained these counts until the end of trial period when compared with control samples (Lauková et al., 1999). The effect of pediocin AcH produced by *Lb. plantarum* WHE 92 applied to sliced cooked sausage was not efficient enough to kill all *L. monocytogenes* (Mattila et al., 2003). The inhibitory effect of nisin towards *L. monocytogenes* in experimentally contaminated Turkish fermented sausages (sucuk) was dependent on the concentrations of the bacteriocin (Hampikyan & Ugur, 2007). The enterocin AS-48 (148 AU/g) caused a drastic decrease in *L. monocytogenes* population (5.5 log CFU/g) in fuet (a low acid fermented sausage) during its maturation (Ananou et al., 2010). The inhibitory effects of pediocin PA-1 (5000 BU/mL) produced by *P. acidilactici* MCH14 was studied in frankfurters, decreasing by 2 and 0.6 log cycles of

the counts of *L. monocytogenes* after storage at 4°C for 60 days and at 15°C for 30 days, respectively, when compared to the control (Nieto-Lozano et al., 2010).

In conclusion, *Lb. curvatus* MBSa2 and MBSa3 isolated from Italian type salami samples produce two bacteriocins (sakacin P and sakacin X) with great stability (heat, pH and NaCl), and remarkable activity against *L. monocytogenes*. The semi-purified bacteriocins extracted from cultures of *Lb. curvatus* MBSa2 strain and applied to the batter for salami production caused a 2 log CFU/ count reduction in the final product when compared to salami not added of bacteriocins, suggesting that application of these bacteriocins can be a supplementary measure to increase the safety of these ready-to-eat products with regards to *L. monocytogenes*.

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References

Ananou, S., Garriga, M., Jofré, A., Aymerich, T., Gálvez, A., Maqueda, M., Martínez-Bueno, M., & Valdivia, E. (2010) Combined effect of enterocin AS-48 and high hydrostatic pressure to control food-borne pathogens inoculated in low acid fermented sausages. *Meat Science*, 84, 594–600.

Aymerich, M.T., Garriga, M., Monfort, J.M., Nes, I., & Hugas, M. (2000) Bacteriocin-producing lactobacilli in Spanish-style fermented sausages: characterization of bacteriocins. *Food Microbiology*, 17, 33–45.

Balciunas, E. M., Castillo Martinez, F. A., Todorov, S. D., Franco, B. D. G. M., Converti, A., & Oliveira, R. P. S. (2013). Novel biotechnological applications of bacteriocins: A review. *Food Control*, 32, 134-142.

Batdorj, B., Dalgarrondo, M., Choiset, Y., Pedroche, J., Métro, F., Prévost, H., Chobert, J.-M., & Haertlé, T. (2006) Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. *Journal of Applied Microbiology*, 101, 1364–5072.

Belgacem, Z.B., Ferchichi, M., Prévost, H., Dousset, X., & Manai, M. (2008) Screening for anti-*Listeria* bacteriocin-producing lactic acid bacteria from “Gueddid” a traditionally Tunisian fermented meat. *Meat Science*, 78, 513–521.

Bhugaloo-Vial, P., Dousset, X., Metivier, A., Sorokine, O., Anglade, P., Boyaval, P., & Marion, D. (1996) Purification and amino acid sequences of Piscicocins V1a and V1b, two Class IIa bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. *Applied and Environmental Microbiology*, 62, 4410–4416.

Borges, M. F., Siqueira, R. S., Bittencourt, A. M., Vanetti, M. C. D., & Gomide, L. A. M. (1999). Occurrence of *Listeria monocytogenes* in Salami. *Revista de Microbiologia*, 30, 362-364.

Bouttefroy, A., Linder, M., & Millière, J.B. (2000) Predictive models of the combined effects of curvaticin 13, 4% de NaCl and pH on the behaviour of *Listeria monocytogenes* ATCC 15313 in broth. *Journal. Applied Microbiology*, 88, 919-929.

Carvalho, K.G., Bambirra, F.H.S., Kruger, M.F., Barbosa, M.S., Oliveira, J.S., Santos, A.M.C., Nicoli, J.R., Bemquerer, M.P., Miranda, A., Salvucci, E.J., Sesma, F.J.M., & Franco, B.D.G.M. (2010) Antimicrobial compounds produced by *Lactobacillus sakei* subsp. *sakei* 2a, a bacteriocinogenic strain isolated from a Brazilian meat product. *Journal of Industrial Microbiology and Biotechnology*, 37, 381–390.

Chaillou, S., Champomier-Vergès, M.C., Cornet, M., Crutz-Le Coq, A.M., Dudez, A.M., Martin, V., Beaufils, S., Darbon-Rongère, E., Bossy, R., Loux, V., & Zagorec, M. (2005). The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nature Biotechnology*, 23, 1527–1533.

Cintas, L. M., Rodriguez, J. M., Fernandez, M. F., Sletten, K., Nes, I. F., Hernandez, P. E., & Holo, H. (1995) Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. *Applied Environmental Microbiology*, 61, 2643–2648.

Coppola, R., Iorizzo, M., Saotta, R., Sorrentino, E., & Grazia, L. (1997). Characterization of micrococci and staphylococci isolated from sopressata mlsana, a Southern Italy fermented sausage. *Food Microbiology*, 14, 47-53.

Coppola, S., Mauriello, G., Aponte, M. Cotter, P. D., Hill, C., & Ross, R. P. (2005) Bacteriocins: Developing Innate Immunity for Food. *Nature Reviews Microbiology*, 3, 777-788.

Criado R, Diep DB, Aakra A, Gutiérrez J, Nes IF, Hernández PE, & Cintas LM. (2006) Complete sequence of the enterocin Q-encoding plasmid pCIZ2 from the multiple bacteriocin producer *Enterococcus faecium* L50 and genetic characterization of enterocin Q production and immunity. *Applied Environmental Microbiology*, 72, 6653-6666.

Deegan, L.H., Cotter, P.D., Hill, C., & Ross, P. (2006). Bacteriocins: biological tools for bio-preservation and shelf-life extension. *International Dairy Journal*, 16, 1058–1071.

Deraz, S. F., Karlsson, E. N., Hedstrom, M., Andersson, M. M., & Mattiasson, Bo. (2005) Purification and characterisation of acidocin D20079, a bacteriocin produced by *Lactobacillus acidophilus* DSM 20079. *Journal of Biotechnology*, 117, 343–354.

Di Pinto, A., Novello, L., Montemurro, F., Bonerba, E., & Tantillo, G. (2010) Occurrence of *Listeria monocytogenes* in ready-to-eat foods from supermarkets in Southern Italy. *New Microbiologica*, 33, 249-252.

Dickson-Spillmann, M., Siegrist, M., & Keller, C. (2011). Attitudes toward chemicals are associated with preference for natural food. *Food Quality and Preference*, 22, 149-156.

Dobson, A., Cotter, P.D., Ross, R.P., & Hill, C. (2012). Bacteriocin production: a probiotic trait? *Applied Environmental Microbiology*, 78, 1–6.

Dortu, C., Huch, M., Holzapfel, W.H., Franz, C.M.A.P., & Thonart, P. (2008) Anti-listerial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. *Letters in Applied Microbiology*, 47, 581–586.

Fadda, S., López, C., & Vignolo, G. (2010). Role of lactic acid bacteria during meat conditioning and fermentation: Peptides generated as sensorial and hygienic biomarkers. *Meat Science*, 86, 66–79.

Felske, A., Rheims, H., Wokerink, A., Stackebrandt, E., & Akkermans, A.D.L. (1997) Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology*, 143, 2983–2989.

Franz, C. M., Schillinger, U., & Holzapfel, W. H. (1996) Production and characterization of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. *International Journal Food Microbiology*, 29, 255-270.

Garcia, M. T., Canamero, M. M., Lucas, R., Omar, N. B., Pulido, R. P., & Galvez, A. (2004) Inhibition of *Listeria monocytogenes* by enterocin EJ97 produced by *Enterococcus faecalis* EJ97. *International Journal of Food Microbiology*, 90, 161– 170.

Hampikyan, H. & Ugur, M. (2007) The effect of nisin on *L. monocytogenes* in Turkish fermented sausages (sucuks). *Meat Science*, 76, 327–332.

Ishibashi, N., Himeno, K., Fujita, K., Masuda, Y., Perez, R. H., Zendo, T., Wilaipun, P., Leelawatcharamas, V., Nakayama, J., & Sonomoto, K. (2012) Purification and characterization of multiple bacteriocins and an inducing peptide produced by *Enterococcus faecium* NKR-5-3 from Thai fermented fish. *Bioscience Biotechnology Biochemistry*, 76, 947-953.

Kaiser, A.L., & Montville, T.J. (1996). Purification of the bacteriocin bavaricin MN and characterization of its mode of action against *Listeria monocytogenes* Scott A cells and lipid vesicles. *Applied and Environmental Microbiology*, 62, 4529–4535.

Kumar, R. (2011). Modern Trends to Investigate Foodborne Listeriosis. *Journal of Food Technology*, 9, 9–17.

Lahtinen, S., Ouwehand, A.C., Salminen, S., & von Wright A. (Eds.). Lactic acid bacteria. Microbiological and functional aspects (4th ed.), CRC Press, Boca Raton (USA) (2011), pp. 1–17

Leroy, F., & De Vuyst, L. (1999) The Presence of Salt and a Curing Agent Reduces Bacteriocin Production by *Lactobacillus sakei* CTC 494, a Potential Starter Culture for Sausage Fermentation. *Applied and Environmental Microbiology*, 65, 5350–5356.

Macwana, S. & Muriana, P.M. (2012) Spontaneous bacteriocin resistance in *Listeria monocytogenes* as a susceptibility screen for identifying different mechanisms of resistance and modes of action by bacteriocins of lactic acid bacteria. *Journal of Microbiological Methods* **88**, 7–13.

Mandal, V., Mandal, N.C. and Sen, S.K. (2011) Isolation and characterization of pediocin NV 5 producing *Pediococcus acidilactici* LAB 5 from vacuum-packed fermented meat product. *Indian Journal of Microbiology* 51, 22–29.

Mangia, N.P., Trani, A., Di Luccia, A., Faccia, M., Ganbacorta, G., Fabcello, F., & Dieana, P. (2013). Effect of the use of autochthonous *Lactobacillus curvatus*, *Lactobacillus plantarum* and *Staphylococcus xylosus* strains on microbiological and biochemical properties of the Sardinian fermented sausage. *European Food Research and Technology*, 236, 557-566.

Martins, E. A., & Germano, P. M. L. (2011). *Listeria monocytogenes* in ready-to-eat, sliced, cooked ham and salami products, marketed in the city of São Paulo, Brazil: Occurrence, quantification, and serotyping. *Food Control*, 22, 297-302.

Messens, W. and de Vuyst, L. (2002) Inhibitory substances produced by Lactobacilli isolated from sourdoughs-a review. *International Journal of Food Microbiology* 72, 31–43.

Mills, S., Stanton, C., Hill, C., & Ross, R.P. (2011). New developments and applications of bacteriocins and peptides in foods. *Annual Review of Food Science and Technology*, 2, 299–329.

Moretro, T., Aasen, I. M., Storro, I., & Axelsson, L. (2000) Production of sakacin P by *Lactobacillus sakei* in a completely defined medium. *Journal of Applied Microbiology*, 88, 536–545.

Müller, D.M., Carrasco, M.S., Tonarelli, G.G. and Simonetta, A.C. (2009) Characterization and purification of a new bacteriocin with a broad inhibitory spectrum produced by *Lactobacillus plantarum* lp 31 strain isolated from dry-fermented sausage. *Journal of Applied Microbiology* 106, 2031–2040.

Nielsen, J.W., Dickson, J.S., & Crouse, J.D. (1990). Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. *Applied and Environmental Microbiology*, 56, 2142–2145.

Nieto-Lozano, J. C., Reguera-Useros, J. I., Peláez-Martínez, M. C., Sacristán-Pérez-Minayo, G., Gutiérrez-Fernández, Á. J., de la Torre, A. H. (2010) The effect of the pediocin PA-1 produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* in Spanish dry-fermented sausages and frankfurters. *Food Control*, 21, 679–685.

Nightingale, K.K., Thippareddi, H, Phebus, R.K., Marsden, J.L.; Nutsch, A.L. (2006). Validation of a Traditional Italian-Style Salami Manufacturing Process for Control of *Salmonella* and *Listeria monocytogenes*, *Journal of Food Protection*, 69 (4), 794-800.

Nishie, M. Nagao, J.-I., & Sonomoto, K. (2012). Antibacterial peptides “Bacteriocins”: an overview of their diverse characteristics and applications. *Biocontrol Science*, 17, 1–16.

Okada, Y., Monden, S., Igimi, S., & Yamamoto, S. (2012) The Occurrence of *Listeria monocytogenes* in Imported Ready-to-Eat Foods in Japan. *Journal Veterinary Medical Science*, 74, 373-375.

Papamanoli, E., Tzanetakis, N., Litopoulou-Tzanetaki, E., & Kotzekidou, P. (2003) Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Science*, 65, 859–867.

Papathanasopoulos, M. A., von Holy, A., Krier, F., Hastings, J. W., Revol-Junelles, A.-M., Lefebvre, G., & Le Caer, J. P. (1997) Multiple Bacteriocin Production by

Leuconostoc mesenteroides TA33a and Other *Leuconostoc/Weissella* Strains. *Current Microbiology*, 35, 331–335.

Petruzzelli, A., Blasi, G., Masini, L., Calza, L., Duranti, A., Santarelli, S., Fisichella, S., Pezzotti, G., Aquilanti, L., Osimani, A., & Tonucci, F. (2010) Occurrence of *Listeria monocytogenes* in Salami Manufactured in the Marche Region (Central Italy). *Journal Veterinary Medical Science*, 72, 499-502.

Reminger, A., Ehrmann, M.A. and Vogel, R.F. (1996) Identification of bacteriocin-encoding genes in Lactobacilli by Polymerase Chain Reaction (PCR). *Systematic Applied Microbiology* 19, 28–34.

Sakate, R. I., Aragon, L. C., Raghianti, F., Landgraf, M., Franco, B. D., & Destro, M. T. (2003). Occurrence of *Listeria monocytogenes* in pre-sliced vacuum-packaged. *Archivos Latinoamericanos de Nutrición*, 53, 184-7.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones, J. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States-major pathogens. *Emerging Infectious Diseases*, 17, 7-15.

Sobrino, J., Rodriguez, J.M., Moreira, W.L., Cintas, L.M., Fernandez, M.F., Sanz, B. and Hernandez, P.E. (1992) Sakacin M, a bacteriocin-like substance from *Lactobacillus sake* 148. *International Journal of Food Microbiology* 16, 215–225.

Sudirman I., Mathier F., Michel M., Lefebvre G. (1993) Detection and properties of curvaticin 13, a bacteriocin-like substance produced by *Lactobacillus curvatus* SB13. *Current Microbiology*, 27, 35–40.

Tahiri, I., Desbiens, M., Benech, R., Kheadr, E., Lacroix, C., Thibault, S., Ouellet D., Fliss, I. (2004) Purification, characterization and amino acid sequencing of divergicin M35: a novel class IIa bacteriocin produced by *Carnobacterium divergens* M35. *International Journal of Food Microbiology*, 97, 123– 136.

Todorov, S. D., Vaz-Velho, M., Gibbs, P. (2004) Comparison of two methods for purification of plantaricin ST31, a bacteriocina produced by *Lactobacillus plantarum* ST31. *Brazilian Journal of Microbiology*, 35, 157-160.

Todorov, S.D., Ho, P., Vaz-Velho, M., & Dicks, L.M.T. (2010). Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Science*, 84, 334–343.

Todorov, S.D., Rachman, C., Fourrier, A., Dicks, L.M.T., van Reenen, C.A., Prévost, H. and Dousset, X. (2011) Characterization of a bacteriocin produced by *Lactobacillus sakei* R1333 isolated from smoked salmon. *Anaerobe* 17, 23–31.

Todorov, S.D., Vaz-Velho, M., Franco, B.D.G.M., Holzapel, W.H. (2013). Partial characterization of bacteriocins produced by three strains of *Lactobacillus sakei*, isolated from *salpicao*, a fermented meat product from North-West of Portugal. *Food Control*, 30 (1). 111-121.

Uteng, M., Hauge, H. H., Brondz, I., Nissen-Meyer, J., & Fimland, G. (2002) Rapid Two-Step Procedure for Large-Scale Purification of Pediocin-Like Bacteriocins and Other Cationic Antimicrobial Peptides from Complex Culture Medium. *Applied and Environmental Microbiology*, 68, 952–956.

van Reenen, C.A., Dicks L.M.T., & Chikindas, M.L. (1998). Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology*, 84, 1131–1137.

Vaughan, A., Eijsink, V.G.H., van Sinderen, D., O'Sullivan, T.F. and O'Hanlon, K. (2001) An analysis of bacteriocins produced by lactic acid bacteria isolated from malted barley. *Journal of Applied Microbiology*, 91, 131–138.

Vermeiren, L., Devlieghere, F., Debevere, J. (2004) Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. *International Journal of Food Microbiology*, 96, 149– 164.

Vogel, B. F., Hansen, L. T., Mordhorst, H., & Gram. L. (2010). The survival of *Listeria monocytogenes* during long term desiccation is facilitated by sodium chloride and organic material. *International Journal of Food Protection*, 140, 192-200.

Xiraphi, N., Georgalaki, M., van Driessche, G., Devreese, B., van Beeumen, J., Tsakalidou, E., Metaxopoulos, J. and Drosinos, E.H. (2006) Purification and

characterization of curvaticin L442, a bacteriocin produced by *Lactobacillus curvatus* L442. *Antonie van Leeuwenhoek* 89, 19–26.

Yamazaki, K., Suzuki, M., Kawai, Y., Inoue, N., Montville, T. J. (2005) Purification and Characterization of a Novel Class IIa Bacteriocin, Piscicocin CS526, from Surimi-Associated *Carnobacterium piscicola* CS526. *Applied and Environmental Microbiology*, 71, 554–557.

Figure 1 RAPD-PCR profiles of *Lactobacillus curvatus* MBSa2 and MBSa3. Lane M: 100 bp marker; Lane 1: *Lb. curvatus* MBSa2; Lane 2: *Lb. curvatus* MBSa3; Lane 3: control, no DNA. (a) OPL-01 primer (GGCATGACCT); (b): OPL-02 (TGGGCGTCAA); (c): OPL-04 (GACTGCACAC); (d): OPL-14 (GTGACAGGCT) and (e): OPL-20 (TGGTGGACCA).

Figure 2. Growth (●-) and bacteriocin-production (bars) by *Lactobacillus curvatus* MBSa2 (A) and *Lactobacillus curvatus* MBSa3 (B) in MRS broth at 25°C, 30°C and 37°C. (▲-) indicates the pH of the MRS broth.

Figure 3 Growth of *Lactobacillus curvatus* MBSa2 (a) and *Lactobacillus curvatus* MBSa3 (b) in MRS broth supplemented with 0% (◇), 2% (Δ), 4% (○), 6% (×), 8% (–) and 10% (□) NaCl, at 30°C and growth of *Lactobacillus curvatus* MBSa2 (c) and *Lactobacillus curvatus* MBSa3 (d) in MRS broth at pH 4 (–■–) and pH 6 (–▲–), at 30°C.

Figure 4 DNA fragments obtained after PCR with genomic DNA from *Lactobacillus curvatus* MBSa2 and MBSa3 using sakacin P specific primers (SakP-F/SakP-R). Lane 1, molecular weight marker (100 bp); lane 2, genomic DNA of MBSa2; lane 3, genomic DNA of MBSa3; lane 4, control, no DNA.

Figure 5 Counts of *Listeria monocytogenes* in salami containing the bacteriocin produced by *Lactobacillus curvatus* MBSa 2 (—●—), in salami containing sterile water instead of the bacteriocin (---■---) and in salami containing only *Listeria monocytogenes* (---●---). Counts were performed in the salami batter (time 0) and in the product up to the end of manufacturing (time 30).

Table 1. Spectrum of activity of the bacteriocins produced by *Lactobacillus curvatus* MBSa2 and MBSa3.

Target microorganism	Source	Diameter of the inhibition zone (mm)	
		MBSa2	MBSa3
<i>Bacillus cereus</i>	ATCC 1178	0	0
<i>Staphylococcus aureus</i>	ATCC 29213	0	0
<i>Staphylococcus aureus</i>	ATCC 25923	0	0
<i>Staphylococcus aureus</i>	ATCC 6538	0	0
<i>Listeria welshimeri</i>	USP ^a	0	0
<i>Listeria seeligeri</i>	USP	0	0
<i>Listeria ivanovii</i> subsp. <i>ivanovii</i>	ATCC 19119	15	16
<i>Listeria innocua</i>	ATCC 33090	18	21
<i>Listeria innocua</i> 225/07 serovar 6a	FIOCRUZ ^b	15	16
<i>Listeria innocua</i> 224/07 serovar 6a	FIOCRUZ	11	15
<i>Listeria innocua</i> 047/07 serovar 6a	FIOCRUZ	15	14
<i>Listeria innocua</i> 588/08 serovar 6a	FIOCRUZ	14	11
<i>Listeria monocytogenes</i> Scott A	USP	13	13
<i>Listeria monocytogenes</i> 602/08 serovar 1/2a	FIOCRUZ	13	13
<i>Listeria monocytogenes</i> 046/07 serovar 1/2c	FIOCRUZ	11	14
<i>Listeria monocytogenes</i> 103 serovar 1/2a	USP	0	15
<i>Listeria monocytogenes</i> 106 serovar 1/2a	USP	13	14
<i>Listeria monocytogenes</i> 104 serovar 1/2a	USP	14	15
<i>Listeria monocytogenes</i> 409 serovar 1/2a	USP	12	14
<i>Listeria monocytogenes</i> 506 serovar 1/2a	USP	14	14
<i>Listeria monocytogenes</i> 709 serovar 1/2a	USP	11	12
<i>Listeria monocytogenes</i> 607 serovar 1/2b	USP	18	17
<i>Listeria monocytogenes</i> 603 serovar 1/2b	USP	10	20
<i>Listeria monocytogenes</i> 426 serovar 1/2b	USP	10	14
<i>Listeria monocytogenes</i> 637 serovar 1/2c	USP	10	14
<i>Listeria monocytogenes</i> 422 serovar 1/2c	USP	12	15
<i>Listeria monocytogenes</i> 712 serovar 1/2c	USP	13	15
<i>Listeria monocytogenes</i> 408 serovar 1/2c	USP	14	15
<i>Listeria monocytogenes</i> 211 serovar 4b	USP	15	16
<i>Listeria monocytogenes</i> 724 serovar 4b	USP	19	16
<i>Listeria monocytogenes</i> 101 serovar 4b	USP	18	18
<i>Listeria monocytogenes</i> 703 serovar 4b	USP	18	20
<i>Listeria monocytogenes</i> 620 serovar 4b	USP	20	20
<i>Listeria monocytogenes</i> 302 serovar 4b	USP	15	14
<i>Escherichia coli</i>	ATCC 8739	0	0
<i>Escherichia coli</i> O157:H7	ATCC 35150	0	0
<i>Enterobacter aerogenes</i>	ATCC 13048	0	0
	ATCCC		
<i>Salmonella</i> Typhimurium	14028	0	0
<i>Salmonella</i> Enteritidis	ATCC 13076	0	0
<i>Enterococcus faecalis</i>	ATCC 12755	0	0

<i>Enterococcus hirae</i> D105	USP	10	13
<i>Enterococcus faecium</i> S5	AGRIS ^c	10	15
<i>Enterococcus faecium</i> S154	AGRIS	0	11
<i>Enterococcus faecium</i> S100	AGRIS	0	0
<i>Enterococcus faecium</i> ST62BZ	USP	10	10
<i>Enterococcus faecium</i> ST211Ch	USP	0	0
<i>Enterococcus faecium</i> ET 12	UCV ^d	0	0
<i>Enterococcus faecium</i> ET 88	UCV	0	0
<i>Enterococcus faecium</i> ET 05	UCV	0	0
<i>Lactococcus lactis</i> V94	USP	0	0
<i>Lactobacillus fermentum</i> ET35	UCV	10	10
<i>Pediococcus pentosaceus</i> ET 34	UCV	0	0
<i>Lactobacillus curvatus</i> ET 06	UCV	0	0
<i>Lactobacillus curvatus</i> ET 31	UCV	0	9
<i>Lactobacillus curvatus</i> ET 30	UCV	0	0
<i>Lactobacillus sakei</i> subsp. <i>sakei</i> 2a	USP	0	0
<i>Lactobacillus sakei</i>	ATCC 15521	10	11
<i>Lactococcus lactis</i> V69	USP	0	0
<i>Lactobacillus delbrueckii</i> B5	USP	0	0
<i>Lactobacillus delbrueckii</i> ET 32	UCV	0	0
<i>Lactobacillus acidophilus</i> La14	Rhodia	0	0
<i>Lactobacillus acidophilus</i> Lac4	Rhodia	0	0
<i>Lactobacillus acidophilus</i> La5	Rhodia	0	0
<i>Lactococcus lactis</i> B16	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> MK02R	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D2	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B1	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D4	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B2	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B15	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D3	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> D5	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> B17	USP	0	0
<i>Lactococcus lactis</i> subsp. <i>lactis</i> R704	Chr. Hansen	0	0

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Table 2 Effect of temperature, pH and presence of NaCl on residual antimicrobial activity of bacteriocins produced by *Lactobacillus curvatus* MBSa2 and MBSa3.

	condition	Residual activity (%)	
		MBSa2	MBSa3
Temperature/time	4, 25, 30, 37, 45, 60, 80, 100° C / 60 min	100	100
	121° C / 15 min	100	100
pH	2, 4, 6, 8	100	100
	10	100	26
NaCl (%)	2, 4, 6, 8, 10	100	100

Table 3. Targeted bacteriocin genes and primers used in the study.

Target bacteriocin	Primer*	Sequence (5'-3')**	Size (bp)	Reference	Initial denaturation	Denaturation	Annealing	Elongation
Sakacin A	SakA-F SakA-R	GAATWTRMANCAATTAYMGGTGG CAGCCGCTAATCATACCACC	150	Dortu <i>et al.</i> , 2008	95°C, 15 min	95°C, 30 s	55°C, 1 min	72°C, 1 min
Sakacin T- α	SakT- α -F SakT- α -R	TCGGTGGCTATACTGTCTAAACA TGTCCTAAAAATCCCAATGC	160	Macwana and Muriana (2012)	95°C, 15 min	95°C, 30 s	58°C, 1 min	72°C, 1 min
Sakacin T- β	SakT- β -F SakT- β -R	AAGAAATGATAGAAATTTTGGAGG TGIGAAATCCCAATCTTGTCCTG	151	Macwana and Muriana (2012)	95°C, 15 min	95°C, 30 s	56°C, 1 min	72°C, 1 min
Sakacin Q	SakQ-F SakQ-R	GAA RTW SYA NCA ATT ADN GGT GG TAC CAC CAG CAG CCA TTC CC	130	Dortu <i>et al.</i> (2008)	95°C, 15 min	95°C, 30 s	53°C, 1 min	72°C, 1 min
Sakacin P	SakP-F SakP-R	ATG GAA AAG TTT ATT GAA TTA TTA T TT ATT CCA GCC AGC GTT	186	Reminger <i>et al.</i> (1996)	94°C, 3 min	94°C, 30 s	40°C, 1 min	72°C, 1 min
Sakacin G	SakGA1-F SakGA1-R	TTA GAA CTA CAC TGA TCG TG TGG AAG AAT GAG TAC TTG TT		Todorov <i>et al.</i> (2011)	94°C, 4 min	94°C, 30 s	38°C, 30 s	72°C, 30 s
Sakacin G	SakGA2-F SakGA2-R	CGT TAC AAC AGA ACT TCA AG TGG AAG AAT GAG TAC TTG TT		Todorov <i>et al.</i> (2011)	94°C, 4 min	94°C, 30 s	38°C, 30 s	72°C, 30 s
Curvacin A	CurA-F CurA-R	GTA AAA GAA TTA AGT ATG ACA TTA CAT TCC AGC TAA ACC ACT	171	Reminger <i>et al.</i> (1996)	94°C, 3 min	94°C, 30 s	40°C, 1 min	72°C, 1 min

*F: Forward; R: Reverse

**deoxymosine; R, T/A; W, A/G; S, C/A; Y, A/C; D, C/T; N, A/C.

Table 4 Purification of bacteriocins produced by *Lactobacillus curvatus* MBSa2 and MBSa3.

Purification stage		Volume (mL)	Activity (AU/mL)	Protein (mg/mL)	Specific activity (AU/mg)	Purification factor	Yield (%)
MBSa2							
Supernatant		200	800	3.10	257.65	1.00	100
Cation-exchange		700	200	2.46	81.20	0.31	87.5
Reversed phase		20	6400	2.54	2519.56	9.78	80
C ₁₈ RP-HPLC	P1	2	16000	2.18	7353.19	28.54	20
	P2	2	8000	1.89	4242.23	16.46	10
MBSa3							
Supernatant		200	800	4.41	181.26	1.00	100
Cation-exchange		700	200	1.93	103.85	0.57	87.5
Reversed phase		20	6400	2.32	2753.78	15.19	80
C ₁₈ RP-HPLC	P1	2	16000	2.14	7491.33	41.33	20
	P2	2	8000	1.88	4263.16	23.52	10

Table 5 Measurements of pH in the batters (time 0) and the four types of salami along 30 days of manufacturing

Product*	Time (Day)				
	0	4	10	20	30
LM free	5.80±0.45	4.82±0.31	4.72±0.71	5.33±0.09	5.31±0.02
LM + BAC	5.87±0.39	4.91±0.04	5.07±0.24	5.38±0.08	5.53±0.31
LM + Water	5.77±0.45	4.84±0.13	5.10±0.16	5.38±0.02	5.46±0.15
LM only	5.81±0.47	4.65±0.41	5.00±0.31	5.35±0.02	5.44±0.06

* LM: *Listeria monocytogenes* Scott A; BAC: bacteriocin produced by *Lactobacillus curvatus* MBSa2

Table 6 Measurements of a_w in the batters (time 0) and the four types of salami along 30 days of manufacturing

Product*	Time (Day)				
	0	4	10	20	30
LM free	0.98±0.00	0.97±0.00	0.96±0.00	0.92±0.00	0.88±0.00
LM + BAC	0.98±0.00	0.97±0.00	0.95±0.01	0.92±0.00	0.89±0.00
LM + Water	0.96±0.00	0.97±0.00	0.95±0.01	0.91±0.00	0.89±0.00
LM only	0.97±0.00	0.94±0.02	0.94±0.01	0.90±0.01	0.88±0.00

* LM: *Listeria monocytogenes* Scott A; BAC: bacteriocin produced by *Lactobacillus curvatus* MBSa2

Capítulo 04

“Bacteriocin production by *Lactobacillus curvatus* MBSa2 entrapped in calcium alginate beads during manufacturing of Italian type salami”

Artigo em preparação para submissão para publicação em

Meat Science

Bacteriocin production by *Lactobacillus curvatus* MBSa2 entrapped in calcium alginate beads during manufacturing of Italian type salami

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Abstract:

Bacteriocins of lactic acid bacteria (LAB) have been extensively studied due to their applications for food preservation. However, components of the food matrix may interfere or inhibit bacteriocin production, and encapsulation of the strains may protect them of the adverse conditions in the food environment. In this study, a bacteriocinogenic LAB (*Lactobacillus curvatus* MBSa2) isolated from salami was encapsulated in calcium alginate, and tested for functionality in MRS broth and in salami experimentally contaminated with *Listeria monocytogenes* AL602/08 (a meat isolate), during 30 days of manufacture, including fermentation and maturation steps. The entrapment process did not affect bacteriocin production by *Lb. curvatus* MBSa2 in MRS broth and in salami. Both free and encapsulated *Lb. curvatus* MBSa2 caused reduction in the counts of *L.monocytogenes* AL602/08 in salami during manufacture, but the counts in salami containing free or alginate encapsulated *Lb. curvatus* MBSa2 did not differ significantly ($p > 0.05$).

Key-words: Encapsulation, bacteriocin, calcium alginate, salami, *Lactobacillus curvatus*.

Introduction

Lactic acid bacteria (LAB) have a long history of application in fermented meat due their beneficial influence on nutritional, organoleptic, and shelf-life characteristics (Ammor and Mayo, 2007; Hammes, 2012). Antimicrobial peptides called bacteriocins produced by LAB have been widely studied for application in foods as natural preservatives (Renye Jr. et al., 2011; Mills et al., 2011; Balciunas et al., 2013; O'Shea et al. 2013). Bacteriocins can be used in foods as *ex-situ* preparations, i.e, the bacteriocin is produced in culture media and then purified and added to the food, or the bacteriocin can be produced *in situ* by a bacteriocinogenic strain added to the food.

One important drawback of the application of pure or semi-purified bacteriocin in food preservation is the difficulty in obtaining large amounts necessary to achieve the expected antimicrobial activity. Added bacteriocins are often used in combination with other antimicrobial hurdles to enhance their bactericidal effects. In counterpart, bacteriocin production by LAB in food matrix is a dynamic process where the different interactions with food compounds can influence the efficacy of the use for food preservation (Aasen et al, 2003). In addition, bacteriocinogenic strains should be carefully selected, as they need to maintain viability and produce bacteriocins in the food, even in less favorable environments, as occurs in acidic and low aw foods and those containing other antimicrobial agents, such as spices and seasonings (Gálvez et al., 2007; Gálvez et al, 2008).

The encapsulation for protection of LAB has been used to improve viability of cells in the intestinal tract and in foods such as yoghurts, cheeses, cream and fermented milk (Krasaekoopt et al., 2003; Rathore et al., 2013). The terms entrapment and encapsulation were used indifferently in most of the studies reported in the literature. One of the main components widely used for encapsulation and entrapment of LAB is

alginate, a nontoxic linear heteropolysaccharide extracted from different types of algae (Cook et al., 2012). The alginate recovers the bacterial cells and forms a barrier, protecting them against environmental instability. The alginate barrier constitutes a semipermeable spherical fine coat, which nutrients and metabolites readily cross (Kailasapathy, 2002; Anal and Singh, 2007).

Some studies have shown that encapsulation of LAB in calcium-alginate improves lactic acid production (Scannell et al., 2000; Garbayo et al., 2004; Idris and Suzana, 2005; Rao et al., 2008), but little is known on bacteriocin production by entrapped LAB. In a previous study, the authors reported that *Lactobacillus curvatus* MBSa2, a bacteriocinogenic strain isolated from salami produced in Brazil, is capable of inhibiting the growth of *L.monocytogenes* Scott A in culture media (Barbosa et al, submitted) and during manufacture of Italian type salami (Barbosa et al, submitted). In this study, *Lb. curvatus* MBSa2 was entrapped in calcium alginate and tested for activity against *L.monocytogenes* AL602/08, a meat product isolate, in conditions simulating those encountered during production of salami and *in situ*, in salami batter experimentally contaminated with this pathogen, up to 30 days of manufacture.

Material and Methods

Bacterial strains

The bacteriocin-producing strain used in this study was *Lactobacillus curvatus* MBSa2 isolated from Italian type salami (Barbosa et al., submitted). *Listeria monocytogenes* AL602/08 sorovar 1/2a isolated from meat product and donated by Dr. Ernesto Hofer of the Laboratory of Bacterial Zoonosis the Institute Oswaldo Cruz, Rio de Janeiro, Brazil, was used as the target pathogen. *Lb. curvatus* MBSa2 and *L.monocytogenes* AL602/08 were maintained at -70°C in MRS broth (Difco, USA) and

BHI broth (Difco, USA) respectively, added of 20% (v/v) glycerol (Synth, Brazil). Before use, the cultures were grown twice in the appropriate broths at 30°C and 37°C, respectively, for 24h.

Entrapment procedure

Entrapment of *Lb. curvatus* MBSa2 was performed according to Ivanova et al. (2000, 2002), with modifications. A culture containing 10^8 - 10^9 CFU/mL, obtained in MRS broth (Difco, USA) at 30°C for 24h was centrifuged at 6000 xg for 15 min at 4°C, washed three times in 0.1% peptone water (w/v) and added to a 2% sodium alginate I-G3-150 (Kimica Chile Ltda, Santiago, Chile) solution. The mixture was dripped in a solution of 100 mM calcium chloride (CAAL, Brazil) using a peristaltic pump. The mixture remained under magnetic stirring during the dipping process. The formed calcium alginate beads were kept for 30 min for gel strengthening and then separated by size using stainless steel sieves of different mesh sizes (250, 355, 500, 710 and 1000 mm). The beads retained in the sieves were washed three times with distilled water. The diameter of the calcium alginate beads was determined using a binocular CBA brightfield microscope (Olympus, USA) with an ocular micrometer. The average diameter was determined measuring 15 beads for each sample.

Release and counts of lactobacilli

One gram of beads was placed in tubes containing 9 ml of phosphate buffered saline pH 7.4 (PBS), i.e., 1 mL of phosphate buffer 0.33M (pH 7.5) mixed with 29 ml of sodium chloride (9 g/L), and then vortexed for five minutes at room temperature (Brachkova et al., 2010). The suspension was submitted to decimal serial dilutions using 0.1% sterile peptone water (Difco, USA), and each dilution was plated in duplicate on

MRS agar (Oxoid, UK) plates and incubated at 30°C for 48 h, for counting of released lactobacilli.

Bacteriocin assay

Bacteriocin assays were performed in cell free supernatants (CFS) of the cultures of free or entrapped *Lb. curvatus* MBSa2, prepared by centrifugation at 6000 xg for 15 min at 4°C of the MRS broth (Difco, USA) incubated at 30°C for 24h. The pH of the CFS was adjusted to 6.0-6.5 with 6N NaOH (Synth, Brazil) and then the CFS was heated at 80°C for 30 min and filter-sterilized through a 0.22 µm membrane filter (Millex GV 0,22 µm [Millipore, USA]). The amount of bacteriocin in the CFS was determined by titration using the spot-on-the-lawn method as described by Reenen et al. (1998), with modifications. The CFS was submitted to serial two-fold dilutions in 100 µL of 5 mmol/L 2-[N-morpholino] ethanesulfonic acid (MES) buffer pH 6.5 (Sigma) in 96-well microtiter-plates (TPP, Switzerland). Ten microliters from each well were transferred to the surface of plates containing two layers of media, constituted of 10-12 mL of 15% agar (w/v) (Difco, USA) overlaid with 5 mL of BHI soft-agar (BHI broth [Oxoid, UK] plus 0.85% [w/v] of bacteriological agar [Difco, USA]) containing *L.monocytogenes* AL602/08 (10^5 - 10^6 CFU/mL). When the drops air-dried, the plates were incubated at 37°C for 12 h and observed for inhibition zones. One arbitrary unit of the bacteriocin in the CFS was defined as the reciprocal of the highest dilution showing a clear inhibition zone. Results were expressed in arbitrary units per millilitre (AU/mL) (Kaiser and Montville, 1996).

Evaluation of the influence of entrapment on the viability and bacteriocin production by

Lb. curvatus MBSa2

The viability and bacteriocin production by *Lb. curvatus* MBSa2 were evaluated before and after entrapment in calcium alginate. Before entrapment, one milliliter of the bacterial suspensions prepared as described in 2.2 was submitted to serial decimal dilutions, plated on MRS agar and incubated 30°C for 48 h for counts of viable cells. One milliliter of the same bacterial suspension was added to 10 mL MRS broth, incubated at 30°C for 24 h and tested for antimicrobial activity as described in 2.4. One gram of entrapped cells were treated as described in 2.4 for release of cells, plated on MRS agar and incubated 30°C for 48 h for counts of viable cells. For assay of bacteriocin production, one gram of entrapped cells were added to 10 mL of MRS broth and incubated at 30°C for 24 h, when the suspension was tested for antimicrobial activity as described in 2.4.

*Evaluation of the influence of the size of the alginate beads on the viability and bacteriocin production by entrapped *Lb. curvatus* MBSa2 in conditions simulating salami manufacturing*

Entrapped or free *Lb. curvatus* MBSa2 was cultivated in MRS broth (Difco, USA) formulated to simulate the environmental conditions during salami manufacture concerning pH and Aw. In separate experiments, the (1) pH of the medium was adjusted to 6.0, 5.5 and 5.0 using an 85% lactic acid solution (Purac, Brazil) and (2) Aw was adjusted to 0.97, 0.90 and 0.85 adding 5%, 13.5% e 22.5% NaCl (Synth, Brazil), respectively. Cultures were incubated at 18°C, 24°C and 30°C up to 14 days, and enumerations of viable were done at days 1, 3, 7 and 14, following procedures described in 2.4.

Salami manufacturing

Salami was prepared in the pilot plant of a meat industry, located in Sao Paulo, SP, Brazil, following the manufacture procedure used in this industry. Salami was formulated with 10% bovine meat, ground through a 3 mm disc, 75% pork shoulder, ground through a 8 mm disc and 15% lard, chopped into cubes of appr. 125 mm³. The meats were added of 1.3% NaCl, 1% Compact Salami 160 (Kraki and Kienast Ltda, Brazil), correspondent to a preformulated mixture of maltodextrin, sugar, garlic powder, onion powder, ground red pepper, ground white pepper, sodium nitrate, sodium erythorbate, garlic essential oil and nutmeg essential oil, and 0.02% Bactoferm™ T-SPX starter culture (*Pediococcus pentosaceus* and *Staphylococcus xylosus*) (CHR Hansen, Denmark). The ingredients were mixed in a stainless steel meat homogenizer (CAF HG 120/114S, Brazil) for 3 to 5 min and the resulting batter was kept under refrigeration until used.

For experimental contamination, a culture of *L.monocytogenes* AL602/08 in BHI broth incubated at 37 °C for 24 h was centrifuged at 6000 x g for 15 min and the pellet was resuspended in sterile 0.1% peptone (w/v) water. This procedure was repeated three times in order to eliminate all components of the BHI medium. The salami batter was divided in six portions: portion 1 was added of a suspension of free *Lb. curvatus* MBSa2 (MBSa2 F); portion 2 was added of a suspension of entrapped *Lb. curvatus* MBSa2 (MBSa2 E); portion 3 was added of *L.monocytogenes* AL602/08 (LM); portion 4 was added of a suspension of free *Lb. curvatus* MBSa2 and *L.monocytogenes* AL602/08 (MBSa2 F + LM); portion 5 was added of a suspension of entrapped *Lb. curvatus* MBSa2 and *L.monocytogenes* AL602/08 (MBSa2 E + LM) and portion 6 was used as control (with no experimental contamination). The batters were transferred into caliber 60 collagen casings (Fibran S.A., Brazil), pre-hydrated in 15% saline solution for 30 min, using a small-scale stainless steel filling machine (Filizola, Brazil). Prior

each use, the cylinder and the piston of the filling machine were autoclaved at 121°C for 15 min. The casings containing the batter (approx.. 20 cm long) were transferred to EL111 chambers (Eletrolab, Brazil) where the temperature and relative humidity (RH) were controlled as follows: 4 days at 20°C and 97% RH (fermentation step), 5 days at 18°C and RH from 95% to 87% and then for 20 days at 15 °C and RH from 87% to 75% (maturation step). These experiments were performed in triplicates.

The pH and the a_w of the batter and salami were measured at times 0, 4, 10, 20 and 30 days of manufacture using a HI1090B6 pH electrode (Hannah Instruments, USA) and Novasina AWC500 (Novasina AG, Switzerland), respectively. Both measurements were made in duplicates.

Viability and bacteriocin production by free and entrapped Lb. curvatus MBSa2 during manufacturing of salami

Counts of lactic acid bacteria and *L.monocytogenes* AL602/08 were performed in the batter (day 0) and in the salami at days 4, 10, 20 and 30. Samples (25g) of batter and salami added of free *Lb. curvatus* MBSa2 were transferred into a sterile stomacher bag and homogenized with 225 mL of 0.1% peptone water. Samples (25g) of batter and salami added of entrapped *Lb. curvatus* MBSa2 were transferred into a sterile stomacher bag and homogenized with 225 mL of PBS pH 7.4. Homogenates were submitted to serial decimal dilutions in the proper diluents and counts of *L.monocytogenes* AL602/08 were performed by plating on Oxford agar, incubated at 37°C for 48 h. Counts of LAB were performed by plating on MRS agar incubated at 30°C for 48 h. Bacteriocin-producing LAB were counted by plating on MRS agar plates overlaid with BHI soft-agar containing *L.monocytogenes* AL602/08 (10^5 - 10^6 CFU/mL), incubated at 37°C for 24 h. Five colonies presenting activity against the target pathogen were selected from

each agar plate and confirmed for bacteriocin production. For these tests, CFS were prepared as described in 2.4 and treated with proteinase K (0.1 mg/mL) for 1 h at 37°C (Noonpakdee et al., 2003). The treated mixtures were heated at 80°C for 5 min for enzyme inactivation, cooled and tested for residual activity against *L.monocytogenes* AL602/08 using the spot-on-the-lawn method (van Reenen et al., 1998). The total counts of *Lb. curvatus* MBSa2 were calculated based on the ratio between the number of bacteriocin-producing LAB and the number of total LAB per plate.

Statistical analysis.

Average counts of *Lb. curvatus* MBSa2 and *L.monocytogenes* AL602/08 were submitted to ANOVA followed by Tukey's test, when appropriate, using $p<0.05$ for significance.

Results and Discussion

Results in Fig 1 indicate that the entrapment in calcium alginate caused a two-log reduction in the viability of *Lb. curvatus* MBSa2 ($p\leq 0.05$). However, the production of bacteriocin was not affected.

Viability of entrapped cells can be affected by the physico-chemical properties of the capsules, such as type and concentration of the coating material, initial cell numbers and bacterial strains (Nazzaro et al., 2012). Moreover, the size of the Ca-alginate beads is an important parameter to be considered, as large beads (diameters of 1 to 3 mm) could adversely affect the textural and sensory quality of the food (Hansen et al., 2002; Nazzaro et al., 2012). In this study, entrapment of *Lb. curvatus* MBSa2 resulted in production of two groups of beads, with average sizes of 266 μm and 473 μm . The influence of the size of alginate beads on production of microbial metabolites

was also observed by Zain et al., (2011), who reported that yeast ST1 produced more ethanol when encapsulated in alginate beads of size of 0.5 cm than in beads of size of 0.3 cm. Similarly, Idris and Suzana (2006) reported that production of lactic acid by *L. delbrueckii* subsp. *delbrueckii* ATCC 9646 was much higher when immobilized in Ca-alginate beads produced using 2.0% sodium alginate concentration (1.0 mm bead diameter).

Tanaka et al. (1984) reported that the molecular weight cut-off point of the Ca-alginate matrix is approximately 20 kDa. Considering that the molecular weight of the two bacteriocins produced by *Lb. curvatus* MBSa2 strain is less than 5 kDa (Barbosa et al., submitted), little interference in diffusion would be expected on the basis of weight.

Results of the evaluation of the influence of beads diameter and environmental conditions during manufacture of salami (pH, Aw and temperature) on the survival and bacteriocin production by free *Lb. curvatus* MBSa2 and *Lb. curvatus* MBSa2 entrapped in two sizes of alginate beads in MRS both are shown in Figs 2, 3 and 4.

The survival of free or entrapped *Lb. curvatus* MBSa2 in MRS both at 18°C was similar, regardless the size of the alginate bead. At 24°C, free MBSa2 presented a 2.56 log reduction in the viable counts from day 7 to day 14, and MBSa2 entrapped in beads of 473 µm had a different behavior when compared to MBSa2 entrapped in smaller beads (Fig. 2). This difference in behavior can be explained by the limitation of the bacterial entrapment. The cells on or near the surface of the matrix beads very often leak out from the matrix and grow in a medium as free cells (Westman et al., 2012). The beads size did not influence the production of bacteriocin by MBSa2.

Growth of MBSa2 in MRS broth with pH 6.0, 5.5 or 5.0 at 30°C (Fig. 3) was similar. After 14 days, a significant 4 log reduction ($p \leq 0.05$) in the counts for free *Lb. curvatus* MBSa2 was observed, while reduction in the counts of encapsulated *Lb.*

curvatus MBSa2 was 2 log, regardless the size of the beads. However, higher levels of bacteriocin were detected for free MBSa2 than for entrapped cells.

The survival of *Lb. curvatus* MBSa2 was influenced by the A_w (0.97, 0.90 and 0.85) of the MRS Broth (Fig. 4). At A_w 0.97, the population of free or entrapped MBSa2 in MRS Broth incubated for 14 days remained stable, but at A_w 0.90, entrapped *Lb. curvatus* MBSa2 survived better than the free cells, which presented a 2 log decrease after 14 days at 30°C. When the A_w was 0.85, both free and entrapped cells presented a decrease in cell viability. Bacteriocin production by MBSa2 was detected only in MRS broth with A_w 0.97, and production was bead size dependent: the larger the diameter of the beads the largest was bacteriocin level. The maximum bacteriocin production in MRS broth (12,800 AU/mL) occurred on the first day of incubation for cells encapsulated in beads with diameter of 473 μm , and remained stable until day 14.

Little work on bacteriocin production by encapsulated LAB has been carried out (Scannell et al., 2000, Ivanova et al., 2000, Ivanova et al., 2002, Sarika et al., 2012). Most studies with encapsulated LAB focused on improving resistance of LAB to hostile environmental conditions (Brachkova et al., 2010; Todorov et al., 2012; Ortakci and Sert, 2012; Shamekhi et al., 2013) or enhancement of lactic acid production (Narita et al., 2004; Göksungur et al., 2005; Rao et al., 2008). Scannell et al, 2000, have shown that production of bacteriocins by *Lactococcus lactis* subsp. *lactis* DPC 3147 and *L. lactis* DPC 496, entrapped in Ca-alginate, in culture medium under controlled temperature (30°C) and pH (6.5) was more effective than production by non-encapsulated cells., Ivanova et al., 2000, Ivanova et al., 2002 and Sarika et al., 2012 reported similar results for encapsulated *Enterococcus faecium* A2000 and *Lactobacillus plantarum* MTCC B1746 and *Lactococcus lactis* MTCCB440.

Listeriosis, caused by *L. monocytogenes*, is a severe disease with high hospitalization and case fatality rates, affecting mainly the elderly, pregnant, newborn and immunocompromised population. *L. monocytogenes* is a foodborne pathogen ubiquitous in the environment and presents unusual physiological properties, capable to adapt to, survive and grow in a wide range of environmental conditions, such as low temperatures and acid or osmotic stress, encountered in many meat products (Gandhi and Chikindas, 2007; Orsi et al., 2011; Carpentier and Cerf, 2011; Milillo et al., 2012). Thus, the control of *L.monocytogenes* in these products is essential to protect human health.

In this first report on application of Ca-alginate entrapped bacteriocinogenic LAB for control of *L.monocytogenes* in salami, it was observed that both free and entrapped *Lb. curvatus* MBSa 2, added to the salami batter, survived well in the product until the end of manufacture period (Fig.5). Many factors can affect survival of LAB in dry fermented meat products, such as time, temperature, relative humidity, ingredients and nature of the starter cultures. Similar stability in the population of LAB during ripening of dry fermented sausages was observed by Erkkilä et al. (2001) for free *L. rhamnosus* LC-705, *L. rhamnosus* GG and *L. rhamnosus* E-97800 and by Ruiz-Moyano et al. (2011) for free *L. fermentum* HL57. However, Wang et al., 2013 observed that the population of *L. sakei* rapidly increased from the initial count of 5.32 log CFU/g to 8.79 log CFU/g in 15 days and then decreased to 6.73 log CFU/g in 30 days. Muthukumarasamy and Holley (2006) reported that counts of *L. reuteri* entrapped in Ca-alginate beads presented a slight reduction while counts of non-encapsulated cells was from 7.12 to 4.54 log CFU/g during manufacture of salami.

Monitoring of pH of salami without added *Lb. curvatus* MBSa 2 or *L.monocytogenes* AL602/08 (control) during the 30 days of manufacture indicated that

pH decreased from 5,92 for the batter to 5,15 for the product on the 4th day (end of fermentation step) and increased again to 5,45 at the 30th day (end of ripening step) (Table 1). The *A_w* dropped from 0,98 in the batter to 0,88 on the 30th day of manufacture (Table 2). Similar pH and *A_w* values were found for the salami containing *Lb. curvatus* MBSa2. Lücke (2000) reported that a rapid pH drop to below 5.3 is important for the inhibition of pathogens, such as *Salmonella* and *Staphylococcus aureus*, and drying of the product to *A_w* below 0.91 prevents post-process acidification.

Bacteriocin production by *Lb. curvatus* MBSa2 strains in the salami containing *L.monocytogenes* AL602/08 is shown in Fig. 6. The decrease in population of the pathogen along time was similar in all types of salami. The counts remained stable during the fermentation period (4 days), and decreased steadily afterwards, for all conditions assayed. At the end of the manufacture period (30th day), the counts of *L.monocytogenes* AL602/08 were 2 log lower in all types of salami, and differences observed for the different types were not significant ($p>0.05$).

There results indicate that encapsulation of bacteriocin-producing LAB in calcium alginate may not be the best strategy for improvement of their protective effect in meat products. Recent studies have shown that encapsulation of semi-purified bacteriocins, instead of bacteriocin-producing LAB, in vesicles composed by one or more phospholipid bilayers (liposomes) is more effective than entrapment in alginate (Teixeira et al., 2008; Taylor et al., 2008; Malheiros et al., 2010a; Malheiros et al., 2010b; Mills et al., 2011; Malheiros et al., 2012; Zou et al., 2012). These materials should be considered as an interesting technological alternative for the control of *L.monocytogenes* in foods.

In conclusion, the entrapment of *Lb. curvatus* MBSa2 in calcium alginate did not improve bacteriocin production in salami. Consequently, no improvement in

inhibition of *L.monocytogenes* in this meat product could be achieved. Other bacteriocins, or other types of entrapment may be required for the effective control of this pathogen in salami.

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References

Aasen, I.M., Markussen, S., Møretrø, T., Katla, T., Axelsson, L. & Naterstad, K. (2003) Interactions of the bacteriocins sakacin P and nisin with food constituents. *International Journal of Food Microbiology*, 87, 35–43.

Ammor, M. S. & Mayo, B. (2007) Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science*, 76, 138–146.

Anal, A. K. & Singh, H. (2007) Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. *Trends in Food Science & Technology*, 18, 240-251.

Balciunas, E. M., Martinez, F. A. C., Todorov, S. D., Franco, B. D. G. M., Converti, A. & Oliveira, R. P. S. (2013) Novel biotechnological applications of bacteriocins: A review. *Food Control*, 32, 134-142.

Brachkova, M. I., Duarte, M. A. & Pinto, J. F. (2010) Preservation of viability and antibacterial activity of *Lactobacillus* spp. in calcium alginate beads. *European Journal of Pharmaceutical Sciences*, 41, 589–596.

Carpentier, B., & Cerf, O. (2011) Review- Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology*, 145, 1–8.

Chen, H. & Hoover, D.G. (2003) Bacteriocins and their Food Applications. *Comprehensive Reviews in Food Science and Food Safety*, 2, 82-100.

Cook, M. T., Tzortzis, G., Charalampopoulos, D. & Khutoryanskiy, V. V. (2012) Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release*, 162, 56–67.

Cotter, P. D., Hill, C. & Ross R. P. (2005) Bacteriocins: developing innate immunity for food. *Nature Review Microbiology*, 3, 777-788.

Deegan, L. H., Cotter, P. D., Hill, C. & Ross, P. (2006) Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *International Dairy Journal*, 16, 1058–1071.

Erkkilä, S., Suihko, M.-L., Eerola, S., Petäjä, E. & Mattila-Sandholm, T. (2001) Dry sausage fermented by *Lactobacillus rhamnosus* strains. *International Journal of Food Microbiology*, 64, 205–210.

Gálvez, A., Abriouel, H., López, R. L. & Omar, N. B. (2007) Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology*, 120, 51–70.

Gálvez, A., López, R. L. Abriouel, H., Valdivia, E. & Omar, N. B. (2008). Application of Bacteriocins in the Control of Foodborne Pathogenic and Spoilage Bacteria. *Critical Reviews in Biotechnology*, 28:125–152.

Gandhi M. & Chikindas M. L. (2007) *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Medical Microbiology*, 113,1-15.

Garbayo, L., Vílchez, C., Vega, J. M., Nava-Saucedo, J. E. & Barbotin, J. N. (2004) Influence of immobilization parameters on growth and lactic acid production by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* co-immobilized in calcium alginate gel beads. *Biotechnology Letters*, 26, 1825-1827.

Göksungur, Y., Gündüz, M. & Harsa, Ş. (2005) Optimization of lactic acid production from whey by *L casei* NRRL B-441 immobilized in chitosan stabilized Ca-alginate beads. *Journal of Chemical Technology and Biotechnology*, 80, 1282–1290.

Hammes, W. P. (2012) Metabolism of nitrate in fermented meats: The characteristic feature of a specific group of fermented foods. *Food Microbiology*, 29, 151-156.

Hansen, L. T., Allan-Wojtas, P. M., Jin, Y.-L. & Paulson, A. T. (2002) Survival of Ca-alginate microencapsulated *Bifidobacterium* spp. in milk and simulated gastrointestinal conditions. *Food Microbiology*, 19, 35-45.

Idris, A. & Suzana, W. (2006) Effect of sodium alginate concentration, bead diameter, initial pH and temperature on lactic acid production from pineapple waste using immobilized *Lactobacillus delbrueckii*. *Process Biochemistry*, 41, 1117–1123.

Ivanova, E., Chipeva, V., Ivanova, I., Dousset, X. & Poncelet, D. (2000-2002) Encapsulation of Lactic Acid Bacteria in Calcium Alginate Beads for Bacteriocin Production. *Journal of Culture Collections*, 3, 53-58.

Kailasapathy, K. (2002) Microencapsulation of Probiotic Bacteria. *Current Issues Intestinal Microbiology* 3, 39-48.

Kaiser, A. L. & Montville, T. J. (1996) Purification of the Bacteriocin Bavaricin MN and Characterization of Its Mode of Action against *Listeria monocytogenes* Scott A Cells and Lipid Vesicles. *Applied and Environmental Microbiology*, 62, 4529–4535.

Krasaekoopt, W., Bhandari, B. & Deeth, H. (2003) Evaluation of encapsulation techniques of probiotics for yoghurt. *International Dairy Journal*, 13, 3–13.

Lücke, F.K. (2000) Utilization of microbes to process and preserve meat. *Meat Science*, 56, 105-115.

Malheiros, P.S., Daroit, D.J., Silveira, N.P. & Brandelli, A. (2010b) Effect of nanovesicle-encapsulated nisin on growth of *Listeria monocytogenes* in milk. *Food Microbiology*, 27, 175-178.

Malheiros, P.S., Micheletto, Y.M.S., Silveira, N.P. & Brandelli, A. (2010a) Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin. *Food Research International*, 43, 1198-1203.

Malheiros, P.S., Sant'Anna, V, Barbosa, M.S., Brandelli, A. & Franco, B.D.G.M. (2012) Effect of liposome-encapsulated nisin and bacteriocin-like substance P34 on *Listeria monocytogenes* growth in Minas frescal cheese. *International Journal of Food Microbiology*, 156, 272-277.

Milillo S. R., Friedly E. C., Saldivar J. C., Muthaiyan A., O'Bryan C., Crandall P. G., Johnson M. G. & Ricke S. C. (2012) A Review of the Ecology, Genomics, and Stress Response of *Listeria innocua* and *Listeria monocytogenes*. *Critical Reviews in Food Science and Nutrition* 52, 712-725.

Mills, S.; Stanton, C., Hill, C. & Ross, R. P. (2011) New Developments and Applications of Bacteriocins and Peptides in Foods. *Annual Review of Food Science and Technology*, 2, 299–329.

Muthukumarasamy, P. & Holley, R.A. (2006) Microbiological and sensory quality of dry fermented sausages containing alginate-microencapsulated *Lactobacillus reuteri*. *International Journal of Food Microbiology*, 111, 164–169.

Narita, J., Nakahara, S., Fukuda, H. & Kondo, A. (2004) Efficient Production of L-(+)-Lactic Acid from Raw Starch by *Streptococcus bovis* 148. *Journal of Bioscience and Bioengineering*, 97, 423–425.

Nazzaro, F., Orlando, P., Fratianni, F. & Coppola, R. (2012) Microencapsulation in food science and biotechnology. *Current Opinion in Biotechnology*, 23, 182-186.

Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K. & Panyim, S. (2003) Isolation of nisin-producing *Lactococcus lactis* WNC 20 strain from nham, a traditional Thai fermented sausage. *International Journal of Food Microbiology*, 81, 137–145.

O' Shea, E. F., Cotter, P. D., Ross, R. P. & Hill, C. (2013) Strategies to improve the bacteriocin protection provided by lactic acid bacteria. *Current Opinion in Biotechnology*, 24,130–134.

Orsi, R.H., den Bakker, H.C. & Wiedmann, M. (2011) *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. *International Journal of Medical Microbiology*, 301, 79–96.

Ortakci, F. & Sert, S. (2012) Stability of free and encapsulated *Lactobacillus acidophilus* ATCC 4356 in yogurt and in an artificial human gastric digestion system. *Journal of Dairy Science*, 95, 6918-6925.

Rao, C. S., Prakasham, R. S., Rao, A. B. & Yadav, J. S. (2008) Functionalized Alginate as Immobilization Matrix in Enantioselective L (+) Lactic Acid Production by *Lactobacillus delbrueckii*. *Applied Biochemistry Biotechnology*, 149, 219–228.

Rathore, S. Desai, P. M., Liew, C. V., Chan, L. W. & Heng, P. W. S. (2013) Microencapsulation of microbial cells. *Journal of Food Engineering*, 116, 369–381.

Renye Jr., J. A., Somkuti, G. A., Garabal, J. I. & Du, L. (2011) Heterologous production of pediocin for the control of *Listeria monocytogenes* in dairy foods. *Food Control*, 22, 1887-1892.

Ruiz-Moyano, S., Martín, A.M., Benito, J., Hernández, A., Casquete, R. & Córdoba, M.G. (2011) Application of *Lactobacillus fermentum* HL57 and *Pediococcus*

acidilactici SP979 as potential probiotics in the manufacture of traditional Iberian dry-fermented sausages. *Food Microbiology*, 28, 839-847.

Sarika, A.R., Lipton, A.P. & Aishwarya, M.S. (2012) Comparative Assessment of Bacteriocin Production in Free and Immobilized *Lactobacillus plantarum* MTCC B1746 and *Lactococcus lactis* MTCC B440. *Journal of Applied Sciences Research*, 8, 2197-2202.

Scannell, A. G. M., Hill, C., Ross, R. P., Marx, S., Hartmeier, W. & Arendt, E. K. (2000) Continuous production of lactacin 3147 and nisin using cells immobilized in calcium alginate. *Journal of Applied Microbiology*, 89, 573-579.

Shamekhi, F., Shuhaimi, M., Ariff, A. & Manap, Y. A. (2013) Cell viability of microencapsulated *Bifidobacterium animalis* subsp. *lactis* under freeze-drying, storage and gastrointestinal tract simulation conditions. *Folia Microbiology*, 58, 91–101.

Tanaka, H., Matsumura, M. & Veliky, I.A. (1984) Diffusion characteristics of substrates in Ca-alginate gel beads. *Biotechnology and Bioengineering*, 26, 53-58.

Taylor, T.M., Bruce, B.D., Weiss, J. & Davidson, P.M. (2008) *Listeria monocytogenes* and *Escherichia coli* O157:H7 inhibition in vitro by liposome-encapsulated nisin and ethylene diaminetetraacetic acid. *Journal of Food Safety*, 28, 183–197.

Teixeira, M.L., Santos, J., Silveira, N.P. & Brandelli, A. (2008) Phospholipid nanovesicles containing a bacteriocin-like substance for control of *Listeria monocytogenes*. *Innovative Food Science and Emerging Technologies*, 9, 49–53.

Todorov, S.D., LeBlanc, J.G. & Franco, B.D.G.M. (2012) Evaluation of the probiotic potential and effect of encapsulation on survival for *Lactobacillus plantarum* ST16Pa isolated from papaya. *World Journal Microbiology Biotechnology*, 28, 973–984.

van Reenen, C. A., Dicks, L. M. T. & Chikindas, M. L. (1998) Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology*, 84, 1131–1137.

Vatanyoopaisarn, S., Prapatsornwattana, K., Kuhakongkeat, T. & Phalakornkule, C. (2011) Potential use of lactic acid bacteria with bacteriocin-like activity against *Staphylococcus aureus* as dual starter cultures in Thai fermented sausage “Sai Krok Prew”. *International Food Research Journal*, 18, 697-704.

Wang, X.H., Ren, H.Y., Liu, D.Y., Zhu, W.Y. & Wang, W. (2013) Effects of inoculating *Lactobacillus sakei* starter cultures on the microbiological quality and nitrite depletion of Chinese fermented sausages. *Food Control*, 32, 591-596.

Westman, J.O., Ylittervo, P., Franzén, C.J. & Taherzadeh, M. J. (2012) Effects of encapsulation of microorganisms on product formation during microbial fermentations. *Applied Microbiology Biotechnology*, 96, 1441–1454.

Zou, Y., Lee, H.-Y., Seo, Y.-C. & Ahn, J. (2012) Enhanced Antimicrobial Activity of Nisin-Loaded Liposomal Nanoparticles against Foodborne Pathogens. *Journal of Food Science*, 77, 165-70.

Table 1. pH of the batter and salami containing free *Lactobacillus curvatus* MBSa2 (MBSa2 F), *Lactobacillus curvatus* MBSa2 encapsulated in beads of calcium alginate (MBSa2 E), *Listeria monocytogenes* (LM) and control (WLC = without laboratorial contamination) during manufacture

Salami	Time (Days)				
	0	4	10	20	30
WLC	5,92±0,01	5,15±0,03	5,17±0,02	5,43±0,05	5,45±0,05
MBSa2 F	5,95±0,01	5,17±0,04	5,19±0,01	5,40±0,05	5,41±0,02
MBSa2 E	5,94±0,02	5,21±0,02	5,27±0,04	5,46±0,06	5,52±0,04
LM	5,97±0,01	5,18±0,03	5,24±0,03	5,34±0,01	5,38±0,03
MBSa2 F + LM	5,96±0,01	5,20±0,02	5,29±0,09	5,35±0,01	5,40±0,01
MBSa2 E + LM	5,97±0,01	5,23±0,02	5,28±0,03	5,47±0,02	5,47±0,03

Table 2. Water Activity (a_w) of the batter and salami containing free *Lactobacillus curvatus* MBSa2 (MBSa2 F), *Lactobacillus curvatus* MBSa2 encapsulated in beads of calcium alginate (MBSa2 E), *Listeria monocytogenes* (LM) and control (WLC = without laboratorial contamination) during manufacture

Salami	Time (Days)				
	0	4	10	20	30
WLC	0,98±0,00	0,97±0,00	0,93±0,02	0,91±0,02	0,88±0,01
MBSa2 F	0,98±0,00	0,98±0,00	0,95±0,01	0,93±0,01	0,89±0,00
MBSa2 E	0,98±0,00	0,98±0,00	0,95±0,01	0,93±0,01	0,89±0,01
LM	0,98±0,00	0,98±0,00	0,95±0,00	0,92±0,00	0,87±0,01
MBSa2 F + LM	0,98±0,00	0,98±0,00	0,95±0,00	0,92±0,01	0,90±0,01
MBSa2 E + LM	0,98±0,00	0,98±0,00	0,96±0,01	0,93±0,01	0,89±0,01

Figure 1 Survival (grey bars) and bacteriocin production (black bars) by free *Lactobacillus curvatus* MBSa2 and entrapped in calcium alginate.

Figure 2 Survival (A) and bacteriocin production (B) by free *Lactobacillus curvatus* MBSa2 (○) and entrapped in calcium alginate beads of 266±3μm diameter (□) and 473±3μm diameter (Δ) in MRS broth, pH 6.5, incubated at 18°C and 24°C for 14 days.

Figure 3 Survival (A) and bacteriocin production (B) by free *Lactobacillus curvatus* MBSa2 (○) and entrapped in calcium alginate beads of 266±3μm diameter (□) and 473±3μm diameter (Δ) in MRS broth with pH adjusted to 6.0, 5.5 and 5.0, incubated at 30°C for 14 days.

Figure 4 Survival (A) and bacteriocin production (B) by free *Lactobacillus curvatus* MBSa2 (○) and entrapped in calcium alginate beads of 266±3µm diameter (□) and 473±3µm diameter (Δ) in MRS broth with Aw adjusted to 0.97, 0.90 and 0.85, incubated at 30°C for 14 days.

Figure 5 Counts of free *Lactobacillus curvatus* MBSa2 (■) and entrapped in calcium alginate beads (▲), in the presence of *Listeria monocytogenes* (LM) (□ and Δ, respectively), in salami batter (time 0) and in salami up to 30 days of manufacture.

Figure 6 Counts of *Listeria monocytogenes* (LM) alone (■) and when in the presence of free (▲) and encapsulated *Lactobacillus curvatus* MBSa2 (●) in salami batter (time 0) and in salami up to 30 days of manufacture.

ANEXOS