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INSTITUTO DE CIÊNCIAS BASICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA

AGRÍCOLA E DO AMBIENTE

DESENVOLVIMENTO DE LIPOSSOMAS CONTENDO PEPTÍDEOS ANTIMICROBIANOS PARA O CONTROLE DE *Listeria monocytogenes* EM PRODUTOS LÁCTEOS

PATRÍCIA DA SILVA MALHEIROS

Porto Alegre, Rio Grande do Sul, Brasil

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Desenvolvimento de lipossomas contendo peptídeos antimicrobianos para o controle de *Listeria monocytogenes* em produtos lácteos

Autor: Patrícia da Silva Malheiros
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RESUMO

Lipossomas são estruturas auto-organizadas adequadas para encapsular e proteger substâncias antimicrobianas de interações com componentes dos alimentos. O presente estudo teve por objetivo desenvolver lipossomas contendo nisina e bacteriocina produzida por *Bacillus* sp. P34 (BLS P34) para o controle de *Listeria monocytogenes* em produtos lácteos. Os lipossomas desenvolvidos nesse trabalho foram preparados utilizando fosfatidilcolina de soja parcialmente purificada. Inicialmente, a encapsulação de nisina foi avaliada através de duas metodologias: fase reversa e hidratação do filme. Com isso, observou-se que a metodologia de hidratação do filme foi a mais adequada para a continuação dos estudos devido à manutenção integral da atividade antimicrobiana da bacteriocina após a encapsulação. BLS P34 foi encapsulada nas mesmas condições. Os lipossomas contendo nisina ou BLS P34 foram estáveis por 24 dias, apresentando as seguintes características: tamanho entre 130 e 160 nm e polidisperdidade entre 0,22 e 0,35, determinados por espalhamento de luz; morfologia esférica, determinada por microscopia eletrônica; eficiência de encapsulação de 94,12% para a nisina e 100% para a BLS P34, determinados por ultra-filtração; potencial zeta de -55 mV para a nisina e -24 mV para a BLS P34. A nisina livre manteve 100% de sua atividade antimicrobiana inicial após 24 dias, enquanto que a nisina encapsulada apresentou 25% de atividade antimicrobiana residual. Não houve diferença na atividade antimicrobiana de BLS P34 livre e encapsulada durante o armazenamento. O modo de ação de BLS P34 encapsulada contra *Listeria monocytogenes* demonstrou que não ocorre fusão entre o lipossoma e a bactéria, sendo necessária a liberação do peptídeo para a ação antimicrobiana. A ação antimicrobiana das bacteriocinas livres e encapsuladas sobre leite e queijo Minas frescal contaminados artificialmente com *L. monocytogenes* foi investigada. A nisina livre foi mais eficiente na inibição do patógeno em leite desnatado armazenado a 30ºC. Entretanto, em temperatura de refrigeração, nisina livre e encapsulada exerceram efeito bactericida. BLS P34 livre e encapsulada mantiveram as contagens de células viáveis de *L. monocytogenes* sempre abaixo do controle em leite desnatado e integral armazenados a 30ºC e 7ºC. Em queijo Minas frescal, todos os tratamentos reduziram a população do patógeno em comparação ao controle, por 21 dias. Porém, a encapsulação de nisina e BLS P34 promoveram melhor efeito inibitório do que as bacteriocinas livres após 10 dias de armazenamento do queijo. A partir desses resultados evidencia-se o potencial da tecnologia de encapsulação de peptídeos antimicrobianos no controle de *L. monocytogenes* em produtos lácteos.

[1]/ Tese de Doutorado em Microbiologia Agrícola e do Ambiente, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil (153 p) Abril, 2011.

Development of liposomes containing antimicrobial peptides for control of *Listeria monocytogenes* in dairy products

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ABSTRACT

Liposomes are promising self-organized structures able to encapsulate and protect antimicrobial substances of the interactions with food components. This study aimed to develop liposomes containing nisin and the bacteriocin produced by *Bacillus* sp. P34 (BLS P34) for control of *Listeria monocytogenes* in dairy products. The liposomes developed in this work were prepared using partially purified soybean phosphatidylcholine. Initially, the encapsulation of nisin was evaluated by two methods: reverse phase and film hydration. It was observed that the film hydration method was the most suitable for further studies due to the overall maintenance of the antimicrobial activity after encapsulation. BLS P34 was encapsulated in the same conditions. The liposomes containing nisin or BLS P34 were stable for 24 days, showing the following characteristics: size between 130 and 160 nm and polydispersity between 0.22 and 0.35 determined by light scattering; spherical morphology, determined by electron microscopy; encapsulation efficiency of 94.12% for nisin and 100% for BLS P34, as determined by ultra filtration; zeta potential of -55 mV for nisin and -24 mV for BLS P34. Free nisin retained 100% of its original antimicrobial activity after 24 days, while the encapsulated nisin showed 25% residual antimicrobial activity. There was no difference in antimicrobial activity of free and encapsulated BLS P34 during storage. The mode of action of encapsulated BLS P34 against *Listeria monocytogenes* showed that no fusion occurs between liposomes and bacteria, being necessary to release the peptide for antimicrobial activity. The antimicrobial action of free and encapsulated bacteriocins on milk and Minas cheese artificially contaminated with *L. monocytogenes* was investigated. Free nisin was more effective in inhibiting of the pathogen in skim milk stored at 30°C. However, under refrigeration, free and encapsulated nisin exerted bactericidal effect. Viable cell counts of *L. monocytogenes* were always below the control in whole and skimmed milk stored at 30 and 7°C in presence of free and encapsulated BLS P34. In Minas cheese, all treatments reduced the pathogen population in comparison to control, for 21 days. However, the encapsulation of nisin and BLS P34 promoted better inhibitory effect than the free bacteriocins after 10 days storage of cheese. These results show the potential of liposomes containing nisin or BLS P34 on the control of *L. monocytogenes* in dairy products.

[1]/ Doctoral thesis in Agricultural and Environmental Microbiology, Institute of Basic Health Sciences, Universidade Federal Rio Grande do Sul, Porto Alegre, Brazil (153 p.), April, 2011.

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LISTA DE ABREVIATURAS E SÍMBOLOS

%: percentual

®: marca registrada

℃: graus Celsius

µl: microlitro

µm: micrômetro

µg: micrograma

AU: arbitrary units

BAL: Bactérias Ácido Lácteas

BHI: *Brain Heart Infusion*

BLS: substância antimicrobiana tipo-bacteriocina

CH: *cholesterol*

DPPC: *dipalmitoyl phosphatidylcholine*

EDTA: *ethylenediamine tetraacetic acid*

EE: eficiência de encapsulação

FAO: *Food and Agriculture Organization*

FC: fosfatidilcolina

FC-1: fosfatidilcolina de soja parcialmente purificada

FG: fosfatidilglicerol

GUV: *giant* unilamellar *vesicles*

ICTA: Instituto de Ciência e Tecnologia de Alimentos

kDa: kiloDalton

KHz: kilohertz

LUV: *large unilamellar vesicles*

mg: miligrama

mL: mililitro

nm: nanômetros

mV: milivolt

MLV: *multilamellar vesicles*

MVV: *multivesicular vesicles*

MET: Microscopia Eletrônica de Transmissão

MEV: Microscopia Eletrônica de Varredura

PBS: *Phosphate Buffered Saline*

PDI: polidispersidade

rpm: rotações por minuto

sp.: espécies

SUV: small unilamellar *vesicles*

TSA: *Trypticase Soy Agar*

UFC: Unidades Formadoras de Colônias

UI: Unidades Internacionais

WHO: *World Health Organization*

YE: *Yeast Extract*

1 INTRODUÇÃO

A crescente demanda dos consumidores por alimentos minimamente processados, preparados sem a adição de conservantes químicos e com longa vida útil impulsiona a indústria alimentícia e as instituições de pesquisas para a busca de novas tecnologias de conservação.

Dentre as alternativas tecnológicas para estender a vida útil e aumentar a segurança de alimentos está a bioconservação por meio do emprego de microbiota protetora e/ou seus peptídeos antimicrobianos, as bacteriocinas.

Bacteriocinas são substâncias antimicrobianas de natureza protéica, que apresentam ausência de letalidade para a célula produtora. Nisina, uma bacteriocina produzida por *Lactococcus lactis* subsp. *lactis*, foi a primeira a ser aprovada para uso em produtos alimentícios, sendo a mais utilizada como bioconservante natural em todo o mundo. No Brasil, o uso de nisina é permitido somente em produtos lácteos, visando especialmente à inibição do patógeno *Listeria monocytogenes*.

Diversas espécies de *Bacillus* produzem bacteriocinas ou substância semelhante à bacteriocina (*bacteriocin-like*) com grande potencial para aplicação em alimentos. Especificamente, *Bacillus* sp. linhagem P34 produz substância semelhante à bacteriocina que já foi caracterizada mostrando inibir

diversos micro-organismos patogênicos e deteriorantes, além de apresentar ausência de toxicidade em experimentos *in vitro*, sugerindo a possibilidade de sua utilização como bioconservante de alimentos.

A aplicação direta de bacteriocina em alimentos pode resultar em diminuição ou perda de sua atividade antimicrobiana devido a problemas decorrentes da interação com componentes da matriz alimentar. Para contornar esse problema, a encapsulação de bacteriocinas em lipossomas pode ser uma alternativa promissora, pois protege o antimicrobiano dessas interações indesejáveis.

Os lipossomas são estruturas coloidais constituídos de um núcleo interno aquoso e uma membrana formada pela auto-associação de moléculas fosfolipídicas em bicamadas. Portanto, estas estruturas podem ser utilizadas para a encapsulação de substâncias hidrofílicas no seu núcleo aquoso e compostos hidrofóbicos na bicamada lipídica oferecendo larga aplicação em diversas áreas, como a alimentícia.

Neste contexto, o objetivo deste trabalho foi desenvolver lipossomas contendo peptídeos antimicrobianos e avaliar sua aplicação no controle de *Listeria monocytogenes* em produtos lácteos.

Os objetivos específicos do estudo foram:

1. Desenvolver lipossomas contendo nisina, através da comparação de dois métodos: fase reversa e hidratação do filme;
2. Desenvolver lipossomas contendo a bacteriocina parcialmente purificada produzida por *Bacillus* sp. linhagem P34 (BLS P34);
3. Caracterizar os lipossomas contendo nisina e BLS P34 através de:

   a. Tamanho e polidispersidade, por espalhamento dinâmico de luz
   b. Morfologia, por microscopia
   c. Eficiência de encapsulação, por ultra-filtração
   d. Carga de superfície, por determinação de potencial zeta
   e. Efeito *in vitro* sobre *Listeria monocytogenes*, por difusão em agar

4. Avaliar o efeito de nisina livre e encapsulada no controle de *Listeria monocytogenes* em leite desnatado e integral;
5. Avaliar o efeito de BLS P34 livre e encapsulada no controle de *Listeria monocytogenes* em leite desnatado e integral;
6. Produzir queijo Minas frescal e avaliar o efeito de nisina livre, nisina encapsulada, BLS P34 livre e BLS P34 encapsulada no controle de *Listeria monocytogenes* nessa matriz alimentar.

2 REVISÃO BIBLIOGRÁFICA

2.1 Definição e classificação das bacteriocinas

Bacteriocinas são substâncias antimicrobianas de natureza protéica, sintetizadas ribossomicamente, que apresentam ausência de letalidade para a célula produtora (Montville e Kaiser, 1993; Jack et al., 1995; Cotter et al., 2005). Tais peptídeos têm sido encontrados em muitos grupos de bactérias e, dentro das espécies, dezenas ou mesmo centenas de diferentes tipos de bacteriocinas são produzidas (Klaenhammer, 1993; Zouhir et al., 2010). As diferentes bacteriocinas apresentam variações no espectro de atividade, modo de ação, peso molecular, origem genética e propriedades bioquímicas (Diep e Nes, 2002).

Quanto à classificação, Klaenhammer (1993) dividiu as bacteriocinas produzidas por bactérias ácido lácticas em quatro classes distintas, ou seja, (I) lantibióticos, (II) pequenos peptídeos termoestáveis, (III) grandes proteínas termolábeis, (IV) grandes complexos peptídicos contendo carboidrato ou lipídio em sua estrutura. As bacteriocinas mais estudadas são as pertencentes às classes I e II (Ennahar et al., 2000). Em 2005, Cotter et al. (2005) propuseram uma modificação nesta classificação, dividindo as bacteriocinas produzidas por bactérias ácido lácticas em apenas duas categorias distintas: os lantibióticos

(classe I) contendo lantionina e os não lantibióticos (classe II), enquanto os peptídeos de alto peso molecular termolábeis, formalmente componentes da classe III, seriam separadamente designados de 'bacteriolisinas'. Os autores sugerem ainda que a classe IV seja extinta.

Drider et al. (2006) propuseram uma nova organização, onde as bacteriocinas estariam distribuídas em três grandes classes de acordo com suas características bioquímicas e genéticas.

A classe I ou lantibióticos, composta por peptídeos contendo lantionina ou β-lantionina, foi dividida em duas subcategorias: tipo A (moléculas lineares) com a nisina e subtilina como seus principais exemplos e tipo B (molécula globular) com a mersacidina e mutacina como integrantes dessa categoria.

A classe II contém pequenos peptídeos (< 10 kDa) heterogêneos e estáveis. Essa classe foi subdividida por Nes et al. (2007) em quatro subclasses: (IIa) bacteriocinas como a pediocina com atividade contra *Listeria*; (IIb) bacteriocinas que requerem a atividade combinada de dois peptídeos; (IIc) bacteriocinas que não apresentam peptídeo guia; (IId) bacteriocinas circulares.

A classe III, composta por grandes proteínas (> 30 kDa) termolábeis, são bacteriocinas complexas quanto à atividade e à estrutura protéica. Seu mecanismo de ação se diferencia das demais bacteriocinas, promovendo a lise da parede celular do micro-organismo alvo. Apresentam uma porção N-terminal homóloga a uma endopeptidase, responsável pela catálise da parede celular, e uma porção C-terminal responsável pelo reconhecimento da célula alvo (Lai et al., 2002).

A classificação mais recente de bacteriocinas produzidas por bactérias Gram positivas foi proposta por Zouhir et al. (2010). Nessa classificação, as bacteriocinas conhecidas até o momento são divididas em grupos, de acordo com as seqüências de consenso dos aminoácidos que fazem parte de sua composição.

Grupo 1: Peptídeos com 20 a 30 aminoácidos, sendo que 13 bacteriocinas compõe esse grupo, incluindo mutacina 2, lacticina 481 e 3147, mersacidina, plantaricina W alpha, mutacina H29B, streptococcina AFF22 e AM49, entre outras.

- Grupo 2: Composto por duramycina C, ancovenina e cinnamycina.
- Grupo 3: Peptídeos com 36 a 50 aminoácidos. Este grupo é representado por 29 bacteriocinas, todas com atividade *antilisterial*. O subgrupo 3a tem como representantes enterocina A, bavaricina MN, sakacina A, entre outras. O subgrupo 3b tem como representantes bavaricina A, sakacina P, pediocina PA1, entre outras.
- Grupo 4: Composto por 8 bacteriocinas destacando-se a subtilina, nisina A e nisina Z.
- Grupo 5: Composto por pep 5, epicidina 280.
- Grupo 6: Composto por citolisina, plantaricina W beta e lacticina 3147 A2.
- Grupo 7: Composto por acidocina J 1132 beta e plantaricina 1.25 beta.
- Grupo 8: Composto por carnobacteriocina A e enterocina B.
- Grupo 9: Composto por plantaricina S alpha, gassericina T e lacticina F.

- Grupo 10: Composto por lacticina F lafX, acidocina LF 221B e lactobina A.
- Grupo 11: Composto por lactocian 705 e divergicina 750.
- Grupo 12: Composto por lactococcina G beta, sublancina 168 e PlnJ.

As classificações vêm sendo modificadas com a descoberta de novas bacteriocinas, devendo ser necessário algum tempo até que um sistema de classificação definitivo seja obtido (De Martinis et al., 2002; Heng et al., 2007).

Existem, por exemplo, as substâncias chamadas de substâncias semelhantes às bacteriocinas (*bacteriocin-like*) que englobam os compostos antimicrobianos de natureza protéica que ainda não estão completamente definidas ou não cumprem com todas as características das bacteriocinas. Estas substâncias geralmente possuem um espectro de ação mais amplo, atuando contra uma variedade de bactérias Gram-positivas e Gram-negativas, sendo que algumas apresentam ainda atividade antifúngica, antiviral e amebicida (De Vuyst e Vandamme, 1994; Messens e De Vuyst, 2002, Benitez et al., 2010a; Todorov et al., 2010, Benitez et al., 2010b).

2.2 Mecanismo de ação das bacteriocinas

As bacteriocinas podem ser bactericidas ou bacteriostáticas. Para que o efeito bactericida ocorra, o micro-organismo alvo deve apresentar sensibilidade ao antimicrobiano que, mesmo em baixas concentrações, é capaz de provocar a morte celular rapidamente. A sensibilidade de bactérias Gram-negativas e Gram-positivas às bacteriocinas tem como base a composição química da parede celular. Para tornarem-se mais sensíveis à ação das bacteriocinas, as

bactérias Gram-negativas precisam ser desestabilizadas por estresse físico e/ou químico (Ennhar et al., 2000; Cotter et al, 2005; Nes et al., 2007).

A maioria das bacteriocinas age formando canais na membrana ou poros transversalmente à bicamada fosfolipídica, o que provoca a saída de pequenos compostos ou a alteração da força protomotriz necessária para a produção de energia e para a síntese de proteínas ou ácidos nucléicos (Montville e Chein, 1998; Oscáris e Pisabarro, 2001).

Para formar os poros, as bacteriocinas precisam interagir com a membrana citoplasmática da célula-alvo. A primeira fase na formação de poros pela bacteriocina envolve as interações eletrostáticas entre a carga positiva e os resíduos polares da bacteriocina com os fosfolipídios aniônicos presentes na bicamada lipídica da membrana alvo (Abee et al.,1995). A segunda fase é irreversível e envolve mudanças letais em cepas sensíveis à bacteriocina (Desmazeaud, 1997).

Dois mecanismos foram propostos para descrever detalhadamente os passos que envolvem a permeabilidade da membrana por peptídios líticos com características anfipáticas.

a. Modelo *Barrel stave*: a bacteriocina se liga como monômero na membrana citoplasmática, insere-se na bicamada lipídica e os monômeros inseridos agregam-se lateralmente para formar o poro. Alguns estudos demonstram que a parte C-terminal da molécula da nisina penetra na porção hidrofóbica da membrana citoplasmática (Martin et al., 1996). Outros estudos indicam que a parte C-terminal é responsável pela ligação da molécula na

membrana das células sensíveis, e a penetração na camada lipídica é realizada pela parte N-terminal.

b. Modelo *Wedge*: a bacteriocina liga-se a bicamada lipídica sendo puxada para dentro da membrana pela força protônica. Sua orientação em relação à bicamada lipídica não se altera, pois o peptídeo não entra em contato com a parte hidrofóbica da membrana (Bruno e Montville, 1993).

Estudos detalhados das bacteriocinas da classe I permitem afirmar que os lantibióticos agem na membrana citoplasmática das bactérias sensíveis. Embora um mecanismo de ação ainda não tenha sido completamente elucidado para todos os lantibióticos, sabe-se que estes compostos utilizam o lipídeo II na membrana celular como receptor. A ligação de bacteriocinas, tais como a nisina, ao lipídeo II facilita um mecanismo de ação duplo envolvendo a prevenção da síntese de peptídeoglicano e formação de poros (Cotter et al., 2005).

As bacteriocinas da classe II não contêm aminoácidos modificados e, dependendo do gradiente elétrico, estas interagem com receptores para se inserirem na membrana citoplasmática. Estas bacteriocinas apresentam uma seqüência comum de aminoácidos, Tirosina-Glicina-Asparagina-Glicina-Valina, provavelmente responsável pela aderência às células sensíveis (Ennahar et al., 2000). Segundo Cotter et al. (2005), os peptídeos dessa classe, em geral, apresentam uma estrutura helicoidal anfifílica a qual permite que elas se insiram na membrana da célula-alvo levando a despolarização e morte.

As classes III e IV são compostas por grande bacteriocinas, cujo mecanismo de ação é pouco estudado (Héchard e Sahl, 2002). Entretanto,

sugere-se que as bacteriolisinas (pertencentes à classe III de Klaenhammer, 1993) atuariam diretamente na parede celular de bactérias Gram-positivas levando à lise e morte das células-alvo (Cotter et al., 2005).

2.3 Nisina e o peptídeo antimicrobiano P34

Nisina é um peptídeo antimicrobiano anfifílico catiônico de 3,5 kDa composto por 34 aminoácidos (Breukink e Kruijff, 1999) produzido por cepas de *Lactococus lactis* subsp. *lactis*. Essa bacteriocina foi considerada de uso seguro para aplicação em alimentos pelo *Joint Food and Agriculture Organization/World Health Organization* (FAO/WHO) em 1969, sendo utilizada como bioconservante em muitos países (Cotter et al., 2005). A FAO/WHO aceita nisina como aditivo alimentar para queijo processado em uma concentração de 12,5 mg de nisina pura por kg de produto (Ross et al., 2002). Nisina tem recebido muita atenção devido a seu amplo espectro inibitório contra bactérias Gram-positivas como *Listeria* e *Staphylococcus,* bem como a inibição efetiva de esporos de espécies de *Bacillus* e *Clostridium* (Arauz et al., 2009).

Duas variantes naturais da nisina são conhecidas. A estrutura delas é semelhante, mas diferem no aminoácido da posição 27: histidina em nisina A e asparagina em nisina Z. Segundo De Vos et al. (1993), nisina Z tem melhores propriedades de difusão em ágar do que a nisina A, o que seria uma característica importante para aplicações em alimentos. Entretanto, somente nisina A é comercializada, sendo disponível na forma de pó, o qual não é completamente solúvel. O produto comercial (Nisaplin®) apresenta 2,5% de nisina pura, sal e leite. Segundo alguns autores (Scott e Taylor, 1981; Cleveland et al., 2002), as proteínas do leite ligam-se à nisina tornando sua

atividade antimicrobiana menor em comparação a uma preparação purificada ou parcialmente purificada.

Além disso, algumas outras limitações foram relatadas em relação ao uso de nisina, como por exemplo, o desenvolvimento de resistência por parte de alguns micro-organismos. Outro obstáculo importante é que algumas bactérias apresentam a capacidade de produzir substâncias proteolíticas que inibem a ação da nisina, já sendo relatada a expressão de nisinase (enzima que degrada nisina) em alguns trabalhos (Lee et al., 2002; Garde et al., 2004; Liang et al., 2010). Portanto, a busca por novas bacteriocinas é justificada, visando à segurança e aumento na qualidade de alimentos.

As bacteriocinas produzidas por *Bacillus* estão se tornando cada vez mais importantes devido ao seu amplo de espetro de ação - quando comparadas com bacteriocinas produzidas por bactérias ácido lácteas - o qual pode incluir bactérias Gram-negativas, fungos ou leveduras, além das espécies de bactérias Gram-positivas (Abriouel et al., 2011). O gênero *Bacillus* apresenta uma grande variedade de espécies produtoras de peptídeos antimicrobianos com diferentes estruturas químicas e, além disso, apresenta um histórico de uso seguro em alimentos (Pedersen et al., 2002; Gebhardt et al., 2002). Em 1977, já existiam 167 peptídeos descritos, dos quais 66 associados a linhagens da espécie *Bacillus subtillis* e 23 eram produtos da espécie *Bacillus brevis* (Katz e Demain, 1977). Outras espécies do gênero *Bacillus* produtoras de bacteriocinas são: *Bacillus polyfermenticus* (Lee et al., 2001), *Bacillus thuringiensis* (Ahern et al., 2003), *Bacillus licheniformis* (Cladera-Oliveira et al.,

2004), *Bacillus cereus* (Bizani et al., 2005), *Bacillus amyloliquefaciens* (Lisboa et al., 2006), entre outros.

O micro-organismo *Bacillus* sp. linhagem P34 foi isolado do intestino do peixe Piau-com-pinta, proveniente do ambiente aquático da bacia amazônica brasileira. Essa bactéria produz substância antimicrobiana semelhante às bacteriocinas (BLS) apresentando atividade contra importantes bactérias patogênicas Gram-positivas, como *Listeria monocytogenes* e *Bacillus cereus* (Motta et al., 2004). A substância antimicrobiana tem massa molecular de 1.456 Da, é relativamente estável ao calor e sensível às enzimas proteolíticas. Além disso, o peptídeo apresenta atividade em um amplo espectro de pH (de 3,0 a 10) e caráter aniônico (Motta et al., 2007a; Motta et al., 2007b). A partir de imagens obtidas por microscopia eletrônica de transmissão, Motta et al. (2008) observaram que a bacteriocina age no envelope celular da bactéria-alvo gerando perda de protoplasma. De acordo com essas características, BLS P34 poderia estar associada com o grupo de bacteriocinas ativas contra *Listeria* da classe I, conforme Klaenhamer (1993).

Segundo Vaucher et al. (2010), o peptídeo antimicrobiano P34 apresentou baixa citotoxicidade *in vitro*, com efeito similar ao observado para a nisina, sendo um indicativo de segurança, do ponto de vista toxicológico, para futuras aplicações dessa bacteriocina em alimentos.

2.4 Aplicações de bacteriocinas em alimentos

A demanda dos consumidores por alimentos minimamente processados ou “alimentos frescos” sem a adição de conservantes químicos tem estimulado

o interesse na pesquisa por agentes antimicrobianos naturais, como as bacteriocinas (Gálvez et al., 2008; García et al., 2010). Atualmente, as bacteriocinas mais estudadas são aquelas produzidas por bactérias láticas (BAL), pois são tradicionalmente associadas aos alimentos e consideradas seguras (Abriouel et al., 2011).

Desde os primeiros usos de nisina, na década de 50, muitos trabalhos já foram desenvolvidos sobre a aplicação de bacteriocinas de BAL em alimentos (García et al., 2010), especialmente em produtos lácteos.

Maisnier-Patin et al. (1995) investigaram a ação antimicrobiana de nisina sobre *L. monocytogenes* em leite desnatado durante aquecimento. A utilização de 25 ou 50 UI/mL de nisina resultou numa redução significativa do tempo necessário para a eliminação do patógeno.

Davies et al. (1997) avaliaram a eficiência de nisina comercial (Nisaplin®) no controle de *L. monocytogenes* em queijo ricota armazenado a 6–8℃ por 70 dias. A adição de 2,5 mg/mL de nisina inibiu o crescimento do patógenos por 8 semanas.

Lauková et al. (1999) estudaram o efeito inibitório de enterocina CCM 4231 sobre o desenvolvimento de *Staphylococcus aureus* em leite. Após 24h, os pesquisadores observaram decréscimo do número de células viáveis do patógeno de 10 log UFC/mL no controle para 2 log UFC/mL na amostra com enterocina. Quando a mesma bacteriocina foi aplicada em queijo *Saint-Paulin* Lauková et al. (2001) observaram redução de 3,6 log UFC/g na contagem de *L. monocytogenes* em relação ao queijo controle após uma semana a 14ºC.

Enan (2006) analisou o efeito da combinação de nisina e plantaricina UGI no crescimento de *L. monocytogenes* resistente a plantaricina UGI. A mistura das bacteriocinas evitou a recuperação e crescimento do patógeno resistente, tanto *in vitro* como em alimentos, sugerindo que o efeito sinérgico poderia estender a vida útil de produtos cárneos e lácteos.

Kykkidou et al. (2007) avaliaram o uso de nisina como tratamento antimicrobiano para estender a vida útil de queijo *Galotyri*. A adição de 50 UI/g e 150 UI/g de nisina aumentaram a *shelf-life* do produto armazenado a 4ºC por 7 e 21 dias, respectivamente, com o queijo mantendo boas características sensoriais e contagem de leveduras dentro do limite aceitável (5 log UFC/g).

Pinto et al. (2011) avaliaram diferentes concentrações de nisina contra *S. aureus* em queijo Minas Serro. Os autores obtiveram diferenças de 1,2 e 2 log UFC/g na contagem do patógeno após 7 dias de maturação para queijos contendo 100 e 500 UI/mL de nisina, respectivamente, em comparação a amostra controle.

Após as bacteriocinas produzidas por BAL, as bacteriocinas e substâncias antimicrobianas semelhantes às bacteriocinas (BLS) produzidas pelo gênero *Bacillus* podem ser consideradas como mais importantes (Abriouel et al., 2011). Entretanto, apesar da diversidade de bacteriocinas produzidas por espécies de *Bacillus* com atrativas propriedades tecnológicas e industriais, existem poucos relatos na literatura sobre a aplicação desses peptídeos em alimentos. Dois exemplos representativos sobre a aplicação de bacteriocinas de *Bacillus* em produtos lácteos são bacillocin 490 e cereína 8A.

Martirani et al. (2002) avaliaram o efeito de bacillocin 490, uma bacteriocina produzida por *Bacillus licheniformis* termofílico, contra *Bacillus smithii* em leite de búfala. Os autores obtiveram 50% de redução na inibição do patógeno após 5 horas de incubação a 25ºC.

Bizani et al. (2008) avaliaram a inibição de *L. monocytogenes* em leite e queijo pela ação de cereína 8A, uma bacteriocina produzida por *B. cereus* isolado de solo brasileiro. Em leite UHT, a bacteriocina reduziu a contagem do patógeno em 3 log UFC/mL por 14 dias a 4ºC. Quando a bacteriocina foi aplicada durante a produção de queijo Minas frescal, houve somente um atraso no inicio da fase de crescimento exponencial. Já na superfície do queijo, cereína 8A reduziu em 2 ciclos logarítmicos a contagem de *L. monocytogenes* durante 30 dias a 4ºC. Os autores atribuem este efeito à inativação da cereina 8A durante a fabricação do queijo por enzimas endógenas ou ligação a componentes da matriz alimentar.

É importante ressaltar que as bacteriocinas podem ser incorporadas nos alimentos de diferentes formas: 1) pela utilização de bactérias capazes de produzir bacteriocinas diretamente no alimento; 2) pelo uso de ingredientes preparados através de fermentação com bactérias produtoras de bacteriocinas e 3) pelo uso de uma preparação purificada (ou semi-purificada) de bacteriocina como ingrediente (Deegan et al., 2006; Gálvez et al., 2008).

Entretanto, vários trabalhos indicam que a aplicação direta de bacteriocinas em alimentos pode resultar em diminuição ou perda de sua atividade antimicrobiana devido a problemas decorrentes da interação com componentes dos alimentos (Jung et al., 1992; Branen e Davison, 2004; Chollet

et al., 2008). Para contornar esse problema, a encapsulação de bacteriocinas em lipossomas pode representar uma alternativa tecnológica promissora.

2.5 Lipossomas

Os lipossomas são estruturas coloidais constituídos de um núcleo interno aquoso e uma membrana formada pela auto-associação de moléculas fosfolipídicas em bicamadas. O mecanismo de formação de lipossomas baseia-se essencialmente nas interações entre fosfolipídios e moléculas de água, onde os grupos polares finais dos fosfolipídios são expostos à fase aquosa (interior e exterior) e as caudas de hidrocarboneto hidrofóbicas são forçadas a formar uma bicamada (Jesorka e Orwar, 2008). A imposição de energia para os fosfolipídios agregados resulta em uma organização em bicamadas fechadas (lipossomas) e, durante esse processo, solutos presentes no meio aquoso podem ser encapsulados (Mozafari et al., 2008a). Devido à presença de fases aquosa e lipídica na estrutura das vesículas, elas podem ser utilizadas na encapsulação e liberação de materiais hidrossolúveis, lipossolúveis e anfifílicos (Khosravi-Darani et al., 2007; Mozafari et al., 2008a). Lipossomas têm sido testados como carreadores de agentes terapêuticos, ferramentas analíticas, além de modelos de membranas biológicas (Sharma e Sharma, 1997; Frézard, 1999; Gómez-Hens e Fernández-Romero, 2005, Edwards e Baeumner, 2006; Date et al., 2007; Jesorka e Orwar, 2008).

Na indústria de alimentos, os lipossomas têm sido investigados para liberação controlada de proteínas, enzimas, vitaminas, antioxidantes e *flavors* (Taylor et al., 2005; Mozafari et al., 2006; Mozafari et al., 2008b). Mais recentemente, os lipossomas foram investigados por sua capacidade de

incorporar compostos bioativos. Os lipossomas podem proteger o material encapsulado contra muitas mudanças ambientais e químicas, como proteção contra pH e temperatura (Mozafari et al., 2008a; Mozafari et al., 2008b). Como os lipossomas podem ser preparados a partir de componentes naturais, barreiras reguladoras que poderiam impedir a sua aplicação em sistemas alimentícios são potencialmente reduzidos ou eliminados, e novas formulações poderiam ser rapidamente implementadas (Taylor et al., 2005; Mozafari et al., 2008).

2.6 Lipossomas contendo peptídeos antimicrobianos

Existem diversos métodos descritos na literatura para a preparação de lipossomas (Mozafari, 2005, Mertins et al., 2005, Teixeira et al., 2008). Entretanto, o mais utilizado para encapsulação de peptídeos antimicrobianos em nanovesículas lipossômicas é a hidratação do filme fosfolipídico (Taylor et al., 2005; Were et al., 2003, Laridi et al., 2003). Esse método consiste na formação de um filme lipídico na superfície interna de um balão de fundo redondo após solubilização do lipídio em solvente e evaporação do mesmo. A bacteriocina em solução tampão é adicionada neste filme, sendo que a dispersão dos fosfolipídios é facilitada por ultra-agitação. Com isso, o filme desprende da superfície do balão sob forma de vesículas com diâmetros elevados e multilamelares (MLV). Para diminuir e homogeneizar o tamanho dos lipossomas uma nova energia deve ser fornecida como ultra-som, extrusão e/ou microfluidificação (Sharma e Sharma, 1997; Jesorka e Orwar, 2008). O objetivo é formar vesículas com tamanho adequado, polidispersidade aceitável, e alta eficiência de encapsulação (Mozafari et al., 2008a).

A concentração de composto que pode ser encapsulado é uma função da estrutura lipídica, e dependente das interações eletrostáticas e hidrofóbicas entre antimicrobianos e fosfolipídios (Were et al., 2003). Especificamente, nisina é um peptídeo antimicrobiano anfifílico catiônico (Breukink e Kruijff, 1999) que pode ser encapsulado no interior da fase aquosa e também ser imobilizado nas membranas dos lipossomas (Laridi et al., 2003). A eficiência de encapsulação da nisina é normalmente maior em lipossomas fabricados a partir de fosfolipídios neutros (zwitterionicos), como a fosfatidilcolina (FC), em comparação com lipossomas contendo lipídios aniônicos, tais como fosfatidilglicerol (FG) (Were et al., 2003, Were et al., 2004; Taylor et al., 2008).

O potencial zeta é geralmente utilizado como um indicador de cargas na superfície dos lipossomas. Por exemplo, lipossomas vazios de FC apresentaram um potencial zeta de -8,3 mV a 25°C, enquanto que FC:FG (8:2) e FC:FG (6:4) mostraram potencial zeta de -52,28 e -72,60 mV, respectivamente (Taylor et al., 2007). Lipossomas compostos de lipídios contendo elevadas cargas elétricas podem ser mais estáveis que os lipossomas neutros, pois quanto maior o potencial zeta (positivo ou negativo) maiores são as interações repulsivas, reduzindo a freqüência das colisões.

A carga do lipossoma também pode influenciar na interação entre a nisina encapsulada e a bactéria. Como a célula bacteriana tem carga negativa, repulsão eletrostática pode ocorrer, por exemplo, entre lipossomas contendo FG e a superfície da célula, impedindo o contato direto entre o lipossoma e o patógeno (Were et al., 2004) Com isso, poderia não ocorrer fusão de

membrana, sugerido como o principal mecanismo de interação entre lipossomas e bactérias (Mugabe et al., 2006; Colas et al., 2007).

A encapsulação de nisina Z resultou em uma significativa redução de tamanho dos lipossomas quando comparado com lipossomas vazios (Laridi et al., 2003), o que poderia ser atribuído ao efeito da nisina sobre a organização da membrana lipídica e/ou hidrofobicidade do lipídio, já que a inserção de nisina pode alterar a permeabilidade e a estrutura de bicamada da membrana (El Jastimi e Lafleur, 1999; El Jastimi et al., 1999). Taylor et al. (2007) mostraram que a encapsulação de nisina em lipossomas de FC resultou em aumento do tamanho das vesículas, enquanto o tamanho das vesículas foi menor quando a encapsulação foi feita em lipossomas de FC:FG (8:2, 6:4). Lipossomas de FC:FG, através da neutralização da carga do lipídio aniônico pela nisina e um realinhamento dos fosfolipídios, pode ter atingido um nível de estruturação mais ideal, o que poderia explicar as reduções nos diâmetros (El Jastimi et al., 1999; Bonev et al., 2000; Taylor et al., 2007).

Silva et al. (2008) demonstraram que a inclusão de um peptídeo sintético contendo lisinas carregadas positivamente distribuídas ao longo da seqüência do peptídeo resultou em lipossomas com um "aspecto amorfo", enquanto que a adição de um peptídeo contendo a carga positiva C-terminal resultou em lipossomas com uma morfologia de vareta. Portanto, a incorporação de diferentes peptídeos em lipossomas pode originar diferenças no diâmetro (tamanho), potencial zeta e na morfologia dos lipossomas.

A presença de colesterol na composição do lipossoma normalmente resulta em aumento no diâmetro médio e diminuição na capacidade de

encapsulação de nisina (Laridi et al., 2003; Were et al., 2003). O colesterol promove o aumento na ordenação das cadeias lipídicas e estreitamento na parede do lipossoma, o que pode levar à redução na afinidade e capacidade de inserção da nisina dentro da membrana lipídica (El Jastimi e Lafleur, 1999; El Jastimi et al., 1999). Entretanto, o colesterol pode ser benéfico agindo como um componente de estabilização do lipossoma, através da diminuição da permeabilidade da membrana lipídica e aumento nas interações coesivas (Deo e Somasundaran, 2003). Resultados semelhantes foram relatados com diferentes peptídeos antimicrobianos (Choi et al., 2004, Zhao et al. 2006; Verly et al., 2008).

A concentração de nisina é também um fator importante para a eficiência de encapsulação. A capacidade de encapsulação da nisina aumenta com o aumento da concentração do antimicrobiano. Essa maior capacidade de encapsulação pode indicar que parte da nisina encapsulada é inserida ou imobilizada na membrana lipídica, sugerindo que a área de membrana lipídica disponível por unidade de massa de nisina não seria um fator limitante para a adsorção do peptídeo sobre a membrana do lipossoma (Laridi et al., 2003).

Outros fatores, tais como o pH da solução de nisina, poderiam apresentar um impacto significativo na quantidade de nisina encapsulada no lipossoma. Larid et al. (2003) mostraram que a diminuição no pH da solução de nisina gerou um aumento na quantidade de bacteriocina encapsulada em lipossomas preparados com diferentes pro-lipossomas (Pro-lipo H e Pro-lipo S). Isso pode ocorrer devido a maior solubilidade da nisina em baixo pH e/ou

aumento na ionização de certos grupos no peptídeo e certos constituinte dos lipídeos.

2.7 Aplicações em alimentos e sistemas modelo de bacteriocinas encapsuladas em lipossomas

Embora vários estudos tenham sido publicados mostrando a produção de lipossomas estáveis contendo peptídeos antimicrobianos e suas propriedades (seção 2.6), existem relativamente poucos dados publicados sobre a aplicação em alimentos ou sistemas modelo de tais bacteriocinas encapsuladas.

A encapsulação de pediocina AcH em lipossomas de FC resultou em um aumento de 27,5% e 28,9% na recuperação da atividade da pediocina nas suspensões de músculo bovino e gordura, respectivamente, comparado com as mesmas suspensões contendo pediocina livre (Degnan e Luchansky, 1992). Em uma investigação similar, Degnan et al. (1993) avaliaram a atividade de pediocina AcH encapsulada em lipossomas nas suspensões de leite em pó desnatado, nata, músculo bovino, ou gordura bovina (preparada em água deionizada, na concentração final de 10%). Os autores observaram um aumento na recuperação de pediocina encapsulada de 29-62% em comparação com o antimicrobiano livre. Tais resultados demonstram a eficácia dos lipossomas em assegurar a atividade de bacteriocinas, minimizando os efeitos negativos observados para a pediocina não encapsulada, tais como a associação da bacteriocina com proteína e/ou gordura presente nas suspensões e proteólise (Degnan e Luchansky, 1992; Degnan et al., 1993).

Nos últimos anos, a maioria das pesquisas avaliando a aplicação em sistemas modelos ou alimentos de bacteriocinas encapsuladas vem sendo desenvolvida com nisina Z, por ser um peptídeo aprovado para uso em alimentos e devido às suas propriedades de difusão em ágar. Dentre os micro-organismos, normalmente *Listeria monocytogenes*, um importante patógeno de origem alimentar, é utilizado como indicador.

Laridi et al. (2003) investigaram a estabilidade e os efeitos de nisina Z encapsulada em lipossomas preparados com fosfatidilcolina hidrogenada de grau alimentício (pro-lipo H) e colesterol (20%, m/m), durante a fabricação de queijo *Cheddar*. Apesar de reduzir a população de *Lactococcus* viáveis, a nisina Z encapsulada não interferiu na fermentação do queijo *Cheddar* e permaneceu estável durante todo o processo de produção desse queijo. Os autores também avaliaram a liberação de nisina Z encapsulada em diferentes meios durante o armazenamento refrigerado (4ºC) por 27 dias. Após 18 dias de armazenamento, a quantidade de nisina Z liberada no soro, tampão fosfato salino (PBS), leites contendo 3,25, 2,0 e 1,0% de gordura, e leite desnatado representaram, respectivamente, 78,2, 71,5, 63,7, 53,9, 45,5 e 39,2% da quantidade inicial de nisina Z encapsulada. No dia 27, a quantidade de nisina Z foi aproximadamente 40-50% menor do que no décimo oitavo dia de armazenamento. Portanto, a estabilidade das vesículas lipossômicas foi influenciada pela composição do ambiente externo, tais como cátions bivalentes no soro e o teor de gordura no leite (Laridi et al., 2003).

Benech et al. (2002a,b, 2003) avaliaram os efeitos de nisina Z encapsulada em lipossomas produzidos com pro-lipo H na atividade

antimicrobiana e também nos atributos físico-químicos e sensoriais durante a maturação de queijo *Cheddar*. Após seis meses, os queijos adicionados de nisina encapsulada apresentaram uma redução de 3,0 log UFC/g na população de *L. innocua* e 90% de recuperação da atividade antimicrobiana inicial. Já nos queijos adicionados de uma cepa produtora de nisina, a redução na população de *L. innocua* foi de 1,5 log UFC/g e a atividade nisinogênica inicial diminuiu progressivamente, correspondendo a apenas 12% em relação à atividade antimicrobiana no dia zero. Isso foi correlacionado com uma maior atividade e estabilidade da nisina encapsulada em comparação com a linhagem produtora de nisina (Benech et al., 2002a,b). Durante a maturação, nisina encapsulada em lipossomas e/ou nisina imobilizada na sua estrutura, estavam localizadas principalmente na interface gordura/caseína e/ou soro. Ao contrário, a nisina produzida pela linhagem nisinogênica estava uniformemente distribuída na matriz do queijo fresco, mas concentrando-se na área de gordura com o passar do tempo. Assim, a encapsulação ou imobilização nas membranas dos lipossomas pode representar uma forma alternativa para a liberação da nisina, melhorando a sua estabilidade, disponibilidade e distribuição na matriz do queijo, e também protegendo a cultura *starter* da ação da nisina (Benech et al., 2002a; Benech et al., 2002b). Além disso, Benech et al. (2003) reportaram que a nisina encapsulada não afetou a proteólise nem as características sensoriais do queijo, enquanto que a adição da cepa nisinogênica junto com a cultura *starter* aumentou a proteólise e lipólise, embora as características reológicas não tenham sido significativamente afetadas.

Lipossomas de FC e de FC:FG (6:4) contendo 5,0 ou 10,0 µg/mL de nisina, com ou sem ácido etilenodiamino tetra-acético (EDTA), mostraram efeito inibitório quase equivalente contra *Listeria monocytogenes*, quando comparado à nisina livre junto ao EDTA. Embora o crescimento de *L. monocytogenes* não tenha sido inibido durante o experimento (48 h) utilizando nisina livre ou encapsulada, houve uma inibição considerável do patógeno em comparação ao controle positivo. Ao contrário, *Escherichia coli* O157:H7 apresentou inibição máxima quando nisina e EDTA foram co-encapsulados (Taylor et al., 2008).

Assim, a encapsulação de peptídeos antimicrobianos em lipossomas pode representar uma alternativa para superar alguns problemas relacionados com a aplicação direta de peptídeos antimicrobianos em alimentos.

3 RESULTADOS E DISCUSSAO

Os resultados obtidos neste trabalho estão apresentados na forma de artigos científicos publicados, aceitos, submetidos ou a serem submetidos para publicação em periódicos especializados. Cada subtítulo desse capítulo corresponde a um destes artigos.

3.1 Food applications of liposome- encapsulated antimicrobial peptides

- Artigo publicado no periódico Trends in Food Science & Technology

3.2 Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin

- Artigo publicado no periódico Food Research International

3.3 Effect of nanovesicle-encapsulated nisin on growth of *Listeria monocytogenes* in milk

- Artigo publicado no periódico Food Microbiology

3.4 Inhibition of *Listeria monocytogenes* growth in Minas frescal cheese by free and nanovesicle-encapsulated nisin

- Artigo submetido para publicação no periódico Brazilian Journal of Microbiology

3.5 Nanovesicle encapsulation of antimicrobial peptide P34: physicochemical characterization and mode of action on *Listeria monocytogenes*

- Artigo publicado no periódico Journal of Nanoparticle Research

3.6 Antilisterial activity and stability of nanovesicle-encapsulated antimicrobial peptide P34 in milk

- Artigo submetido para publicação no periódico Food Control

3.7 Effect of nisin and bacteriocin-like substance P34 liposome-encapsulated on *Listeria monocytogenes* growth in Minas Frescal cheese

- Artigo a ser submetido para publicação no periódico International Journal of Food Microbiology

3.1 ARTIGO 1

3.1.1 Food applications of liposome-encapsulated antimicrobial peptides

ELSEVIER

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Review

Food applications of liposome-encapsulated antimicrobial peptides

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Antimicrobial peptides have been extensively examined as potential biopreservatives in hurdle technology. However, stability issues like proteolytic degradation and the potential interaction of the antimicrobial peptide with food components might result in decreased antimicrobial activity. The entrapment of bacteriocins into liposomes might represent an alternative to overcome the problems related to the direct application of these antimicrobial peptides in food. Encapsulation of bacteriocins into liposomes is mainly reported to be achieved by the thin-film hydration method, and phosphatidylcholine is the most common phospholipid employed in liposome manufacture. This article reviews the main characteristics of liposomes, such as size, zeta-potential, and encapsulation efficiency. A detailed up-to-date summary of potential application of bacteriocin-loaded liposomes, with particular emphasis on nisin encapsulation, is presented.

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Introduction

Food safety is a major issue for both food industry and consumers. In this perspective, the food industry faces a technological challenge due to the increased concern on utilization of chemical preservatives and the increased demand for more natural and minimally processed foods. Among the proposed technologies, biopreservation is considered as a promising perspective. Particularly, bacteriocins have been extensively examined as potential biopreservatives in hurdle technology, which utilizes the synergy of combined treatments to more effectively preserve foods (Abee, Krockel, & Hill, 1995; Cleveland, Montville, Nes, & Chikindas, 2001; Deegan, Cotter, Hill, & Ross, 2006; Gálvez, Abriouel, López, & Omar, 2007).

Bacteriocins are ribosomally-synthesized bacterial peptides that inhibit, in most cases, strains closely related to the producer strain (Papagianni, 2003). These antimicrobial peptides have been isolated from different bacteria and, especially, lactic acid bacteria (LAB) have a widespread ability to produce bacterocins (Gálvez *et al*., 2007; Jack, Tagg, & Ray, 1995; Riley & Wertz, 2002). Since the majority of bacteriocin-producing LAB are natural food isolates, their antimicrobial peptides could be exploited by the food industry as a tool to control undesirable bacteria in a food-grade and natural manner, which is likely to be more acceptable to consumers (Cleveland *et al*., 2001; Deegan *et al*., 2006). Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* was the first bacteriocin approved for food use and is the only bacteriocin widely employed as a food preservative (Arauz, Jozala, Mazzola, & Vessoni Penna, 2009; Delves-Broughton, Blackburn, Evans, & Hugenholtz, 1996; Sobrino-López & Martín-Belloso, 2008). However, stability issues like proteolytic degradation and the potential interaction of the bacteriocins with food components, such as fat and proteins, might result in decreased antimicrobial activity (Aasen *et al*., 2003; Branen & Davidson, 2004; Chollet, Sebti, Martial-Gros, & Degraeve, 2008; Glass & Johnson, 2004).

Liposomes are basically vesicles composed by one or more phospholipid bilayers encapsulating a volume of aqueous media. The mechanism of liposome formation is essentially based on the unfavorable interactions occurring between phospholipids and water molecules, where the polar headgroups of phospholipids are exposed to the aqueous phases (inner and outer), and the hydrophobic hydrocarbon tails are forced to face each other in a bilayer

(Jesorka & Orwar, 2008). Energy input to the aggregated phospholipids results in organized, closed bilayer vesicles (liposomes) and, during this process, the entrapment of solutes present in the aqueous media occurs (Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008). Due to the presence of both lipid and aqueous phases in the structure of lipid vesicles, they can be utilized in the entrapment, delivery, and release of water-soluble, lipid-soluble, and amphiphilic materials (Khosravi-Darani, Pardakhty, Honarpisheh, Rao, & Mozafari, 2007; Mozafari, Johnson *et al*., 2008). Liposomes have been examined as carriers for therapeutic agents, as analytical tools, as well as models of biological membranes (Date, Joshi, & Patravale, 2007; Edwards & Baeumner, 2006; Frézard, 1999; Gómez-Hens & Fernández-Romero, 2005; Jesorka & Orwar, 2008; Lian & Ho, 2001; Ropert, 1999; Sharma & Sharma, 1997).

In the food industry, liposomes have been investigated to deliver proteins, enzymes, vitamins, antioxidants and flavors (Mozafari *et al*., 2006; Mozafari, Khosravi-Darani *et al*., 2008; Taylor, Davidson, Bruce, & Weiss, 2005a, 2005b). The great advantage of liposomes over other encapsulation technologies (spray-drying, extrusion, fluidized beds) is the stability that liposomes impart to water-soluble material in high water activity applications (Desai & Park, 2005). Liposome entrapment has been shown to stabilize the encapsulated materials against a range of environmental and chemical changes, including enzymatic and chemical modification, as well as buffering against extreme pH and temperature (Mozafari, Johnson *et al*., 2008; Mozafari, Khosravi-Darani *et al*., 2008). As liposomes could be prepared from naturally occurring components, regulatory hurdles that may prevent their application in food systems are potentially reduced or eliminated, and new formulations could be quickly implemented (Gibbs, Kermasha, Alli, & Mulligan, 1990; Mozafari, Johnson *et al*., 2008; Taylor *et al*., 2005a). Thus, the entrapment of bacteriocins into liposomes might represent an alternative to overcome some problems related to the direct application of bacteriocins in food, such as proteolytic degradation or interaction with food components. In this context, the present article discusses the characteristics and the potential applications of bacteriocin-loaded liposomes, with particular emphasis on nisin encapsulation.

Characteristics of bacteriocin-loaded liposomes

While the thorough discussion of methods employed in liposome manufacture is out of the scope of this article, various excellent reviews on this topic are available (see, for instance, Jesorka & Orwar, 2008; Mozafari, 2005; Taylor *et al*., 2005a). Depending on the preparation method, several types of liposomes can be distinguished. Usually, liposomes are classified according to their structure as multilamellar vesicles (MLVs, >400 nm), large unilamellar vesicles (LUVs, 80 nm-1 μm), small unilamellar vesicles (SUVs, 20–80 nm), giant unilamellar vesicles (GUVs, >1 μm) and multivesicular vesicles (MVVs, >1 μm) (Gómez-Hens & Fernández-Romero, 2005). Most techniques yield LUVs and MLVs and, without further steps, such as membrane extrusion, heating or sonication, almost all the above methods produce heterogeneous mixtures of lipid vesicles (Mozafari, 2005). Hence, the emphasis in making liposomes is not toward assembling bilayer membranes randomly, but the formation of vesicles with the right size, acceptable polydispersity, elasticity, structure, and encapsulation efficiency (Mozafari, Johnson *et al*., 2008).

Encapsulation of bacteriocins into liposomes is mainly reported to be achieved by the thin-film hydration method (Fig. 1). By this technique, a pre-formed lipid film is hydrated with an aqueous buffer containing the bacteriocin, at a temperature above the phase transition temperature of lipids. The resulting heterogeneous population of MLVs can be further processed (membrane extrusion, sonication), resulting in SUVs of uniform size (Jesorka & Orwar, 2008; Sharma & Sharma, 1997). Table 1 summarizes some properties of bacteriocin-loaded liposomes, such as encapsulation efficiency and size (diameter).

The concentration of compounds that can be entrapped is a function of lipid composition and may be attributed to electrostatic and hydrophobic interactions between antimicrobials and phospholipids (Were, Bruce, Davidson, & Weiss, 2003). Specifically, nisin is a cationic amphiphilic antimicrobial peptide of 3.5 kDa composed of 34 amino

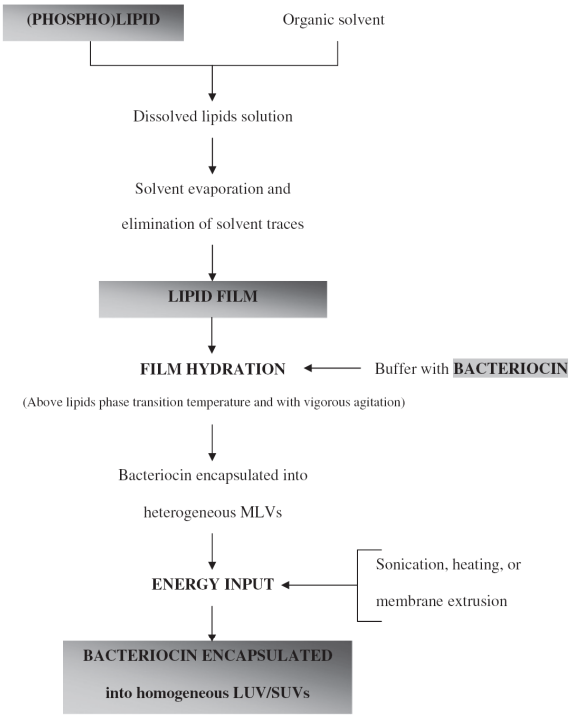

Fig. 1. Thin-film hydration method for encapsulation of antimicrobial peptides into liposomes.

Table 1. Summary of preparation methods and some characteristics of bacteriocin-loaded liposomes.[a]

Antimicrobial peptide	Liposome constitution	Preparation method	Characteristics of bacteriocin-loaded liposomes		Reference
			Size	*Encapsulation efficiency*	
Nisin (commercial)	PC, PC:PG (8:2), PC-PG (6:4)	Thin-film hydration + freeze-thaw + membrane extrusion	At 25 °C, 5 μg/mL nisin: 122.7–310.1 nm; 10 μg/mL nisin: similar to 5 μg/mL	NR[b]	Taylor *et al.*, 2007
Nisin (commercial) co-encapsulated with calcein	PC, PC:CH (7:3), PC:PG:CH (5:2:3)	Thin-film hydration + sonication	144, 223, and 167 nm for PC, PC:CH (7:3), and PC:PG:CH (5:2:3); Nisin only: 131.3–181.9 nm	63, 54, and 59% for PC, PC:CH, and PC:PG:CH, respectively	Were *et al.*, 2003
Nisin (commercial)	PC, PC:CH (7:3), PC:PG:CH (5:2:3)	Thin-film hydration + sonication	NR	> 54% (not specified)	Were *et al.*, 2004
Nisin Z	Proliposome H (Pro-lipo® H)	Lipids heated above phase transition temperature	Mainly SUVs (80–120 nm)	47%	Benech, Kheadr, Lacroix *et al.*, 2002; Benech *et al.*, 2003; Benech, Kheadr, Laridi *et al.*, 2002
Bacteriocin-like substance P40	PC	Reverse phase evaporation + sonication	570 nm (558–582 nm)	NR	Teixeira *et al.*, 2008
Nisin Z	Proliposomes (Pro-lipo® C, Duo, S and H) with or without CH (0–20%)	Heating above phase transition temperature	740 nm (140–2400 nm) for Pro-lipo® H + 20% CH	9.5–47%	Laridi *et al.*, 2003
Nisin Z	DPPC; phospholipon 90H; phospholipon 100H; with or without CH, DCP, SA	Mozafari (based on heating method) + membrane extrusion	190–284 nm	11.7–54.2%	Colas *et al.*, 2007
Nisin (commercial)	PC; PG; PC:PG (8:2, 6:4)	Thin-film hydration + agitation (vortex) + freeze-thaw + membrane extrusion	SUV	NR	Taylor *et al.*, 2008
Pediocin AcH	PC	Thin-film hydration + agitation (hand-shake)	MLV	18% (16–20%)	Degnan & Luchansky, 1992; Degnan *et al.*, 1993
Nisin (commercial)	DPPC; DSCP; DPPC:DSCP (1:1)	Thin-film hydration + agitation (vortex) + freeze-thaw + sonication	MLV	NR	Taylor *et al.*, 2005b

[a] Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; DPPC, dipalmitoyl-PC; DSPC, distearoyl-PC; CH, cholesterol; SA, stearylamine; DCP, dicetylphosphate; MLV: multilamellar vesicles; SUV: small unilamellar vesicles.
[b] Not reported.

acids (Breukink & de Kruijff, 1999). Thus, nisin might be encapsulated in the inner aqueous phase of liposome and also be immobilized into liposome membranes (Laridi *et al*., 2003). Nisin entrapment efficiency is usually reported to be higher in liposomes manufactured from neutral (zwitterionic) phospholipids, such as phosphatidylcholine (PC), in comparison to liposomes containing anionic lipids, such as phosphatidylglycerol (PG) (Taylor, Bruce, Weiss, & Davidson, 2008; Were, Bruce, Davidson, & Weiss, 2003, 2004). Accordingly, highest nisin encapsulation efficiency (34.6%) was achieved by using proliposome H, which contained the lower content of negatively charged phosphatidylinositol (1% of total phospholipid content) and the higher content of the neutral zwitterionic PC (85% of total phospholipid content) when compared to other proliposomes (9.5–26%) (Laridi *et al*., 2003). Since PC liposomes show properties of uncharged monolayers at near neutral pH values, electrostatic interactions with peptides might be excluded. Thus, the insertion of peptides into PC vesicles could possibly be due to hydrophobic interactions and association with PC bilayer structures (Teixeira, Santos, Silveira, & Brandelli, 2008; Were *et al*., 2003). Cationic vesicles, containing stearylamine, showed lower encapsulation efficiency (11.7–13.6%) when compared to other liposomes, which could result from electrostatic repulsion between the positively charged nisin and the cationic vesicles. In contrast, anionic vesicles containing dicetylphosphate showed the highest encapsulation efficiency (50.1–54.2%) for nisin (Colas *et al*., 2007). If the same assumption is valid for anionic vesicles, electrostatic interactions could be responsible for the observed increase in encapsulation efficiency.

Surface charge of liposomes depends primarily on phospholipid composition (Taylor, Gaysinsky, Davidson, Bruce, & Weiss, 2007). In model membranes, the association of nisin with phospholipid bilayers is strongly dependent on membrane surface charge, being reported to bind more efficiently to membranes containing anionic than neutral phospholipids (Bonev, Chan, Bycroft, Roberts, & Watts, 2000; El Jastimi & Lafleur, 1997). Analogous results were reported for different peptides (Choi *et al*., 2004; Ringstad, Nordahl, Schmidtchen, & Malmsten, 2007). Specifically, the C-terminus contains the major part of the positive charge in the nisin molecule, mediating the electrostatic interactions of nisin with membranes (Breukink & de Kruijff, 1999). Such electrostatic interactions are considered as the initial step for the subsequent events leading to membrane pore formation (Breukink & de Kruijff, 1999; Deegan *et al*., 2006). Membrane leakiness parallels the extent of peptide binding, and nisin Z proved to have high penetration ability for membranes containing anionic lipids and low penetration ability for neutral lipids (Breukink, Ganz, de Kruijff, & Seelig, 2000). Hence, the binding of nisin with the negatively charged headgroups of phospholipids, such as PG, might lead to the formation of unstable pores on liposomes (Were *et al*., 2003).

In this context, PC liposomes containing nisin (5 and 10 μg/mL) demonstrated the slowest apparent release of the antimicrobial, whereas PG-containing liposomes appeared to release their contents very efficiently, suggesting that in PC:PG (6:4) liposomes nisin effectively effluxes from such vesicles *via* pore formation (Taylor *et al*., 2008). For those authors, while PC liposomes did not inhibited significantly the target pathogens (32 °C for 48 h) in comparison to free nisin, PC:PG (8:2 and 6:4) liposomes produced a significant inhibition. Accordingly, Laridi *et al*. (2003) reported that liposomes prepared from proliposomes with lower contents of negatively charged phospholipids were less susceptible to the nisin-membrane destabilizing action in comparison with other liposomes tested. Also, a concentration-dependent effect of nisin-induced leakage might occur in PC and PG liposomes, since the percentage calcein released upon addition of 375 μM of nisin followed the order: PC > PC:PG:cholesterol (5:2:3) > PC:cholesterol (7:3), whereas with 150 μM of nisin the calcein release followed the order: PC:PG:cholesterol (5:2:3) > PC:cholesterol (7:3) > PC (Were *et al*., 2003). The entrapment of nisin into dipalmitoyl-PC (DPPC), distearoyl-PC (DSPC) and DPPC:DSPC (1:1) liposomes increased the gel-to-liquid crystalline phase transition temperature (T_M) in all liposomes tested, suggesting that nisin insertion, at low concentrations, might stabilize PC vesicles *via* a possible lowering of curvature stresses. Therefore, the interaction of nisin with lipids not commonly found in prokaryotic membranes (such as PC) is likely to produce a different consequence than permeabilization, for example a stabilizing effect (Taylor *et al*., 2005b).

The zeta-potential is usually utilized as an indicator for accessible charges in liposomal surfaces. For instance, empty PC liposomes presented a zeta-potential of −8.3 mV at 25 °C, whereas PC:PG (8:2) and PC:PG (6:4) showed zeta-potentials of −52.28 and −72.60 mV, respectively (Taylor *et al*., 2007). Similar results were reported elsewhere (Ringstad *et al*., 2007; Willumeit *et al*., 2005), since PC has a slightly anionic character (Zhao *et al*., 2006). Liposomes composed of charged polar lipids with higher electrical charges can be expected to be more stable than liposomes composed of neutral polar lipids, since high (negative or positive) zeta-potentials increase the repulsive interactions, reducing the frequency of liposome collisions. Taylor *et al*. (2007) reported that the introduction of nisin into PC and PC:PG (6:4) liposomes caused a reduction in the net surface charge of liposomes, when compared with the empty counterparts at 25 °C; but the same effect was not observed in PC:PG (8:2) liposomes. Ringstad *et al*. (2007) reported that the interaction with positively charged peptides decreased the electrostatic potential (zeta-potential) of both zwitterionic and anionic liposomes. The cationic peptide NK-2 caused charge neutralization in dipalmitoyl-PG (DPPG) liposomes. However, the addition of NK-2 had no influence on the zeta-potential of DPPC liposomes, indicating that the peptide does not

interact with this lipid matrix (Willumeit *et al*., 2005). Those authors hypothesized that no charge interaction occurred since, as indicated by a zeta-potential close to zero (−2.3 mV), the negatively charged phosphate group of PC seem to be completely shielded by the large trimethylamine umbrella of the choline group (Willumeit *et al*., 2005).

Liposome charge may also influence the interaction between nisin-loaded liposomes and bacteria. As the bacterial cell has a negative charge, electrostatic repulsion might occur between PG-containing liposomes and the cell surface, preventing direct contact between liposomes and pathogens, and the subsequent release of antimicrobials (Were *et al*., 2004). This assumption gains further importance since the mechanism of interaction between liposomes and bacteria seems to involve membrane fusion (Colas *et al*., 2007; Mugabe, Halwani, Azghani, Lafrenie, & Omri, 2006).

Liposomes entrapping nisin Z (740 nm on average) presented a size reduction of approximately 58% when compared to empty liposomes (1741 nm on average) (Laridi *et al*., 2003), which could be attributed to the effect of nisin on membrane lipid organization and/or lipid hydrophobicity, as nisin insertion is reported to perturb the permeability and the structure of membrane bilayers (El Jastimi, Edwards, & Lafleur, 1999; El Jastimi & Lafleur, 1999). Taylor *et al*. (2007) showed that encapsulation of 5 μg/mL nisin into PC liposomes resulted in vesicle sizes of about 310 nm, which corresponded to an increase of approximately 300% when compared to empty liposomes (103 nm on average) at 25 °C, and similar results were observed for encapsulation of 10 μg/mL nisin. However, nisin encapsulation into PC:PG (8:2; 6:4) liposomes only tended to reduce vesicle sizes. The liposome sizes were not directly responsive to the level of incorporated nisin (Taylor *et al*., 2007). PC:PG liposomes, through the charge neutralization of the anionic lipids by nisin and a realignment of the phospholipids, may have attained a more optimal packing, which could explain the reductions in diameters (Bonev *et al*., 2000; El Jastimi *et al*., 1999; Taylor *et al*., 2007). In contrast, nisin encapsulation was reported to increase the diameter of liposomes (Were *et al*., 2003). Nisin incorporation was also showed to induce the deformation of the liposome (proliposome H plus 20% cholesterol), or increase membrane thickness (Laridi *et al*., 2003). Silva, Little, Ferreira, and Cavaco-Paulo (2008) demonstrated that the inclusion of a synthetic peptide containing positively charged amino acid lysine distributed along the peptide sequence resulted in DPPC liposomes with an ‘‘amorphous’’ aspect, while the addition of a peptide containing the positive charge at C-terminal resulted in liposomes with a defined rod shape. Therefore, the incorporation of different peptides into liposomes might have variable effects on diameter (size), zeta-potential of membrane surface, and shape of liposomes (Silva *et al*., 2008).

Addition of cholesterol to liposome compositions typically results in decreased encapsulation capacity (Laridi *et al*., 2003; Were *et al*., 2003). However, liposomes manufactured with hydrogenated PC:cholesterol (8:2) presented higher nisin encapsulation efficiency when compared to liposomes of hydrogenated PC only (Colas *et al*., 2007). Were *et al*. (2004) observed that although concentrations of nisin encapsulated into PC liposomes were higher than that in PC:cholesterol (7:3) liposomes, antimicrobial activities against *Listeria monocytogenes* were similar. Nevertheless, the leakage of nisin from liposomes is generally reported to be inhibited by cholesterol. A comparison of nisin-containing PC and PC:cholesterol (7:3) liposomes showed that the addition of cholesterol reduced leakage (Were *et al*., 2003). Thus, cholesterol could be beneficial as a stabilizing component in liposome compositions (Were *et al*., 2004), and the frequently observed effects on encapsulation efficiency and nisin leakage might originate from the same mechanism. As cholesterol promotes increased ordination of lipid chains, such tighter lipid packing may reduce the affinity and the insertion of nisin into the lipid membrane, decreasing the ability of nisin to perturb the permeability and the structure of liposomes (El Jastimi *et al*., 1999; El Jastimi & Lafleur, 1999). Similar results were reported with different antimicrobial peptides, such as temporin L (Zhao & Kinnunen, 2002), the synthesized antimicrobial decapeptide KSL (Choi *et al*., 2004), plantaricin A (Zhao *et al*., 2006) and the amphibian peptide DD K (Verly *et al*., 2008).

Other factors, such as the pH of nisin solutions might present significant effects on the amount of liposome-entrapped nisin. For instance, a pH reduction (pH range: 3.6–6.6) showed a positive effect in the amount of nisin entrapped into liposomes manufactured from some proliposome preparations (Laridi *et al*., 2003). Those authors suggested that a lower pH improved the solubility of nisin and/or increased the ionization of certain groups in the peptide and certain lipid constituents, which favored interactions between the peptide and lipid membrane and, therefore, increased the amount of entrapped and/or liposome-immobilized nisin Z. Indeed, nisin is around 228 times more soluble at pH 2.0 than at pH 8.5 (Liu & Hansen, 1990), and thus nisin’s antimicrobial activity is high at acid pH, but lost at pH values above 7 (Deegan *et al*., 2006). Liposomes can be expected to retain the encapsulated material if the phospholipids used to formulate the liposomes maintain their charge regardless of the pH of systems in which they are applied. Hence, the knowledge on the pK_a values of the phospholipids utilized for liposome manufacture is essential (Taylor *et al*., 2007).

Nisin concentration is also an important factor affecting encapsulation efficiency, as increased encapsulation capacity was achieved by increasing nisin concentration. Such increased entrapment may indicate that part of the encapsulated nisin is inserted or immobilized on the lipid membrane, suggesting that the lipid membrane area available

per mass unit of nisin would not be a limiting factor for nisin adsorption onto liposome membrane (Laridi *et al*., 2003). Bower, McGuire, and Daeschel (1995) showed a general increase in the amounts of nisin adsorbed to silica surfaces with increasing solution concentrations of nisin, which was suggested to result from the formation of nisin multilayers.

Application of bacteriocin-loaded liposomes in foods and model systems

Although various studies have been published showing the production of stable antimicrobial-containing liposome vesicles and its properties, there are relatively few published data regarding the application of such bacteriocin-loaded liposomes in foods, or even in model food systems.

The encapsulation of pediocin AcH within PC liposomes resulted in a 27.5% and 28.9% average increase in the recovery of pediocin activity in slurries of heated beef muscle and tallow, respectively, compared to otherwise similar beef slurries containing free pediocin (Degnan & Luchansky, 1992). In a similar investigation, Degnan, Buyong, and Luchansky (1993) evaluated liposomes as pediocin AcH vectors in slurries of nonfat dry milk, butterfat, beef muscle tissue, or beef tallow (prepared in deionized water, at final concentration of 10%, w/v), and a 29–62% increase in pediocin recovery (average over all pediocin concentrations tested; 0–110000 AU) was reported over free pediocin AcH. Interestingly, a greater improvement of pediocin activity was observed in dairy-based than meat-based slurries (Degnan *et al*., 1993). Such results demonstrate the efficacy of liposomes in enhancing bacteriocin activity, minimizing the negative effects observed for non-encapsulated pediocin, such as association of the bacteriocin with protein and/or fat present in slurries and proteolysis (Degnan *et al*., 1993; Degnan & Luchansky, 1992).

Since nisin is approved for food use, a greater amount of investigation is directed towards encapsulation and application of this antimicrobial peptide, particularly nisin Z (Benech, Kheadr, Lacroix, & Fliss, 2002, 2003; Benech, Kheadr, Laridi, Lacroix, & Fliss, 2002; Laridi *et al*., 2003). Two natural variants of the lantibiotic nisin are known and have a similar structure but differ in a single amino acid residue at position 27; histidine in nisin A and asparagine in nisin Z. According to De Vos, Mulders, Siezen, Hugenholtz, and Kuipers (1993), nisin Z has better diffusion properties than nisin A in agar; which is an important characteristic for food applications. In studies of liposome-bearing nisin, *L. monocytogenes*, an important foodborne pathogen, is usually selected as indicator strain.

Laridi *et al*. (2003) investigated the stability and the effects of nisin Z entrapped into liposomes, prepared with proliposome H and polyoxyethanyl-cholesterol sebacate (20%, w/w), during Cheddar cheese manufacture. Encapsulated nisin Z, despite lowering the viable counts of lactococci, did not severely disturb Cheddar cheese fermentation and was stable throughout the Cheddar cheese-making temperature cycle. Additionally, the amount of nisin Z released in non-inoculated milk was higher than in milk inoculated with starter lactococci probably due to partial degradation of released nisin Z by starter bacteria and/or fixation of released nisin to the bacterial cell wall (Laridi *et al*., 2003). Those authors also evaluated the release of encapsulated nisin Z in different media during refrigerated storage at 4 °C for 27 days. At 18 days storage, the amount of released nisin Z in whey, phosphate-buffered saline (PBS), milks containing 3.25, 2.0 and 1.0% fat, and skim milk represented, respectively, 78.2, 71.5, 63.7, 53.9, 45.5 and 39.2% of the initial quantity of encapsulated nisin Z. At day 27, the amount of nisin Z estimated was 40–50% lower than that at 18 days of storage. Therefore, the stability of liposome vesicles was influenced by the composition of the external environment, such as bivalent cations in whey and the fat content in milk (Laridi *et al*., 2003).

Benech, Kheadr, Lacroix *et al*. (2002) and Benech *et al*. (2003) added nisin Z-loaded Pro lipo H liposomes to cheese milk, evaluating the antimicrobial activity and physicochemical and sensory attributes during 6 months Cheddar cheese ripening. After six months, less than 10 CFU of *L. monocytogenes* per g of cheese were found and 90% of the initial nisin activity was recovered in nisin-loaded liposomes, whereas 10^3–10^4 CFU/g and only 12% recovery were reported in cheeses manufactured with a nisin-producing mixed starter culture. This was correlated with a high activity and stability of encapsulated nisin compared to nisin produced by the nisinogenic strain (Benech, Kheadr, Lacroix *et al*., 2002). During ripening, liposome-encapsulated nisin and/or nisin immobilized in linear liposome structures were observed, located mainly at the fat/casein interface and/or in whey pockets, whereas nisin produced by nisinogenic strain was uniformly distributed in the fresh cheese matrix, but concentrated in the fat area as the cheeses aged. Thus, entrapment/immobilization into liposome membranes might represent an alternative way for nisin delivery, improving its stability, availability, and distribution in the cheese matrix, and also protecting the cheese starter from detrimental action of nisin (Benech, Kheadr, Lacroix *et al*., 2002; Benech, Kheadr, Laridi *et al*., 2002). Further supporting the promising application of bacteriocin-loaded liposomes in cheeses, Benech *et al*. (2003) reported that liposome-encapsulated nisin did not affected cheese proteolysis, rheology and sensory characteristics, whereas the incorporation of a nisinogenic strain into cheese starter culture increased the proteolysis and lipolysis, although rheology was not significantly affected.

Were *et al*. (2004), evaluating the antimicrobial activity of free and encapsulated nisin against *Listeria monocytogens*, observed a ~2 log greater inhibition for nisin encapsulated in PC and PC: cholesterol liposomes.

PC and PC:PG (6:4) liposomes containing 5.0 or 10.0 μg/mL nisin, with or without ethylene diaminotetraacetic acid (EDTA), showed an almost equivalent inhibitory

effect against *L. monocytogenes* when compared to the non-encapsulated nisin plus EDTA, and a slightly greater effect than inhibition by PC:PG (8:2) liposomes. Likewise, after 12 h incubation at 32 °C, *Escherichia coli* O157:H7 was less inhibited by PC:PG (8:2) than PC and PC:PG (6:4) liposomes. Nevertheless, the control non-encapsulated nisin and EDTA presented the maximum inhibition, whereas at 24 and 48 h, inhibition by encapsulated and free nisin and EDTA was equivalent (Taylor *et al*., 2008). Although the growth of *L. monocytogenes* was not inhibited over the entire course of experimentation (48 h) by the free or liposome-entrapped nisin, a considerable inhibition of pathogens versus the positive controls was observed. Also, the inhibition of *E. coli* O157:H7 by free and liposomal antimicrobial varied greatly, but maximum inhibition was observed in liposomes co-encapsulating nisin and EDTA (Taylor *et al*., 2008).

Normally, nisin is not active against Gram-negative cells due to lipopolysaccharidic (LPS) composition of its outer membrane which acts as a barrier to the action of the nisin on the cytoplasmatic wall (Arauz *et al*., 2009). On the other hand, chelating agents, such as EDTA, confine the divalent magnesium and calcium ions of the LPS causing destabilization of the layer, broadening the spectrum of activity of nisin (Abee *et al*., 1995; Arauz *et al*., 2009). Particularly, low levels of EDTA synergistically enhanced the activity of nisin against *E. coli* O157:H7 and *E. coli* O104:H21; and a similar effect was observed for *L. monocytogenes* (Branen & Davidson, 2004). Thus, the co-encapsulation of antimicrobial and chelator might increase the inhibitory potential of liposomes against Gram-positive and Gram-negative bacteria (Taylor *et al*., 2008), possibly through the above mechanism.

Perspectives and conclusion

Nisin-loaded liposomes may be suitable for controlling spoilage and pathogenic bacteria in food, withstanding the exposure to different environmental and chemical stresses typically encountered in foods and food processing operations, such as low- or high-pH and moderate heat treatments (Taylor *et al*., 2007), thus improving nisin stability, efficacy, and also its distribution in food matrices (Laridi *et al*., 2003). As antimicrobials could be present both in the aqueous and lipids phases of liposomes, a complementary effect could be expected, providing both short-term (by release of encapsulated nisin) and long-term (desorption of membrane-immobilized nisin) antibacterial actions, adding to the effective safety and shelf-life of some food products (Benech, Kheadr, Lacroix *et al*., 2002; Taylor *et al*., 2008).

The main limitation for the entrapment of nisin and other antibacterial peptides in liposomes is the ability of these peptides to interact with and disrupt liposomal membranes. However, both nisin-loaded vesicles and membrane-immobilized nisin may provide a powerful tool for controlling spoilage and pathogenic organisms in food (Laridi *et al*., 2003). Although cholesterol is reported to stabilize the liposome vesicles, there is concern for cholesterol consumption through liposomes (Hsieh, Chen, Wang, Chang, & Chang, 2002), which warrant further investigations on the replacement of cholesterol by other lipids.

Since the most common phospholipid in lecithin, PC, is inexpensively isolated from natural sources, such as soy or egg yolk, liposomes could be obtained by an acceptable cost (Desai & Park, 2005; Mertins, Sebben, Schneider, Pohlmann, & Silveira, 2008). Also, as these components are safe, encapsulated nisin might obtain regulatory approval to be used as food-grade products (Mozafari, Johnson *et al*., 2008). Perhaps, a main issue to be addressed for industrial production is the scaling-up of the liposome encapsulation process at acceptable cost-in-use levels (Desai & Park, 2005).

In our laboratory, commercial nisin A was encapsulated in liposomes of partially purified soybean PC as a cheap and easily available product. Nisin-loaded liposomes, manufactured by the thin-film hydration method followed by bath-type ultrasound, presented high encapsulation efficiency, displaying enhanced antimicrobial activity. These liposomes have been applied in milk as food model, inhibiting *L. monocytogenes* growth (Malheiros, Daroit, Silveira, & Brandelli, 2010). Furthermore, liposome encapsulation of other antimicrobial peptides, such as cerein 8A, which presents important inhibition of *L. monocytogenes* in dairy products when applied in non-encapsulated form (Bizani, Morrissy, Dominguez, & Brandelli, 2008), is under investigation.

Investigations on liposome-encapsulated antimicrobial peptides generally show advantages in comparison to free bacteriocins. These promising results should encourage intensive efforts focusing on liposome research, aiming the development of a potentially valuable tool to be implemented in hurdle technology, thus improving food shelf-life and safety.

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3.2 ARTIGO 2

3.2.1 Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin

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Development and characterization of phosphatidylcholine nanovesicles containing the antimicrobial peptide nisin

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ABSTRACT

Two methodologies were compared to encapsulate nisin in liposomes of partially purified soybean phosphatidylcholine: reversed-phase and hydration film. In the hydration film method, both probe-type and bath-type ultrasound were evaluated. The size of liposomes was evaluated by light scattering analysis and residual antimicrobial activities by agar diffusion assay using *Listeria monocytogenes* ATCC 7644 as indicator strain. The size of liposomes prepared by reversed-phase, hydration film using probe-type and bath-type ultrasound were 190, 181 and 148 nm with residual antimicrobial activities after encapsulation of 25%, 50% and 100%, respectively. The methodology of film hydration using bath-type ultrasound was chosen for assessment of its physicochemical characteristics. Nisin had entrapment efficiency of 94.12%. Measured Zeta potentials for unfiltered and filtered (0.22 μm) liposomes were −55.8 and −54.5 mV, respectively. The antimicrobial activity of free nisin, encapsulated nisin and filtered was evaluated for a period of 24 days. It was observed that the free nisin remained 100% of residual activity while the liposomes containing nisin were losing their antimicrobial activity over time reaching 25% residual activity after 10 days. The size (132–149 nm) and pH (4.5) remained constant over time. It was observed by microscopy that the liposomes maintained their spherical morphology. The stability observed by size and pH was not the same regarding antimicrobial activity and Zeta potential, indicating that the liposomes should be applied shortly after its preparation.

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1. Introduction

Bacteriocins are antibacterial proteins produced by bacteria that kill or inhibit the growth of other bacteria (Cleveland, Montville, Nes, & Chikindas, 2001). Because of their potential use as natural preservatives, bacteriocins produced by lactic acid bacteria have been subject of many studies (Arauz, Jozala, Mazzola, & Penna, 2009). Nisin is a 3.5 kDa cationic polypeptide produced from *Lactococcus lactis* strains approved for specific uses in foods in more than 40 countries (O'Sullivan, Ross, & Hill, 2002). This bacteriocin is effective at inhibiting a variety of gram-positive bacteria, including food pathogens such as *Listeria monocytogenes*. Nisin kills susceptible bacteria through a multi-step process that destabilize the phospholipid bilayer of the cell and creates transient pores (Bonev, Chan, Bycroft, Roberts, & Watts, 2000).

For application of natural compounds such as nisin in foods, two aspects are often overlooked: (1) the changes in the organoleptical and textural properties of the food when the substance is added and (2) the interaction of these compounds with food ingredients, including the influence of this interaction on their efficacy (Devlieghere, Vermeiren, & Debevere, 2004). Encapsulation of antimicrobial substances in liposomes may offer a potential solution to protect antimicrobials, enhance their efficacy and stability in food application (Laridi et al., 2003; Taylor, Bruce, Weiss, & Davidson, 2008; Teixeira, Santos, Silveira, & Brandelli, 2008; Were, Bruce, Davidson, & Weiss, 2003). Encapsulation and controlled delivery of bacteriocins and other bioactive agents are among the applications for the emerging field of nanobiotechnology.

Liposomes are colloidal structures having an internal aqueous pool formed by self-assembly of amphiphilic lipid molecules in solution (Mertins, Sebben, Pohlmann, & Silveira, 2005). A major lipidic component of the membrane bilayer is phosphatidylcholine (PC), which consists of a mixture of natural phospholipids made of a polar end formed by a choline and a phosphate group linked to the hydrophobic portion (two long acyl chains from 16 to 22 carbons) linked by ester bonds with the glycerol. The phospholipids can be obtained from waste oil called lecithin, which are a cheap and rich source of PC (Mertins, Sebben, Schneider, Pohlmann, & Silveira, 2008).

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Liposomes can be used in a wide range of applications due to their physical and chemical characteristics, and their ability to incorporate lipophilic, amphiphilic and/or hydrophilic compounds (Khosravi-Darani, Pardakhty, Honarpisheh, Rao, & Mozafari, 2007; Woodle, 1995). Several types of liposomes can be distinguished. A liposome composed of a number of concentric bilayers is known as a multilamellar vesicle (MLV), while one composed of a single lipidic bilayer is known as a uni-lamellar vesicle (ULV) (Khosravi-Darani et al., 2007). MLV have high encapsulation efficiency, but also great variation in the vesicles size, size distribution and lamellarity. The use of mechanical treatments such as sonication can change the MLV suspension into large uni-lamellar vesicles (LUVs) and small uni-lamellar vesicles (SUVs) (Silva, Little, Ferreira, & Cavaco-Paulo, 2008).

Manufacture of liposome requires input of energy to dispersion of lipid/phospholipid molecules in an aqueous medium (Mozafari, 2005). Sonication, or exposure to ultrasound, is used widely to manufacture artificial liposomes, however yet little is know about the mechanism by which liposomes are affected by ultrasound (Richardson, Pitt, & Woodbury, 2007). Cavitation, a principal effect of low-frequency sonication, has been shown to be responsible for many biophysical effects of ultrasound on cells (Nybor, 2001).

Liposomes are under intensive investigation and development by the pharmaceutical, cosmetic and food industries as micro- and nanocarrier systems for the protection and delivery of bioactive agents (Acosta, 2009; Khosravi-Darani et al., 2007). Nanovesicles to delivery of bioactive food components with substantiated health benefits of the foods will be required to meet the challenges in developing healthy foods which are aimed at reducing the risks of target diseases in a population (Sanguansri & Augustin, 2006). Despite these technological developments, there is a lack of information regarding the basis of design for such nanoparticle systems (Acosta, 2009). The main goal of the present study was to compare methodologies to encapsulate nisin in liposomes of partially purified soybean phosphatidylcholine and assess their physicochemical characteristics.

2. Materials and methods

2.1. Purification of crude soybean lecithin

Samples of crude soybean lecithin and PC of high purity were provided by Solae S.A. (Esteio, Brazil). The crude soybean lecithin (10 g) was dissolved in 50 ml of ethyl acetate (Merck, Darmstadt, Germany). Then, slowly and under agitation, 2 ml of distilled water were added, resulting formation of two phases. The upper phase was separated from the lower phase and discarded. The lower phase, having a gel aspect, was dispersed in 30 ml of acetone, forming clusters that were crushed using a glass stick. Then, the acetone was separated by decanting and a new aliquot of 30 ml acetone was added, repeating the shredding process. The precipitate was vaccum filtered and dried in a dissecator, providing a mass of 6.3 g. The sample was designated as PC-1.

2.2. Phospholipid analysis

All purification procedures were monitored by thin layer chromatography using chloroform, methanol and MilliQ water (6.5:2.5:0.4), and plates of silica gel 60 F_{254} (Merck). The plates were revealed in saturated atmosphere of iodine. The PC of high purity provided by the industry served as standard for comparison (Mertins et al., 2008).

2.3. Liposome production by reversed-phase

Nanovesicles were prepared by the reversed-phase evaporation method as follows (Teixeira et al., 2008): 60 mg of PC-1 were dissolved in 10 ml of chloroform; 200 μl of nisin solution (0.5 mg ml^{-1}) were dropped into the solution to form a water in oil emulsion, which was sonicated (2–4 min) using a bath sonicator (Unique USC 700, Indaiatuba, Brazil) yielding a homogeneous opalescent dispersion of reverse micelles. The organic solvent was evaporated in an evaporator at 35 °C under vacuum, giving a highly viscous organogel. The organogel was reverted to nanovesicles after addition of 5 ml of water (MilliQ) under shaking. Samples were filtered though 0.45 μm pore membrane and placed into dust-free vials for light scattering measurements.

2.4. Liposome production by film hydration

Appropriate amounts of the PC-1 were dissolved in chloroform in a round-bottom flask and the organic solvent was removed by rotary evaporator until a thin film was formed on the walls. Traces of organic solvents were removed by storage for 18 h in desiccator under vacuum. The resulting dried lipid film was dispersed by the addition of phosphate buffer (10 mM) at pH 6.4 containing nisin (0.5 mg ml^{-1}). These mixtures were then vortexed above their phase transition temperature (60 °C) to produce MLVs. Sonication of the preparation (in order to reduce the size and homogenize liposomes) was carried out in a two ultrasound: (1) bath-type ultrasound (40 kHz, Unique USC 700) with temperature of 55–45 °C for 40 min (2) probe-type ultrasound (50–60 kHz, Sonics & Materials Inc. VCX 400, Danbury, CT, USA) where the MLV were exposed to five cycles of sonication for 1 min, followed by 3 min stopped to allow the cooling of the sample in the ice bath. The sample was resting for 15 min and then centrifuged (1000g/15 min/25 °C) to precipitate the particles of titanium released from the tip of the ultrasound.

2.5. Light scattering analysis

The mean particles size and polydispersity (PDI) were evaluated by light scattering performed on a Brookhaven Instruments standard setup (BI-200 M goniometer, BI-9000AT digital correlator) with a He–Ne laser (λ = 632.8 nm) as light source. An interference filter was used before detecting the signal on the photomultiplier. The sample cell was placed in the indexmatching liquid decahydronaphthalene (Aldrich). The apparent values of hydrodynamic radius R_h (related to the diffusional dynamics of a vesicle) were obtained in this work by Dynamic Light Scattering (DLS) at 90°. The time correlation functions were measured in the multi-τ mode using 224 channels and data treatment was made by means of the cumulant analysis provided by Brookhaven. The R_h determination was made by means of the Stokes–Einstein relation using the diffusion coefficient D_{app} determined by:

$$D_{app} = \frac{k_B T}{6\pi\eta_0 R_h}$$

where k_B is the Boltzmann constant, T is the absolute temperature, η_o is the viscosity of the solvent and R_h is the hydrodynamic radius.

For each liposome preparation, DLS measurements were performed immediately after the liposomes were prepared. Change in effective diameter of filtered (0.22 μm) and unfiltered liposomes produced by film hydration using bath-type ultrasound were monitored periodically for up to 24 days for liposomes stored at 4 °C (Were et al., 2003).

2.6. Antimicrobial activity assay

The antimicrobial activity was detected by agar diffusion assay. An aliquot of 10 μl of the free nisin and nisin entrapment in liposomes produced by methods descript were applied on BHI agar plates previously inoculated with a swab submerged in indicator strain (*L. monocytogenes* ATCC 7644) suspension, which corresponded to a 0.5 McFarland turbidity standard solution, (approximately 10^7 CFU ml^{-1}). Plates were incubated at 37 °C for 24 h. The reciprocal value of the highest dilution that produced an inhibition zone was taken as the activity unit (AU) per ml (Motta & Brandelli, 2002).

To determine whether apparent decreases in liposome-encapsulated nisin activity were due to physical interactions of nisin with liposome components, 500 μl of vesicles suspension was mixed with 500 μl of 2% (v/v) Triton X-100 in order to disrupt the membrane and release the encapsulated nisin (Were et al., 2003). The mixture was heated at 98 °C for 5 min to desorb nisin from phospholipid vesicle.

2.7. pH and Zeta-potential measurements

The pH values of free nisin and encapsulated nisin produced by film hydration were determined at room temperature using a pH stripe (Machery-Nagel, Germany). The Zeta potential analyses of filtered and unfiltered liposomes were carried out after dilution of the formulations in 1 mM NaCl using a Zetasizer®nano-ZS ZEN 3600 equipment (Malvern Instruments, Herrenberg, Germany). The measurements were made in triplicate.

2.8. Entrapment efficiency

The entrapment efficiency (EE) of liposomes produced by film hydration was determined using agar diffusion method. Encapsulated nisin was separated from unencapsulated nisin by ultrafiltration (Ultracel YM-10 Membrane, Millipore). EE was calculated using the following equation (Laridi et al., 2003):

$$EE(\%) = \frac{\text{Encapsulated nisin (AU ml}^{-1})}{\text{Encapsulated nisin (AU ml}^{-1}) + \text{unencapsulated nisin (AU ml}^{-1})} \times 100$$

2.9. Microscopy

Morphological examination of liposomes was performed using a high resolution light microscope (Olympus BVPM Series Model 8, Olympus, Tokyo, Japan) before and during sonication. After sonication in a bath-type ultrasound, the morphological examination was performed using a scanning electron microscope (model JEOL JSM-6400, Jeol Ltd., Tokyo, Japan). The sample preparation included the drying and covering with a gold layer (Silva et al., 2008).

3. Results

3.1. Comparison of methodologies to encapsulate nisin

Soy lecithin was used for the production of PC liposomes because it is a cheap and easily available product. In addition, soy-derived lecithin presents compatibility with the ultimate application in food systems. The soy lecithin was partially purified resulting in significant elimination of fatty acids present in the original sample (data not show). The resulting soy PC was used in the development of liposomes. The molecular composition of the soybean PC (PC-1) is around 75% distearoylphosphatidylcholine (DSPC), 12% dioleoylphosphatidylcholine (DOPC) and 8% dipalmitoylphosphatidylcholine (DPPC) (Mertins et al., 2008).

Two methodologies, reversed-phase and hydration film, were compared for encapsulation of nisin in liposomes by evaluating the size, PDI and antimicrobial activity. In addition, we tested two types of ultrasound in the film hydration (Table 1). Film hydration using bath-type ultrasound resulted in liposomes of smaller size and with full maintenance of antimicrobial activity. Although the PDI has been higher than those observed for other techniques, it can be considered small. Therefore, the methodology of film hydration using bath-type ultrasound was the most suitable for further studies of the production of liposomes.

3.2. Physicochemical characteristics of liposomes

The antimicrobial activity of free nisin (0.5 mg ml^{-1}) was 1600 AU ml^{-1}. After encapsulation in liposomes, using the technique of film hydration, the residual activity was 100%, i.e., there was maintenance of antimicrobial activity (Table 1). The entrapment efficiency of nisin in liposomes prepared by film hydration using ultrasound bath-type was 94.12% and the pH was 4.5 maintaining constant for 24 days.

The antimicrobial activities of free nisin and encapsulated nisin filtered through a 0.22 μm nylon membrane were evaluated for a period of 24 days. It was observed that the free nisin maintained its initial activity while the liposomes containing nisin were losing their antimicrobial activity over time reaching 25% residual activity after 10 days (Fig. 1). To assess whether there was an actual loss of activity or a stronger association between the nisin and the membrane phospholipids a treatment with Triton X-100 was made at days 10 and 20. The antimicrobial activity (400 AU ml^{-1}) increased to 800 AU ml^{-1} indicating that a fraction of nisin was unavailable.

Filtered and unfiltered liposomes presented sizes of approximately 140 nm, which has remained constant over time. The change in the size of liposome population was lesser than 0.4 indicating that the liposomes were physically stable over the 24 day period (Table 2).

The Zeta potential, as an indicator for accessible surface charges, was determined as -55.8 ± 3.1 and -54.5 ± 1.9 mV for unfiltered and filtered liposomes, respectively. After 20 days, the Zeta potential was determined as -48.1 ± 4.6 and -37.2 ± 1.2 mV for unfiltered and filtered liposomes, respectively. Therefore, there was an increase in the values of Zeta potential. Furthermore, the curves were not reproducible after 20 days indicating high degradation and instability of liposomes over time.

Images of giant (micrometer-range) liposomes were observed by light microscopy before and during sonication. It was observed that the liposomes had high variation in size (Fig. 2). After 30 min of sonication, micron-scale liposomes were no longer visible and subsequent microscopic characterization was carried out using scanning electron microscopy (SEM, Fig. 3). As viewed with SEM, liposomes were agglomerated, which may be an artifact of the drying process used in preparation of these slides prior to coating with gold. In both cases, we observed that the liposomes showed spherical morphology.

4. Discussion

To produce liposomes with high residual antimicrobial activity three strategies were tested for encapsulation of nisin in nanovesicles. In the reversed-phase evaporation method, after evaporation of the organic solvent, all other components are in the organogel. Dispersion of the organogel in pure water under shaking leads to nanovesicle formation (Mertins et al., 2005; Teixeira et al., 2008). In this work, nisin solution was added after dilution of PC-1 using

Table 1
Comparison of size, polydispersity index (PDI) and antimicrobial activity of nisin after encapsulation by different techniques.

Method	Size (nm)	PDI	Residual antimicrobial activity (%)
Reversed-phase	190 ± 3.5	0.30 ± 0.069	25
Film hydration: probe-type ultrasound	181.4 ± 5.12	0.27 ± 0.082	50
Film hydration: bath-type ultrasound	148.0 ± 9.2	0.392 ± 0.004	100

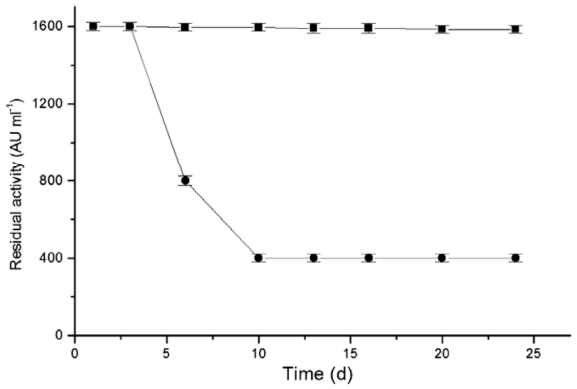

Fig. 1. Effective antimicrobial activity of free and encapsulated nisin as a function of storage time at 4 °C. (■) Free nisin, (●) encapsulated nisin. Values are means of three independent experiments.

Table 2
Effective diameter and PDI of liposomes containing nisin as a function of time.

Day	Size (nm)		PDI	
	Unfiltered	Filtered	Unfiltered	Filtered
1	148.0 ± 9.2	137.7 ± 6.1	0.392 ± 0.004	0.346 ± 0.066
3	149.0 ± 1.7	137.3 ± 11.6	0.282 ± 0.020	0.290 ± 0.056
6	144.7 ± 3.5	139.3 ± 6.4	0.301 ± 0.085	0.305 ± 0.010
10	144.7 ± 4.0	138.7 ± 9,1	0.339 ± 0.008	0.274 ± 0.035
13	141.0 ± 5.6	142.3 ± 9.8	0.400 ± 0.025	0.351 ± 0.036
16	146.3 ± 3.2	137.3 ± 9.8	0.390 ± 0.076	0.355 ± 0.002
20	135.7 ± 2.9	138.3 ± 1.5	0.371 ± 0.069	0.296 ± 0.062
24	143.7 ± 9.9	132.0 ± 6.2	0.396 ± 0.012	0.290 ± 0.027

chloroform, i.e., before its evaporation. As the chloroform is a strong organic solvent part of nisin may have been inactivated leading a reduction of its antimicrobial activity. This is undesirable for subsequent application of liposomes in food. Most techniques are not suitable for the encapsulation of sensitive substances, including antimicrobial peptides, because of their exposure to mechanical stresses and potentially harmful chemicals, such as volatile organic solvents (Colas et al., 2007; Taylor, Davidson, Bruce, & Weiss, 2005).

In the preparation of liposomes made by the thin film hydration method, LUVs were obtained by sonication using two types of ultrasound. PDI decreased with higher power intensity using probe-type ultrasound, since with this power it is possible to achieve a more rapid mixing of solution (Silva et al., 2008). However, this high intensity may be associated to nisin denaturalization, reducing its antimicrobial activity. In addition, nisin may have been strongly linked to the wall of the liposome becoming unavailable. The encapsulation by film hydration using ultrasound bath-type was the most effective, because the liposomes were smaller than the other methods tested and retained 100% of antimicrobial activity. This result is very interesting for food applications, which we want to accomplish later. PDI was higher, but the size of the liposomes remained stable over time. To the best of our knowledge, this is the first time that nisin encapsulation has been compared for different methods. According to Taylor et al. (2005) ultrasound bath-type is less destructive to liposomes, presents greater reproducibility and more homogenous product vs. probe sonicator.

Taylor et al. (2008) reported the use of liposomes within 72 h of production, but the examination of antimicrobial activity over time has not been previously investigated. In this work, the residual antimicrobial activity was 50% in the sixth day (Table 2). After, the residual antimicrobial activity remained at 25% for up to 24 days. However, the size and PDI of the liposomes remained constant over time (132–149 nm by 24 days). The effective diameter of liposomes and PDI was not affected by filter sterilization. These results are in agreement with the study of size as function of time reported by Were et al. (2003), which showed physically stable liposomes over the 2-week period investigated. Filter sterilization is a suitable method to remove foreign particles such as microorganisms from liposome dispersion while maintaining their integrity (Were et al., 2003).

Many studies showed higher EE in PC liposomes than those also containing phosphatidylglycerol and/or cholesterol (Laridi et al., 2003; Taylor et al., 2008; Were, Bruce, Davidson, & Weiss, 2004; Were et al., 2003). The electrostatic interaction of nisin with negatively charged membrane phospholipids such as PC has been recognized to be more pronounced than the interaction with neutral

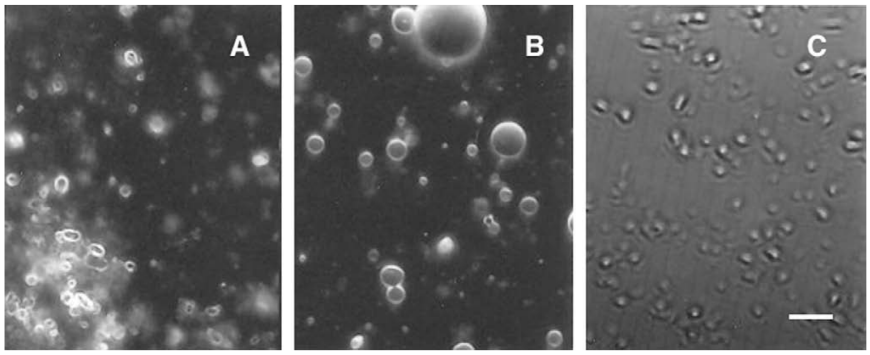

Fig. 2. Optical microscopy images obtained: (A) before sonication, (B) after 10 min of sonication, and (C) after 20 min of sonication. Bar, 10 μm.

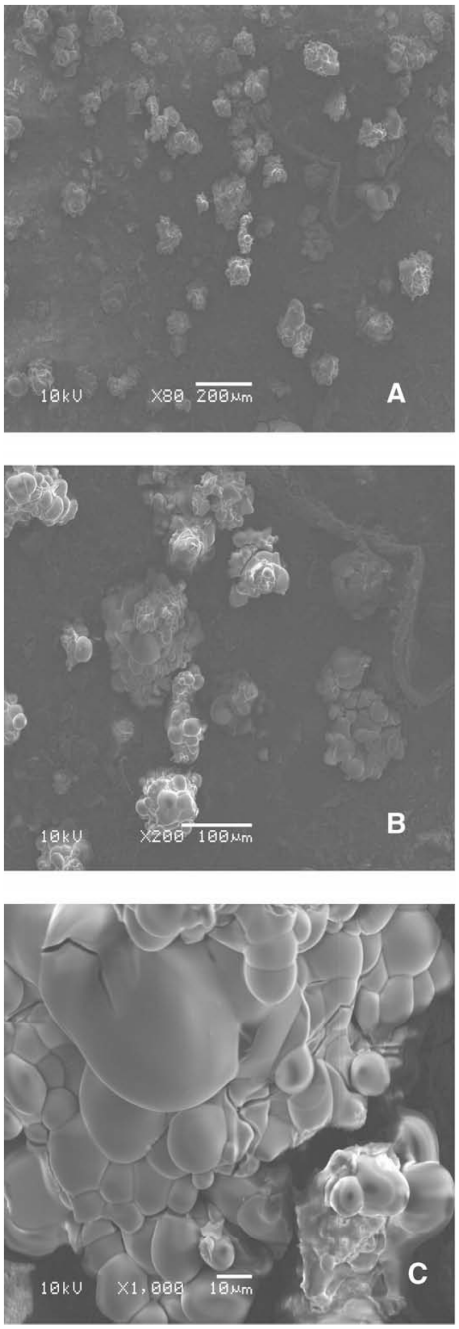

Fig. 3. SEM microphotographs after sonication (the samples filtered after ultrasound treatment), using different magnifications: (A) 80×, (B) 200×, and (C) 1000×.

lipids such as PG (Bonev et al., 2000). In this study, the entrapment efficiency in PC-1 was 94.12% using ultrafiltration and the pH of liposomes solution was 4.5. Were et al. (2003) found EE to co-encapsulated nisin and calcein of 63%, 54% and 59% for the PC, PC/cholesterol (70:30), and PC/PG/ cholesterol (50:20:30) using fluorescence quenching. Degnan and Luchansky (1992) reported EE of 18% for pediocin AcH in MLV made of PC. Laridi et al. (2003) investigated the effects of fatty acid compositions, pH, cholesterol content, and nisin Z content on the EE of different commercial preparations of proliposomes. Those authors found higher EE for hydrogenated PC liposomes (34.6% EE) for the encapsulation of nisin Z versus other unsaturated phospholipids (11.6% EE) or phospholipid mixtures (26% EE). Increasing cholesterol concentration in liposomes led to a decrease in nisin Z encapsulation. An increase in pH from 3.6 to 6.6 resulted in reduction in EE for hydrogenated liposomes. Higher percentages of unsaturated fatty acids did not display large fluctuation in EE. Nisin entrapped in different nanoliposomes by Mozafari's method result EEs ranging from 12% to 54% (Colas et al., 2007). For those authors anionic vesicles possessed the highest EE for nisin while increase in cholesterol content in lipid membranes up to 20% molar ratio resulted in a reduction in EE.

An analysis of size distributions of liposomes before applying ultrasound (MLVs) was performed by Silva et al. (2008) and it was observed that these MLVs presented a large size (2400 nm) with a high polydispersity (0.910). This result was attributed to the polydispersity of MLVs population presented in the sample. The size (nm) and the polydispersity (PDI) decreased after sonication using a probe-type ultrasound with a rapid drop in the Z-average.

The Zeta potential of unfiltered and filtered PC liposomes was similar (−55.0 mV approximately). Taylor, Gaysinsky, Davidson, Bruce, and Weiss (2007) described PC liposomes with a Zeta potential of −8.3 mV at 25 °C, whereas PC:PG (8:2) and PC:PG (6:4) showed Zeta potentials of −52.28 and −72.60 mV, respectively. The Zeta potential was compensated at very low [peptide]:[lipid] molar ratios which indicates an electrostatically driven interaction (Willumeit et al., 2005). The electrostatic repulsion between the particles is responsible for the low sedimentation volume (Arias, López-Viota, Clares, & Ruiz, 2008). In this study sedimentation was not observed for up to 24 days. After 20 days, the Zeta potential increased and the curves were not reproducible indicating degradation and instability of liposomes. This may be related to the decrease of antimicrobial activity observed over time.

The microscopy evaluation revealed that the liposomes showed a rounded morphology. Encapsulation of positively charged peptides (LLLLK LLLLK LLLLK LLLLK) presents an increase in Zeta-potential values after using probe-type ultrasound and an "amorphous" aspect. Conversely, the liposomes that incorporated other peptides (LLLLL LCLCL LLKAK AK) presented a defined shape (rod shape) and the potential surface of liposome did not change significantly by the use of ultrasound (Silva et al., 2008). In the SEM the liposomes agglomerate similarly to the morphology observed by Arias et al. (2008). Therefore, the incorporation of different peptides into liposomes might originate differences on shape of liposomes (Silva et al., 2008).

Our results indicate that the nisin was easily incorporated, with high EE, in liposomes made of partially purified PC obtained from industrial soy byproduct. Antimicrobial activity decreased over time and Zeta potential was also affected. However, size, PDI and pH were constant by 24 days incubation. In stability studies, Zeta potential and antimicrobial activity should be evaluated over time, because the analysis of size alone can generate incorrect conclusions.

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3.3 ARTIGO 3

3.3.1 Effect of nanovesicle-encapsulated nisin on growth of *Listeria monocytogenes* in milk

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Short communication

Effect of nanovesicle-encapsulated nisin on growth of *Listeria monocytogenes* in milk

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ABSTRACT

Commercial nisin was encapsulated in nanovesicles (mean diameter 140 nm) prepared from partially purified soy lecithin. Nisin-loaded liposomes and unencapsulated (free) nisin were initially tested in BHI medium and skim milk inoculated with *Listeria monocytogenes* and incubated for 48 h at 30 °C. At such abuse temperature conditions, free nisin showed better inhibitory than the liposomal counterparts. Subsequently, the effect of encapsulated or free nisin was evaluated in combination with refrigeration (7 ± 1 °C) in both whole (3.25% fat) and skim (0% fat) milk for up to 14 day. A decrease of 3–4 log cycles in *L. monocytogenes* counts was observed for free and encapsulated nisin at 0.5 mg/ml concentration. Liposome encapsulation of antimicrobial peptides may be important to overcome stability issues and interaction with food components. The utilization of nanovesicle-encapsulated nisin in combination with low temperatures appeared to be effective to control *L. monocytogenes* in milk, emphasizing the importance of hurdle technology to assure food safety.

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1. Introduction

Nisin is an antimicrobial peptide produced by strains of *Lactococcus lactis* subsp. *lactis*, recognized as safe for food applications by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additivies. The FAO/WHO Codex Committee on milk and milk products accepted nisin as a food additive for processed cheese at a concentration of 12.5 mg pure nisin/kg of product (Ross et al., 2002). Nisin has received much attention because of its broad inhibitory spectrum against Gram-positive bacterial strains like *Listeria* and *Staphylococcus*, and also the effective inhibition of the growth of Bacilli and Clostridia spores (Arauz et al., 2009).

Nisin has numerous applications as a natural food preservative, including dairy products, canned foods and processed cheese (O'Sullivan et al., 2002). However, to apply nisin in foods it is important to consider the interactions of this bacteriocin with food ingredients, and the influence of such interactions on its efficacy (Devlieghere et al., 2004). Several studies demonstrate that proteolytic degradation and the interaction of nisin with food components might result in decreased antimicrobial activity (Jung et al., 1992; Gänzle et al., 1999; Bhatti et al., 2004; Chollet et al., 2008).

Encapsulation of antimicrobial peptides into liposomes may offer a potential alternative to protect antimicrobials, enhancing their efficacy and stability for food applications (Laridi et al., 2003; Were et al., 2003; Teixeira et al., 2008).

Liposomes are vesicles composed of one or more phospholipid bilayers encapsulating a volume of aqueous media. Liposome manufacture requires input of energy for dispersion of lipid/phospholipid molecules in an aqueous medium, and the main objective of such process is to obtain vesicles with the right size, acceptable polydispersity, elasticity, structure, and encapsulation efficiency (Mozafari, 2005; Mozafari et al., 2008). Liposomes can be used in a wide range of applications due to their ability to incorporate lipophilic, amphiphilic and/or hydrophilic compounds (Khosravi-Darani et al., 2007). The bacteriocin nisin is a 34 amino acid peptide with cationic and hydrophobic characteristics (Breukink and de Kruijff, 1999); hence, nisin might be encapsulated in the inner aqueous phase of liposome and also be immobilized into liposome membranes (Laridi et al., 2003).

L. monocytogenes is a foodborne pathogen of concern to the dairy industry due to its capability to grow under refrigeration (Gandhi and Chikindas, 2007). The aim of the present study was to assess the effect of liposome-encapsulated nisin against *L. monocytogenes*

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in milk. Initially, the effect of free and encapsulated nisin was tested in BHI media and skim milk at 30 °C. Thereafter, the combined effect of nisin and refrigeration temperature against *L. monocytogenes* was evaluated in skim and whole milks.

2. Materials and methods

2.1. Nisin, milk and medium

Commercial nisin (Nisaplin) was purchased from Danisco Brasil Ltda. According to the manufacturer, the formulation contains NaCl and denatured milk solids as fillers, and 2.5% pure nisin. Nisin solution was prepared with 0.01 M HCl and was filter-sterilized through 0.22 μm membranes (Millipore). Before each experiment, nisin was diluted with 10 mM phosphate buffer (pH 6.4) to reach concentrations of 0.1 and 0.5 mg/ml of pure nisin. UHT whole milk (3.25% fat) and UHT skim milk (0% fat) were purchased from a local market. BHI (Brain Heart Infusion; HiMedia, India) medium was prepared according to manufacturer instructions.

2.2. Bacterial culture and sample inoculation

L. monocytogenes ATCC 7644, utilized in the present study, was maintained on BHI agar plates at 4 °C, and subcultured periodically. Before each experiment, this strain was grown in BHI medium at 37 °C for 18–24 h in an orbital shaker, reaching about 9 log CFU/ml. Subsequently, the culture was diluted in saline solution (8.5 g/l NaCl), and added to BHI medium or milk samples to result an initial inoculum of 4 log CFU/ml.

2.3. Nisin encapsulation

Encapsulation of nisin in liposomes of partially purified soybean phosphatidylcholine (PC-1; Mertins et al., 2008) was carried out by the thin-film hydration method. Briefly, 0.076 g of PC-1 was dissolved with 10 ml chloroform in a round-bottom flask and the organic solvent was removed by a rotary evaporator until a thin film was formed on the flask walls. Traces of organic solvents were removed by storage for 18 h in a vacuum desiccator. The resulting dried lipid film was dispersed by the addition of phosphate buffer containing nisin. These mixtures were then mixed exceeding their phase transition temperature (60 °C). Sonication of the preparation, in order to reduce the size and homogenize liposomes, was carried out in a bath-type ultrasound (40 kHz, Unique USC 700) for 30 min. The size of nanovesicles was determined by light scattering as described elsewhere (Teixeira et al., 2008). The entrapment efficiency (EE) was determined using agar diffusion method. Encapsulated nisin was separated from unencapsulated nisin by ultrafiltration (Ultracel YM-10 Membrane, Millipore) and the EE was calculated according to Laridi et al. (2003).

2.4. Antimicrobial activity assay

The antimicrobial activity was detected by agar diffusion assay. Aliquots (10 μl) of free nisin or liposome-encapsulated nisin were applied on BHI agar plates previously inoculated with a swab submerged in a suspension of indicator strain (*L. monocytogenes* ATCC 7644), which corresponded to a 0.5 McFarland turbidity standard solution (approximately 7 log CFU/ml). Plates were incubated at 37 °C for 24 h. The reciprocal value of the highest dilution that produced an inhibition zone was taken as the activity unit (AU) per ml (Motta and Brandelli, 2002).

2.5. Effect of free and encapsulated nisin in milk and BHI

Free and encapsulated nisin (0.5 mg/ml) were added to tubes containing 10 ml of BHI broth or milk. *L. monocytogenes* ATCC 7644 cells were added to produce an initial count of 4 log CFU/ml. Controls were inoculated with *L. monocytogenes* and saline solution (8.5 g/l NaCl). The tubes were incubated for 0, 0.16, 2, 4, 8, 24, 30 and 48 h at 30 °C.

To assess the effect of free and encapsulated nisin in whole and skim milk, the tubes were incubated for 0, 5, 10 and 14 day at refrigeration temperatures (6–8 °C). The quantification of *L. monocytogenes* was performed by the drop culture method, which has a detection limit of approximately 1.69 log CFU/ml (Milles and Misra, 1938; Malheiros et al., 2009). The colonies were counted in BHI agar plates and Listeria Oxford medium after 24 and 48 h of incubation at 37 °C, respectively (Bizani et al., 2008).

Each experiment was performed in duplicate and repeated at least twice. Mean values and S.E.M. were calculated.

3. Results and discussion

The antimicrobial activity of free and encapsulated nisin (0.5 mg/ml) was 1.600 AU/ml, and nisin-loaded liposomes presented a mean diameter of 140 nm. The entrapment efficiency of nisin in liposomes was 94%. The effect of unencapsulated (free) and liposome-encapsulated nisin on *L. monocytogenes* growth was first investigated in BHI medium and skim milk.

Free nisin caused a decrease in *L. monocytogenes* counts below the detection limit of the method on BHI medium at 30 °C during the experiment (48 h); however, when encapsulated nisin was tested, *L. monocytogenes* were reduced to counts below the detection limit only during the first 8 h, increasing thereafter (Fig. 1A). Similar results for *L. monocytogenes* growth were observed in skim milk (Fig. 1B). The results presented in Fig. 1 indicate that nisin-loaded liposomes were less efficient in controlling *L. monocytogenes* growth, when compared to free nisin, in both BHI medium and skim milk at 30 °C. Usually, the efficiency of liposome-encapsulated bacteriocins is higher when compared to the free counterparts (Degnan et al., 1993; Were et al., 2004). However, Taylor et al. (2008) observed that the inhibition of *L. monocytogenes* by phosphatidylcholine (PC) liposomes containing nisin was nearly equivalent to the unencapsulated nisin in BHI medium. In an investigation with a bacteriocin-like antimicrobial peptide produced by *Bacillus licheniformis* P40, encapsulation within PC nanovesicles resulted in smaller inhibitory zones on agar plates inoculated with *L. monocytogenes* than those observed for the free peptide; hence, a higher dose of encapsulated bacteriocin was required to obtain the same inhibitory effect observed for the unencapsulated counterpart (Teixeira et al., 2008).

Nisin-loaded liposomes, manufactured from partially purified soy PC, presented a zeta-potential of approximately −55 mV (Malheiros et al., unpublished results), which was lower (−6.89 to −7.61 mV) than that obtained for nisin encapsulated into liposomes produced from purified PC (Taylor et al., 2007). Due to its cationic character, nisin associates more strongly with negatively charged than neutral membranes (El Jastimi and Lafleur, 1997); thus, it could be suggested that nisin was strongly linked to the liposome structure, becoming unavailable to act against *L. monocytogenes*. Although electrostatic interactions between nisin and negatively charged phospholipids are reported to induce membrane destabilization and pore formation, the amount of bound nisin increased considerably only beyond an anionic lipid content beyond 40% (Breukink and de Kruijff, 1999). For instance, nisin was showed to efficiently efflux from liposomes manufactured with PC:phosphatidylglycerol (60:40), while nisin encapsulated within PC liposomes

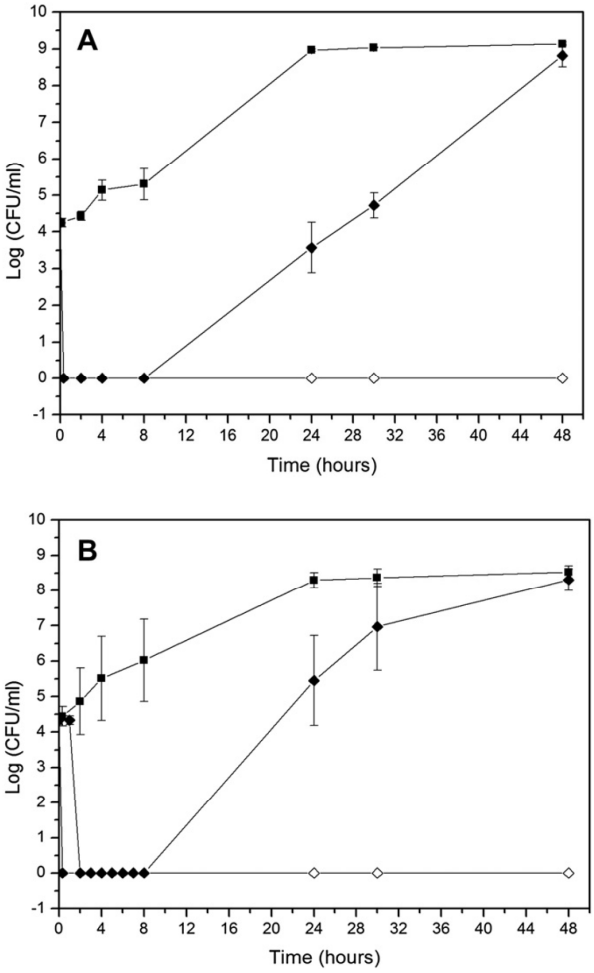

Fig. 1. Survival of *Listeria monocytogenes* in BHI medium (A) and skim milk (B) at 30 °C tested with free nisin (◇), encapsulated nisin (◆) and control (■).

(100%) demonstrated the slowest apparent release of antimicrobial (Taylor et al., 2008). Taylor et al. (2005) reported that nisin insertion, at low concentrations, might act to stabilize PC liposomes. It was suggested that the interaction of nisin with lipids not commonly found in prokaryotic membranes (e.g., PC) is likely to produce a different effect than permeabilization, such as a stabilizing effect (Taylor et al., 2005).

Gänzle et al. (1999) showed that addition of 1% egg yolk lecithin completely abolished nisin activity towards *Lactobacillus curvatus* DSM20019 and *Listeria innocua* DSM20649, and that at a level of 0.1%, the antimicrobial activity was reduced by 40–70%. The effect of lecithin was attributed to the formation of stable complexes between nisin and the zwitterionic phospholipids (Gänzle et al., 1999). Strong nisin–liposome interactions might reduce the rate of nisin release to a level inferior to that required for the control of *L. monocytogenes* growth. Alternatively, the highly negative zeta-potential of nisin-loaded liposomes may have influenced nisin interaction with bacteria. As the bacterial cell has a negative charge, there may have been electrostatic repulsion between liposomes and the cell surface, preventing direct contact between liposomes and pathogens, and the subsequent release of antimicrobials (Were et al., 2004).

Nisin is currently employed as a food biopreservative in hurdle technology, implying that it should not be used as the only barrier to prevent the growth or survival of food pathogens. Thus, nisin is likely to be utilized as one of a number of hurdles selected to increase microbial inactivation, reducing the likelihood of food-borne diseases (Deegan et al., 2006; Gálvez et al., 2007). The combined effect of nisin (in both free and encapsulated forms) and refrigeration (~7 °C) on *L. monocytogenes* growth was investigated in whole and skim milk. Without nisin addition (controls), *L. monocytogenes* counts increased from approximately 4.5 log CFU/ml at time zero to above 8.5 log CFU/ml after 14 days in both skim and whole milks. In skim milk, encapsulated and free nisin (0.5 and 0.1 mg/ml) treatments consistently lowered *L. monocytogenes* counts below the detection limit of the method during a period of 14 days (Fig. 2A). However, in whole milk, only the high concentrations (0.5 mg/ml) of both free and encapsulated nisin reduced bacterial counts (Fig. 2B). When free nisin in the lower concentration was added to whole milk, a decrease in bacterial counts (~3 log CFU/ml) was observed at day 5, increasing afterwards and achieving values as high as 5 log CFU/ml. This result was somewhat expected, since the negative effect of fat on nisin antimicrobial activity is extensively reported, caused by the adsorption of nisin onto fat globules

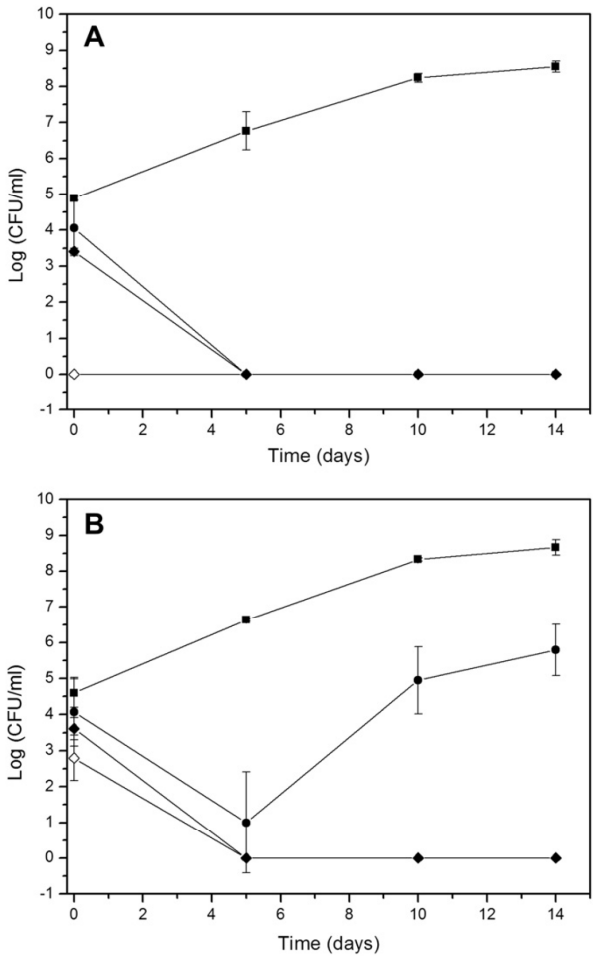

Fig. 2. Survival of *Listeria monocytogenes* in skim milk (A) and whole milk (B) at refrigeration temperature (6–8 °C) tested with 0.5 mg/mL of free nisin (◇), 0.1 mg/mL of free nisin (●), 0.5 mg/mL of encapsulated nisin (◆) and control (■).

(Jung et al., 1992; Bhatti et al., 2004; Chollet et al., 2008). Also, since partially purified soy PC was utilized in liposome production, the interaction between nisin and residual fatty acids might have influenced the antimicrobial activity of nisin-loaded liposomes at 30 °C (Fig. 1). Branen and Davidson (2004) showed that nisin had no effect against *L. monocytogenes* growth in 2% fat UHT milk held at 25 °C for 24 h to simulate abuse temperature; demonstrating that the activity of antimicrobials in food systems may be affected by a number of factors, including fat, protein, and cation concentrations, as well as storage temperature.

Liposome encapsulation technologies may allow for the maintenance of antimicrobial activity by protecting the antimicrobial against cross-reactions with food components (Taylor et al., 2008). Also, as antimicrobials could be present both in the aqueous and lipids phases of liposomes, a complementary effect could be expected, providing both short-term (by release of encapsulated nisin) and long-term (desorption of membrane-immobilized nisin) antibacterial actions (Benech et al., 2002).

In agreement with the current study, Taylor et al. (2008) reported that *L. monocytogenes* (strains Scott A and 310) cultures were not inhibited from growth over the entire course of experimentation (48 h) by nisin-loaded PC liposomes. However, these authors observed, through measurements of optical density at 630 nm, a substantial inhibition (~60%) of pathogens versus the positive controls after 48 h of incubation at 32 °C on double-strength BHI medium. The combined utilization of nisin (free or encapsulated) with the low temperature commonly utilized in retail markets (~7 °C) seemed to be effective in controlling *L. monocytogenes* growth during 14 days, reinforcing the significance of the hurdle technology concept for food quality and safety.

It is important to note that most of the investigations on nisin-loaded liposomes are performed with purified phospholipids and/or purified nisin Z (Benech et al., 2002; Laridi et al., 2003; Were et al., 2004; Taylor et al., 2008). In this study, partially purified soy phosphatidylcholine and commercial nisin A were utilized. Thus, some conflicting results might be, to a certain extent, explained by these differences. The production of stable and effective liposome-encapsulated bacteriocins, for successful utilization in the microbiological stabilization of food products, depends on selection of suitable lipid–antimicrobial combinations (Were et al., 2003). The present investigation might be valuable to address the actual viability of application of nisin-loaded liposomes in foods, since the food industry require technologies that are available at low costs to be implemented in products which usually have a low or moderate profit margin.

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3.4 ARTIGO 4

3.4.1 Inhibition of *Listeria monocytogenes* growth in Minas frescal cheese by free and nanovesicle-encapsulated nisin

ABSTRACT

The effectiveness of unencapsulated (free) and nanovesicle-encapsulated nisin to control *Listeria monocytogenes* in Minas Frescal cheese was assessed. Commercial nisin was encapsulated into liposomes of partially purified soy lecithin. Free (0.1 mg/mL and 0.25 mg/mL) and nanovesicle-encapsulated nisin (0.25 mg/mL) were applied onto the surface of commercial cheese samples, and *L. monocytogenes* was inoculated before incubation at 6-8°C for 28 days. A bactericidal effect was observed with 0.25 mg/mL free nisin; a bacteriostatic effect was observed for liposome-encapsulated nisin and 0.1 mg/mL free nisin. Free nisin was more efficient than nisin-loaded liposomes in controlling *L. monocytogenes*. Possible reasons for this behavior, and also the significance of nisin to fresh cheeses are discussed. Nisin acted as a suitable barrier within hurdle technology, potentially extending the shelf-life and safety of fresh cheeses.

Keywords: nisin, liposome, encapsulation, cheese, *Listeria monocytogenes*

Food preservation and microbiological quality represent major concerns and challenges to the food industry. Much effort has been focused on the application of antimicrobial peptides as potential biopreservatives in hurdle

technology. The most extensively studied bacteriocin is nisin, as it is the only one approved for food applications, and it has gained a widespread industrial significance. This bacteriocin, produced by strains of *Lactococus lactis* subsp. *lactis*, shows a broad inhibitory spectrum against Gram-positive bacterial strains like *Listeria* and *Staphylococcus*, also inhibiting the outgrowth of spores of Bacilli and Clostridia (1). Nisin is frequently added directly to food systems as commercial products, an application in which loss of antimicrobial activity usually occurs over time due to enzymatic degradation and interactions with food components, such as proteins and lipids (19).

Nanotechnology is recognized as a potential source of novel products and processes for the food industry (17). However, only limited researches in nanotechnology have been performed in foods and food-related products, and the global development of nanofoods seems to be on its initial stage (6). Thus, encapsulation technology is exploited aiming to offer an alternative to protect antimicrobials, potentially enhancing their efficacy and stability in foods (11). Encapsulation into liposome, composed by one or more phospholipid bilayers encapsulating a volume of aqueous media (13), appears as promising alternative.

Fresh cheeses generally have a short shelf-life, even under refrigeration. Minas Frescal is an example of such cheese, presenting high pH (4.9-6.7), high moisture content (>55%), and low percentage of salt (1.4-1.6%) (4, 5, 18). Such characteristics favor the development of bacteria that, in addition to limiting the product shelf-life, may pose a health hazard to consumers, since pathogenic bacteria could be found in such cheeses (15). Particularly, the ability to grow

under refrigeration makes *Listeria monocytogens* a foodborne pathogen of special concern to the dairy industry, and this bacterium was previously found in fresh and soft cheeses (4, 5, 8). The aim of this study was to evaluate the effectiveness of free (unencapsulated) and nanovesicle-encapsulated nisin to control *Listeria monocytogenes* in Minas Frescal fresh cheese surfaces at 6-8°C.

Commercial nisin (Nisaplin), containing 2.5% pure nisin A, was purchased from Danisco Brasil Ltda. A nisin solution was prepared with 0.01 M HCl and was filter-sterilized through 0.22 µm membranes (Millipore). Before each experiment, nisin was diluted with 10 mM phosphate buffer (pH 6.4) to reach concentrations of 0.1 and 0.25 mg/mL pure nisin. *L. monocytogenes* ATCC 7644 was maintained on Brain-Heart Infusion (BHI) agar plates at 4ºC, and subcultured periodically. Before each experiment, this strain was grown in BHI broth at 37 ºC for 18-24 h in an orbital shaker (125 rpm). This culture was diluted in saline solution (8.5 g/L NaCl), and added to cheese surface.

Antimicrobial activity of free and encapsulated nisin was detected by the agar diffusion assay performed in BHI agar plates inoculated with *L. monocytogenes* ATCC 7644, and expressed as arbitrary units per mL (AU/mL) (9).

Nisin encapsulation into partially purified soybean phosphatidylcholine liposomes was carried out by the thin-film hydration method (9). The size of the unilamellar nanovesicles was determined by light scattering (22). Encapsulated and unencapsulated nisin were separated by ultrafiltration through 10-kDa cut-off membranes (Ultracel YM-10 Membrane, Millipore). Entrapment efficiency

(EE) was determined using the agar diffusion method, and calculated as previously reported (9).

Free nisin (0.1 mg/mL and 0.25 mg/mL) and liposome-encapsulated nisin (0.25 mg/mL) were applied onto the surface of commercial Minas frescal cheese cubes of approximately 10 g, and after 30 min, 500 µL of a *L. monocytogenes* suspension containing ~4.5 log CFU/mL was inoculated into the cubes surface. Non-inoculated controls and experimentally inoculated cheeses were kept under refrigeration at 6-8 ℃ for 28 days. At defined intervals, one cube of each type of cheese was homogenized with 90 ml of Listeria Enrichment Broth (Acumedia, Baltimore, MA, USA) in a blender for 60 s, and plated on Oxford Listeria selective agar (Acumedia) plates for enumeration of viable *L. monocytogenes*. All experimental treatments were tested in duplicates for three independent preparations, and averages were calculated for treatments at each time point.

For recovery of nisin activity, free and nanovesicle-encapsulated nisin (0.25 mg/mL) were applied onto the surface of 5 g cheese cubes and two methods were tested: (a) the cubes were homogenized with 10 mM phosphate buffer (pH 6.4); and (b) the cubes were homogenized with 10 mM phosphate buffer (pH 6.4) containing 0.2% (w/v) Tween 80 and the mixture was heated for 15 min at 60 ℃ in a water bath (2). Then, for both methods, the homogenates were centrifuged at 10,000 × g for 30 min, and the antimicrobial activity was assessed by the agar diffusion assay.

The antimicrobial effect of nisin and other bacteriocins in cheeses is mainly investigated through the direct addition of the bacteriocin to the product during manufacture or post-production, or by inoculation of cheese milk with

bacteriocinogenic starters (3, 16, 19). However, the successful utilization of encapsulated nisin in reducing *L. monocytogenes* counts in fluid model systems (10, 20, 22) also suggests its potential usefulness in (semi-)solid matrices, such as cheeses. Liposomes were selected as nisin carriers due to the presence of both lipid and aqueous phases in its structure, allowing the entrapment, delivery, and release of water-soluble, lipid-soluble, and amphiphilic materials (13). As nisin is a cationic amphiphilic peptide, this bacteriocin could be both encapsulated in the inner aqueous phase of liposomes and immobilized into liposome membranes (11).

The antimicrobial activity of 0.25 and 0.1 mg/mL free nisin was 400 and 200 AU/mL, respectively, and liposome-encapsulated (0.25 mg/mL) nisin showed an antimicrobial activity of 400 AU/mL. Nisin-loaded liposomes presented a mean diameter of 140 nm, with an EE of 100%. Initial counts of *L. monocytogenes* inoculated into Minas frescal cheese were around 2.7 log CFU/mL. In control cheese (without nisin), counts increased to approximately 5 log CFU/mL after 28 days of incubation under refrigeration (Table 1). When free (0.1 mg/mL) or encapsulated (0.25 mg/mL) nisin were applied onto cheese prior the inoculation with *L. monocytogenes* resulted in lower counts when compared to the controls. Regarding free nisin, the higher concentration (0.25 mg/mL) exhibited a bactericidal effect against *L. monocytogenes*. The inferior detection limit of the method employed in microbial counts (~1.69 log CFU/mL) (12) was reached after 7 days, and such effect was maintained until day 28 (Table 1). At lower concentration (0.1 mg/mL), free nisin presented a bacteriostatic effect throughout the experiment, although *L. monocytogenes* counts tended to slowly

increase as time progressed. Similarly to 0.1 mg/mL free nisin, liposome-encapsulated nisin also presented a bacteriostatic effect (Table 1).

Differences in the effect of free (bactericidal) and encapsulated nisin (bacteriostatic) at 0.25 mg/mL might indicate a strong association of nisin to the phospholipidic vesicles, as previously suggested for fluid systems (10), followed by a gradual release from liposomes. Insertion of nisin into liposomes of PC, a lipid not commonly found in prokaryotic membranes, might cause the stabilization of the nanovesicles, possibly through a lowering of curvature stresses (21). Accordingly, nisin-loaded PC liposomes showed the slowest apparent release of the antimicrobial when compared to liposomes manufactured with PC:phosphatidylglycerol (60:40) (20). Additionally, lecithin inhibits nisin activity, possibly through the formation of stable nisin-phospholipid complexes (7). Although attempts to recover nisin from the cheese samples were not successful, the rate of nisin release from liposomes seemed to be sufficient to inhibit *L. monocytogenes* growth in comparison to the control (Table 1).

Presence of *L. monocytogenes* in soft, fresh cheeses is usually related to the utilization of unpasteurized contaminated milk or, when pasteurization is employed, postprocessing contamination occurs (8, 18). As counts of *L. monocytogenes* found in such cheeses are probably lower than that employed in this experiment, nisin in its free or encapsulated forms might inhibit the growth of this pathogen during refrigerated storage. In this context, control of *L. monocytogenes* growth for 28 days is well correlated with the shelf-life of Minas Frescal cheeses (25-33 days) (5).

Nascimento *et al* (15) reported that nisin application in Minas Frescal cheese gains further importance since counts of *L. monocytogenes* inoculated into this cheese, manufactured using a commercial starter with adjunct bacteriocinogenic cultures, showed no significant differences when compared to cheeses produced without bacteriocinogenic cultures. On the other hand, according to Naldini *et al* (14), *L. monocytogenes* counts remained almost unchanged during storage (25 days at 5 or 10°C) of Minas Frescal cheeses manufactured with the addition of starter culture (traditional process), whereas an increase of 2-3 log cycles was observed in cheeses manufactured by direct acidification (14). These results were attributed to pH lowering through lactic acid production, and competition of the lactic culture with *L. monocytogenes*. *Listeria* spp. was found in 22.6% and 12.9% of Minas Frescal cheeses manufactured by direct acidification and by the traditional process, respectively (5). Therefore, both free and encapsulated nisin forms might be employed in Minas Frescal cheeses manufactured either by the traditional process or direct acidification, aiming to restrict *L. monocytogenes* growth if contamination occurs, and adding to the effective safety of these products. Also, the storage of Minas Frescal cheese at 10°C increased *L. monocytogenes* growth when compared to storage at 5°C (14), reinforcing the significance of nisin's antimicrobial activity within the hurdle technology concept.

There are few reports on the effect of antimicrobial loaded liposomes in cheese. Previously, Benech *et al.* (2) have reported that liposome-entrapped nisin improved nisin stability and its inhibitory action against *L. monocytogenes* in Cheddar cheese matrix during ripening. These results, divergent from those

obtained in the current study, might be related to differences in the materials and protocols employed. For instance, Benech *et al.* (2) utilized purified phospholipids and purified nisin Z to produce nisin-loaded liposomes, and the nisin-loaded liposomes were added to milk during cheese manufacture. Also, those authors recovered 90% of the initial activity of nisin-loaded liposomes after 6 months of cheese ripening. Contrarily, in the present study no antimicrobial activity could be detected in liposome-entrapped nisin applied onto commercial cheese cubes.

Due to the scarce information available on this topic, the current investigation might contribute to assess the feasibility and suitability of applying nisin-loaded nanovesicles in (semi-)solid food matrices, such as cheeses. As encapsulation was observed to protect the starter cultures from the detrimental action of nisin, not affecting the fermentation process during cheese production (2), the possibility to apply nisin-loaded liposomes to milk during the manufacture of Minas Frescal cheese through the traditional process (which employs starter cultures) warrants further investigations.

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Table 1. Counts of *Listeria monocytogenes* in experimentally contaminated Minas Frescal cheese treated with free or liposome-encapsulated nisin[a], stored under refrigeration for 28 days

Time (days)	Control	Free nisin (0.10 mg/ml)	Free nisin (0.25 mg/ml)	Encapsulated nisin (0.25 mg/ml)
0	2.87 ± 0.10	2.79 ± 0.00	2.87 ± 0.10	2.87 ± 0.10
7	3.03 ± 0.40	1.98 ± 0.14	< 1.69	1.97 ± 0.04
14	3.75 ± 0.76	2.60 ± 0.22	< 1.69	2.19 ± 0.28
21	4.15 ± 0.30	2.48 ± 0.57	< 1.69	3.02 ± 0.12
28	5.07 ± 0.41	3.21 ± 0.98	< 1.69	3.42 ± 0.60

[a] The values represent mean log CFU/mL ± SD of two replicates.

3.5 ARTIGO 5

3.5.1 Nanovesicle encapsulation of antimicrobial peptide P34: physicochemical characterization and mode of action on *Listeria monocytogenes*

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RESEARCH PAPER

Nanovesicle encapsulation of antimicrobial peptide P34: physicochemical characterization and mode of action on *Listeria monocytogenes*

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Abstract Antimicrobial peptide P34, a substance showing antibacterial activity against pathogenic and food spoilage bacteria, was encapsulated in liposomes prepared from partially purified soybean phosphatidylcholine, and their physicochemical characteristics were evaluated. The antimicrobial activity was estimated by agar diffusion assay using *Listeria monocytogenes* ATCC 7644 as indicator strain. A concentration of 3,200 AU/mL of P34 was encapsulated in nanovesicles and stocked at 4 °C. No significant difference ($p > 0.05$) in the biological activity of free and encapsulated P34 was observed through 24 days. Size and PDI of liposomes, investigated by light scattering analysis, were on average 150 nm and 0.22 respectively. Zeta potential was −27.42 mV. There was no significant change ($p > 0.05$) in the physicochemical properties of liposomes during the time of evaluation. The liposomes presented closed spherical morphology as visualized by transmission electron microscopy (TEM). The mode of action of liposome-encapsulated P34 under *L. monocytogenes* cells was investigated by TEM. Liposomes appeared to adhere but not fuse with the bacterial cell wall, suggesting that the antimicrobial is released from nanovesicles to act against the microorganism. The effect of free and encapsulated P34 was tested against *L. monocytogenes*, showing that free bacteriocin inhibited the pathogen more quickly than the encapsulated P34. Liposomes prepared with low-cost lipid showed high encapsulation efficiency for a new antimicrobial peptide and were stable during storage. The mode of action against the pathogen *L. monocytogenes* was characterized.

Keywords Antimicrobial · Bacteriocin · Liposome · Nanovesicles · Morphology · Mode of action · Microbiology

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Introduction

Antimicrobial peptides are produced all over by different groups of bacteria. *Bacillus* species can synthesize many antimicrobial peptides representing diverse chemical structures (Stein 2005). *Bacillus* sp. P34, a bacterium isolated from Brazilian Amazon basin, produces an antimicrobial peptide that shows antibacterial activity against pathogenic and food spoilage bacteria such as *Listeria monocytogenes*,

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Bacillus cereus, *Aeromonas hydrophila*, *Erwinia carotovora*, *Pasteurella haemolytica*, *Salmonella gallinarum*, among others (Motta et al. 2007a). Peptide P34 has a molecular mass of 1,456 Da, is susceptible to several proteases and is stable within a broad range of pH and temperature (Motta et al. 2007b). Furthermore, according to in vitro cytotoxicity tests, the antimicrobial peptide P34 shows similar behavior compared to nisin, a bacteriocin recognized as safe for food applications by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (Vaucher et al. 2010). Therefore, peptide P34 shows potential for use as a biopreservative in food and pharmaceutical industries.

Bacteriocins are antimicrobial peptides that can be added to foods in the form of concentrated preparations as preservatives, shelf-life extenders, additives, or ingredients (Gálvez et al. 2007). However, stability issues like proteolytic degradation and the potential interaction of the bacteriocins with food components, such as fat, might result in decrease of the biological activity (Aasen et al. 2003; Glass and Johnson 2004; Chollet et al. 2008). Also, the potential of peptides for medical uses is mainly limited due to their poor stability to proteolysis, low permeability across barriers, and short shelf-life in the circulatory system (Reis et al. 2006).

Phospholipid nanovesicles have been investigated for protection of antimicrobial peptides, enhancing their efficacy and stability for food applications (Malheiros et al. 2010a; Sant'Anna et al. 2011). Phospholipid vesicles, often called liposomes, are colloidal structures having an internal aqueous pool formed by self-assembly of amphiphilic lipid molecules in solution. Owing to the presence of both lipid and aqueous phases in the structure of lipid vesicles, they can be utilized in the entrapment, delivery, and release of water-soluble, lipid-soluble, and amphiphilic materials (Khosravi-Darani et al. 2007; Mozafari et al. 2008a). Liposome entrapment may stabilize encapsulated bioactive materials against a range of environmental and chemical stresses, including the presence of enzymes or reactive chemicals and exposure to extreme pH, temperature, and high ion concentrations (Mozafari et al. 2008b). As liposomes could be prepared from naturally occurring components, such as lecithin, which are a cheap and rich source of phosphatidylcholine (PC) (Mertins et al. 2008), regulatory hurdles that may prevent their application in food systems are reduced (Taylor et al. 2005; Mozafari et al. 2008a).

Encapsulation of bacteriocins into liposomes is reported to be achieved mainly by the thin-film hydration method (Malheiros et al. 2010a). The important and desired characteristics are the formation of vesicles with the right size, acceptable polydispersity, structure, and encapsulation efficiency (Mozafari et al. 2008a). Knowledge on liposome properties is required to develop nanovesicles that have optimal entrapment efficiencies and allow the controlled release of antimicrobials (Were et al. 2003). Furthermore, the size and morphology of liposome has a strong influence on their distribution within food systems (Mozafari et al. 2008b).

The objectives of this study were to formulate liposomes prepared from partially purified PC bearing the antimicrobial peptide P34 and determine their stability during storage, encapsulation efficiency, and morphology. The mode of action of encapsulated peptide P34 on *Listeria monocytogenes* was also evaluated.

Materials and methods

Production of peptide P34

The antimicrobial peptide P34 was produced by *Bacillus* sp. P34. This strain was isolated from the intestinal contents of Piau-com-pinta (*Leporinus* sp.) fish of the Amazon basin, Brazil (Motta et al. 2004). In brief, *Bacillus* sp. P34 was grown in BHI broth (Oxoid, Basingstoke, UK) in a rotary shaker for 24 h at 30 °C. The cells were harvested by centrifugation, and the supernatant was sterilized with 0.22-μm filter membranes (Millipore, Bedford, MA, USA). The peptide was purified by precipitation with 20% (w/v) ammonium sulfate and gel filtration chromatography in a Sephadex G-100 column (Pharmacia Biotech, Uppsala, Sweden). The active fractions were pooled and stored at 4 °C (Motta et al. 2007a).

Peptide P34 encapsulation

Peptide P34 was encapsulated by the thin-film hydration method (Malheiros et al. 2010b) in liposomes of partially purified soybean phosphatidylcholine (PC-1),

composed of 75% distearoylphosphatidylcholine, 12% dioleoylphosphatidylcholine, and 8% dipalmitoylphosphatidylcholine (Mertins et al. 2008). The lipid was dissolved in chloroform in a round-bottom flask, and the organic solvent was removed by a rotary evaporator until a thin film was formed on the flask walls. The lipid film was suspended in phosphate buffer containing peptide P34 and vigorously vortexed at 60 °C. Then, the preparation was sonicated in a bath-type ultrasound (Unique USC 700, Indaiatuba, Brazil) for 30 min. The liposome-encapsulated BLS P34 was sterilized by filtration with 0.22 µm membranes.

Antimicrobial activity assay

The antimicrobial activity was detected by agar diffusion assay. Aliquots (10 µl) of free and liposome-encapsulated P34 were applied on BHI agar plates previously inoculated with a swab submerged in a suspension of *L. monocytogenes* ATCC 7644 (7 log colony-forming units (CFU) per mL). Plates were incubated at 37 °C for 24 h. The reciprocal value of the highest dilution that produced an inhibition zone was taken as the activity units (AU) per mL (Motta and Brandelli 2002). Antimicrobial activity was evaluated immediately after the liposomes preparation and was monitored periodically for 24 days for free and encapsulated P34 stored at 4 °C (Malheiros et al. 2010b).

Characterization of nanovesicles

Liposome size and polydispersity index (PDI) were determined by Dynamic Light Scattering (DLS) in a Brookhaven Instruments standard setup (BI-200M goniometer, BI-9000AT digital correlator) (Mertins et al. 2006). DLS measurements were performed immediately after the liposomes preparation and were monitored periodically for 24 days for filtered liposomes stored at 4 °C. The pH values of free and encapsulated P34 were determined at room temperature using a pH stripe. The zeta potential of encapsulated P34 were carried out after dilution of the formulations in 1 mM NaCl using a Zetasizer® nano-ZS ZEN 3600 equipment (Malvern Instruments, Herrenberg, Germany). The entrapment efficiency (EE) of liposome-encapsulated P34 was determined by measuring the antimicrobial activity by agar diffusion method as described above. Encapsulated P34 was separated from non-encapsulated P34 by ultrafiltration (Ultracel YM-30 and YM-50 Membrane, Millipore). EE was calculated according to Laridi et al. (2003).

Transmission electron microscopy

Transmission electron microscopy (TEM) was employed to monitor interactions between liposome and bacteria. Overnight cultures (10^9 CFU/mL) of *L. monocytogenes* ATCC 7644 were mixed with liposome-encapsulated BLS P34 and incubated for 1 h at 30 °C with agitation. Cells were harvested by centrifugation and washed twice and 0.1 M phosphate buffered (pH 7.3). The cells were fixed with 2.5% (v/v) glutaraldehyde for 10 days; (2.5% glutaraldehyde + 2% paraformaldehyde + 0.12 M phosphate buffer) and then postfixed in 2% (w/v) osmium tetroxide in the same buffer for 45 min before dehydration. Dehydration was done in a graded acetone series (30–100%) and embedding in Araldite-Durcupan for 72 h at 60 °C. Thin sections were mounted on grids, covered with collodion film, and post-stained with 2% uranyl acetate and Reynold's lead citrate.

Furthermore, morphological examination of liposomes was performed by TEM with negative staining. Liposome-encapsulated P34 suspension was diluted 10-fold in phosphate buffer, and the sample was deposited on a sample grid and negatively stained with uranyl acetate solution (2%, w/v).

All preparations were observed using a JEOL JEM 1200ExII transmission electron microscope (JEOL, Tokyo) operating at 120 kV.

Effect of free and encapsulated P34 on *L. monocytogenes*

An overnight culture of *L. monocytogenes* was obtained in BHI medium at 37 °C for 18 h. Kinetics of the effect of free and encapsulated P34 on *L. monocytogenes* was determined at 30 °C in aeration with a final P34 concentration of 800 AU/mL. Viable cell counts were determined during 4.5 h of incubation. The control was taken with addition of 10 mM sodium phosphate buffer pH 7.0. Each experiment was run two separate times with duplicate analysis in each replicate.

Statistical analysis

Results of independent experiments were compared using Tukey's test by Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA). Means were considered significantly different at $p < 0.05$.

Results and discussion

The bacterial resistance to conventional antimicrobial agents is a major problem for the pharmaceutical and food industries. In this context, a lot of research is being conducted to formulate new classes of natural antimicrobials. Peptide P34 was purified and characterized, showing excellent properties to control pathogenic and spoilage microorganisms (Motta et al. 2007a). The encapsulation and targeting of bacteriocins using nanovesicles is a current approach of great interest to science and industry. In a previous study, nisin was easily encapsulated in liposomes prepared from partially purified PC-1 (Malheiros et al. 2010b), an inexpensive product with low risk of toxicity, encouraging continued research for the encapsulation of new antimicrobial peptides such as P34.

In this study, the antimicrobial activities of free and nanovesicle-encapsulated P34 were evaluated during storage at 4 °C (Table 1). A concentration of 3,200 AU/mL of P34 was encapsulated in PC-1 liposomes. After encapsulation the antimicrobial activity was 2,600 AU/mL in average. Differences in the biological activity of free and encapsulated bacteriocin stored under refrigeration temperature were not significant ($p > 0.05$) for up to 24 days. The residual antimicrobial activity of liposome-encapsulated nisin was 50% by the sixth day and remained at 25% for up to 24 days (Malheiros et al. 2010b). Taylor et al. (2008) reported the use of liposomes containing nisin within 72 h of their production. In contrast, a bacteriocin-like substance from *Bacillus licheniformis*, encapsulated by reverse-phase method remained approximately 90% of its initial activity after 30 days, whereas free bacteriocin maintained its activity for only 14 days (Teixeira et al. 2008).

The size stability of nanovesicles containing the antimicrobial substance stored for 24 days under refrigeration is presented in Table 2. The liposomes presented average sizes of 150 nm, acceptable PDI and were relatively monodisperse according to light scattering analysis. PDI values around 0.2 indicated low liposomes polydispersity. This value is expected for systems prepared from biological materials, in which PDI ranges from 0.2 to 0.3. Nanovesicles containing the peptide P34 were physically stable, which corroborate our previous study (Malheiros et al. 2010b), where the size and PDI of the liposome-encapsulated nisin remained constant (132–149 nm by 24 days). Furthermore, in this study, liposomes were filtered through 0.22 μm membranes because previous results demonstrated the maintenance of the integrity of vesicles and their sterilization, which are very important requirements for further application in food systems (Malheiros et al. 2010c).

Table 1 Antimicrobial activity of free and encapsulated peptide P34 as a function of storage time at 4 °C

Day	Antimicrobial activity (AU/mL)	
	Free	Encapsulated
0[a]	3,200	2,600
3	1,600	1,400
6	2,000	2,000
10	1,400	1,200
13	1,600	1,400
16	1,600	1,200
20	2,000	1,200
24	1,400	1,000

[a] Means between free and encapsulated P34 were not significantly different ($p > 0.05$)

Table 2 Effective diameter, PDI and zeta potential of liposomes containing peptide P34 as a function of storage time at 4 °C

Day	Size (nm)	PDI	Zeta potential (mV)
0[a]	162.9 ± 13.3	0.218 ± 0.049	−27.42 ± 9.39
3	154.8 ± 16.1	0.254 ± 0.049	ND
6	149.8 ± 11.2	0.262 ± 0.041	−24.90 ± 5.30
9	157.7 ± 17.6	0.229 ± 0.040	ND
12	155.3 ± 12.0	0.269 ± 0.047	ND
15	148.5 ± 11.8	0.237 ± 0.038	−25.52 ± 9.08
18	153.3 ± 15.2	0.221 ± 0.039	ND
21	144.6 ± 15.1	0.245 ± 0.052	−25.72 ± 8.38
24	145.9 ± 15.1	0.228 ± 0.052	ND

ND not determined

[a] Means of size, PDI and zeta potential were not significantly different ($p > 0.05$) through the time

The pH of the solution of free and encapsulated BLS P34 was close to neutrality (6.5–7.0) and remained constant for up to 24 days (data not shown).

The zeta potential was −27.42 mV (Table 2). Zeta potential measures the surface charge of particles. As the zeta potential increases, the surface charge of the particles will be also increased. Zeta potential can greatly influence particle stability in suspension through the electrostatic repulsion between particles (Hans and Lowman 2002; Taylor et al. 2007). In this study, zeta potential was negative and remained constant over a period confirming the stability of nanovesicles containing P34 (Table 2). The stability of the appetite-stimulating peptide hormone ghrelin was evaluated after liposome encapsulation (Moeller et al. 2010). The authors showed that to empty neutral dipalmitoylphosphatidylcholine liposomes the polydispersity was larger, and sedimentation occurred. However, when ghrelin was encapsulated in the same liposome the sedimentation disappeared and the polydispersity decreased. Zeta potential increased from −0.34 to 8.34 mV after ghrelin encapsulation. According to the authors, the positively charged peptide would induce an electrostatic repulsion between the vesicles on binding to the liposome, and the decrease in the polydispersity indicated that some degree of binding takes place (Moeller et al. 2010). In fact, the incorporation of different peptides into liposomes might have variable effects on size, zeta potential, and shape of liposomes (Silva et al. 2008).

The encapsulation efficiency of P34 in PC-1 was 100%. Peptide P34 has a molecular mass of about 1.5 kDa, and although aggregates of this peptide can be formed in aqueous solution (Motta et al. 2007b), the use of ultrasound on the preparation of liposomes probably would separate the molecules leaving them in small sizes than 30 kDa. To separate free from encapsulated P34, a 30 KDa filter was tested, and after a 50 kDa filter. The encapsulation and/or binding of P34 to nanovesicles was the same (100%) for both filters. The entrapment efficiency is a function of lipid composition and may be attributed to electrostatic and hydrophobic interactions between antimicrobials and phospholipids (Were et al. 2003). Many studies report that nisin, a cationic peptide, presents higher EE in neutral phospholipids, such as PC, than liposomes containing anionic lipids, such as phosphatidylglycerol (Laridi et al. 2003; Were et al. 2004; Taylor et al. 2008). The lipid used for encapsulation of P34 has a higher amount of PC; however it contains other lipids that may modify the membrane charge. Encapsulated nisin prepared with the same lipid (PC-1) used in this study showed zeta potential of −52.28 mV and EE of 94.12% (Malheiros et al. 2010b). Similarly, the zeta potential of liposome-encapsulated P34 showed a negative value. The peptide P34 presents some characteristics resembling the antimicrobial peptide fengycin (Motta et al. 2007b). The interaction of fengycin with dipalmitoylphosphatidylcholine monolayers was studied, and it is found that they form a partially miscible mixed monolayer at a molar ratio $\leq$0.5 (Deleu et al. 2005). Therefore, the insertion of peptide P34 into PC vesicles could possibly be due to hydrophobic interactions and association with PC structures (Were et al. 2003; Teixeira et al. 2008).

The nanovesicles presented an almost spherical morphology and were possible to observe three-dimensional structures in images obtained by TEM (Fig. 1). The morphology of liposomes containing P34 observed by TEM is in agreement with that typically defined for liposomes (Mozafari et al. 2002). Chetanachan et al. (2008) showed the benefit of using TEM with negative staining technique to investigate the morphology of liposomes produced by thin film method. The size of liposomes containing P34 seems to be approximately 150 nm, and is in agreement with the light scattering analysis (Table 2). Moreover, the nanovesicles showed similar size in the evaluation by TEM reinforcing the suggestion of a monodisperse system. In contrast, some authors found higher size when the liposome was evaluated by DLS in comparison with TEM (Wang et al. 2010). The authors attribute that this may have occurred because the size measured by DLS is related to the hydrodynamic diameter while evaluation by TEM is related to the dry state. Liposomes developed in this study were sonicated to make them smaller, more homogeneous, and unilamellar, as evidenced by TEM images. When the liposomes are not subjected to several steps, such as membrane extrusion, heating or sonication, multilamellar vesicles are formed (Mozafari 2005; Malheiros et al. 2010a).

The interaction between nanovesicles containing P34 and *L. monocytogenes*, a gram-positive pathogen of great importance in the food industry, was evaluated by TEM. The liposome-encapsulated BLS P34 surrounded *L. monocytogenes* cells after 1 h of

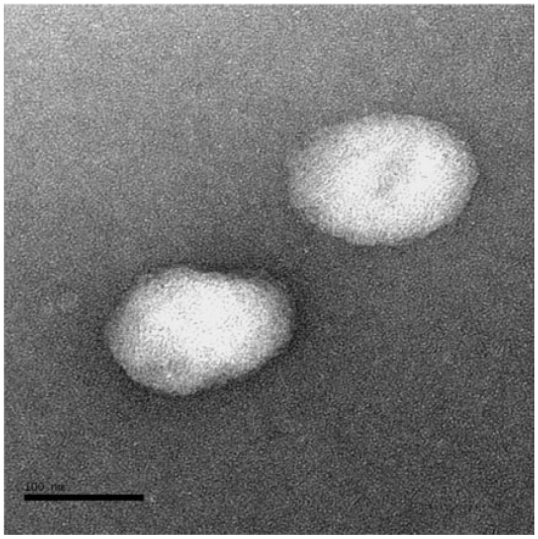

Fig. 1 Transmission electron microscopy image of liposome-encapsulated peptide P34 prepared with partially purified soybean phosphatidylcholine. Bar = 100 nm

incubation at 30 °C. Liposomes appeared to be adhered but not to fuse with the bacteria (Fig. 2). In this sense, it is suggested that the peptide needs to be released from the liposomes to act in the cell membrane of target bacteria. Free peptide P34 acts through vesiculization of the protoplasm, pore formation, and disintegration of *L. monocytogenes* cells (Motta et al. 2008). Colas et al. (2007) demonstrated that empty nanoliposomes surrounded *B. subtilis* cells, and a few of them fused with the bacterial membrane after 2 h of incubation at 37 °C.

The incubation of encapsulated P34 did not cause a decrease in the number of viable cells of *L. monocytogenes* during 4.5-h incubation (Fig. 3). However, an increase in the viable counts of *L. monocytogenes* was observed in control group and the differences with the cells treated with encapsulated P34 were significant from 2-h incubation. After 4.5 h, the control group presented viable counts of about 1 log higher than the encapsulated P34. In the case of free peptide, pathogen inhibition was fast, although the bactericidal effect was not observed until 4.5 h. The release of encapsulated P34 and consequent inhibition of target pathogen may occur at a longer period in comparison with the free P34. In this context, a feasible alternative could be the combined food application of the free and encapsulated P34, allowing a rapid inhibitory effect against the target pathogens caused by free P34 and a

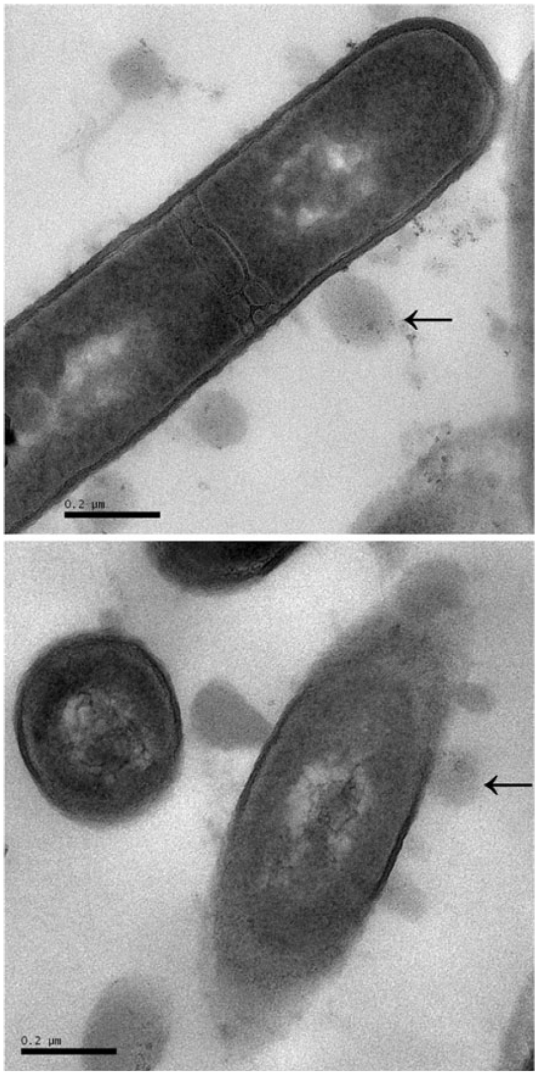

Fig. 2 Transmission electron microscopy showing the interaction of nanovesicle encapsulated P34 (*arrows*) with *L. monocytogenes*. *Upper panel* longitudinal section; *bottom panel* transversal section. Bar = 200 nm

prolonged inhibition to be achieved by encapsulated P34. This alternative would be interesting to increase the shelf-life of foods without the addition of high doses of antimicrobials aiming at a bactericidal effect.

Conclusion

This study provides further evidence that the liposome encapsulation of bacteriocins can be performed using

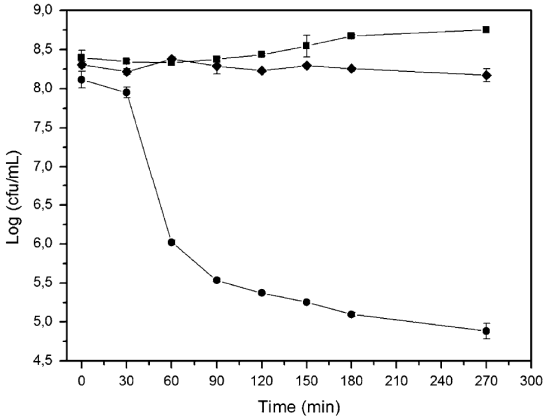

Fig. 3 Effect of free peptide P34 (*filled circle*), encapsulated peptide P34 (*filled diamond*), and control (*filled square*) on the growth of *L. monocytogenes* incubated for 270 min. Each point represents the means of duplicates of two independent experiments

a inexpensive source of lipid. The liposomes containing the antimicrobial peptide P34 developed in this study showed excellent physicochemical characteristics demonstrated by high encapsulation efficiency and stability during storage. Although the fusion of liposome-encapsulated P34 and the membrane of target bacteria was not observed, the antimicrobial peptide is able to be released from the vesicles, as evidenced by the antimicrobial activity. In this sense, it is suggested that the encapsulation this new antimicrobial in liposomes may have a true potential for use as food preservative in hurdle technology. Experiments to test its potential applications in milk products are under investigation in our laboratory.

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3.6 ARTIGO 6

3.6.1 Antilisterial activity and stability of nanovesicle-encapsulated antimicrobial peptide P34 in milk

3.6.2 Abstract

Bacillus sp. P34, a strain isolated from aquatic environments of Brazilian Amazon basin, produces a bacteriocin-like substance (BLS) which was encapsulated in nanovesicles prepared from partially purified soy lecithin. The efficiency of free and encapsulated BLS P34 to control the development of *L. monocytogenes* and maintenance of antimicrobial activity was assessed over time in UHT milk. The antimicrobial activity of free and encapsulated BLS P34 decreased approximately 50% after 4 days of storage (<4 ºC) in skim and whole milk. After this period there was not significant loss of activity up to 21 days. The viable counts of *L. monocytogenes* in skim and whole milk containing 3200 AU/ml of free or encapsulated BLS P34 were always lower than those observed in controls without bacteriocin at both 30 ºC and 7 ºC. At 1600 AU/ml concentration, free and encapsulated BLS P34 were inhibitory to *L. monocytogenes* in skim milk, when compared with the control at 7 days. Nanovesicle-encapsulated and free BLS P34 shows potential use as biopreservative for application in milk-derived products.

Keywords: liposome; nanovesicles; bacteriocin; *Listeria monocytogenes*; milk; *Bacillus*

3.6.3 Introduction

Bacteriocins are antimicrobial peptides widespread produced among bacteria that may show varied antimicrobial spectra (Cotter, Hill, & Ross, 2005). Those produced by Gram-positive bacteria, particularly by lactic acid bacteria, have been largely studied with the perspective of food protection against pathogenic and spoilage microorganisms (Cleveland, Montville, Nes, & Chikindas, 2001; Arauz, Jozala, Mazzola, & Penna, 2009). Their proteinaceous nature implies a putative degradation in the gastro-intestinal tract of human and animals, suggesting that some bacteriocin-producing lactic acid bacteria or purified bacteriocins could be used as natural preservatives in foods (Cleveland et al., 2001). Bacteriocins are also produced by several other classes of bacteria (Riley & Wertz, 2002).

Bacillus has been reported as a safe and interesting bacterial genus for utilization in food industry (Pedersen, Bjørnvad, Rasmussen, & Petersen, 2002). Production of bacteriocins or bacteriocin-like substances (BLS) has been described for many species of the genus *Bacillus* including *B. subtilis* (Zheng, Yan, Vederas, & Suber, 1999), *B. thuringiensis* (Kamoun et al., 2005), *B. amyloliquefaciens* (Lisboa, Bonatto, Bizani, Henriques, & Brandelli, 2006), *B. licheniformis* (Cladera-Olivera, Caron, & Brandelli, 2004) and *B. cereus* (Bizani & Brandelli, 2002). *Bacillus* sp. strain P34 was isolated from aquatic environments of Brazilian Amazon basin, and its antimicrobial activity was described as a BLS active against important Gram-positive pathogenic bacteria like *Listeria monocytogenes* and *Bacillus cereus* (Motta, Cladera-Olivera, & Brandelli, 2004). This antimicrobial substance has a molecular mass of 1,456

Da, was relatively heat stable and sensitive to proteolytic enzymes (Motta, Cannavan, Tsai, & Brandelli, 2007a). In cytotoxicity tests, the peptide P34 shows similar behavior to nisin, a bacteriocin wide accepted for utilization in food industry (Vaucher, Motta, & Brandelli, 2010).According to its properties of size and protein stability data, BLS P34 could be associated with the group of Listeria-active class Ib bacteriocins (Motta, Lorenzini, & Brandelli, 2007b). This antimicrobial substance has the cell membrane as target of action, promoting loss of protoplasmic material and consequently the death of the cell (Motta, Flores, Souto, & Brandelli, 2008).

Bacteriocins or bacteriocin-like substances can lose their antimicrobial activity in many food products for a variety of reasons. Interference and cross-reactions of the antimicrobial with various food constituents, such as protein and fat, are difficult to overcome and often require large amounts of antimicrobial in order to gain significant reductions in the pathogen load in a product (Taylor, Bruce, Weiss, & Davidson, 2008). The efficiency of these peptides in food products may be increased by their incorporation into liposome, enhancing the stability by a protective effect against endogenous food proteases or binding to food compounds (Laridi et al., 2003; Were, Bruce, Davidson, & Weiss, 2003; Teixeira, Santos, Silveira, & Brandelli, 2008; Malheiros, Daroit, Silveira, & Brandelli, 2010a, Malheiros, Daroit, & Brandelli, 2010b). Liposome, a closed phospholipid bilayer membrane, has a nano-order interface harboring hydration layer and non-polar (hydrophobic) layer on its surface. Due to the presence of both lipid and aqueous phases in the structure of lipid vesicles, they can be utilized in the entrapment, delivery, and release of water-soluble, lipid-soluble,

and amphiphilic materials (Khosravi-Darani, Pardakhty, Honarpisheh, Rao, & Mozafari, 2007; Mozafari, Johnson, Hatziantoniou, & Demetzos, 2008).

Nanovesicles to delivery of bioactive components with substantiated health benefits of the foods will be required to meet the challenges in developing healthy foods, which are aimed at reducing the risks of target diseases in a population (Sanguansri & Augustin, 2006). A microorganism that causes disease is *Listeria monocytogenes,* which is capable of surviving environmental conditions that are normally fatal to many other bacteria, enabling this pathogen to remain active in foods and eventually establish infection after consumption (Cunningham, O'Byrne, & Oliver, 2009). The aim of the present study was to assess the effect of free and nanovesicles-encapsulated BLS P34 against *L. monocytogenes* in milk. Thereafter, the maintenance of antimicrobial activity was assessed over time in milk.

3.6.4 Materials and methods

Bacterial strains and media

Bacillus sp. P34, an isolate from intestine of the teleost Piau-com-pinta (*Leporinus* sp.) from Amazon basin, was the producer strain and was previously characterized (Motta et al., 2004). *Listeria monocytogenes* ATCC 7644 was used as the indicator organism for the bacteriocin activity assay. The strains were maintained on BHI agar plates at 4 ºC, and subcultured periodically. Before each experiment, this strain was grown in BHI medium at 37 ºC for 18-24 h in a rotary shaker (180 rpm).

Production of BLS P34

For the production of BLS, the producer strain was aerobically cultivated in 150 ml BHI-medium in orbital shaker at 180 rpm for 24h. The culture was centrifuged at 10,000 *g* for 15 min at 4 ºC, and the supernatant was sterilized with a 0.22 µm membranes. The filtrate was precipitated with ammonium sulfate at 20% saturation. The precipitate was dissolved in 10 mmol/l sodium phosphate buffer pH 7.0. This solution was further purified by gel filtration chromatography on a Sephadex G-100 column, and active fractions were pooled. The partially purified BLS P34 was stored in sterile flasks at 4℃ until used (Motta et al., 2007b).

BLS P34 encapsulation

Encapsulation of BLS P34 in nanovesicles of partially purified soybean phosphatidylcholine (PC-1; Mertins, Sebben, Schneider, Pohlmann, & Silveira, 2008) was carried out by the thin-film hydration method according to Malheiros et al. (2010c). Summarizing, PC-1 was dissolved in chloroform and the solvent was removed by a rotary evaporator until a thin film was formed on the flask walls. The dried lipid film was dispersed by the addition of sodium phosphate buffer containing BLS P34. These mixtures were then agitated (60 ºC) and sonicated in bath-type ultrasound (40 kHz, Unique USC 700). The size of nanovesicles was determined by light scattering (Teixeira et al., 2008). The entrapment efficiency (EE) was determined using antimicrobial activity assay. Discrimination of encapsulated from non-encapsulated BLS P34 was performed

by ultrafiltration (Ultracel YM-50 Membrane, Millipore) and the EE was calculated (Malheiros et al., 2010c).

Antimicrobial activity assay

Antimicrobial activity was determined essentially as described elsewhere (Motta and Brandelli, 2002). BLS P34 was diluted by serial two-fold dilution method (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128), and an aliquot of 10 ml each dilution was applied onto BHI agar plates inoculated with a swab submerged in *L. monocytogenes* ATCC 7644 (approximately 7 log CFU/ml). Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and was expressed as activity unit (AU) per milliliter.

Determination of BLS P34 activity in milk

UHT skim milk (0% fat) and UHT whole milk (3.25% fat) were purchased from a local market. Free and nanovesicles-encapsulated BLS P34 was independently applied to skim and whole milk to produce an initial activity of 800 AU/ml. The samples were kept under refrigeration (4 ºC) and the antimicrobial activity was evaluated by 21 days as described above.

Inhibitory effect of free and encapsulated BLS P34 in milk

L. monocytogenes culture (9 log CFU/ml) was diluted in saline solution (8.5 g/l NaCl) to count approximately 6 log CFU/ml, and 500 µl of this suspension was added to 10 ml of whole and skim milk. After, 1 ml of free and nanovesicles-encapsulated BLS P34 (3200 AU/ml) was added to tube. Controls

were inoculated with *L. monocytogenes* and sodium phosphate buffer (10 mmol/l pH 7.0). The tubes were incubated for 0, 2, 4, 6 and 24 h at 30 ºC.

The effect of 1600 AU/ml and 3200 AU/ml of free and encapsulated BLS P34 was assessed in whole and skim milk at refrigeration temperature (7 ± 1 ºC). Counts of *L. monocytogenes* were estimated at 0, 7 and 14 days for 1600 AU/ml and 0, 2, 5, 8, 10 and 14 days for 3200 AU/ml (Malheiros et al., 2010a). The quantification of *L. monocytogenes* was performed by the drop culture method (Malheiros, Brandelli, Noreña, & Tondo, 2009). The colonies were counted in BHI agar plates after 24 h of incubation at 37 ºC.

Statistical analysis

All experimental treatments were tested in duplicate, and averages were calculated for treatments at each time point. Obtained counts were compared using test *t* of Tukey. Data analyses were performed with the software Statistica 7.1, and differences were considered significant at $P<0.05$.

3.6.5 Results

Encapsulation of BLS P34

BLS P34 was encapsulated into nanovesicles of partially purified soybean phosphatidylcholine by hydration film method. Nanovesicles presented a mean diameter of 160 nm and the entrapment efficiency was 100%.

Antimicrobial activity of free and encapsulated BLS P34 in milk

The antimicrobial activities of free and encapsulated BLS P34 were evaluated for a period of 21 days in milk (Fig. 1). It was observed that there was no significant difference (P>0.05) in antimicrobial activity between skim and whole milk. There was a loss of approximately 50% of antimicrobial activity after 4 days of storage at 4 ºC in both samples containing free or encapsulated BLS P34. After this period there was no significant loss of activity for up to 21 days for all samples and treatments.

Inhibitory effect of free and encapsulated BLS P34 in milk

Free BLS P34 (3200 AU/ml) caused a decrease in *L. monocytogenes* counts below the detection limit of the method on skim milk at 30 ºC during 6 h; however, after 24 h of incubation the microbial counts were of approximately 4 log CFU/ml. When encapsulated BLS P34 was tested, it was observed 2 log reduction in counts of *L. monocytogenes* at 6 h without significant increase until 24 h (Fig. 2A). A decrease of 4 log cycles in *L. monocytogenes* counts was observed for free and encapsulated BLS P34 versus control at 24 h in skim milk at 30 ºC. In the case of whole milk at 30 ºC, it was not observed significant growth of *L. monocytogenes* for free and encapsulated BLS P34 until 6 h (Fig. 2B). Only at 24 h there was significant (P<0.05) difference on growth of *L. monocytogenes* for both treatments compared to control, showing 1-2 log cycles lower than the not treated milk.

Two concentrations of BLS P34 were tested for refrigeration temperature (7 ± 1 ºC). At lower concentration (1600 AU/ml) in skim milk, free and encapsulated BLS P34 were inhibitory to *L. monocytogenes* cells

compared to the control (~2.5 log CFU/ml reduction) at 7 days (Fig. 3A). In whole milk both treatments tested showed no inhibitory effect on the pathogen for up to 14 days (Fig. 3B).

A concentration of 3200 AU/ml of free or encapsulated BLS P34 was very effective in reducing viable counts in skim milk (Fig. 4A). In the second day of incubation encapsulated BLS P34 showed a decrease of 2.5 log CFU/ml compared to the control, whereas free BLS P34 caused a decrease in *L. monocytogenes* counts below the detection limit of the method. After, there were no significant differences ($P>0.05$) between the treatments. Free and encapsulated BLS P34 treatments consistently lowered *L. monocytogenes* counts below the detection limit of the method at days 5 and 8, increasing thereafter. In whole milk, free and encapsulated BLS P34 presented no significant reduction ($P<0.05$) in bacterial population during the first 2 days. However, after 5 days both treatments decreased the population of *L. monocytogenes* by 1-2 log cycles compared to the control (Fig 4B).

3.6.6 Discussion

We have earlier reported that BLS P34 was inhibitory to a broad spectrum of indicator strains, including several spoilage and pathogenic microorganisms (Motta et al., 2007a). Moreover, the purified antimicrobial substance P34 presented low *in vitro* toxicity to eukaryotic cells with similar effect to that observed for nisin, suggesting to be safe for food use (Vaucher, Motta, & Brandelli, 2010).

In this study, the antimicrobial activity of free and nanovesicles-encapsulated BLS P34 was assessed in skim and whole milk as a function of storage time at 4 °C. The initial decrease (after 4 days) of antimicrobial activity of BLS P34 in skim and whole milk may be associated to interaction with the milk proteins, which can bind somehow with BLS P34 as suggested for nisin (Jung, Bodyfelt, & Daeschel, 1992). It was observed that the milk fat content and the encapsulation in nanovesicles did not influence the antimicrobial activity of BLS P34. In contrast, the negative effect of fat on antimicrobial activity of nisin is extensively reported, caused by the peptide adsorption onto fat globules (Jung et al., 1992; Bhatti, Veeramachaneni, & Shelef, 2004; Chollet, Sebti, Martial-Gros, & Degraeve, 2008). Jung et al. (1992) reported that nisin activity against *L. monocytogenes* dropped by 33% in skim milk, by 50% in milk with 1.2% fat, and by 88% in milk with 12.9% fat. More recently, Chollet et al. (2008) observed that a 30% fat (w/w) level in gel gave rise to a significant decrease ($P<0.01$) in nisin antimicrobial activity against *K. rhizophila.* Encapsulation of BLS P34 in liposomes maintains the same inhibitory effect against *L. monocytogenes* than free BLS P34. Teixeira et al. (2008) suggest controlled release of the antimicrobial peptide from the vesicle and/or slower diffusion on semisolid agar medium, since to these authors a higher dosage of liposome-encapsulated bacteriocin was necessary to obtain the same inhibitory effect observed for the free bacteriocin.

Although the residual antimicrobial activity of free and encapsulated BLS P34 was similar in skim and whole milk, the inhibition of *L. monocytogenes* by free and encapsulated BLS P34 was especially observed in skim milk. In

accordance, the antimicrobial peptide nisin was more effective in reducing the initial population of *L. monocytogenes* in brain-heart infusion broth and in skim milk than fat milk (Jung et al., 1992). Therefore, higher concentration of nisin it is necessary to control the growth this bacteria in whole milk (Malheiros et al., 2010a).

Bacteriostatic effect was observed in skim milk at 30 ºC using encapsulated BLS P34 (3200 AU/ml) treatment. However, when free BLS P34 was tested, *L. monocytogenes* were reduced to counts below the detection limit during the first 6 h. Therefore, the free BLS P34 inhibited the target pathogen in the first moment possibly by injuring the bacteria, while encapsulated BLS P34 was released over time maintaining the same initial count by 24 h. Nisin, an antimicrobial peptide recognized as safe for food applications by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additivies, was encapsulated in liposomes and tested against *L. monocytogenes* in BHI medium and skim milk at 30 ºC (Malheiros et al., 2010a). At such abuse temperature conditions, these authors found that free nisin showed better inhibitory than the liposomal counterparts. Taylor et al. (2008) evaluated the effect of nisin encapsulated in purified phosphatidylcholine liposomes to inhibition of *L. monocytogenes* growth. Those authors observed inhibition of about 60% of pathogens by free and encapsulated nisin after 48 h of incubation at 32 ºC on double-strength BHI medium. Antimicrobial activity of bacteriocins in food matrix is affected by several factors, such as temperature to storage, fat and protein content and cation concentrations. This was

demonstrated in the work from Branen and Davidson (2004), when nisin did not inhibited *L. monocytogenes* in 2% fat milk stored at 25 ºC for 24 h.

Bacteriocins have been used to inhibit *L. monocytogenes* in fluid systems. The addition of the antimicrobial peptide cerein 8A (final concentration of 160 AU/ml) to UHT milk resulted in a decrease of 3 log cycles in viable cells of *L. monocytogenes* within the 14-day period at 4 ºC (Bizani, Morrissy, Dominguez, & Brandelli, 2008). In this study free and encapsulated BLS P34 at final concentration of 160 AU/ml was bacteriostatic by 7 days at 7 ºC in skim milk. However, according to Motta et al. (2007a) this same antibacterial substance was bactericidal and bacteriolytic (final concentration of 160 AU/ml) to *L. monocytogenes* after 6 h of growth in TSB medium at 37 ºC. In whole milk the treatments tested were not effective on the decrease of *L. monocytogenes* growth at 7 ºC.

Free and encapsulated BLS P34 at 3200 AU/ml consistently lowered *L. monocytogenes* counts below the detection limit of the method at days 5 and 8 in skim milk. In whole milk, after 5 days both treatments decreased the population of *L. monocytogenes* by 1-2 log cycles compared to the control. Malheiros et al. (2010a) observed that in skim milk, encapsulated and free nisin (0.5 and 0.1 mg/ml) treatments consistently lowered *L. monocytogenes* counts below the detection limit (<1.69 log CFU/ml) during a period of 14 days. However, in whole milk, only the highest concentration (0.5 mg/ml) of nisin reduced bacterial counts. It is important to note that antilisterial activities of nisin are well documented; however chemical composition and treatment of a food may play an important role in its antilisterial effects (Bhatti et al., 2004).

Antimicrobials peptides could be present both in the aqueous and lipids phases of liposomes. Then, might be able to effectively provide both short-term (by release of encapsulated bacteriocin) and long-term (desorption of membrane-immobilized bacteriocin) antibacterial actions (Benech, Kheadr, Lacroix, & Fliss, 2002; Taylor et al., 2008).

3.6.7 Conclusions

The present study demonstrated that the milk fat had not a significant effect on the activity of free and encapsulated BLS P34, but influenced on the ability of bacteriocin to inhibit *L. monocytogenes* growth in fluid milk. The encapsulation of BLS P34 in nanovesicles of partially purified soy phosphatidylcholine was relatively inexpensive as required by the food industry. In general, free and encapsulated BLS P34 appeared to be effective to control *L. monocytogenes* in milk, especially skim milk, suggesting its potential use as biopreservative in food products. Therefore, the use of new bacteriocin in combination with nanotechnology, suggested as the new revolution in many industries, was useful to control an important pathogen in the dairy industry. This research addresses the feasibility of the use of new antimicrobial peptides and nanotechnology to improve the safety of food. Further study, regarding the maintenance of antimicrobial activity and ability of free and encapsulated BLS P34 to inhibit *L. monocytogenes* growth over time, will be conducted using fluid milk and frescal cheese.

3.6.8 References

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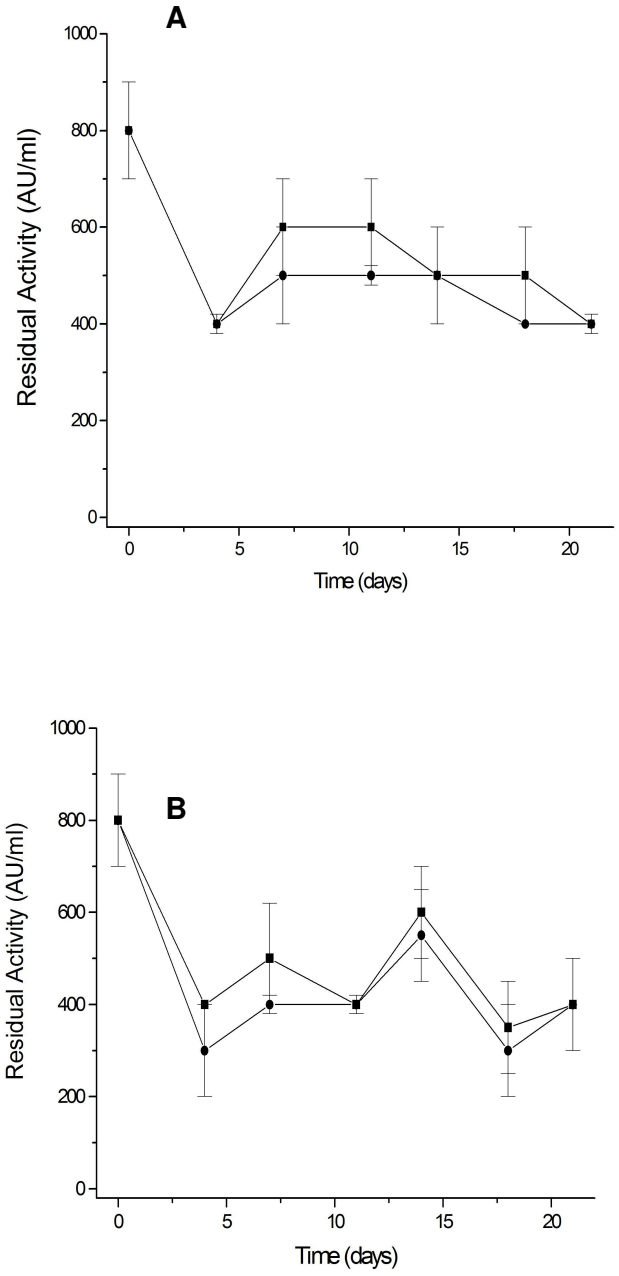

Figure 1. Residual antimicrobial activity of free and encapsulated BLS P34 in skim milk (A) and whole milk (B) as a function of storage time at 4°C. (■) Free BLS P34 and (●) encapsulated BLS P34. Values are means of two independent experiments.

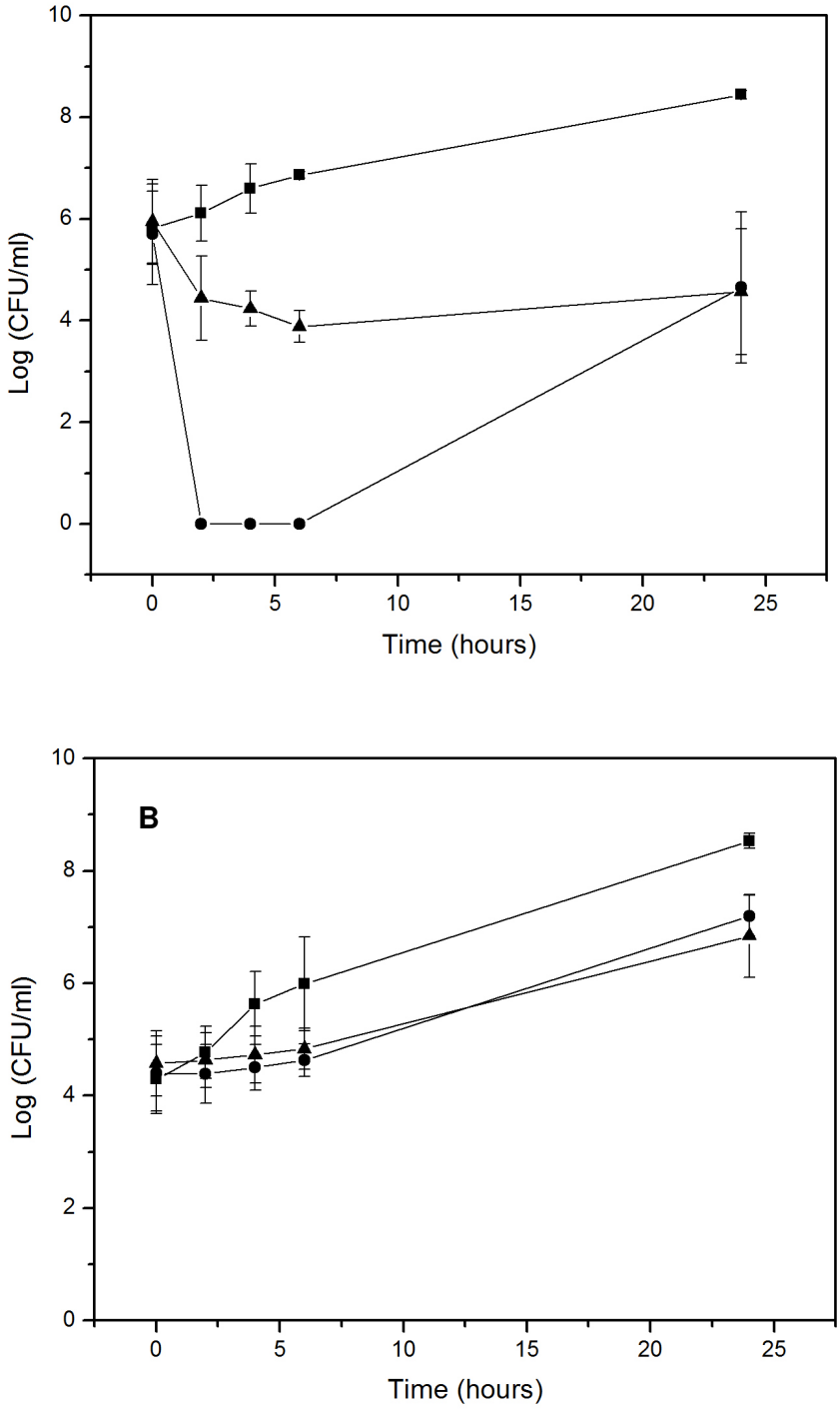

Figure 2. Viable cell counts of *Listeria monocytogenes*, confronted to 3200 AU/ml of free BLS P34 (●), encapsulated BLS P34 (▲) and control (■) in skim milk (A) and whole milk (B) at 30 ºC.

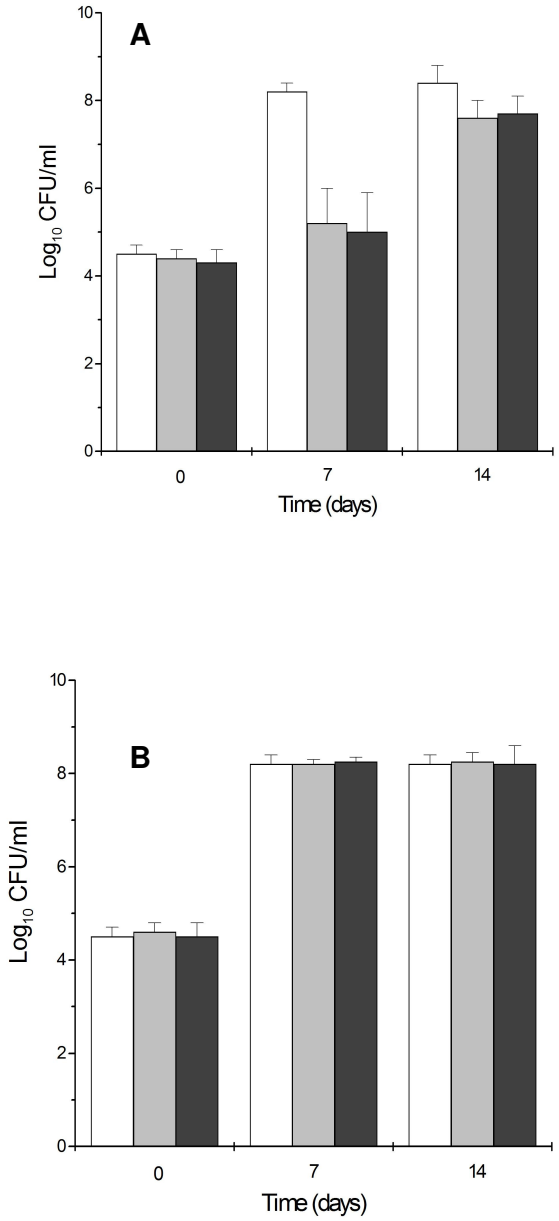

Figure 3. Viable cell counts of *Listeria monocytogenes* in skim milk (A) and whole milk (B) at 7 ± 1°C, confronted to 1600 AU/ml of free BLS P34 (grey bars), encapsulated BLS P34 (black bars) and control (white bars).

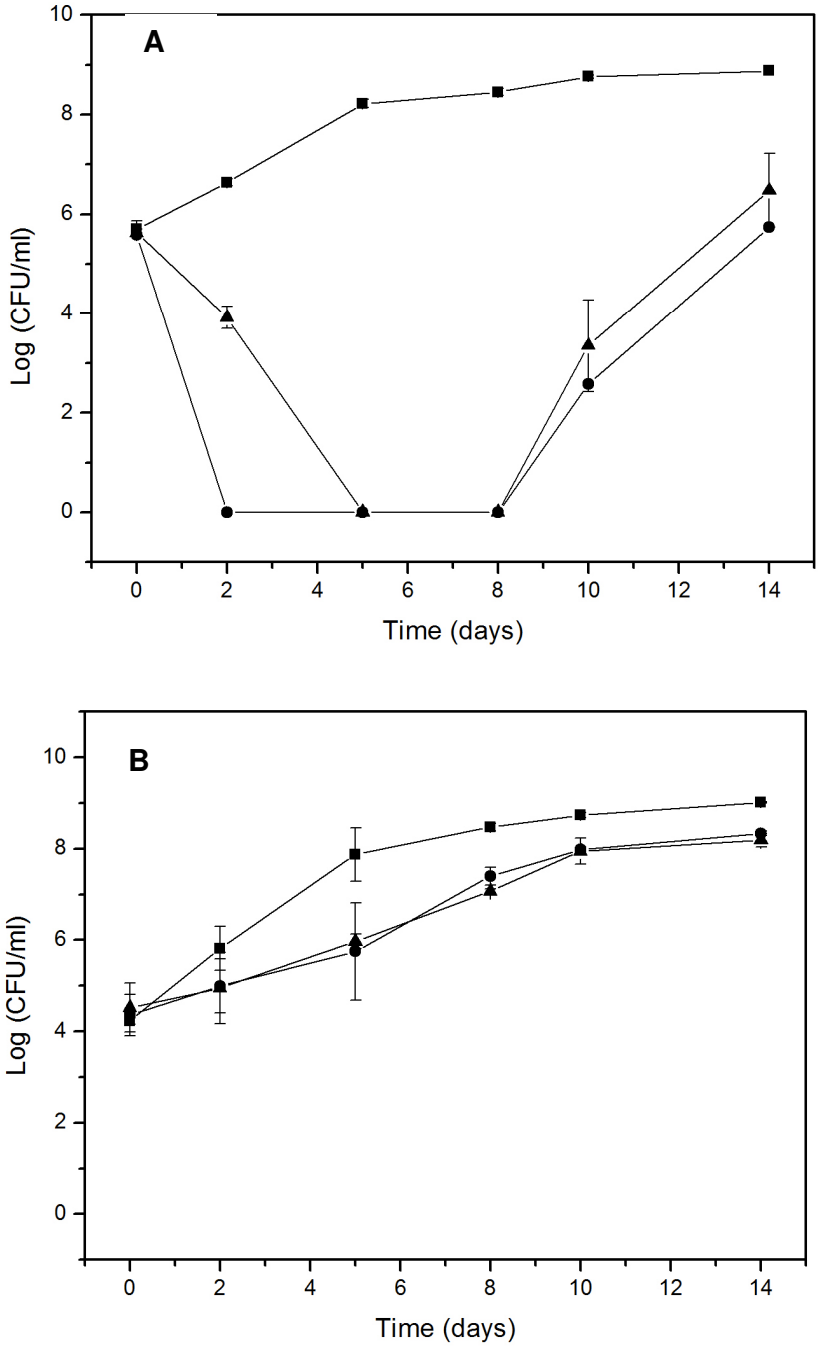

Fig 4. Viable cell counts of *Listeria monocytogenes* in skim milk (A) and whole milk (B) at 7 ± 1°C, confronted to 3200 AU/ml of free BLS P34 (●), encapsulated BLS P34 (▲) and control (■).

3.7 ARTIGO 7

3.7.1 Effect of nisin and bacteriocin-like substance P34 liposome-encapsulated on *Listeria monocytogenes* growth in Minas Frescal cheese

3.7.2 Abstract

The efficacy of liposome-encapsulated nisin and bacteriocin-like substance (BLS) P34 to control the development of *Listeria monocytogenes* in Minas frescal cheese was investigated. Nisin and BLS P34 were encapsulated in partially purified soybean phosphatidylcholine (PC-1) liposomes and PC-1-cholesterol (7:3) liposomes. Nisin and BLS P34 encapsulated in PC-1-cholesterol liposomes presented, respectively 218 nm and 158 nm, zeta potential of -64 mV and -53 mV, and entrapment efficiency of 88.9% and 100%. All treatments reduced the population of *L. monocytogenes* compared to the control during 21 days of storage of Minas frescal cheese to 7 ºC. However, nisin and BLS P34 encapsulated in PC-1-cholesterol liposomes were less efficient in controlling *L. monocytogenes* growth in comparison with free and PC-1 liposome-encapsulated bacteriocins. The higher inhibitory effect was observed through of encapsulation of nisin and BLS P34 in PC-1 liposomes after 10 days of storage of the product. The encapsulation of bacteriocins in liposomes of partially purified soybean phosphatidylcholine may be a promising technology for the control of foodborne pathogens in cheeses.

Keywords: bacteriocin, encapsulation, liposome, cheese, *Listeria monocytogenes,* nisin

3.7.3 Introduction

Encapsulation technology has been shown to protect antimicrobials of interfering food components, potentially enhancing their efficacy and stability. Among the materials used for the encapsulation of antimicrobials, such as bacteriocins, liposomes appear as the most studied (Were et al., 2003; Laridi et al., 2003; Malheiros et al., 2010a). Liposomes are colloidal particles consisting of a membranous system formed by lipid bilayers encapsulating aqueous space(s). As liposomes contain both lipid and aqueous phases, they can be utilized for the entrapment, delivery, and release of water soluble, lipid-soluble, and amphiphilic materials (Mozafari et al., 2008).

Bacteriocins are antimicrobial substances of polypeptidic nature that are not lethal to the producing cells (Cotter et al., 2005). These antimicrobial substances differ from traditional antibiotics by mechanisms of synthesis, mode of action, antimicrobial spectrum, toxicity and resistance mechanism (Montville and Chen, 1998; Sang and Blecha, 2008). The bacteriocin nisin is recognized as safe for food use by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives. Nisin is produced by strains of *Lactococus lactis* subsp. *lactis*, and its importance is due to its wide spectrum of activity against Gram-positive bacteria and also on spores of Bacilli and Clostridia (Sobrino-López and Martín-Belloso, 2008; Arauz et al., 2009).

However, other bacteriocins are being evaluated with respect to their potential for use in food. Production of bacteriocins or bacteriocin-like

substances (BLS) has been described for many species of the genus *Bacillus* (Motta et al., 2004; Kamoun et al., 2005; Lisboa et al., 2006). *Bacillus* sp. P34, a strain isolated from the fish Piau-com-pinta of Brazilian Amazon basin, produces a bacteriocin like substance (BLS P34) that inhibits a broad range of bacterial strains, including *Listeria monocytogenes* (Motta et al., 2007a). Purified peptide P34 presented low *in vitro* toxicity to eukaryotic cells with similar effect to that observed for nisin, showing great potential to be safely used in foods (Vaucher et al., 2010).

L. monocytogenes is a foodborne pathogen that grows under refrigeration and is of special concern to the dairy industry. This bacterium was previously isolated from Minas Frescal cheese, a fresh, soft, white cheese, which presents high pH (4.9-6.7), high moisture content (>55%), and low percentage of salt (1.4-1.6%) (Carvalho et al. 2007; Brito et al. 2008). Bacteriocins can be an additional hurdle to reduce the levels of pathogenic microorganisms in cheese. However, several studies indicate that direct application of bacteriocins in foods may result in decrease or loss of antimicrobial activity due to problems related to interaction with food components (Jung et al. 1992; Aasen et al. 2003; Chollet et al., 2008). To circumvent this problem, encapsulation of bacteriocins in liposomes may represent a promising alternative technology.

In this context, the aim of this study was to evaluate the inhibitory effect of commercial nisin and BLS P34 encapsulated in nanometric liposomes against *L. monocytogenes* in Minas Frescal cheese.

3.7.4 Material and methods

Nisin solution preparation

Commercial nisin (Nisaplin) was purchased from Danisco Brasil Ltda. Nisin solution was prepared with 0.01 M HCl and was filter sterilized through 0.22 µm membranes (Millipore). Before each experiment, nisin was diluted with 10 mM phosphate buffer (pH 6.4) to reach concentrations of 0.25 mg ml^{-1} of pure nisin.

Bacterial strains and growth conditions

The producer strain of BLS P34 bacteriocin was *Bacillus* sp. P34, an isolate from fish intestine (*Leporinus* sp.) from the Brazilian Amazon basin (Motta et al., 2004). *L. monocytogenes* ATCC 7644 was used as the indicator organism for the bacteriocin activity assay. The strains were maintained on BHI agar plates at 4ºC, and subcultured periodically.

For application in cheese, cultures of *L. monocytogenes* ATCC 7644 obtained in TSB-YE (37 ºC/18h) were centrifuged at 5,000 g for 10 min to 4 ºC. The supernatant fluid was discarded and the pellet was suspended in 0.1% peptone water, followed by centrifugation. After supernatant removal, the pellet was suspended in 0.1% peptone water and the suspension was then diluted in 0.1% peptone water to reach approximately 10^4 CFU ml^{-1}. This suspension was added to 200 g of cheese mass in order to obtain the concentration of 10^2 CFU g^{-1} approximately.

Production of BLS P34

Partial purification of BLS P34 was described by Motta et al. (2007b). *Bacillus* sp. P34 was cultivated aerobically in BHI broth at 30 ºC, 180 cycles min^{-1} for 24 h. Cells were harvested by centrifugation, and the resulting supernatant was filtered through 0.22 μm membranes. The cell free culture filtrate was submitted to precipitation with ammonium sulfate to 20% saturation. The resulting pellet was ressuspended in 10 mM sodium phosphate buffer pH 7.0 and applied to a gel filtration column (Sephadex G-100, Pharmacia Biotech, Uppsala, Sweden) and eluted with 10 mM sodium phosphate buffer pH 7.0. Fractions with antimicrobial activity were stored at 4 ºC until used for antimicrobial assay.

Nisin and BLS P34 encapsulation

Encapsulation of nisin and BLS P34 in liposomes of partially purified soybean phosphatidylcholine (PC-1; Mertins et al., 2008) and PC-1-cholesterol (7:3) was carried out by the thin-film hydration method (Malheiros et al., 2010b). PC-1 or PC-1-cholesterol was dissolved with chloroform in a round-bottom flask and the solvent was removed by a rotary evaporator until a thin film was formed on the flask walls. The resulting dried lipid film was dispersed by the addition of phosphate buffer containing nisin or BLS P34 under vigorous stirring to 60 ºC. The preparation was sonicated in a bath-type ultrasound (40 kHz, Unique USC 700) for 30 min and, filtered through 0.22 μm membranes. Size and polydispersity (PDI), encapsulation efficiency (EE), and zeta potential of bacteriocins encapsulated in PC-1-cholesterol liposomes were determined

according a previous study described to PC-1 liposomes (Malheiros et al., 2010b).

Antimicrobial activity assay

The antimicrobial activity was detected by agar diffusion assay. Aliquots of 10 µl were applied on BHI agar plates previously inoculated with a swab submerged in a suspension of *L. monocytogenes* ATCC 7644, which corresponded to a 0.5 McFarland turbidity standard solution (10^7 CFU ml^{-1}). Plates were incubated at 37 ºC for 24 h. The reciprocal value of the highest dilution where an inhibition zone was observed was taken as activity units per mL (AU ml^{-1}).

Minas Frescal Cheese preparation

Minas cheese was prepared according to Scholz (1995). Six liters of pasteurized cow's milk, purchased in the local market, was heated to 34±1 ºC, and added of 2.5 ml of acid lactic, 3 ml de $CaCl_2$ and 5.4 ml of commercial rennet Estrella (85% bovine pepsin + 15% bovine chymosin, Chr. Hansen). After coagulation (about 40 min), curd cutting was performed for 30 min under low agitation. Sodium chloride (2.0 g l^{-1}) was also added to the curd. The curd was transferred to perforated sterile plastic circular cheese containers (appr. 15 cm diameter) and maintained at 21 ^{o}C for 1 h for dripping. The cheeses were unmolded, packed in plastic bags, and stored under refrigeration (8-10 ^{o}C). Each package contained approximately 200 g of cheese.

Five milliliters of solution of 0.25 mg ml^{-1} of pure nisin was added to 200 g of cheese until reaches 6.25 mg of pure nisin per gram of cheese. After 30 min, a population of 4 log CFU ml^{-1} of *L. monocytogenes* was inoculated in the curd.

The cheese experimental groups were:

- cheese containing only *L. monocytogenes*;
- cheese containing free nisin and *L. monocytogenes*;
- cheese containing encapsulated nisin in PC-1-cholesterol liposomes and *L. monocytogenes*;
- cheese containing encapsulated nisin in PC-1 liposomes and *L. monocytogenes*;
- cheese containing free BLS P34 and *L. monocytogenes*;
- cheese containing encapsulated BLS P34 in PC-1-cholesterol liposomes and *L. monocytogenes*;
- cheese containing encapsulated BLS P34 in PC-1 liposomes and *L. monocytogenes*;
- cheese without any addition.

Determination of pH, water activity and L. monocytogenes counts in the cheeses during storage

These parameters during storage of the cheeses under refrigeration (7 ºC) were monitored for 21 days, which corresponds to the shelf-life of this product (Buriti et al, 2005). The pH values of cheeses controls (without addition of *L. monocytogenes* and bacteriocins) were determined on triplicate samples (three different cheeses of the same trial) with a pH meter Analyser Model 300 M

(Analyser, São Paulo, Brazil) equipped with a penetration electrode model DME-CF (Digimed, São Paulo, Brazil). Water activity (aw) at 25 ºC was determined on triplicate samples using the Novasina aw-Center AWC 503-C equipped with a three-compartment a_w box (Novasina, Pfäikon, Switzerland).

The enumeration of *L. monocytogenes* was performed using the method of Hartman et al (1975), with modifications. Portions of 10 g of cheese were homogenized with 90 ml of 0.1% peptone water using a stomacher for 60s and submitted to decimal serial dilution in 0.1% peptone water. *L. monocytogenes* was counted by pour-plating 1 ml of each dilution in 15 ml of TSA-YE (Trypticase Soy *Agar* supplemented with 0.6% Yeast Extract). After homogenization and solidification of the medium, an overlayer of 5 ml of selective media for *Listeria* spp. (*Listeria* Selective Agar Base - Oxford Formulation) was added and the plates were incubated at 37 ºC for 48 hours.

Statistical analysis

The software Statistica 7.1 (Statsoft, Tulsa, OK, USA) was used for statistical comparison analysis of the experimental groups. All experimental treatments were tested in triplicate for three independent preparations, and averages were calculated for treatments at each time point. Bacteria counts were compared using Tukey's *t* test. Data analyses were performed with the software and differences were considered statistically significant at $P<0.05$.

3.7.5 Results and discussion

Nisin and BLS P34 encapsulation

Size, PDI and zeta potential of nisin and BLS P34 encapsulated in PC-1-cholesterol liposomes are presented in Table 1. Nisin and BLS P34 encapsulated in PC-1 liposomes were previously characterized (Malheiros et al 2010b; Malheiros et al., 2011). The addition of cholesterol with phosphatidylcholine for the encapsulation of nisin and BLS P34 in liposomes did not affect the polydispersity of the samples. However, it did affect the average diameter of liposomes containing nisin by increasing from 137 to 218 nm and zeta potential of -55 to -64 mV (Malheiros et al., 2010b). Cholesterol could be beneficial as a stabilizing component in the liposome composition by decreasing membrane permeability and increasing cohesive interactions (Deo and Somasundaran, 2003). According to Were et al. (2003), the presence of cholesterol generally increased liposome size containing nisin. The liposome size depends of lipid composition and the encapsulated compound (Were et al., 2003). The EE of nisin encapsulated in PC-1-cholesterol liposomes was 88.9%. The EE decreased with the addition of cholesterol in comparison to encapsulated nisin in PC-1 liposomes (94.12%). This decrease in EE of nisin in liposomes was reported by Laridi et al. (2003) and Were et al. (2003), which described that the presence of cholesterol in the liposome membranes reduces the affinity of nisin for the lipid membrane. For BLS P34, the addition of cholesterol did not change the size of the liposomes, but the zeta potential increased from -27 to -53 mV (Malheiros et al., 2011). Liposomes with high zeta-potential can be expected to be more stable because the repulsive interactions increase, reducing the frequency of liposome collisions (Malheiros

et al., 2010a). The EE of BLS P34 encapsulated in PC-1-cholesterol liposomes was 100%. In this case, the EE was not affected by addition of cholesterol.

Inhibition of L. monocytogenes in Minas Frescal cheese

Minas Frescal cheese, a typical Brazilian fresh cheese, is one of the most highly consumed lactic products in Brazil, with wide acceptance in the national market (Souza and Saad, 2009). It presents high water activity, pH above 5.0, low salt content and absence of preservatives (Buriti et al., 2005a). The cheeses prepared in this study presented similar characteristics, ie, water activity ranged from 0.989 to 0.990 and pH ranged from 6.49 to 6.56 during 21 days.

The antimicrobial effect of bacteriocins in cheeses is mainly investigated by inoculation of cheese milk with bacteriocinogenic starters or through the direct addition of the bacteriocin during cheese manufacture or post-production (Bizani *et al.* 2008; Sobrino-López and Martín-Belloso 2008; Mojgani et al., 2010). The addition of lactocin RN 78 in experimental cheese reduced more effectively the population of *L. monocytogenes* than the producer strain *Lactobacillus casei* RN 78 (Mojgani et al. 2010), demonstrando que o tratamento de queijo com bacteriocina pode ser mais eficaz na inibição de patógenos do que a utilização de bacteriocinogenic starters.

The FAO/WHO Codex Committee on milk and milk products accepted nisin as a food additive for processed cheese at a concentration of 12.5 mg pure nisin/kg of product (Ross et al., 2002). In this work, the final concentration of nisin applied to cheese corresponded to half of the maximum permissible concentration. This concentration was used because of interest in assessing

whether the encapsulated nisin present better inhibitory effect against *L. monocytogenes* than nisin free. Free nisin, in maximum permissible concentration, effectively inhibited the growth of *L. monocytogenes* for a period of 55 days or more in ricotta-type cheeses (Davies et al., 2007). Bactericidal action could cover up the comparison between the effect of free and encapsulated nisin. As the antimicrobial activity of 0.25 mg ml^{-1} of free and encapsulated nisin was 800 AU ml^{-1}, the same activity was used for BLS P34.

The initial population of *L. monocytogenes* in the cheeses was 2.6 log CFU g^{-1} (Fig 1 and 2). The counts of *L. monocytogenes* in the cheeses containing free nisin, nisin encapsulated in PC-1-cholesterol liposomes and nisin encapsulated in PC-1 liposomes in the cheeses were below the detection limit (10 CFU g^{-1}) for 5 days. On the eighth day, the counts were between 3 and 4 log CFU g^{-1}, indicating that those cells that resisted the action of nisin started to multiply after the 5^{th} day. However, there was no difference between treatments (P>0.05). Moreover, on 8^{th} day *L. monocytogenes* control (without treatment) reached the stationary phase of growth reaching populations of 8-8.5 CFU g^{-1}. After 8 days, nisin encapsulated in PC-1 liposomes showed higher inhibitory effect (Fig 1), but at statistically significant levels only in day 14. There was no increase in *L. monocytogenes* population between 8^{th} and 10^{th} days using nisin encapsulated in PC-1 liposomes as treatment. In addition, at day 14 the pathogen was reduced 4.6 logs CFU g^{-1} compared to control and approximately 2 log CFU g^{-1} compared with free nisin and encapsulated nisin in

PC-1-cholesterol liposomes. All treatments reduced the population of the pathogen compared to the control during the shelf life of the product (Fig 1).

In a previous work, commercial nisin (0.5 mg ml^{-1}), free and encapsulated in PC-1 liposomes, consistently lowered *L. monocytogenes* counts below the detection limit below detection limit of the method used by the authors (1.69 log CFU ml^{-1}) during a period of 14 days in skim and whole milk (Malheiros et al, 2010c).

Benech et al (2002) added nisin Z-loaded Pro lipo H liposomes (300 IU g^{-1} of cheese) to milk prior to renneting, evaluating the inhibition of *Listeria innocua in* Cheddar cheese during 6 months ripening. Immediately after cheese production, 3 log-unit reductions in viable counts of *L. innocua* were obtained in cheeses with encapsulated nisin, while for nisinogenic starter the reduction was 1.5 log-unit. After 6 months, the viable counts of *L. innocua* in liposome-containing cheeses were below the detection limit of the method (10 CFU g^{-1}), whereas they were 3.7×10^4 and 4.3×10^4 CFU g^{-1}, respectively, in nisinogenic starter and nisin-free cheeses. This was correlated with a high concentration and purity of encapsulated nisin compared to nisin produced by the nisinogenic strain. Furthermore, the authors demonstrated by transmission electron microscopy that the liposomes were present in the fat-casein interface and whey pockets, where bacteria are concentrated, resulting in higher accessibility of nisin to bacterial cells. However, the authors did not compare the effect of encapsulated nisin in relation to free nisin.

Figure 2 shows the effect of BLS P34 on *L. monocytogenes* growth in Minas frescal cheese. The counts of *L. monocytogenes* in control cheeses

(without bacteriocin) differed (P<0.05) of all cheeses treated for 21 days, excepting for 14 and 18 days in which the pathogen was not inhibited by the action of encapsulated BLS P34 in PC-1-cholesterol liposomes. In the decimo day of storage, BLS P34 encapsulated in PC-1 liposomes showed better inhibitory effect with significant differences in comparison with other treatments. After, there was no significant difference in the growth of *L. monocytogenes* by the action of free BLS P34 and encapsulated BLS P34 in PC-1 liposomes. However, is important to note that the 10^{th} -18^{th} day encapsulated BLS P34 in PC-1 liposomes inhibited the pathogen at least 0.5 log CFU g^{-1} more than free BLS P34 (Fig 2).

Bizani et al. (2008) added the antimicrobial peptide cerein 8A (final concentration 400 AU ml^{-1}) to Minas frescal cheese during manufacturing at the same time of commercial rennet and on surface of manufactured cheese to control the development of *L. monocytogenes.* The authors observed that cerein 8A showed less effective activity in the manufactured cheese in comparison with surface application. This effect was associated to inactivation of cerein 8A during cheese manufacturing by endogenous food enzymes or binding to components of the food matrix (Bizani et al., 2008).

In general the encapsulation of nisin and BLS P34 in PC-1-cholesterol liposomes was less efficient in controlling *L. monocytogenes* growth, when compared to other treatments. The addition of cholesterol may have hindered the release of bacteriocins from the liposome acting as a barrier. According to Were et al (2003) the addition of cholesterol decreases leakage of PC liposomes while maintaining a high concentration of antimicrobials. Moreover,

the zeta potential became more negative with the addition of cholesterol to PC-1, which may result in increased electrostatic repulsion among liposomes and bacteria. The addition of cholesterol in liposome membranes may firm the lipid chain, resulting in a shifting of hydrophobic interactions between the peptides and the liposome membrane and inhibiting the ability of bacteriocins to perturb the membrane structure (El Jastimi & Lafleur, 1999).

The encapsulation of nisin and BLS P34 in PC-1 liposomes showed higher inhibitory effect after 10 days of storage in comparison with other treatments. In this context, it is suggested that the bacteriocins are being released from the PC-1 liposomes especially in the latter half of the shelf-live of this product. This release of bacteriocin from the PC-1 liposomes is needed for action against the target microorganism. As evidenced by zeta potential and discussed in previous work (Malheiros et al., 2011), the high negative charge of liposomes can prevent the fusion between the liposome and the wall of the bacterium, which has negative charge.

The utilization of nanotechnology to improve control of microorganisms in the food is quite recent. There are few reports on the effect of antimicrobial loaded liposomes in food. In this work, we used partially purified soybean phosphatidylcholine in the liposomes formulation, being a cheap product and probably of low toxicity. Therefore, this research can be useful to add information about the effect of encapsulation of different bacteriocins (one commercial and one produced in our laboratory) in liposomes to control of *L. monocytogenes* in fresh cheeses.

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Table 1

Entrapment encapsulation (EE), size, polydispersity (PDI) and zeta potential of nisin and BLS P34 encapsulated in phosphatidylcholine (PC-1) liposomes and PC-1-cholesterol (7:3) liposomes

Liposome*	EE (%)	Size (nm)	PDI	Zeta potential (mV)	Reference
PC-1					
Nisin	94.12	137.7 ± 6.1	0.346 ± 0.06	-55.8 ± 3.1	Malheiros et al. 2010b
BLS P34	100	162.9 ± 13.3	0.218 ± 0.05	-27.42 ± 9.39	Malheiros et al. 2011
PC-1-Cho					
Nisin	88.9	218.8 ± 9.3	0.312 ± 0.06	-64.2 ± 0.28	This work
BLS P34	100	157.9 ± 8.4	0.242 ± 0.01	-53.0 ± 4.45	This work

* Liposomes prepared with phosphatidilcholine (PC-1) or PC-1-cholesterol (7:3).

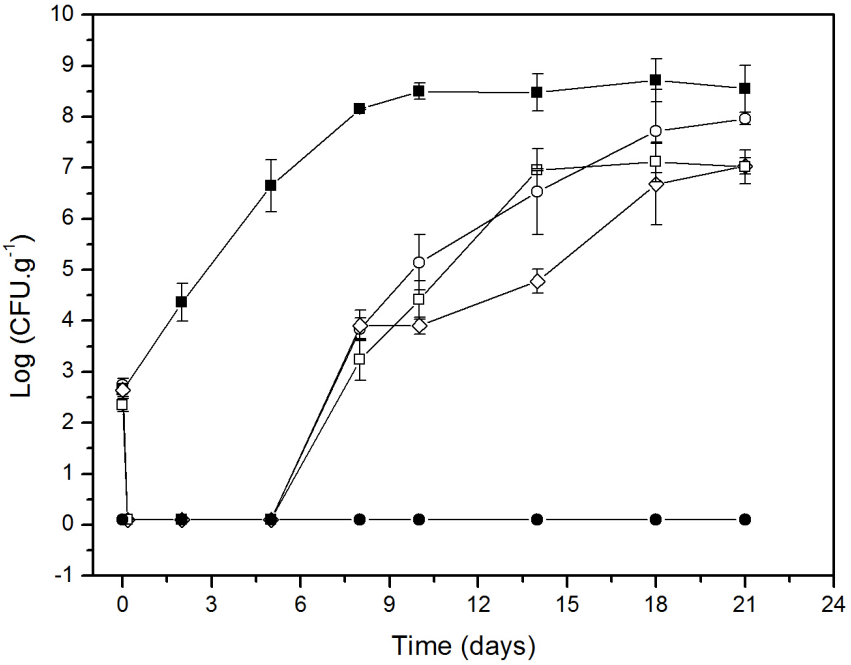

Fig 1. Growth of *Listeria monocytogenes* in Minas Frescal cheese containing phosphatidylcholine encapsulated nisin (◊), phosphatidylcholine/cholesterol encapsulated nisin (○), free nisin (□) and without the addition of nisin (■). Control cheese (without nisin and *L. monocytogenes*) (●)

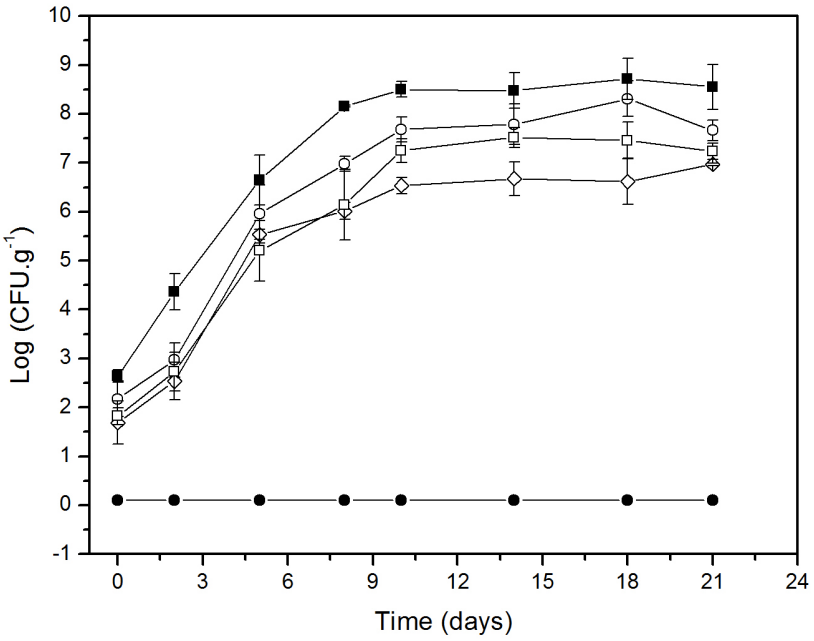

Fig 2. Growth of *Listeria monocytogenes* in Minas Frescal cheese containing phosphatidylcholine encapsulated BLS P34 (◊), (b) phosphatidylcholine/cholesterol encapsulated BLS P34 (○), free BLS P34 (□) and without the addition of BLS P34 (■). Control cheese (without BLS P34 and *L. monocytogenes*) (●)

4 DISCUSSÃO GERAL

O campo da nanociência e nanotecnologia vem crescendo de maneira significativa nos últimos anos. Define-se como nanociência o estudo de sistemas com o tamanho de nanômetro. A nanotecnologia, por sua vez, contempla o desenho, a caracterização, a produção e a aplicação de estruturas, dispositivos e sistemas por meio do controle da sua forma e tamanho na escala nanométrica (RSRAE, 2004; Bouwmeester et al., 2009).

A ciência e a tecnologia de nanoestruturas possuem inúmeras aplicações em diversas áreas do conhecimento e as perspectivas a médio e longo prazos oferecidas pelo uso da nanotecnologia têm sido consideradas muito promissoras (Chen et al., 2006; Fronza, 2006). Na área de alimentos, uma de suas aplicações é a encapsulação e liberação controlada de peptídeos antimicrobianos. Dentre as formas de encapsulação, os lipossomas são considerados de grande interesse, pois podem ser preparados utilizando-se lipídios naturais com baixo risco de toxicidade (Mozafari et al., 2008a).

Neste estudo, foram desenvolvidos lipossomas à base de fosfatidilcolina de soja parcialmente purificada (FC-1) obtida a partir de lecitina, um subproduto de baixo valor da indústria de soja. Esses lipossomas foram empregados para

encapsulação de nisina comercial (Nisaplin®) através de duas metodologias: fase reversa e hidratação do filme. Observou-se que a metodologia de hidratação do filme usando ultra-som de banho - para diminuir e homogeneizar o tamanho dos lipossomas - foi a mais conveniente, pois a bacteriocina manteve 100% de sua atividade antimicrobiana após a encapsulação, o que não foi observado na hidratação do filme usando ultra-som de ponta (50%) e na metodologia de fase reversa (25%). Na metodologia de fase reversa, a bacteriocina entra em contato direto com o solvente (clorofórmio) utilizado na solubilização da FC-1, o que pode levar a inativação do peptídeo (Colas et al., 2007). Na hidratação do filme usando ultra-som de ponta, a alta freqüência ultra-sônica pode ter desnaturado a bacteriocina, reduzindo sua atividade antimicrobiana. O ultra-som de banho, por usa vez, é menos destrutivo aos peptídeos, apresenta maior reprodutibilidade e gera produtos homogêneos (Taylor et al., 2005; Colas et al., 2007).

Em função destes resultados, a hidratação do filme usando ultra-som de banho foi a metodologia de encapsulação escolhida para a continuação dos estudos.

Neste contexto, nisina foi encapsulada em lipossomas de FC-1, obtendo-se uma eficiência de encapsulação de 94,12%, superior à obtida em outros trabalhos que descrevem valores entre 18 e 63% (Degnan e Luchansky, 1992; Were et al., 2003, Laridi et al., 2003). O potencial zeta foi de aproximadamente -55 mV, o diâmetro dos lipossomas variou entre 132 e 149 nm e a polidispersidade entre 0,29 e 0,40 durante 24 dias, sugerindo estabilidade da preparação. Tamanho e potencial zeta foram avaliados antes e após a

esterilização dos lipossomas por filtração em membranas de 0,22 µm. Observou-se que não houve alterações nessas características após a filtração, o que é bastante promissor já que os lipossomas precisam estar livres de micro-organismos para posterior aplicação em alimentos (Were et al., 2003).

Conforme demonstrado por microscopia óptica de alta resolução e microscopia eletrônica de varredura (MEV), os lipossomas contendo nisina apresentaram morfologia esférica, embora aglomerados tenham se formado durante o processo de secagem da amostra para visualização por MEV. Segundo Silva et al. (2008), a encapsulação de diferentes peptídeos pode gerar diferenças na morfologia dos lipossomas. Porém, a maioria dos trabalhos relata lipossomas com morfologia arredondada (Colas et al., 2007; Arias et al., 2008), assim como observado neste estudo.

A atividade antimicrobiana de nisina livre e encapsulada foi avaliada por 24 dias através da metodologia de difusão em agar (Motta e Brandelli, 2002) usando *L. monocytogenes* como micro-organismo indicador. Observou-se que nisina livre manteve 100% de sua atividade antimicrobiana inicial enquanto que a nisina encapsulada teve sua atividade reduzida ao longo do tempo, indicando que os lipossomas devem ser aplicados logo após a sua preparação, como sugerido por Taylor et al. (2008).

Nisina livre e encapsulada foram aplicadas em leite UHT artificialmente contaminado com *Listeria monocytogenes*. Primeiramente, foi avaliada a eficiência de nisina livre e encapsulada no controle de *L. monocytogenes* em meio de cultura BHI e em leite desnatado mantidos a 30ºC por 48 horas.

Nestas condições de abuso de tempo e temperatura, nisina livre mostrou-se mais eficiente do que a nisina encapsulada.

No entanto, nisina vem sendo empregada na bioconservação de alimentos como uma das tecnologias de barreira, ou seja, combinada com outros métodos de inibição, tais como redução de pH, altas pressões, baixas temperaturas, entre outros (Deegan et al., 2006; Gálvez et al., 2007; Sobrino-López e Martín-Belloso, 2008). Neste sentido, os efeitos de nisina livre (0,1 mg/mL e 0,5 mg/mL) e nisina encapsulada (0,5 mg/mL), em associação com manutenção em baixa temperatura, foram avaliados no controle de *L. monocytogenes* em leite desnatado e integral armazenados a 7ºC por 14 dias. Em leite desnatado, todos os tratamentos inibiram o patógeno atingindo contagens abaixo do limite de detecção do método (1,69 log UFC/g). Em leite integral, somente a concentração mais elevada de nisina livre e encapsulada (0,5 mg/mL) apresentou efeito bactericida. A concentração de 0,1 mg/mL de nisina livre não exerceu esse mesmo efeito, já que o patógeno voltou a multiplicar-se após 5 dias de armazenamento, provavelmente em decorrência da adsorção da bacteriocina aos glóbulos de gordura presentes no leite (Jung et al., 1992; Bhatti et al., 2004; Chollet et al., 2008). Além da gordura e temperatura de armazenamento, outros fatores como proteínas e concentração de cátions, também podem afetar a atividade antimicrobiana de nisina (Branen e Davidson, 2004).

Portanto, observou-se que tanto nisina livre quanto nisina encapsulada em combinação com o armazenamento em baixas temperaturas foram

eficientes para controlar *L. monocytogenes* em leite, enfatizando a importância das tecnologias de barreira para garantir a segurança dos alimentos.

É importante ressaltar que a aplicação de bacteriocinas em leite UHT não é um procedimento usual, entretanto esse resultado pode ser útil para avaliar as influências de alguns componentes na atividade de bacteriocinas (Bizani et al., 2008). Muitos pesquisadores utilizam leite como modelo alimentar (Mathieu et al., 1994; Maisnier-Patin et al., 1995; García et al., 2010; Arqués et al., 2011), mas o produto lácteo com maior aplicação prática para o uso de bacteriocinas como bioconservante é o queijo.

Dentre as variedades de queijo, o Minas frescal é um dos mais populares do Brasil, sendo consumido por todas as camadas da população. O queijo Minas frescal é um produto de massa crua, com alto teor de umidade (46 a 55%), não maturado e altamente perecível, mesmo sob refrigeração (Buriti et al., 2005). Os queijos de massa mole com pH alto e umidade elevada permitem o desenvolvimento de muitos micro-organismos, como *Listeria monocytogenes* (Silva et al., 2003; Brito et al., 2008; Nascimento et al., 2008).

Listeria monocytogenes é o agente etiológico da listeriose, doença importante para indivíduos imunocomprometidos, mulheres grávidas, idosos, neonatos e pacientes com câncer (Goulet et al., 2008). Os sintomas clínicos da doença envolvem infecção do sistema nervoso central e bacteremia, podendo causar também endocardite, aborto, parto pré-maturo e septicemia neonatal.

Contaminação de queijos por *L. monocytogenes* tem recebido atenção especial devido a muitos surtos relacionados ao consumo deste produto, alguns envolvendo mortes em diversos países (De Buyser et al., 2001). O leite

cru, em particular, é amplamente reconhecido como uma fonte de contaminação por *L. monocytogenes* e um veículo de listeriose (Bemrah et al., 1998). *L. monocytogenes*, uma bactéria que pode ser isolada do solo, silagem e outras fontes ambientais, resiste aos efeitos do congelamento, secagem e calor (Barocci et al., 2008). Esse micro-organismo cresce em temperaturas entre 1ºC e 45ºC e pH de 4,1 a 9,6, sendo capaz de sobreviver em alimentos por longos períodos de tempo (Jay, 2005). A dificuldade em eliminar esse micro-organismo das indústrias é potencializada pelas condições de umidade, temperatura e presença de matéria orgânica nas plantas de processamento, que aliadas à capacidade do patógeno em produzir biofilmes, podem desencadear a colonização de superfícies de equipamentos e utensílios (Uhitil et al., 2004).

O efeito antilisteria de nisina livre e encapsulada foi avaliado através de testes *in vitro* e também na superfície de queijo Minas frescal comercial experimentalmente contaminado com *L. monocytogenes*. Quando nisina livre e encapsulada foram utilizadas para controlar o crescimento de *L. monocytogenes* na superfície de queijo Minas frescal armazenado a 7ºC por 28 dias, nisina livre foi mais eficiente. Nisina livre, na concentração de 0,25 mg/mL apresentou efeito bactericida, enquanto nisina livre na concentração de 0,1 mg/mL e nisina encapsulada na concentração de 0,25 mg/mL apresentaram atividade bacteriostática.

Durante a produção de queijo Minas frescal, nisina livre e encapsulada foram adicionadas de modo a atingir 6,25 mg por kg de produto, ou seja, metade da concentração máxima permitida pela WHO/FAO. Essa concentração

foi utilizada devido ao interesse em avaliar se a nisina encapsulada apresentaria melhor efeito inibitório contra *L. monocytogenes* do que nisina livre. A bacteriocina livre, na concentração máxima permitida, apresentou efeito bactericida em queijo frescal produzido com leite de cabra (Furtado, 2010).

Neste trabalho, a população de *L. monocytogenes* permaneceu abaixo do limite de detecção da técnica (10 log UFC/g) por 5 dias utilizando como tratamento nisina livre e encapsulada. Após 8 dias, nisina encapsulada apresentou melhor efeito inibitório do que nisina livre. Uma parte da nisina livre pode ter sofrido a ação de enzimas endógenas ou ligação com componentes da matriz alimentar diminuindo sua atividade antimicrobiana (Bizani et al., 2008). A encapsulação da bacteriocina, por sua vez, pode protegê-la dessas interações mantendo sua atividade antimicrobiana por um período mais prolongado (Were et al., 2003; Laridi et al., 2003).

Nisina é a única bacteriocina aprovada para uso em alimentos, sendo de grande interesse o estudo de novas tecnologias, como a encapsulação, para aumentar e/ou estender a sua ação antimicrobiana.

Entretanto, outros peptídeos antimicrobianos, tais como a BLS P34, vêm demonstrando potencial para uso em alimentos (Motta et al., 2007a,b; Motta et al., 2008; Vaucher et al., 2010; Sant'Anna et al., 2011). Neste sentido, a encapsulação de BLS P34, visando melhorar sua ação antimicrobiana, pode ser de grande interesse para a bioconservação de alimentos.

BLS P34 foi encapsulada em lipossomas de fosfatidilcolina de soja parcialmente purificada utilizando a metodologia de hidratação do filme. Os lipossomas contendo BLS P34 foram armazenados a 4ºC por 24 dias. Durante

esse período, as características físico-químicas foram avaliadas determinando-se que o tamanho dos lipossomas variou de 144 a 163 nm, a polidispersidade foi de aproximadamente 0,22 e o potencial zeta variou de -24.90 mV a -27,42 mV. A manutenção do tamanho e do potencial zeta dos lipossomas contento BLS P34 ao longo do tempo indica estabilidade dessa preparação. O potencial zeta pode influenciar fortemente a estabilidade dos lipossomas através da repulsão eletrostática entre as partículas (Taylor et al., 2007), responsável pelo baixo volume de sedimentação (Arias et al., 2008). A atividade antimicrobiana de BLS P34 livre e encapsulada manteve-se sem alterações significativas (P>0.05) durante o armazenamento.

A eficiência de encapsulação (EE) da BLS P34 nos lipossomas foi de 100%. Neste caso, a EE pode estar relacionada tanto com a bacteriocina que foi encapsulada no interior do lipossoma quanto à bacteriocina que se ligou à parede da nanovesícula por interação eletrostática. Fosfatidilcolina é um fosfolipídio neutro. Embora o fosfolipídio utilizado nesse trabalho apresente alta concentração de fosfatidilcolina, outros lipídeos presentes na FC-1 podem modificar a carga da membrana tornando-a carregada negativamente. Devido à natureza catiônica das bacteriocinas (Jack et al., 1995), pode-se sugerir que houve interação entre a BLS P34 e o lipídio usado na formulação do lipossoma, explicando a alta EE. Isso também pode estar correlacionado com a elevada EE da nisina em lipossomas (94,12%).

A análise morfológica de lipossomas utilizando microscopia eletrônica de transmissão (MET) foi considerada a mais benéfica por Chetanachan et al. (2008). Resultados semelhantes foram obtidos por este estudo, onde

lipossomas contendo nisina, visualizados por MEV, apresentaram aspecto aglomerado, enquanto os lipossomas contendo BLS P34, visualizados por MET, apresentaram morfologia arredondada e monodispersa.

O modo de ação das nanovesículas contendo BLS P34 frente a *L. monocytogenes* foi investigado por MET. Os lipossomas contendo BLS P34 cercaram as células de *L. monocytogenes* após 1 hora de incubação (30ºC), mas não ocorreu fusão. Já no estudo conduzido por Colas et al. (2007), lipossomas vazios circundaram células de *B. subtilis* e alguns fundiram com a membrana externa bacteriana após 2 h de incubação a 37ºC. Com isso, sugere-se que BLS P34 precisa ser liberada dos lipossomas para atuar na membrana celular da bactéria alvo. Quando livre, BLS P34 age através da formação de poros e desintegração da célula de *L. monocytogenes* (Motta et al., 2008).

O efeito de BLS P34 livre e encapsulada foi avaliado em leite desnatado, leite integral e durante a produção de queijo Minas frescal.

Em leite desnatado artificialmente contaminado com *L. monocytogenes* armazenado a 30ºC, BLS P34 livre reduziu a população do patógeno para níveis abaixo do limite de detecção da técnica (1,69 log UFC/mL) por 6 horas, provavelmente devido a injuria bacteriana. Entretanto, BLS P34 encapsulada possivelmente foi sendo liberada ao longo do tempo, mantendo a mesma contagem inicial do patógeno por 24 horas. Durante o armazenamento a 7ºC, 3200 UA/mL de BLS P34 livre e encapsulada inibiram a população de *L. monocytogenes* para níveis abaixo do limite de detecção do método entre os

dias 2 e 8 e 5 e 8, respectivamente. Após esse período, o patógeno voltou a crescer, porém mantendo níveis abaixo do controle.

Em leite integral não houve diferença significativa (P>0.05) entre BLS P34 livre e encapsulada. Ambos os tratamentos reduziram a população de *L. monocytogenes* entre 1 e 2 log UFC/mL em comparação ao controle durante o armazenamento a 30ºC e 7ºC.

A manutenção da atividade antimicrobiana de BLS P34 livre e encapsulada foi avaliada em leite desnatado e integral estocados a 4ºC por 21 dias. Observou-se redução de 50% na atividade antimicrobiana após 4 dias de armazenamento em leite desnatado e integral, sendo que não houve diferença entre os tratamentos. Essa diminuição na atividade antimicrobiana pode ser associada à interação da BLS P34 com as proteínas do leite, assim como sugerido para a nisina (Jung et al., 1992). Neste caso, a gordura do leite não influenciou a atividade antimicrobiana de BLS P34. Entretanto, o efeito negativo da gordura sobre a atividade antimicrobiana de nisina é amplamente estudado (Jung et al., 1992; Bhatti et al., 2004; Chollet et al., 2008).

Embora a atividade antimicrobiana residual de BLS P34 livre e encapsulada tenha sido semelhante em leite desnatado e integral, o melhor efeito inibitório sobre a população de *L. monocytogenes* foi observado em leite desnatado. Da mesma maneira, nisina foi mais efetiva em inibir *L. monocytogenes* em meio de cultura BHI e leite desnatado do que em leite integral (Jung et al., 1992), demonstrando a importância da interação entre peptídeos bioativos e componentes dos alimentos na atividade antimicrobiana para a conservação desses produtos.

A aplicação de BLS P34 livre e encapsulada em queijo Minas frescal armazenado a 7ºC por 21 dias resultou em redução no crescimento de *L. monocytogenes* em comparação ao controle. BLS P34 encapsulada apresentou melhor efeito inibitório entre os dias 10 e 18 do que a bacteriocina livre. Estes dados vão ao encontro dos obtidos com nisina encapsulada que também demonstrou ser mais eficiente nestes dias. Sugere-se então, que as bacteriocinas são liberadas dos lipossomas nestes dias, especialmente. Conforme demonstrado através do estudo sobre o modo de ação de BLS P34, essa liberação é necessária para a ação da bacteriocina contra o patógeno alvo. Componentes da matriz alimentar, tais como as enzimas endógenas, podem ter desnaturado parte das bacteriocinas livres, tornando-as inativas. A encapsulação provavelmente protegeu as bacteriocinas de componentes do queijo Minas frescal exercendo melhor efeito após 10 dias de armazenamento.

A utilização de nanotecnologia para melhorar a qualidade microbiológica de alimentos é recente e ainda pouco explorada. As vantagens e limitações de seu uso na indústria de alimentos ainda não são conhecidas (Sanguasri e Augustine, 2006). Portanto, esse trabalho adiciona informações importantes sobre a encapsulação, caracterização e aplicação de duas bacteriocinas - nisina e BLS P34 - em produtos lácteos.

5 CONCLUSÕES

De acordo com resultados obtidos no presente estudo, é possível concluir que:

- O método de hidratação do filme usando ultra-som de banho foi considerado o mais eficiente para a encapsulação de nisina comercial.
- As bacteriocinas nisina e BLS P34 foram encapsuladas eficientemente em nanovesículas lipídicas preparadas com fosfatidilcolina de soja parcialmente purificada, apresentando estabilidade, alta eficiência de encapsulação e morfologia arredondada.
- A atividade antimicrobiana de nisina livre *in vitro* foi constante, mas a nisina encapsulada perdeu metade de sua atividade antimicrobiana inicial durante a estocagem. Entretanto, BLS P34 livre e encapsulada não apresentaram diferença na atividade antimicrobiana durante o armazenamento.
- A partir de imagens obtidas por microscopia eletrônica de transmissão, observou-se que não ocorreu fusão entre os lipossomas contendo BLS P34 e a parede celular de *Listeria monocytogenes*, sendo necessária a liberação de BLS P34 de dentro dos lipossomas para a inibição do patógeno alvo.

- Nisina livre foi mais eficiente do que nisina encapsulada na inibição de *L. monocytogenes* em leite desnatado armazenado a 30ºC. Entretanto, na temperatura de 7ºC, tanto nisina livre quanto encapsulada apresentaram o mesmo efeito bactericida em leite desnatado e também em leite integral, enfatizando a importância da tecnologia de barreiras no controle de patógenos em alimentos.
- A diferença dos componentes presentes no leite desnatado e no leite integral não afetou de forma significativa a atividade antimicrobiana de BLS P34 livre e encapsulada. No entanto, a atividade antilisteria tanto de BLS P34 livre quanto de BLS P34 encapsulada foi mais pronunciada em leite desnatado do que em leite integral.
- O tratamento de leite desnatado e leite integral com BLS P34 livre e BLS P34 encapsulada levou à redução na contagem de células viáveis de *L. monocytogenes* em comparação ao controle, durante 24 horas e 14 dias de armazenamento a 30ºC e 7ºC, respectivamente.
- Em queijo Minas frescal, as bacteriocinas nisina e BLS P34 na forma encapsulada promoveram maior inibição de *L. monocytogenes* do que as bacteriocinas adicionadas na forma livre, após 10 dias de armazenamento a 7ºC.
- A encapsulação de bacteriocinas em lipossomas preparados com fosfatidilcolina de soja parcialmente purificada representa uma tecnologia promissora para o controle de *L. monocytogenes* em produtos lácteos e, potencialmente, em outras matrizes alimentares.

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