UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM MICRIBIOLOGIA AGRÍCOLA E DO AMBIENTE

INFLUÊNCIA DO PEPTIDEO P34 NA EXPRESSÃO GÊNICA EM *LISTERIA SPP*. E ESTUDO DA CITOTOXICIDADE DOS PEPTÍDEOS P34 E P40

RODRIGO DE ALMEIDA VAUCHER

Porto Alegre - RS
2010

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Autor: Rodrigo de Almeida Vaucher

Tese submetida ao Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente da Universidade do Rio Grande do Sul como requisito parcial à obtenção do grau de Doutor em Microbiologia Agrícola e do Ambiente.

Orientador: Prof. Dr. Adriano Brandelli
Instituto de Ciências e Tecnoligia de Alimentos – ICTA/UFRGS.

Porto Alegre – RS
2010

Catalogação na Publicação
UFRGS/ICBS/Biblioteca Setorial

V357i Vaucher, Rodrigo de Almeida

Influência do peptídeo P34 na expressão gênica em Listeria spp. e estudo da citotoxicidade dos peptídeos P34 e P40 / Rodrigo de Almeida Vaucher. – 2010.

Tese (doutorado) – Universidade Federal do Rio Grande do Sul. Instituto de Ciências Básicas da Saúde. Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente. Porto Alegre, BR-RS, 2010.

Orientação: Prof. Adriano Brandelli

1. Peptídeos catiônicos antimicrobianos 2. Listeria monocytogenes 3. Listeria seeligeri 4. Expressão gênica 5. Toxicidade I. Brandelli, Adriano, orient. II. Título.

CDU 579.8 (043)

RODRIGO DE ALMEIDA VAUCHER
FARMACÊUTICO BIOQUIMICO
PUCRS

MEC - UFRGS
ICBS
Protocolo - Secretaria
Nº 035 9523
Data 28-04-10
Assinatura

TESE

Submetida como parte dos requisitos
Para obtenção do Grau de

DOUTOR EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

Programa de Pós-Graduação em Microbiologia Agrícola e do Ambiente

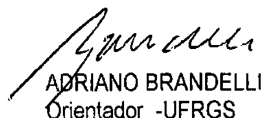
Instituto de Ciências Básicas da Saúde

Universidade Federal do Rio Grande do Sul

Porto Alegre (RS), Brasil

Aprovada em: 17/ 03 / 2010
Pela Banca Examinadora

Homologado em: 29/ 04/ 2010
Por:



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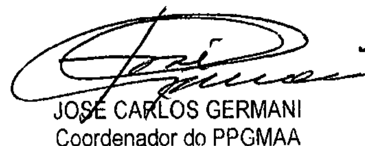

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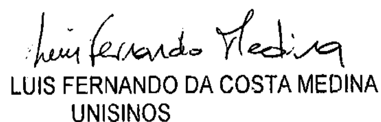

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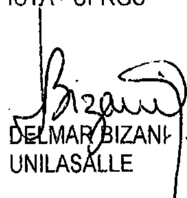

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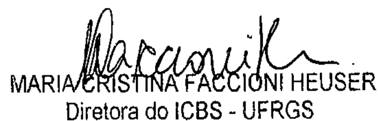

MARIA CRISTINA FACCIONI HEUSER
Diretora do ICBS - UFRGS

AGRADECIMENTOS

Ao professor Adriano Brandelli pela oportunidade, cofiança, amizade e orientação.

A todos meus familiares, em especial minha esposa Janice e minha filhinha Giulia, pelo amor, carinho, companheirismo e incentivo nos momentos mais difíceis.

A doutora e amiga Amanda de Souza da Motta, pelo incentivo e parceria na realização de alguns experimentos.

Aos companheiros do Laboratório 218 do ICTA/UFRGS, Ana Paula, Daniel, Rosiele e Volteir, que apesar do pouco tempo de convívio, sempre estiveram à disposição contribuindo de alguma forma para o bom andamento desse trabalho. Também a minha colega e amiga Camila pela ajuda nos experimentos e cuidados com os camundongos.

Um agradecimento especial ao professor Dr. Juliano Ferreira do Departamento de Química da UFSM/RS e ao colega Sérgio Salla Chagas do Laboratório de Biologia Molecular do LANAGRO/RS, por me acolherem durante parte da realização desse trabalho.

Ao LANAGRO/RS, Universidade da Região da Campanha (URCAMP-Bagé) e Exército Brasileiro pelo suporte pessoal financeiro.

Ao Cnpq pelo suporte financeiro para execução do projeto.

Aos funcionários e professores do Programa de Pós Graduação em Microbiologia Agrícola e do Ambiente.

Obrigado!!!!!!

INFLUÊNCIA DO PEPTIDEO P34 NA EXPRESSÃO GÊNICA EM *LISTERIA SPP.* E ESTUDO DA CITOTOXICIDADE DOS PEPTÍDEOS P34 E P40

Autor: Rodrigo de Almeida Vaucher

Orientador: Adriano Brandelli

RESUMO

Neste estudo foram realizados inicialmente, experimentos para avaliar a ação sinérgica do peptídeo antimicrobiano P34 com sobrenadantes de culturas de algumas bactérias lácticas selecionadas e isoladas de queijo Minas Frescal. Foi investigada a influência deste peptídeo na expressão de genes em *L. monocytogenes* e *L. seeligeri*, sua citotoxicidade em diferentes células eucarióticas e toxicidade "*in vivo*". Também foram realizados alguns testes para avaliar a citotoxicidade do peptídeo antimicrobiano P40. A adição do peptídeo P34 no queijo provocou uma diminuição de até 3 ciclos logarítmicos na contagem de células viáveis de *L. monocytogenes* inoculada artificialmente. Um aumento significativo na expressão dos genes *dlt*A, *lmo 1695* e *mpt*A de *L. monocytogenes* foi observado após 96 h com a presença do peptídeo P34 no queijo. A influência do peptídeo P34 na expressão de genes associados aos componentes do envelope celular de *L. monocytogenes* e *L. seeligeri*, promoveu um aumento não significativo nos níveis de transcrição de genes *dlt*A, *lmo*1695 e *mpt*A observados em *L. monocytogenes* após inoculação em placas e incubação por 24 h a 37°C ou 240 h a 4°C. Em *L. seeligeri* uma diminuição significativa na expressão do gene *dltA* foi observada. Os genes *lmo1695* e *mpt*A demonstraram uma diminuição significativa de sua expressão (2000 e 31872 vezes, respectivamente) na presença do peptídeo P34 e incubação por 24 h a 37°C. A inoculação da placa com o peptídeo P34 e incubação por 240 h a 4ºC não promoveu diminuição significativa da expressão do gene *mpt*A. A citotoxicidade dos peptídeos P34 e P40 foi avaliada em células VERO, tratadas com diferentes concentrações (0,02 - 2,5 μg ml^{-1}). Nos ensaios de MTT, NRU e LDH as EC_{50} para o peptídeo P34 foram 0.60, 1.25, 0.65 μg ml^{-1} e do peptídeo P40 foram 0,30, 0,51 e 0,57 μg ml^{-1}, respectivamente. A atividade hemolítica em eritrócitos humanos foi de (5,8%) e (19%), respectivamente. Os efeitos sobre a viabilidade, motilidade e exocitose acrossomal de espermatozóides humanos também foram avaliadas para o peptídeo P34. Não houve reações de hipersensibilidade ou aumento significativo de títulos de anticorpos durante os experimentos imunogenicidade ou morte dos animais durante experimentos de toxicidade aguda ou subcrônica. A DL_{50} foi superior a 332,3 ± 0,76 mg/kg. Não foram observadas alterações significativas nos parâmetros bioquímicos séricos nos animais tratados com o peptídeo P34. Não foram detectados sinais de possível toxicidade nos animais do grupo tratado com 0,825 mg/ kg/dia do peptídeo P34. Neste grupo apenas alterações histológicas no baço com a presença de megacariócitos foram observadas. A partir destes resultados evidencia-se o potencial do peptídeo P34 para ser utilizado como bioconservante em alimentos.

Palavras chaves: peptídeo antimicrobiano, *Listeria monocytogenes*; *Listeria seeligeri*, expressão gênica, citotoxicidade *in vitro* e toxicidade *in vivo*.

[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. (196p) Março, 2010.

INFLUENCE OF PEPTIDE P34 IN GENE EXPRESSION IN *LISTERIA SPP*. AND STUDY OF CYTOTOXICITY OF PEPTÍDES P34 AND P40

Author: Rodrigo de Almeida Vaucher

Advisor: Prof. Adriano Brandelli

ABSTRACT

In this study initial experiments were performed to evaluate synergistic action of the antimicrobial peptide P34 and culture supernatants of some selected lactic acid bacteria isolated from Minas Frescal cheese. The influence of this peptide in the expression of genes in *L. monocytogenes* and *L. seeligeri*, their cytotoxicity in differents eukaryotic cells and "*in vivo*" toxicity was investigated. Also, some tests were carried out o evaluate the cytotoxicity of the antimicrobial peptide P40. The peptide P34 caused a decrease of up to 3 log cycles in viable counts of *L. monocytogenes* artificially inoculated in cheese. A significant increase in expression of genes *dlt*A, *Imo1695 mpt*A of *L. monocytogenes* was observed after 96 h incubation of the peptide P34 in cheese. The influence of peptide P34 on the expression of genes associated to components of cell envelope of *L. monocytogenes* and *L. seeligeri*, promoted a non significant increase in the levels of transcription of genes *dltA, Imo1695* and *mptA* were observed after incubation of *L. monocytogenes* for 24 hs at 37°C and 240 hs at 4°C in plates. In *L. seeligeri* a significant decrease was observed in gene expression *dltA*. The gene *Imo1695* showed a significant decrease in its expression (2000-fold) after inoculation with the peptide P34. A significant decrease of expression was also observed for the gene *mpt*A (31872 - times) after inoculation with the peptide P34 and incubation for 24 hours at 37°C. The inoculation of the plate with the P34 peptide and incubated for 240 hrs at 4°C, showed a non-significant decrease of gene expression. The cytotoxicity of the peptide P34 and P40 was assessed in VERO cells treated with different concentrations (0.02 - 2.5 μg ml^{-1}). In MTT, NRU and LDH assays the EC_{50} to the peptide P34 were 0.60, 1.25, 0.65 μg ml^{-1} and the peptide P40 were 0.30, 0.51 and 0.57 μg ml^{-1}, respectively. The hemolytical activity on human erythrocytes was of (5.8%) and (19%), respectively. The effects on viability, motility and acrosomal exocytosis of humam sperm were also evaluated for peptideP34. There were no hypersensitivity reactions or significant increase in antibody titer during the immunogenicity experiment or death of animals during the acute or sub-chronic toxicity tests. The LD_{50} was more the 332.3 ± 0.76 mg/kg. No significant changes in the serum biochemical parameters were observed in the animals treated with the peptide P34. Signs of possible toxicity were no detected in animals in the group treated with 0.825 mg/kg day of peptide P34. In this group only histological changes in the spleen with the presence of megakaryocytes were observed. From these results show the potential o peptide P34 to be used in future as biopreservative in foods.

Key words: antimicrobial peptide; *Listeria monocytogenes*; *Listeria seeligeri,* gene expression, cytotoxicity *in vitro* and toxicity *in vivo*.

[1]/Thesis of Doctor in Agricultural and Enviromental Microbiology, Institute of Basic Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (196p), March, 2010.

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LISTA DE ABREVIATURAS

ALT: Aspartato aminotransferase

AST: Alanina aminotransferase

AR: Reação acrossomal

ATCC: American Type Culture Collection

BHI: Infusão de cérebro e coração

BLS: Substância tipo-bacteriocina

DL_{50}: Dose letal 50%

EC_{50}: Efeito da citotoxicidade capaz de diminuir a viabilidade celular em 50%

E-MEM: Meio mínimo essencial de Eagle`s

FAO: Organização de Agricultura e Alimentos

FDA: Food and Drug Administration

GRAS: generaly regarded as safe

ICBS: Instituto de Ciência Básicas da Saúde - UFRGS

ICTA: Instituto de Ciência e Tecnologia de Alimentos – UFRGS

IDA: Ingestão Diária Aceitável

LAB: Bactérias do Ácido Lático

LDH: Lactato Desidronase

LANAGRO: Laboratório Nacional Agropecúario – Ministério da Agricultura

MTT: Brometo de difeniltetrazolium 3-(4,5-dimetiltiazole-2-yl)

NRU: Corante vermelho neutro

OECD: Organisation for Economic Cooperation and Development

OMS: Organização Mundial da Saúde

PCR: Reação em Cadeia da Polimerase

rpm: Revoluções por minuto

SDS: Dodecil sulfato de sódio

UFC: unidades formadoras de colônias

UFRGS: Universidade Federal do Rio Grande do Sul

UFSM: Universidade Federal de Santa Maria

1 INTRODUÇÃO

Grande importância tem sido direcionada a avaliação de novas substâncias antimicrobianas na indústria de alimentos, sendo observado um aumento do número de linhagens bacterianas reportadas com relevância biotecnológica nos últimos anos.

Muitos esquemas de caracterização, purificação e aplicação de bacteriocinas ou ainda de substâncias denominadas como *bacteriocin-like*, sintetizadas principalmente por bactérias lácticas (LAB), vêm sendo propostos. Estas se enquadram no princípio de bioconservação, por inibirem bactérias deteriorantes e/ou patógenos, tais como *Staphylococcus aureus, Bacillus cereus e Listeria monocytogenes*. Estas substâncias podem ser empregadas individualmente, ou de forma sinérgica com o alimento. Apesar do conhecimento das características físicas, químicas, moleculares e possível mecanismo de ação de bacteriocinas como a nisina e pediocina PA-1, ainda existem poucos relatos de como novas substâncias antimicrobianas poderiam agir ao interagir com um microrganismo alvo em alimentos ou até fora dele.

Uma forma sugerida para avaliar a interação entre microrganismo e alimento seria através da investigação da expressão de genes envolvidos em processos essenciais para a formação da estrutura celular, bem como em processos de resistência bacteriana frente à utilização de certas bacteriocinas. Além disso, para aplicação de novas substâncias antimicrobianas em alimentos com finalidade de conservação, se faz necessário estudo de suas propriedades toxicológicas. Para isso, é necessário primeiramente, a avaliação da

imunotoxicidade, citotoxicidade *in vitro* e toxicidade aguda, subcrônica e crônica *in vivo*, respectivamente.

Estudos de toxicidade realizados com algumas bacteriocinas produzidas por microrganismo do gênero *Bacillus* demonstraram que estas não são tóxicas, nem provocam reações imunológicas e, por isso, apresentam grande potencial de aplicabilidade em alimentos. *Bacillus sp*. linhagem P34 foi a bactéria alvo deste estudo, sendo realizado com a mesma, grande parte dos experimentos apresentados a seguir, uma vez que possivelmente produza um peptídeo antimicrobiano com atividade contra diversos microrganismos patogênicos e deteriorantes de alimentos. Este peptídeo vem sendo amplamente estudado, tendo sua caracterização e purificação bem definidas. Além deste, também realizamos um breve estudo da citotoxicidade *in vitro* do *Bacillus licheniformes* P40, outro microrganismo caracterizado anteriormente, e que produz possivelmente outro peptídeo antimicrobiano com potencial biotecnológico.

Dentro deste contexto, o objetivo deste trabalho foi investigar a influência do peptídeo antimicrobiano P34 após sua aplicação, na expressão de genes em *Listeria monocytogenes* e *Listeria seeligeri* em diferentes condições, verificar a citotoxicidade *in vitro* em diferentes linhagens eucarióticas dos peptídeos antimicrobianos P34 e P40 e determinar a toxicidade aguda e subcrônica do peptídeo antimicrobiano P34 *in vivo*.

2 REVISÃO BIBLIOGRÁFICA

2.1 Antimicrobianos

Segundo MEYER *et al.* (1984), os antibióticos são "substâncias antimicrobianas e citostáticas extraídas de organismos vivos, principalmente de origem microbiana, capazes de atuar como tóxicos seletivos, em pequenas concentrações, matando ou inibindo o crescimento microbiano patogênico". Esse conceito abrange todas as substâncias produzidas por organismos vivos (KURYLOWICZ, 1981).

Contudo, o desenvolvimento de métodos sintéticos promoveu uma modificação nesta definição. Agora, o termo antimicrobiano se refere às substâncias produzidas por microrganismos ou por síntese química total ou parcial que, em baixas concentrações, inibe o crescimento de outros microrganismos (TRIPATHI, 2006).

Os antimicrobianos são metabólitos secundários sintetizados por enzimas que apresentam diversos mecanismos de ação, elevada taxa de multiresistência por microrganismos, não sendo digeridos por enzimas proteolíticas do sistema digestivo e atualmente, são aplicados na clínica médica (JOERGER, 2003). Estes quimioterápicos interferem em diversos sítios vulneráveis na célula procariótica, como a síntese da parede celular, as funções da membrana citoplasmática, a síntese protéica, o metabolismo de ácidos nucléicos e o metabolismo intermediário (HARDMAN *et al.,* 2003).

2.2 Peptídeos antimicrobianos

A produção de pequenos peptídeos não está restrita somente às bactérias, mas parece ocorrer em todos os organismos estudados até o presente momento. Tais peptídeos naturais, bem como peptídeos artificiais podem ser derivados por química combinatória ou pelo desenho racional tendo atraído grande atenção nos últimos anos (HANCOCK, 1997; HANCOCK e LEHRER, 1998). Numerosas publicações, tais como as de GENNARO e ZNETTI (2000) e VAN`T HOF *et al.*, (2001) têm analisado os aspectos gerais e específicos de peptídeos antimicrobianos, como sua bioquímica e uso potencial.

Em geral, os peptídeos antimicrobianos são moléculas catiônicas, de natureza anfifílica, podendo ser lineares ou cíclicos, e com tamanho que varia de 30 a 100 resíduos e uma grande variedade de aminoácidos em sua composição (DE SIMONE & SOUZA, 2002). Sua estrutura normalmente contém elementos que facilitam a interação com as membranas carregadas negativamente, e seu modo de ação envolve as membranas das células dos organismos-alvo (HANCOCK e ROZEK, 2002). Por estas características muitos peptídeos antimicrobianos lembram algumas bacteriocinas, como a nisina.

Os peptídeos antimicrobianos são divididos em duas classes: classe I, composta por peptídeos sintetizados não-ribossomalmente, como os glicopeptídeos e a polimixina, e classe II, constituída por peptídeos sintetizados ribossomalmente produzidos por todas as espécies de vida, incluindo as bactérias, como o componente principal do sistema de defesa do hospedeiro (BABA e SCHNEEWIND, 1998; HANCOCK e CHAPPLE, 1999). Até o presente

momento, os peptídeos antimicrobianos foram divididos em vários grupos baseados em sua massa molecular, estrutura secundária e terciária e presença ou ausência de pontes de dissulfeto. Esses peptídeos podem exibir ações bactericida, fungicida, viricida e antitumoral (REDDY *et al.*, 2004a).

Os peptídeos antimicrobianos, ao contrário de muitos antimicrobianos, parecem ser bactericidas ao invés de bacteriostáticos e exigem um curto tempo de contato para induzir a eliminação de patógenos. Tem sido demonstrado que estas substâncias apresentam um número de funções imunomoduladoras que podem estar envolvidas na depuração/controle da infecção, capazes de alterar a expressão gênica do hospedeiro. Também podem atuar como quimiocinas e/ou induzir a produção de quimiocinas, inibir lipopolissacarídio indutor de produção de citocinas pro-inflamatórias, promover a cicatrização de feridas e modular as respostas das células dentríticas e células da resposta imune adaptativa (CHEN e LUO, 2009).

O desenvolvimento de cepas resistentes aos peptídeos antimicrobianos vem sendo pouco relatada (HANCOCK, 1997; VAN`T HOF *et al.*, 2001), entretanto alguns estudos demonstram que certos genes podem conferir resistência aos peptídeos antimicrobianos (ROBEY *et al.*, 2001; PESCHEL e COLLINS, 2001). Seja este, ou qualquer outro fator determinante para a resistência, a natureza oblíqua dos peptídeos antimicrobianos, sugere que o seu papel na natureza tem sido longo e deve ter contribuído para uma adequação de muitos organismos.

Assim, uma maior investigação será necessária para identificar novos peptídeos antimicrobianos com capacidade de substituir alguns

antimicrobianos. Contudo, o uso mais provável para os peptídeos antimicrobianos, deverá ser mesmo como inibidor do crescimento microbiano em superfícies, materiais biológicos ou alimentos (JOEGER, 2003).

2.3 Bacteriocinas

2.3.1 Histórico e definição

Os primeiros registros sobre bacteriocinas datam de 1925, quando GRATIA publicou um estudo referente ao antagonismo promovido por uma linhagem de *Escherichia coli* sobre outras linhagens da mesma espécie. As substâncias responsáveis por esse efeito inibitório foram chamadas de colicinas, em referência ao microrganismo produtor. ROGERS (1928) havia observado a capacidade de certas cepas de *Lactococcus* de promover a inibição de outras bactérias láticas. Somente, em 1947, MATTICK e HIRSCH concentraram uma substância inibidora produzida por uma cepa de *Lactococcus lactis* subsp *lactis*, que apresentava um amplo espectro de atividade, denominada nisina. Mais tarde, JACOB *et al.* (1953) propuseram o termo bacteriocina, para as proteínas antimicrobianas produzidas por microrganismos Gram-negativos e Gram-positivos (NASCIMENTO *et al*., 2008).

Bacteriocinas são proteínas biologicamente ativas, que apresentam propriedades antimicrobianas contra espécies intimamente relacionadas com o organismo produtor (CLEVELAND *et al*., 2001). Dentre as propriedades merecem destaque, seu efeito bactericida ou bacteriostático, dependente da concentração, o pequeno ou amplo espectro de ação, a produção mediada por plasmídeo ou cromossomo, a interação com sítios receptores específicos ou

inespecíficos de ligação na bactéria sensível e o modo de ação similar, desestabilizando a força protônica das membranas das células sensíveis (BRUNO e MONTIVILLE, 1993; EIJISINK, 1998).

Existem inúmeras substâncias com atividade antimicrobiana descobertas, atuando contra uma variedade de bactérias Gram-positivas, Gram-negativas e fungos, que apresentam natureza protéica. Muitas delas, ainda não foram completamente caracterizadas e não cumprem com todas as características para serem classificadas como bacteriocinas. Estas tem sido chamadas de substâncias semelhantes à bacteriocinas (*bacteriocin-like*) ou simplesmente peptídeos antimicrobianos (DE VUYST e VANDAMME, 1994).

Atualmente, as bacteriocinas produzidas por bactérias ácido-lácticas (LAB) são amplamente estudadas, devido ao grande potencial biotecnológico e aplicabilidade na indústria de alimentos. Uma bacteriocina que tem sido empregada há décadas, com a finalidade de bioconsevação de alimentos, é a nisina. Foi inicialmente purificada e comercializada na Inglaterra, sendo considerada segura para uso em alimentos pelo JECFA/OMS desde 1969. Na Europa, tem sido utilizada desde 1983 após ser incluída à lista de aditivos alimentares e em 1988, nos EUA, o FDA autorizou sua utlização em queijos processados (APHA, 1992; CODEX ALIMENTARIUS, 1995). No Brasil, foi autorizada em 1996 para ser empregada em queijos na concentração de até 12,5 mg Kg^{-1} (BRASIL, 2000a, 2000b).

2.3.2 Classificação das bacteriocinas

Atualmente, as bacteriocinas tem sido divididas em três ou quatro grupos, dependendo do microrganismo produtor e dos critérios de classificação de diferentes autores. Uma das classificações amplamente empregada durante muitos anos foi realizada por KLAENHAMMER (1993), que dividia as bacteriocinas em quatro classes distintas, levando em consideração sua estrutura primária, estabilidade térmica, peso molecular e mecanismo de ação. Entretanto, novos sistemas de classificação de bacteriocinas tem sido sugeridos (NES *et al*., 1996; COTTER *et al*. 2005a), além da inclusão de novas categorias (KEMPERMAN *et al*., 2003), sendo necessário mais algum tempo até que um sistema definitivo seja obtido (DE MARTINIS e FREITAS, 2003).

Para KLAENHAMMER (1993), as bacteriocinas da classe I são constituídas por pequenos peptídeos (<5 kDa), compostos geralmente por um ou dois aminoácidos. Estes peptídeos são termoestáveis e contém aminoácidos incomuns como lantionina e b-metillantionina.

A classe II é composta por pequenos peptídeos (<10 kDa), hidrofóbicos, termoestáveis, ativos em membrana e que não contém lantionina. As bacteriocinas dessa classe formam hélices anfifílicas com hidrofobicidade variável, estrutura de b-folha e estabilidade térmica moderada (100 ºC) a alta (121 ºC).

A classe II foi dividida em três subclasses distintas: subclasse IIa, onde estão representados os peptídeos ativos contra *Listeria* spp., que apresentam uma sequência N-terminal comum, composta por Tyr-Gly-Asn-Gly-Val-Xaa-Cys. A subclasse IIb constituída por peptídeos formadores de poros na membrana celular, compostos por duas subunidades importantes para a

atividade. E por último a subclasse IIc representada por peptídeos ativados por tiol, que requerem resíduos de cisteína reduzida para tornarem-se ativos.

A classe III é composta por grandes proteínas termolábeis (>30 kDa).

A classe IV é composta por bacteriocinas bastante complexas, constituídas por uma fração protéica e frações lipídicas ou glicídicas necessárias para a atividade.

Recentemente, um novo sistema de classificação para as bacteriocinas foi proposto por COTTER *et al*. (2005a), que pouco alteram as definições das classes em relação a KLAENHAMMER (1993), mas reagruparam as bacteriocinas em apenas duas categorias: a classe I composta pelos lantibióticos contendo lantionina e que apresentam 11 subclasses proposta por COTTER *et al.* (2005b), além das bacteriocinas composta por um peptídeo, como exemplo, a nisina, mersacidina e lacticina 481 ou dois peptídeos, exemplo lacticina 3147 e citolisina, respectivamente. A Classe II, composta por bacteriocinas que não contém lantionina, apresentando distribuição heterogênea de pequenos peptídeos. Esta classe foi subdividida em quatro subclasses:

Classe IIa representada por substâncias semelhantes a pediocina (pediocina-like), exemplo são a pediocina PA1 e leucocina A.

Classe IIb representada por bacteriocinas com dois peptídeos, exemplo é a lactacina F.

Classe IIc representada por bacteriocinas com estrutura cíclicas, exemplo são a enterocina AS48 e reuterina.

Classe IId representada por peptídeos lineares simples não pediocina, exemplos são lactococcin A e divergicina A.

As proteínas líticas reconhecidas por serem grandes proteínas termolábeis, mureína hidrolases (anteriormente classificada como classe III) foram reagrupadas separadamente, e esta categoria chamada de bacteriolisinas. Exemplo são a lisostafina e enterolisina A.

COTTER *et al.* (2005a), sugerem que as bacteriocinas de *Clostridium* identificas e caracterizadas por KEMPERMAN *et al.* (2003), chamadas de circularina A e closticina 574, sejam reagrupadas na categoria das bacteriocinas que não contém lantionina (classe IIc). Por fim, a classe IV proposta por KLAENHAMMER (1993) não foi incluída na nova proposta, por que segundo COTTER *et al.* (2005a) as bacteriocinas anteriormente classificadas nesta classe não tem sido convincentemente caracterizadas.

2.3.3 Bacteriocinas de bactérias Gram-negativas

Um grande número de bactérias Gram-negativas presentes no ambiente podem produzir bacteriocinas, entre estes os mais amplamente pesquisados estão: *E. coli, Salmonela enterica, Hafnia alvei, Citrobacter freundii, Klebsiella oxytoca, Klebsiella pneumoniae e Enterobacter cloacae* (GORDON *et al.*, 2007; RILEY *et al.* 2003). Dentre as bacteriocinas mais estudadas, merecem destaque as colicinas e as microcinas, ambas de *E. coli*. Entretanto, piocinas e outros peptídeos antimicrobianos também tem sido descritos para cepas de *Pseudomonas aeruginosa*, a partir de isolados clínicos

e amostras de ambiente (MICHEL-BRIAND e BAYSSE, 2002; FONTOURA *et al.*, 2009).

As colicinas são bacteriocinas produzida por *E. coli*, encontradas em 30-50% das cepas isoladas de hospedeiros humanos e muitas vezes referidas como fatores de virulência (RILEY e GORDON, 1992). Desde sua descoberta, foram caracterizados 25 tipos diferentes, sendo consideradas as bacteriocinas mais estudadas de bactérias Gram-negativas e agora muitas servem como sistema modelo para desvendar os mecanismos de estrutura/função, organização genética, ecologia e evolução de outras bacteriocinas (CASCALES *et al.*, 2007).

As colicinas são proteínas de elevada massa molecular que matam as células-alvo através de diferentes mecanismos de ação. A colicina E1 e K inibem a síntese de macromoléculas, a colicina E2 pode causar fragmentação de DNA, e a colicina E3 inibe a síntese de proteínas (NOMURA, 1967). Entretanto, outros estudos também demostraram a capacidade das colicinas de matar a célula alvo, devido à permeabilização da membrana ou degradação dos ácidos nucléicos pela atividade específica de nucleases (BRAUN *et al.*, 1994; SMARDA e SMAJS, 1998; RILEY e WERTZ, 2002b).

Embora as colicinas e microcinas sejam produzidas por cepas de *E.coli*, e apresentem características diferentes, novas bacteriocinas de bactérias Gram-negativas estão sendo estudadas. Um exemplo é a piocina produzida por *Pseudonomas aeroginosa*, que assim como as bacteriocinas produzidas por *Serratia marcenses*, são codificadas exclusivamente, por genes presentes em seu cromossomo. Tem sido observado que as piocinas mostram

similaridade de sequência com as colicinas e outras bacteriocinas, ainda não caracterizadas completamente (RILEY & WERTZ, 2002a).

2.3.4 Bateriocinas de bactérias Gram-positivas

As bacteriocinas de bactérias Gram-positivas são mais abundantes e diversificadas que as encontradas nas bactérias Gram-negativas. Elas diferem das bacteriocinas de bactérias Gram-negativas em dois aspectos fundamentais. Primeiro, as bacteriocinas produzidas por bactérias Gram-positivas não são necessariamente letais para a célula como ocorre com as bactérias Gram-negativas. Esta diferença crítica é devido aos mecanismos de transporte das bactérias Gram-positivas. Normalmente, sua biossíntese é auto-regulada, com mecanismos específicos de transporte e que facilita sua liberação, apesar de algumas poderem usar uma via de exportação *sec*-dependente (DRIDER *et al.*, 2006; EIJSINK *et al.*, 2002; MAQUEDA *et al.*, 2008). Em segundo lugar, as bactérias Gram-positivas têm desenvolvido uma forma de regulação específica, mais complexa que as bactérias Gram-negativas (NES *et al.*, 1996).

A produção de bacteriocinas em bactérias Gram-positivas está geralmente associada com a mudança de fase log para fase estacionária. A regulação da expressão não é dependente do ciclo celular, por si, mas sim a cultura dependente da densidade (DUFOUR *et al.*, 2003). A nisina A age como um feromônio de proteínas na regulação da sua própria expressão, que é controlado por um ou dois componentes do sistema de transdução de sinal típico de muitos sistemas de "*quorum sensing*" (HECHARD e SAHL, 2002).

As bacteriocinas de bactérias Gram-positivas, em particular os lantibióticos, necessitam de muito mais genes para sua expressão do que bactérias Gram-negativas (NAGAO *et al*., 2006). Um exemplo seria o agrupamento de genes necessários para a síntese de nisina, que incluem o gene para o pré-peptídeo (*nisA*), os genes para enzimas que codificam aminoácidos (*nisB e nisC*), o gene para clivagem do peptídeo líder (*nisP*), o gene para secreção (*nisT*), os genes para a imunidade (*nisI* e *nisFEG*) e os genes para a regulação da expressão (*nisR* e *nisK*). Estes conjuntos de genes são encontrados frequentemente em plasmídeos, mas podem também ser encontrados no cromossomo (CHEIGH e PYUN, 2005). Inúmeras bacteriocinas de bactérias Gram-positivas, incluindo nisina, estão localizados em transposons (KIM e DUNN, 1997).

O espectro de ação destas bacteriocinas abrange bactérias Gram-positivas e, com menor intensidade bactérias Gram-negativas. Por exemplo, alguns lantibióticos do tipo A, como nisina A e a mutacina B-Ny266, foram capazes de inibir uma variedade de microrganismos, incluindo espécies dos gêneros *Actinomyces*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Gardnerella, Lactobacillus, Listeria, Micrococcus, Mycobacterium, Propionibacterium, Streptococcus* e *Staphylococcus*. Enquanto, as bactérias Gram-negativas que são geralmente susceptíveis a ação de bacteriocinas, incluem espécies dos gêneros *Campylobacter*, *Haemophilus*, *Helicobacter* e *Neisseria* (MOTA-MEIRA *et al*., 2000, MORENCY *et al*., 2001).

2.3.5 Peptídeos antimicrobianos produzidos por *Bacillus*

Tradicionalmente, as bacteriocinas são descritas como sendo peptídeos antimicrobianos produzidas por bactérias láticas, porém vários estudos estão direcionados a produção de bacteriocinas ou substâncias semelhantes à bateriocinas (*Bacteriocin-like*) por bactérias do gênero *Bacillus*.

Segundo o Manual Bergey's de Bacteriologia Sistemática, os *Bacillus* são bactérias com forma de bastonetes, Gram-positivas (ou positivas nos primeiros estágios de crescimento), formadoras de esporos, aeróbias ou anaeróbias facultativas e a maioria é catalase positiva. São agrupadas de acordo com as exigências físicas, químicas, nutricionais ou genéticas.

Membros do gênero *Bacillus* são conhecidos como produtores de peptídeos antimicrobianos, sendo muitas substâncias produzidas por *Bacillus spp.* extraídas e caracterizadas sendo estas reconhecidas como industrialmente importantes. Como exemplo, pode-se citar *Bacillus subtilis, Bacillus thuringiensis*, *Bacillus stearothermophilus, Bacillus licheniformis, Bacillus megaterium* e *Bacillus cereus.* (JANSEN e HIRSCHMANN, 1944; IVÀNOVICS, 1962; SHAFIA, 1966; BRADLEY, 1967; TAGG *et al.*, 1976; FAVRET e YOUSTEN, 1989; NACLERIO *et al.*, 1993; PAIK *et al.*, 1997; HYRONIMUS *et al.*, 1998; OSCÁRIZ *et al.*, 1999; CHERIF *et al.*, 2001, NAZINA *et al.*, 2001; BIZANI e BRANDELLI, 2002; STEIN, 2005).

Dentre os principais estudos relatados sobre a identificação de bacteriocinas produzidas por *Bacillus spp.*, destacam-se os com *Bacillus subtilis* produtores de diversas bacteriocinas, algumas delas sintetizadas ribossomalmente e produzidas durante a fase ativa de crescimento como a

subtilosina, a subtilina, a sublancina, o TasA, e outros sintetizados não ribossomalmente durante a fase estacionária, como a surfactina, a bacilocina 22 e as iturinas (KLEIN e ENTIAN, 1994; ZHENG e SLAVIK, 1999; TAMEHIRO *et al.*, 2002).

A cereína 7 produzida por uma cepa de *Bacillus cereus* foi caracteizada por OSCÁRIZ *et al.* (1999). Esta bacteriocina apresentou massa molecular de 3,9 kDa apresentando amplo espectro de atividade. NACLERIO *et al.* (1993) já haviam reportado anteriormente, a produção de outra cereína, entretanto, esta apresentou peso molecular de aproximadamente 9 kDa e espectro limitado de atividade. BIZANI e BRANDELLI (2002) reportaram a caracterização de uma nova cereína, denominada de cereína 8A, a partir de uma cepa de *Baillus cereus*.

CLADERA-OLIVERA *et al.* (2004a) também relataram a purificação de uma substância tipo bacteriocina a partir de uma cepa de *Bacillus lincheniformis.* Outros autores que detectaram e caracterizaram bacteriocinas produzidas por *Bacillus licheniformis* foram MARTIRANI *et al.* (2002) que denominaram a mesma de bacillocina 490. Essa bacteriocina é inativada quando tratada com pronase E e proteinase K. PATTNAIK *et al.* (2005), estudaram o efeito de fatores ambientais na produção de lichenina, um composto codificado cromossomicamente como uma bacteriocina, produzido por *Bacillus lichenifornis* 26L-10/3RA, isolado do rúmem de búfalo. Segundo este estudo, a lichenina representa o primeiro composto antimicrobiano de amplo espectro produzido em ambiente de anaerobiose (PATTNAIK *et al.*, 2001).

A thuricina 439, uma bacteriocina produzida por *Bacillus thurigienses* B439 foi caracterizada por AHERN *et al.* (2003). Os resultados obtidos neste estudo confirmaram que essa bacteriocina permanece ativa numa ampla faixa de pH não perdendo a atividade antimicrobiana quando submetida a temperaturas superiores a 80°C. CHERIF *et al.* (2003), observaram que *Bacillus thuringienses ssp. entomocidus* HD9 produz uma bacteriocina denominada de entomocina 9 ativa contra *Listeria monocytogenes*, *Pseudonomas aeroginosas* e vários fungos.

Finalmente, estudos recentes sobre bacteriocinas de bactérias do gênero *Bacillus*, demonstram a possivel utilização destas substâncias como biocontroladores de fitopatógenos e patógenos humanos. Um exemplo dessas substâncias é a iturina A, produzida por *Bacillus amyloliquefaciens* B94 utilizada para controle do fitopatógeno *Rhizoctonia solani*, responsável pela redução do rendimento da produção de soja. KIM e CHUNG (2004) caracterizaram uma potente proteína antifúngica isolada de uma cepa de *Bacillus amyloliquefaciens* MET0908. BATISTA (1993), também verificou uma substância em uma cepa de *Bacillus amyloliquefaciens* capaz de inibir o desenvolvimento de *Listeria monocytogenes.*

2.3.6 Peptídeos antimicrobianos P34 e P40

O peptídeo antimicrobiano P34 utilizado neste estudo, foi isolado de uma bactéria do gênero *Bacillus*, selecionada entre 86 cepas triadas para atividade antimicrobiana contra microrganismos indicadores, sendo oriundo do intestino de um peixe da região Amazônica. Em estudo anterior apresentou um

amplo espectro de ação, sendo capaz de inibir o crescimento de bactérias do mesmo gênero como *Bacillus cereus* e bactérias de outros gêneros como *Listeria*, em especial *Listeria monocytogenes* é um importante patógeno do ponto de vista de segurança alimentar. A atividade antimicrobiana também foi observada sobre *Erwinia carotovora, Pasteurella haemolytica e Rhodococcus sp.* (MOTTA *et al*., 2004).

Testes adicionais para caracterização da cepa P34 foram realizados com sucesso, além de ser verificado o espectro inibitório e sua atividade frente à *Listeria monocytogenes* e *Bacillus cereus* (MOTTA *et al*., 2007a). Posteriormente, o peptídeo antimicrobiano P34 foi purificado (MOTTA *et al*., 2007b), sendo também estudado o modo de ação desse peptídeo no envelope de células bacterianas de *Listeria monocytogenes* (MOTTA *et al*., 2008).

Suas características como estabilidade térmica, atividade em um amplo espectro de pH (intervalo de pH 3,0 a pH 10), caráter aniônico, hidrofobicidade e baixa massa molecular (1498,68 Da), permitiu aos autores sugerir uma classificação deste peptídeo, como possivelmente pertencente a classe I, conforme KLAENHAMER (1993).

O peptídeo antimicrobiano produzido por *Bacillus licheniformis* P40 foi purificado e parcialmente caracterizado. Este peptídeo demonstrou um amplo espectro de ação, com atividade bactericida frente a bactérias patogênicas e deteriorantes de alimentos, tais como *Bacillus cereus*, *Listeria monocytogenes*, *Erwinia carotovora* e *Staphylococcus aureus*, entre outros, bem como possuidor da capacidade de resistir a altas temperaturas (CLADERA-OLIVERA *et al*., 2004a).

Outro estudo realizado com esse peptídeo antimicrobiano foi realizado por TEIXEIRA *et al.* (2007), que incorporaram o mesmo em nanovesículas de fosfatidilcolina, com a finalidade de inibir o crescimento de *Listeria monocytogenes*. Estes autores observaram uma redução do número de células viáveis após 12 minutos de incubação, demonstraram uma estabilidade de até 28 dias a 4ºC, para esta forma encapsulada. Posteriormente, os mesmos autores realizaram a purificação e caracterização do peptídeo P40 de *Bacillus licheniformis*, além de demonstrar a atividade emulsificante do mesmo (TEIXEIRA *et al.*, 2008).

2.4 Aplicações das bacteriocinas

2.4.1 Aplicações em alimentos

Alguns dos desafios que a indústria de alimentos enfrenta na atualidade incluem o aumento da demanda por alimentos livres de microrganismos patogênicos, com uma longa vida de prateleira e que não contenham conservantes químicos. Neste contexto, as bacteriocinas apresentam-se como uma alternativa atrativa que poderia oferecer ao menos parte da solução para estes problemas, já que são produtos bacterianos naturais que são inativados facilmente por proteases intestinais, cumprindo com as características requeridas em um produto para ser utilizado como bioconservante. Além disso, estas moléculas inibem numerosos microrganismos patogênicos presentes em alimentos, são estáveis ao calor e ativas em intervalos amplos de pH (COTTER *et al.*, 2005a).

As bacteriocinas tem sido utilizadas em diferentes alimentos, tais como carnes, derivados do leite, e produtos vegetais (HUGAS, 1998; BARRETO *et al.*, 2004; MATARAGAS *et al.*, 2002). Também são aplicadas, de diferentes formas, sendo as mais comuns através da utilização direta de linhagens produtoras, culturas “starters” em alimentos fermentados, por preparações concentradas de bacteriocinas como aditivo, no desenvolvimento de embalagens com bacteriocinas imobilizadas e recentemente encapsuladas em nanovesículas fosfolipídicas (SCANNELL *et al.*, 2000; PAPAGIANNI, 2003; TEIXEIRA *et al.*, 2007).

Atualmente, a nisina tem sido utilizada de maneira comercial como bioconservante de alimentos, estando presente em pequenas concentrações, apenas (2,5%) em uma preparação comercializada e conhecida como Nisaplin® (Danisco), constituída ainda, de NaCl (7,5%), proteínas do leite (12%) e carboidratos (6%). Esta foi reconhecida como aditivo alimentar pela Organização de Alimentos e Agricultura/Organização Mundial da Saúde (FAO/OMS) em 1969 com limite máximo de ingestão de 33.000 Unidades Internacionais de peso corpóreo. Muitos países permitem o uso da nisina em produtos como leite, queijo, produtos lácteos, tomates, vegetais enlatados, sopas enlatadas, maionese e alimentos infantis (DE MARTINS *et al.*, 2003). Além disso, a nisina tem sido utilizada por mais de 50 anos, em mais de 48 países, sem o desenvolvimento substancial de resistência (DEEGAN *et al.*, 2006).

No Brasil, esta bacteriocina é aprovada para uso em todos os tipos de queijo, no limite de 12,5 mg/Kg e é também permitido o uso em produtos

cárneos com aplicação na superfície externa de diferentes tipos de salsichas (BRASIL, 2000a, BRASIL 2000b). Outra bacteriocina mencionada na literatura é a pediocina PA1 que vem sendo produzida através da adição de ingredientes baseados no fermentado de uma cultura *Pediococcus acidilactici,* estando presente em uma preparação comercial conhecida como ALTA 2431(Quest). O uso de ambas as bacteriocinas esta coberto por diversas patentes norte-americanas e européias (ENNAHAR *et al*., 2000; RODRÍGUEZ *et al*., 2002).

Apesar das duas bacteriocinas serem utilizadas comercialmente, a descoberta de novas bacteriocinas com capacidade de aplicação biotecnológica e que apresentem mecanismos de ação bem caracterizados ainda vem sendo investigada (COTTER *et al*., 2005b).

2.4.2 Aplicações biomédicas

Devido à baixa toxicidade de muitas bacteriocinas as células eucarióticas e sua atividade contra grande número de bactérias patogênicas, estas tem despertado o interesse para aplicação terapêutica na biomedicina. Por isso, estudos nesta área tem sido realizados com o objetivo de elucidar os mecanismos de ação das bacteriocinas e sua atividade contra patógenos resistentes as terapias com antimicrobianos convencionais (COTTER *et al*., 2005a; COTTER *et al*., 2005b).

As bacteriocinas de amplo espectro vêm sendo utilizadas contra bactérias Gram positivas patogênicas a humanos e animais. Um exemplo, a lacticina 3147 de *L. lactis* tem demonstrado atividade *in vitro* contra cepas de *S. aureus* (resistentes à meticilina - MRSA), enterococos (resistentes à

vancomicina), estreptococos (*S. pneumoniae, S. pyogenes, S agalactiae, S. dysgalactiae, S. uberis e S. mutans*), *Clostridium botulinum* e *Propionibacterium acnes* (GALVIN *et al*., 1999).

Experimentos *in vivo* utilizando modelos animais, demonstraram resultados positivos, após o uso de lantibióticos como a mersacidina no tratamento de infecções causadas por *S. pneumoniae* (GOLDSTEIN *et al*., 1998) e *S. aureus* resistentes à meticilina (KRUSZEWSKA *et al*., 2004), bem como em terapias orais, para a prevenção da queda de dentes, mau hálito e gengivite (HOWELL *et al*., 1993). Pesquisas tem proposto a utilização de nisina para fins terapêuticos, para o tratamento de dermatite atópica (VALENTA *et al*., 1996) e úlceras estomacais em pacientes imunodeficientes (DUBOIS, 1995; KIM *et al*., 2003).

A indústria farmacêutica vem realizando experimentos com a incorporação da nisina em pasta de dentes e cremes, estes serão administrados em humanos por via tópica para combater infeccões causadas por cepas de *S. aureus* (COTTER *et al*., 2005a). Outra aplicação biomédica importante da nisina seria na prevenção de mastite. Recentemente, FERNÁNDEZ *et al*. (2008) estudaram a aplicação da nisina para tratamento de mastite estafilocócica em mulheres durante a lactação. Com aplicação biomédica para uso veterinário a nisina tem sido empregada como sanitizante contra patógenos causadores de mastite como espécies de *Staphylococcus* e *Streptococcus* (CAO *et al*., 2007; WU *et al*., 2007). Além disso, a nisina também tem demostrado atividade contraceptiva (ARANHA *et al*., 2004) e de

proteção vaginal em estudos *in vitro* utilizando coelhos e camundongos como modelo (REDDY *et al*., 2004b).

Finalmente, existem perspectivas para aplicação biomédicas diferentes para as bacteriocinas, não sendo estas utilizadas apenas para fins de terapêutica antimicrobiana. Um exemplo seria a cinamicina, que apresenta capacidade inibitória da enzima fosfolipase A2 e da enzima conversora de angiotensina, envolvidas no sistema imune e de manutenção da pressão sanguínea em humanos, respectivamente. Assim, tem sido sugerido que as bacteriocinas poderiam ser utilizadas em processos inflamatórios e na regulação da pressão sanguínea (ENNAHAR *et al*., 2000), embora ainda sejam necessários novos estudos.

2.5 Toxicidade das bacteriocinas

Os primeiros estudos toxicológicos para avaliar o potencial citotóxico de uma bacteriocina foram realizados com a nisina por FRAZER *et al.* (1962). Estes autores determinaram a toxicidade aguda por via oral em animais, demonstrando que a DL_{50} em ratos era superior a 25 mg/Kg de peso, enquanto que em camundongos a DL_{50} era de aproximadamente 174 mg/kg de peso do animal. Também realizaram estudos de toxicidade sub-crônica em ratos, observando uma variação na ingestão de doses de 0,125 mg/Kg/dia a 60 mg/Kg/dia comparadas ao grupo controle. Outro estudo importante realizado por HARA *et al*. (1962) demonstrou o efeito de enzimas gástricas sobre a nisina, sendo evidenciado a inativação do peptídeo antimicrobiano pela tripsina,

direcionando os autores a concluir que a ingestão de nisina não interferia na microbiota gastrointestinal.

CLAYPOOL *et al.* (1966) realizaram estudos para avaliar o efeito da nisina sobre a microbiota oral. Foi evidenciado que um minuto após o consumo de leite achocolatado contendo nisina, somente 40% de sua atividade foi detectada quando comparada ao controle de saliva. No mesmo estudo, foi demonstrado que no leite achocolatado que continha penicilina, a saliva apresentava atividade antimicrobiana por um maior tempo.

Atualmente, a nisina é amplamente utilizada como aditivo natural em alimentos e sabe-se que sua ingestão não causa efeitos tóxicos ao organismo humano, sendo reportada uma DL_{50} de 6950 mg/Kg, similar ao cloreto de sódio, quando administrada oralmente. A partir dessas avaliações, a Organização Mundial da Saúde determinou que a Ingestão Diária Aceitável - IDA, que representa a quantidade máxima de aditivo que poderia causar quaisquer danos a saúde do consumidor é de 33000 Unidades Internacionais ou 0,825mg/Kg de peso corpóreo (HOOVER e STEENSON, 1993). Além disso, recentes estudos demonstraram a rápida inativação da nisina pela quimiotripsina, enzima produzida no pâncreas e liberada no intestino delgado, e sua sensibilidade à ptialina, não sendo detectada na saliva humana após 10 minutos do consumo de líquidos (JOZALA *et al.*, 2007).

Assim, os principais estudos toxicológicos desenvolvidos até o presente momento, se referem aos testes realizados para a aprovação do uso da nisina como bioconservante em alimentos (FDA, 1988).

Novos estudos tem sido realizados para avaliar outras bacteriocinas com potencial de aplicação em alimentos. Devido à sensibilidade de algumas bacteriocinas à degradação por enzimas proteolíticas, sua aplicação parece bastante interessante com relação a segurança alimentar, uma vez que sua ingestão não provoca alterações na microflora gastrointestinal (MORENO *et al.*, 2000). Segundo MARUGG (1991), estudos realizados com diferentes bacteriocinas indicaram que elas não são tóxicas nem provocam reações imunológicas, assim apresentam grande potencial para serem utilizadas como conservantes naturais em alimentos. Entretanto, para que uma bacteriocina, com exceção da nisina, possa ser utilizada para essa finalidade deverá estar identificada quimicamente e bem caracterizada. Além disso, estudos da cinética da molécula após ingestão são necessários, assim como dados toxicológicos (CLEVELAND *et al.*, 2001).

2.6 Gênero *Listeria* e alimentos

O gênero *Listeria* é composto por seis espécies: *L monocytogenes, L seeligeri, L. ivanovvi, L. innocua, L.welshimeri e L. grayi* (HICTHINS, 2003). No entanto, somente *L. monocytogenes* é patogênica para humanos, enquanto *L. ivanovvi* pode ser às vezes patogênicas para animais.

Os representantes deste gênero são bastonetes Gram-positivos, aeróbios e anaeróbios facultativos, possuem em média 1-2 µm de comprimento 0,5 µm de largura, móveis à temperatura de 10°C a 25°C, em ágar semi-sólido e produzem motilidade característica conhecida como “guarda chuva” (JAY, 2005). Apresentam flagelos peritríquios que permitem a motilidade por

movimentos rotatórios ou tombamento (SEELINGER *et al.*, 1986). Esses microrganismos podem ser classificados pela soro tipificação baseada nas diferenças sorológicas de polissacarídeos capsulares, que permite a separação em sorotipos distintos.

Além destas características, são não formadores de esporos, possuem a capacidade de crescer em uma ampla faixa de pH (4.3 - 9,6), temperatura (1°C a 45 °C) e concentrações de sal acima de 10% (SEELINGER *et al.*, 1986). São psicrotróficos, podendo sobreviver e crescer em temperaturas de refrigeração (4°C) e atividade de água abaixo da capacidade inibitória de crescimento da maioria de outros patógenos, bem como resistir ao congelamento e descongelamento (SILVA *et al.*, 2003; ROBERTS *et al.*, 2003; GERMANO *et al.*, 2001).

A *Listeria monocytogenes* esta distribuída de forma uniforme no meio ambiente, sendo responsável pela contaminação de alimentos crus e processados, causando uma das mais graves infecções alimentares, a listeriose ou listeríase. Esta espécie apresenta características bioquímicas peculiares, como a capacidade de produzir compostos ácidos a partir da L-ramnose e α-metil-D-manosídeo. Apresenta também atividade β-hemolítica em ágar sangue, fator de CAMP positivo com *Staphylococcus aureus*, mas não com *Rhodococcus equi* (HOFER *et al.*, 2000).

O controle da *Listeria monocytogenes* tem sido reportado como de fundamental importância para a prevenção de infecções alimentares. Este controle geralmente tem sido realizado pela aplicação de barreiras físicas (irradiação, ultra-alta pressão e calor), químicas, (ácidos orgânicos, sais e

compostos fenólicos) ou biológicas (bacteriocinas), este último considerado um método alternativo na conservação de alimentos (KANG *et al*., 1999; NIKU-PAAVOLA *et al*., 1999, JUNCHER *et al*., 2000; SAMELIS *et al*., 2002; SEMAN *et al*., 2002).

2.7 Identificação, quantificação e expressão gênica em bactérias do gênero *Listeria*

Métodos bacteriológicos convencionais ainda são empregados para a identificação de bactérias do gênero *Listeria*, contudo, são considerados demorados, trabalhosos, pouco seguros, além de requerer tempo para confirmação bioquímica individual da espécie, quando se tem um grande número de colônias isoladas (DONNELLY, 1999). Estas desvantagens são superadas pela substituição destes métodos por técnicas moleculares baseadas na extração e amplificação do DNA alvo de *Listeria* (WIEDMANN, 2002).

Ultimamente, uma inovação tecnológica resultante da técnica da Reação em Cadeia da Polimerase (PCR), denominada de PCR em tempo real (Real-time PCR), vem ganhando espaço nos diagnósticos clínicos e nos laboratórios de pesquisa em alimentos por apresentar a capacidade de gerar resultados quantitativos. Essa técnica permite o acompanhamento da reação e a apresentação dos resultados de forma mais rápida e precisa, em relação à PCR convencional que apresenta somente resultados qualitativos (NORTAN, 2002).

PCR em tempo real vem sendo empregado com a finalidade de identificar e quantificar a presença de *L. monocytogenes* em alimentos ou outros materiais (NOGVA *et al.*, 2000; RUDI *et al.*, 2005; GUILBAUD *et al.*, 2005; RODRÍGUEZ-LÁZARO *et al.*, 2005; LONG *et al.*, 2008). Pode ser empregada para inferir a quantidade inicial de um determinado produto (cDNA/DNA) através do comportamento da cinética de amplificação, ou seja, através da análise das diferentes fases de um ciclo da Reação em Cadeia da Polimerase (PCR). Esta quantificação é baseada na emissão de um sinal fluorescente por uma substância específica que se intercala no DNA bacteriano ou por uma sonda fluorescente que anela de forma específica entre dois primers no DNA alvo durante a reação (ESPY *et al.*, 2006).

Por este motivo, a técnica de PCR em tempo real é utilizada com sucesso para triagem de *Listeria monocytogenes* diretamente do alimento ou no ambiente onde são processados, sendo considerada confiável e oferecendo resultados rápidos e eficientes (NORTON, 2002). Além disso, PCR em tempo real é empregada com sucesso em estudos de expressão gênica em *L. monocytogenes* (KLEIN, 2002). Existem inúmeros estudos de identificação e quantificação de *L. monocytogenes* em alimentos por PCR em tempo real, utilizando genes de virulência como o *hly*, *iap* e *lin 02483* (RODRÍQUEZ-LÁZARO *et al.*, 2004; RODRÍGUEZ-LÁZARO *et al.*, 2005), bem como alguns anteriormente identificados, como a listeriolisina O (MENGAUD *et al.*, 1988) e a proteína de invasão de superfície p60 (KOHLER *et al.*, 1990).

Outros importantes estudos têm investigado a capacidade de certas bacteriocinas, de inibir o crescimento de bactérias do gênero *Listeria* ou outros

microrganismos, bem como de verificar a existência de cepas resistentes a certas classes de bacteriocinas (CRANDALL *et al*., 1998). Finalmente, estudo de expressão gênica utilizando PCR em tempo real, busca compreender melhor os mecanismos envolvidos na diminuição ou aumento da resistência as bacteriocinas (GRAVESEN *et al*., 2002; VADYVALOO *et al*., 2004, XUE *et al*., 2005; DUBAIL *et al*., 2006; BEGLEY *et al*., 2006; XUE e MILLER, 2007).

3. RESULTADOS E DISCUSSÃO

Os resultados deste trabalho estão apresentados na forma de artigos submetidos ou publicados em revistas científicas. Cada subtítulo deste capítulo corresponde a um destes artigos, e os já publicados encontram-se em anexo.

3.1 Antimicrobial peptide P34 influences gene expression of *Listeria monocytogenes* growing in soft cheese

- Artigo submetido para publicação no periódico **International Journal Food Microbiology**.

3.2 Investigation of the influence of antimicrobial peptide P34 on gene expression of *Listeria monocytogenes* and *Listeria seeligeri*

- Artigo a ser submetido ao periódico **Journal of Applied Microbiology**.

3.3 Evaluation of the *in vitro* cytotoxicity of the antimicrobial peptide P34

- Artigo aceito para publicação pelo periódico **Cell Biology International**.

3.4 Investigation of the cytotoxicity of antimicrobial peptide P40 on eukaryotic cells

- Artigo publicado no periódico **Current Microbiology**.

3.5 Evaluation of the toxicity acute and sub-chronic *in vivo* and immunogenicity of the antimicrobial peptide P34

- Artigo a ser submetido ao periódico **Food and Chemical Toxicology**.

Os artigos apresentados neste trabalho fazem parte dos estudos para verificar futura aplicabilidade do peptídeo antimicrobiano P34 como conservante em alimentos, considerando uma possível influência ou não do mesmo, na expressão gênica em *Listeria monocytogenes* e *Listeria seeligeri* em diferentes condições de cultivo e ambientes, além de sua toxicidade *in vitro* e *in vivo*.

Assim, avaliou-se inicialmente a atividade antimicrobiana de LAB isoladas de queijo tipo minas em sinergismo com bactericina P34 e sua influência na expressão gênica em *Listeria monocytogenes*.

Posterior a estes experimentos, observou-se a influência do peptídeo antimicrobiano P34 na expressão de diferentes genes em *Listeria monocytogenes* e *Listeria seeligeri* após ensaios de atividade antimicrobiana em placas.

Em um segundo momento, realizaram-se estudos preliminares sobre a citotoxicidade *in vitro* de dois peptídeos antimicrobianos, produzidos por duas linhagens diferentes de *Bacillus* demoninados de P34 e P40. Foram utilizadas diferentes linhagens celulares eucarióticas e a citotoxicidade de ambos foi comparada com a nisina pela utilização de diferentes ensaios.

Finalmente, foram realizados ensaios *in vivo* para avaliar a toxicidade aguda e determinar a provável LD_{50} do petídeo antimicrobiano P34. Além desses ensaios foi investigada a imunogenicidade e toxicidade subcrônica desse pedtídeo com a finalidade de compreender sua ação após administração da dose diária aceitável por via oral em camundongos, sendo os resultados

comparados a um grupo controle e um grupo tratado com nisina. Esses experimentos tiveram duração de 21 dias e após procedeu-se as analíses bioquímicas de sangue e estudo histológicos de órgãos alvo.

3.1 ARTIGO 1

3.1.1 Antimicrobial peptide P34 influences gene expression of *Listeria monocytogenes* growing in soft cheese

3.1.2 Abstract

Bacillus sp. P34 produces a bacteriocin-like peptide active against important foodborne pathogenic bacteria, such as *Listeria monocytogenes* and *Bacillus cereus*. The bacteriocin P34 showed increased antilisterial activity when combined with culture supernatants of some selected lactic acid bacteria isolated from Minas Frescal cheese. Addition of bacteriocin P34 to cheese caused a decrease of up to 3 log cycles in viable counts of artificially inoculated *L. monocytogenes*. The influence of bacteriocin P34 on the expression of genes associated to components of cell surface of *L. monocytogenes* was investigated by real-time PCR. A significant increase in the expression of the genes *dltA, lmo 1695* and *mptA* was observed after 96 h in the presence of bacteriocin P34. These results suggest that the bacteriocin P34 affect the expression of genes involved in D-alanylation of teichoic acids and lipoteichoic acids, and lysination of the cell-membrane of phospholipids.

Key words: bacteriocin; lactic acid bacteria; *Listeria monocytogenes*; Minas Frescal cheese, gene expression

3.1.3 Introduction

Lactic acid bacteria (LAB) have been extensively used in food fermentation. They contribute to the organoleptic properties of the final product, as well as to its preservation. Their antimicrobial activity is mainly because the production of a range of metabolites such as ethanol, diacetyl, lactic acid, hydrogen peroxide and bacteriocins (Twomey et al., 2002; Deegan et al., 2006).

Bacteriocins are ribosomally-synthesized antimicrobial peptides or proteins with major activity against low-GC Gram-positive species. These peptides are grouped into three major classes, as suggested by Cotter *et al.* (2005): Lanthionine-containing bacteriocins/lantibiotics (Class I), non-lanthionine-containing bacteriocins (Class II) and non-bacteriocin lytic proteins (bacteriolysins) or in pairs (Class IIb); and large, heat-labile proteins (Class III). The identification of LAB bacteriocins with broad inhibitory spectra against spoilage and pathogenic microorganism has gained increasing interest toward their application as food biopreservative (Galvez et al., 2007). Despite the intensive investigation on LAB, interesting antimicrobial peptides are produced by other bacterial classes (Parret and De Mot, 2002; Motta and Brandelli, 2009).

Studies on structure, activity and gene expression of *Listeria* species indicated that the use of bacteriocins as antilisterial agents has been associated with the development of resistance in target bacterial strains (Crandall et al., 1998; Naghmouchi et al., 2007). Most research in this area is focused on specific bacteriocins, such as nisin and some peptides of the class IIa. For example, the low-level resistance to class IIa bacteriocins may be caused by alterations in membrane lipid composition (Crandall et al., 1998; Vadyvaloo et

al., 2004). Some studies have suggested that the interaction of class IIa bacteriocins is highly dependent of the mannose structural family (EII_t^{Man}) and the mechanism of resistance to class II bacteriocins in *Listeria monocytogenes* seems to be linked to reduced expression of a mannose-specific phosphotransferese system ($EIIAB^{Man}$ PTS, encoded by *mptA*) (Cotter et al., 2005).

Other important mechanisms for modulating the charge of the cell envelope are D-alanylation of teichoic acid (TA) and lipoteichoic acid (LTA) in the cell wall, and lysinylation of cell-membrane phospholipids (Vadyvaloo et al*.,* 2004). The lysinylated phospholipids L-lysyl-PG and L-lysyl-cardiolipin are among the four major phospholipids of *Listeria* spp. (Fisher and Leopold, 1999), and it is conceivable that these phospholipids could display differences in lysinylation in the different strains.

Changes of the cell-surface charge by D-alanyl-esterification of TA and lysinylation of membrane phospholipids have been investigated by expression of genes that can be potentially influenced such modifications (*dltA* and *lmo1695*). The cross-resistance to class I and class II bactericions has been described and the combined use of different bacteriocins can be successful in this case (Crandall et al*.,* 1998; Bouttefroy et al., 2000; Gravesen et al., 2004).

Bacillus sp. P34 produces a novel antimicrobial peptide that inhibits important food pathogens like *L. monocytogenes* and *Bacillus cereus* (Motta et al., 2007a). The mode of action of this peptide appears to cause perturbation in

the cell envelope of the target bacteria (Motta et al., 2008), and it may therefore influence the expression of the above mentioned genes of *L. monocytogenes*.

The aim of the present work was to evaluate whether antimicrobial substances produced by autochthonous LAB of Minas Frescal cheese are able to enhance the activity of bacteriocin P34 against *L. monocytogenes,* as well as to investigate the influence of P34 in specific gene expression of this bacterium after the inoculation in Minas Frescal cheese.

3.1.4 Material and methods

3.1.4.1 Bacterial strains

The producer strain was *Bacillus* sp. P34, characterized as described elsewhere (Motta et al., 2007a). *Bacillus* sp. P34 and *Listeria monocytogenes* ATCC 7644 were stored in Brain Heart Infusion Broth (BHI; Oxoid, Basingstoke, UK) containing 20% glycerol (v/v) at -21°C, and propagated twice in BHI broth before use.

3.1.4.2 Production of bacteriocin P34

Bacillus sp. P34 was grown in 100 ml of BHI broth in an orbital shaker at 180 rpm for 24 h at 30°C. The culture was centrifuged at 10,000 x *g* for 10 min, and the supernatant was sterilized with a 0.22 mm membrane (Millipore, Bedford, MA). The bacteriocin was purified by ammonium sulfate precipitation and gel filtration chromatography as previously described (Motta et al., 2007b).

3.1.4.3 Addition of P34 to Minas Frescal cheese

Minas Frescal cheese produced without addition of starter cultures was obtained from a local dairy. A sample was used to isolate autochthonous LAB by spread and pour-plate procedures (Cogan et al. 1997). Isolated LAB strains were maintained in MRS agar (Vetec, Brazil) at 5°C until used. The remaining cheese was divided in four treatment groups. Application of bacteriocin P34 (0, 800 or 6400 AU/ml) was done on cheese surface and then *L. monocytogenes* was inoculated by submerging the product in a suspension of 10^4 CFU/ml. A control group without bacteriocin and *L. monocytogenes* was done by replacing the treatments by 10 mM phosphate pH 7.0. After inoculation, the packages were sealed and kept at 5ºC for up to 7 days. Each treatment was repeated for two independent cheese samples. Samples were removed at time zero, after 96 h and 168 h for evaluation of *L. monocytogenes* growth. Cheese samples (10 g) were homogenized with 90 ml of 0.1% peptone water in a blender for 60 s. Decimal dilutions were prepared and plated on MOX Agar (Modified Oxford *Listeria* agar base added of Oxford *Listeria* Selective Supplement, Merck). Colony counting was performed after 48 h incubation at 37°C.

3.1.4.4 Detection of antimicrobial activity

Antimicrobial activity was detected using the agar diffusion technique (Skalka et al. 1983). Briefly, LAB (*n*=40) were inoculated on MRS agar and incubated at 30°C for 24 h. Then, the plates were overlaid with 10 ml of BHI soft agar (broth plus 0.6% w/v agar) inoculated with 200 µl of the overnight culture of *L. monocytogenes* and incubated at 37°C for 24 h. Inhibition zones around LAB

colonies were measured. The strains that showed antimicrobial activity were selected to synergism test with the bacteriocin P34.

3.1.4.5 Synergism between bacteriocin P34 and crude supernatants of LAB

LAB were cultivated in MRS broth for 48 h at 30°C. After growth, cultures were centrifuged at 10,000 x *g* for 15 min and the pH of the supernatants was measured. Then half volume of the supernatants was neutralized to pH 7.0 with 1.5M NaOH. The synergism was assessed by agar disc diffusion assay (Motta and Brandelli, 2002). Aliquots of 20 µl of neutralized supernatants, non-neutralized supernatants, bacteriocin P34 (6400 AU/ml) and combination of supernatants with the bacteriocin P34 were applied onto 6-mm cellulose discs on BHI agar plates previously inoculated with a suspension of *L. monocytogenes*. The plates were incubated at 37°C for 24 hours, when inhibition zones were measured. Inhibitory activity was expressed relative to the inhibitory zone observed for 1000 IU of commercial nisin (Nisaplin™, Danisco).

3.1.4.6 RNA extraction

Total RNA of *L. monocytogenes* was directly isolated from bacterial populations obtained in cheese groups treated with 0 and 6400 AU/ml bacteriocin P34, using the TRIZOL® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase (Invitrogen, Carlsbad, CA), and its quality was assessed by running samples on a 1% formaldehyde agarose gel. RNA was quantified spectrophotometrically.

3.1.4.7 cDNA synthesis and real-time PCR

The primers used for the real-time PCR were synthesized by Invitrogen (São Paulo, Brazil) and are listed in Table 1. The real-time PCR amplification reaction was carried using SYBR® Green One-Step qRT-PCR with Rox (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized from 0.5 µg of total RNA, using the specific forward and reverse primers (100 µM) for each gene. PCR reactions were run on the *ABI Prism* 7500 sequence detector (Applied Biosystems) under the following conditions: 48°C for 30 min, 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The assay was accomplished for each gene and included cDNA of the samples treated and control without template.

3.1.4.8 Data analysis of real-time PCR

The amplification results were visualized using the *ABI Prism* 7500 sequence detector and the data were analyzed using Sequence Detection System (SDS) software version 1.6.3. Results were obtained as C_T (threshold cycle) values. The software determines a threshold line on the basis of the baseline fluorescent signal, and the data point that meets the threshold is given as C_T, which is inversely proportional to the starting template copy number. The difference in C_T values between the control group, bacteriocin-treated group and endogeneous control (*rpo*D gene encoding the σ^{70} subunit of the RNA polymerase) for each reactions (ΔC_T) was converted into expression units of

*rpo*D equal to 1000 and dividing by $2^{\Delta CT}$. The relative expression genes after incubation were determined by dividing expression units of bacteriocin-treated group by control group. All measurements were performed in duplicate in two independent experiments.

3.1.4.9 Statistical analysis

Treatment comparisons were performed using Tukey's *t*-test and the values were considered significantly different each other at $P<0.05$.

3.1.5 Results and discussion

3.1.5.1 Inhibition of *L. monocytogenes* by autochthonous cheese bacteria

Forty different LAB strains were isolated from Minas Frescal cheese, according to morphologic characteristics, and named LB1 to LB40. Initial screening was realized by direct antagonism against *L. monocytogenes* and eighteen strains demonstrated antibacterial activity against the indicator microorganism. These LAB strains were selected for additional tests against *L. monocytogenes*. Crude supernatants of selected LAB had pH between 4.2 and 5.0, but neither non-neutralized nor neutralized supernatants inhibited *L. monocytogenes* excepting the strain LB38. Preliminary tests on the nature of LB38 activity suggested this strain produces a bacteriocin-like compound (data not shown).

Table 1. Primers used to evaluate the gene expression in *Listeria monocytogenes* by real-time PCR.

Target/Specificity	Primer	Sequence (5'- 3')	Reference
D-Alanine-D-alanyl carrier protein ligase	*dltA* F	CACAAGATCAGCTAATGGACGC	This study
	dltA R	CTGGAACTTCTCCGAAATGTTT	This study
Putative phospholipid lysinylation	*lmo1695* F	GGGATTGACTATCCGTCGCTA	Vadyvaloo et al. (2004)
	lmo1695 R	TTCCGGGCTTTGAGAAGTTAA	Vadyvaloo et al. (2004)
$EIIAB^{Man}$ of mannose-specific PTS	*mptA* F	CAGGACTTAATTTGCCAATGTTG	Vadyvaloo et al. (2004)
	mptA R	CGCGAACACCTTCTTGAGCT	This study
Σ^{70} Subunit of RNA polymerase	*rpoD* F	ACTGAAAAAGTTCGGGAAATCCT	Vadyvaloo et al. (2004)
	rpoD R	TCGCCTAGATGTGAATCGTCTTC	Vadyvaloo et al. (2004)

3.1.5.2 Synergism among culture supernatants and P34

An increased antimicrobial activity was observed for the combination of bacteriocin P34 and non-neutralized supernatants of six LAB strains, but not with neutralized supernatants (Table 2). These results may be associated with the production of organic acids by these LAB cultures. It seems that the concentration of antimicrobial substances in the crude supernatants of selected LAB were not enough to inhibit listerial growth, but adequate to intensify bacteriocin P34 activity. Some studies showed that the antibacterial activity of bacteriocins can be increased in combination with organics acids (Soo Yeon et al., 2001; Gi-Seong et al., 2002).

3.1.5.3 Inhibition of *L. monocytogenes* in Minas cheese

The application of bacteriocin P34 in Minas Frescal cheese caused a decrease of *L. monocytogenes* growth, and the major inhibition was correlated with the bacteriocin concentration of 6400 AU/ml (Fig. 1). A reduction of 1 log cycle was observed using 800 AU/ml of P34, but it tends to reach control values

at the end of incubation. The higher dose necessary to reduce *L. monocytogenes* counts in the cheese may be related with inactivation by endogenous food enzymes or binding to components of the food matrix, which has been described for other bacteriocins (Cleveland et al, 2001; Aasen et al., 2003). Inhibition of *L. monocytogenes* by nisin has been demonstrated in some soft cheeses, like ricotta (Davies *et al*., 1997), Camembert (Mainsnier-Patin et al., 1992) and cottage (Ferreira and Lund, 1996). However, increasing tolerance or resistance of *L. monocytogenes* strains to nisin has been reported (Martinez et al. 2005), indicating the importance of research on new substances showing antilisterial activity. The bacteriocin cerein 8A was recently investigated, causing significant reduction of surface contamination of *L. monocytogenes* in Minas Frescal cheese (Bizani et al., 2008). Similarly, the results of bacteriocin P34 suggest its potential for use as a biopreservative in Minas Frescal cheese.

Table 2. Inhibitory activity of bacteriocin P34 and culture supernatants (CS) of lactic acid bacteria isolated from Minas Frescal cheese against *Listeria monocytogenes*.[a]

		Inhibitory activity[b]		
Isolate	Ph	P34	CS + P34	Neutralized CS + P34
LB1	4.7	3.5 ± 0.1	4.8 ± 0.2	3.0 ± 0.1
LB2	5.0	3.5 ± 0.1	4.3 ± 0.1	3.5 ± 0.2
LB3	4.7	3.5 ± 0.1	4.7 ± 0.1	3.6 ± 0.1
LB5	4.7	2.2 ± 0.1	2.6 ± 0.2	2.0 ± 0.1
LB7	4.7	2.2 ± 0.1	2.4 ± 0.0	2.1 ± 0.1
LB33	4.2	2.2 ± 0.1	2.5 ± 0.1	2.2 ± 0.1

[a]Culture supernatants (CS) alone showed no activity. [b]Inhibitory activity was expressed relative to the inhibitory zone observed for 1000 IU of nisin.

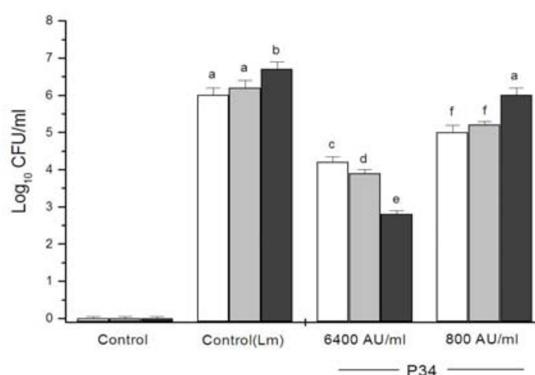

Fig. 1. Effect of bacteriocin P34 on growth of *L. monocytogenes* in Minas Frescal cheese. Cheese samples were treated with 10 mM phosphate buffer pH 7.0 (control); inoculated with *L. monocytogenes* (control Lm), or treated with bacteriocin P34 (6.400 AU/ml or 800 AU/ml) and inoculated with *L. monocytogenes*. Samples were stored at 5°C and analyzed at 0 (white bars), 96 (gray bars) and 168 h (black bars). Bars are the means ± standard deviations of duplicates for two independent samples. Different letters indicate significant differences at *P*<0.05.

3.1.5.4 Influence of P34 on gene expression of *L. monocytogenes*

Based on the inhibition of *L. monocytogenes* in cheese by P34, the expression of three different genes of *L. monocytogenes*, namely *dltA, imo1695* and *mptA,* was evaluated by real-time PCR. These genes have been described as coding for possible targets of class IIa bacteriocins, to which some strains of *L. monocytogenes* present a high level of resistance (Gravesen et al., 2002). In this study the gene *rpoD* was utilized as an endogenous control to standardize the different conditions used in real-time PCR as it is a constitutive gene with homogenic expression between groups. In addition, its C_T values were used to calculate the ΔC_T of the groups (Table 3).

Table 3. Analysis of relative transcript levels of selected genes after inoculation in Minas Frescal cheese by real-time PCR quantification.

Gene	Time (h)	$1000/2^{\Delta C_T}$	Transcript levels (relative to control)
dltA	0 (control)	271.68 ± 0.349	
	0	440.74 ± 0.175	1.62 ± 0.5
	96	3575.14 ± 0.184	13.15 ± 0.52
	168	1104.19 ± 0.293	4.06 ± 0.83
lmo 1695	0 (control)	161.09 ± 0.324	
	0	319.74 ± 0.181	1.98 ± 0.55
	96	1845.48 ± 0.183	11.42 ± 0.56
	168	1016.77 ± 0.213	6.31 ± 0.65
mptA	0 (control)	386.89 ± 0.343	
	0	624.59 ± 0.22	1.61 ± 0.64
	96	6307.2 ± 0.191	16.3 ± 0.55
	168	1969.73 ± 0.173	5.09 ± 0.5
rpoD	Endogenous control	1000	1

It was observed a significant increase in the transcription of *mptA, dltA* and *lmo1695* after 96h inoculation on cheeses containing the bacteriocin P34 at a concentration of 6400 AU/ml, when compared to the control cheese (without bacteriocin addition). The *mptA* gene showed approximately 16-fold greater expression than the control, followed by *dltA* and *lmo1695* with 13-fold and 11-fold greater expression, respectively (Fig. 2). It can be seen that after 96h of inoculation there was a decrease in bacterial growth in bacteriocin-treated samples (Fig. 1). At time zero, there was a non-significant increase in gene transcription, where *lmo1695*, the most expressive, was approximately 2 times higher than control, followed by *dltA* and *mptA*. At 168h, transcription levels of all genes decreased significantly (Fig. 2), accompanied by a decrease in the counts of *L. monocytogenes*. Once again, *lmo1695* from bacteriocin-treated group was slightly more expressive when compared to control.

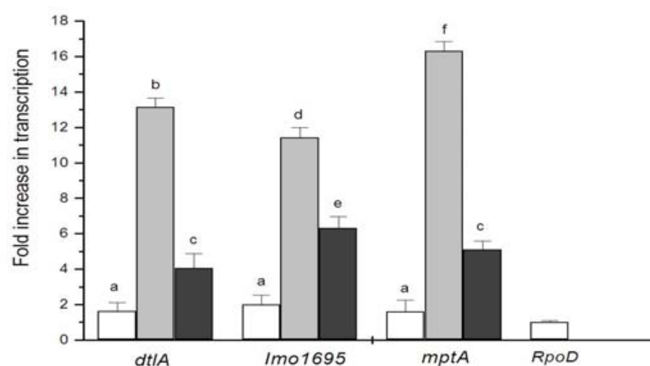

Fig. 2. Expression levels of *dltA, lmo 1695* and *mptA* mRNAs in bacteriocin-treated *Listeria monocytogenes* growing in Minas Frescal cheese. Samples were analyzed at time zero (white bars), 96 (gray bars) and 168 h (black bars) after inoculation. All transcript levels were measured in duplicate for two independent RNA preparations. The *rpoD* gene was used as endogeneous control. Different letters indicate significant differences at $P<0.05$.

The results found for *mptA* gene expression were contrary to those found with class IIa-resistant strains of *L. monocytogenes*, where a clear correlation between *mptA* transcription and the decrease in the expression of mannose permease from the phosphotransferase system was observed (Gravesen et al., 2000; 2002). The present results also differed from those presented by Vadyvallo et al. (2004), which observed no alteration in the expression of *dltA* and *lmo1695*. It is known that the strain of *L. monocytogenes* utilized in this study is a collection strain and that decrease in *mptA* expression has been observed in food-borne class IIa-resistant or mutant strains of *L. monocytogenes* (Gravesen et al., 2000, 2002; Vadyvaloo et al., 2004).

Previous studies have shown that a 1000-fold increased resistance to class IIa bacteriocins in *L. monocytogenes* and *Enterococcus faecalis* resulted

from a loss of *mptA* expression (Ramnath et al., 2000; Dalet et al., 2001; Hechard et al., 2001; Gravessen et al., 2002).

The cell wall of *L. monocytogenes* is composed by thick peptidoglycans containing two types of anionic polymers: teichoic acids (TA), which are covalently linked to peptidoglycan and lipoteichoic acids (LTAs), which are polyphosphoglycerols substituted with a D-alanyl (D-Ala) ester or a glycosyl residue and anchored in the membrane by their glycolipid moiety (Weidenmaier and Peschel, 2008).

Some authors suggest that bacteriocin-induced lysis could be due to the liberation of autolytic enzymes that are usually electrostatically bound to anionic polymers (teichoic and lipoteichoic acids) of the cell wall, which are displaced by cationic bacteriocins from their binding sites (Jack et al., 1995; Maisnier-Patin et al., 1996; Vadyvaloo et al., 2002).

In this study, the bacteriocin P34 influenced the expression of genes that potentially influence cell-surface/cell-membrane structure (*dltA* and *lmo1685* genes). Bacteriocin P34 caused injury to the cell envelope and loss of protoplasmic material in *L. monocytogenes*, which is consistent with the bactericidal and bacteriolytic effect on this indicator bacterium (Motta et al., 2008).

Based on these results, it seems that the damage caused by P34 in the cell surface induces a mechanism that affect the expression of genes involved in D-alanylation of teichoic acids (TA) and lipoteichoic acids (LTAs) or lysination of the cell-membrane of phospholipids. It should be emphasized that

the influence of bacteriocin P34 on gene expression of *L. monocytogenes* was observed in finished cheese.

This approach may properly reflect the results expected to a real condition encountered in food, since the influence of physical conditions and chemical composition of the food in the effectiveness of the bacteriocin was considered. Future studies to evaluate the effect of bacteriocin P34 on resistant strains of *L. monocytogenes* may be useful to further understand the mechanisms of resistance of this important food-borne pathogen.

3.1.6 Acknowledgements

Authors thank Sérgio Salla Chagas from the Laboratory of Molecular Biology of LANAGRO/RS, Ministry of the Agriculture (Brazil), to facilitate the equipment for real-time PCR studies. This work received financial support of CNPq, Brazil.

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3.2 ARTIGO 2

3.2.1 Investigation of the influence of antimicrobial peptide P34 on gene expression of *Listeria monocytogenes* and *Listeria seelegeri*

3.2.2 Abstract

Studies on the structure, activity and gene expression of *Listeria* species, indicate that the use of bacteriocins as food preservative has caused the development of resistance in some target bacterial strains. The influence of bacteriocin P34 on the expression of genes associated to components of cell surface of *Listeria monocytogenes* and *Listeria seeligeri* was investigated by real-time PCR. A non significant increase in the levels of transcription of genes *dltA, lmo1695* and *mptA* were observed after incubation of *L. monocytogenes* for 24 hs at 37°C and 240 hs at 4°C in plates with peptide P34 and nisin. For *L. seeligeri* a significant decrease was observed in gene expression *dltA* similar to the peptide P34 and nisin. The gene *lmo1695* showed a significant decrease in its expression (2000-fold) after inoculation with the peptide P34. A decrease in *mpt*A gene expression was also observed with peptide P34 (31872 - fold) and nisin (16047 - fold) incubated for 24 hs at 37°C. The inoculation of the plate with the peptide P34 and nisin and further incubated for 240 hs at 4ºC showed a non-significant decrease of gene expression with a lower intensity. These results suggest that the bacteriocin P34 may affect the expression of genes involved in D-alanylation of teichoic acids and lipoteichoic acids, and lysination of the cell-membrane of phospholipids in *Listeria spp*.

Key words: bacteriocin; *Listeria monocytogenes; Listeria seeligeri* and gene expression

3.2.3 Introduction

Bacteriocins and bacteriocin-like substance (BLS) are antimicrobial peptides produced by a number of different bacteria that are often effective against closely related species (Riley and Wertz 2002b). These substances have received increasing attention due to their potential use as natural preservatives in food industry, many of them showing highest activity against *Listeria* species (Eijsink et al., 1998; Twomey et al., 2002). A successful example used as preservatives in food is nisin, a non-toxic bacteriocin, inactivated by digestive enzymes (De Martins, 2001) that has been used for over 50 years in different countries without the substantial development of bacterial resistance (Deegan et al, 2006).

Until a few years ago, it was believed that bacteria could not acquire resistance to antimicrobial peptides, so these substances would be strong candidates for new preservative in foods (Scott and Hancock, 2000; Hancook and Rozek, 2002; Bradshaw, 2003). However, the development of strains resistant to antimicrobial peptides from previously sensitive strains has been viewed as difficult but not impossible (Hancock and Chapple, 1999). Studies on the structure, activity and gene expression of *Listeria* species, indicate that the use of bacteriocins as food preservative has caused the development of resistance in some target bacterial strains (Crandall et al., 1998; Peschel et al., 1999, Rodríguez-Lázaro et al., 2004). These studies have suggested that the interaction of class II bacteriocins is highly dependent on EII^{Man} and that the mechanism of resistance to class II bacteriocins can be observed in strains of *Listeria monocytogenes*, in which resistance seems to be linked to reduced expression of a manose-specific phosphotransferese system ($EIIAB^{Man}$ PTS,

encoded by *mptA*) (Cotter *et al.,* 2005). Changes of the cell-surface charge by D-alanyl-esterification of teichoic acid (TA) and lysinylation of membrane phospholipids have been investigated by expression of genes that can be potentially influenced (*dltA* and *lmo1695*). As cross-resistance to class I and class II bacteriocins has been described (Crandall et al., 1998; Bouttefroy et al., 2000; Gravesen et al., 2004) and the combined use of different bacteriocins can be successful.

The genus *Bacillus* contains a number of bacteriocinogenic species such as *Bacillus subtilis, Bacillus thuringiensis*, *Bacillus stearothermophilus, Bacillus licheniformis, Bacillus megaterium* and *Bacillus cereus* that has been recognized as industrially important (Paik et al. 1997; Hyronimus et al. 1998; Oscáriz et al 1999; Cherif et al. 2001, Bizani and Brandelli, 2002 and Stein, 2005). A novel antimicrobial peptide P34 with the potential to be used as food preservative was isolated of a *Bacillus* sp. from aquatic environments of Brazilian Amazon basin (Motta et al., 2007a). This peptide was characterized as a bacteriocin-like substance and purified as described elsewhere (Motta *et al.,* 2007b). The antimicrobial peptide P34 was active against Gram-positive and Gram-negative bacteria, and it seems to disturb the cell envelope of *L. monocytogenes* (Motta et al., 2008).

The aim of this study was to investigate the influence of antimicrobial peptide P34 in the gene expression of *Listeria monocytogenes* and *Listeria seeligeri* after direct inoculation in agar plates.

3.2.4 Material and methods

3.2.4.1 Bacterial strains and media

Listeria monocytogenes ATCC 7644, *Listeria seeligeri* AC 82/4 and *Bacillus* sp. P34 were grown in Brain Heart Infusion broth or agar (BHI; Oxoid, Basingstoke, UK). Strains were maintained as frozen stock cultures at -21ºC in BHI broth supplemented with 20% glycerol.

3.2.4.2 Minimal inhibitory concentration (*MIC*)

The *MIC* of the strains *L. monocytogenes* ATCC 7644 and *L. seeligeri* AC 82/4 against standard antimicrobials was verified through the use of panels PosCombo Type 21 of the semi-automated Micro-Scan-autoScan-4 (Dade Behring Inc., West Sacramento, CA) by the broth microdilution method.

3.2.4.3 Antimicrobial peptides

The antimicrobial peptide P34 was purified as described previously (Motta et al., 2007b). *Bacillus* sp. P34 was cultivated aerobically in 500 ml Erlenmeyer flasks containing 200 ml of BHI broth at 30ºC, 180 rpm for 24 h. Cells were harvested by centrifugation at 10,000 x *g* for 15 min at 12ºC, and the resulting supernatant was filtered through 0.22 µm membranes (Millipore, Bedford, MA, USA). The cell free supernatant was precipitated with ammonium sulfate and subjected to sequential liquid chromatography on Sephadex G-100 and DEAE-Sepharose. The purification factor was 175-fold and the concentration used was 12.5 µg ml^{-1}. Nisin (Nisaplin®; Danisco, Copenhagen, Denmark) was suspended in 0.02 mol l^{-1} HCl and further diluted in phosphate buffer saline (PBS; 35 mmol l^{-1} phosphate, 150 mmol l^{-1} NaCl, pH 7.4) to obtain

a solution of 12.5 μg ml^{-1}. The peptide concentration was determined using the Folin-phenol reagent method (Lowry et al., 1951), using a calibration curve developed with bovine serum albumin as protein standard.

3.2.4.4 Detection of antimicrobial activity

Antimicrobial activity was determined essentially as described elsewhere (Motta and Brandelli, 2002). Briefly, aliquots of 20μl of peptide P34 and nisin (each solution with concentration of 12.5 μg ml^{-1}) were applied on cellulose disks (6 mm) on BHI agar plates previously inoculated with indicator strain suspension (*L. monocytogenes* ATCC 7644 or *L. seeligeri* AC 82/4) which corresponded to 0.5 McFarland turbidity standard solution. The plates were incubated in two different conditions: for 24 hours at 37 °C or 240 hours at 4 ºC. Elapsed time inhibition zones around the disks were measured. PBS was used as negative control.

3.2.4.5 RNA extraction

Bacterial cells of *L. monocytogenes* ATCC 7644 and *L. seeligeri* AC 82/4 were removed directly at the border around inhibition zones in plates after 24h and 240h of incubation of negative control, peptide P34 and nisin, respectively. The concentration of cells was adjusted spectrophotometrically (O.D. 600nm) to a final concentration of $2x10^4$ cells/ml. The TRIZOL® (Invitrogen, Carlsbad, CA) reagent was utilized for extraction total RNA of

according to the manufacturer's instructions. After, the total RNA was treated with DNase-RNase free (Invitrogen, Carlsbad, CA), its quality was assessed by running samples on a 1% formaldehyde agarose gel and quantified spectrophotometrically.

3.2.4.6 *cDNA synthesis and real-time PCR*

The primers used for the real-time PCR were previously described for Vadyaloo *et al.,* (2004) with modifications and synthesized by Invitrogen (São Paulo, Brazil) (**Table 1**). The real-time PCR amplification reaction was carried using SYBR® Green One-Step qRT-PCR with Rox (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA was synthesized from 0.5 µg of total RNA of *L. monocytogenes* ATCC 7644 or *L. seeligeri* AC 82/4 extracted as described above, using the forward and reverse primer (100 µM) specific for each target gene. PCR reactions were run on the *ABI Prism* 7500 sequence detector (Applied Biosystems) under the following conditions: 48°C for 30 min, 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The assay were accomplished in duplicated for each genes, and include cDNA of the samples and control without template.

3.2.4.7 *Data analysis of real-time PCR*

The amplification results were visualized using the *ABI Prism* 7500 sequence detector and the data were analyzed using Sequence Detection System (SDS) software version 1.6.3. Results were obtained as C_T (threshold cycle) values. The software determines a threshold line on the basis of the

baseline fluorescent signal, and the data point that meets the threshold is given as threshold cycle (C_T). The (C_T) values are inversely related to the target cDNA copy number. The mean (C_T) value was calculated for each triplicate reaction in two independent experiments. The (ΔC_T) values were calculated and normalized by subtracting the mean (C_T) value of *L. monocytogenes* ATCC 7644, *L. seeligeri* AC 82/4 and control by mean (C_T) values endogenous control *(rpo*D gene encoding the σ70 subunit of the RNA polymerase) to give the ($\Delta\Delta C_T$) value. The ($\Delta\Delta C_T$) value was calculated subtracting (ΔC_T) value of *L. monocytogenes* ATCC 7644 or *L. seeligeri* AC 82/4 by (ΔC_T) value control. The relative expression of each gene can be quantitatively calculated as ($2^{-\Delta\Delta C_T}$).

Table 1. Primers used to evaluate the gene expression in *Listeria monocytogenes* and *Listeria seeligeri* by real-time PCR.

Target/Specificity	Primer	Sequence (5'- 3')	Reference
D-Alanine-D-alanyl carrier protein ligase	*dltA* F	CACAAGATCAGCTAATGGACGC	This study
	dltA R	CTGGAACTTCTCCGAAATGTTT	This study
Putative phospholipid lysinylation	*lmo1695* F	GGGATTGACTATCCGTCGCTA	Vadyvaloo et al. (2004)
	lmo1695 R	TTCCGGGCTTTGAGAAGTTAA	Vadyvaloo et al. (2004)
$EIIAB^{Man}$ of mannose-specific PTS	*mptA* F	CAGGACTTAATTTGCCAATGTTG	Vadyvaloo et al. (2004)
	mptA R	CGCGAACACCTTCTTGAGCT	This study
σ^{70} Subunit of RNA polymerase	*rpoD* F	ACTGAAAAAGTTCGGGAAATCCT	Vadyvaloo et al. (2004)
	rpoD R	TCGCCTAGATGTGAATCGTCTTC	Vadyvaloo et al. (2004)

3.2.4.8 *Statistical analysis*

Treatment comparisons were performed using Tukey's *t*-test and the values were considered significantly different each other at $P<0.05$.

3.2.5 Results and discussion

Some *L. monocytogenes* strains isolated from foods have become resistant to conventional bacteriocins such as nisin, pediocin PA-1 and bavaricin A and antibiotics used for clinical treatment such as clindamycin, linezolid, ciprofloxacin, ampicillin, rifampicin, trimethoprim, sulphamethoxazole, tetracycline and vancomycin (Rasch and Knochel, 1998; van Schaik et al. 1999; Conter et al. 2009). Thus, the susceptibility of *L. monocytogenes* ATCC 7644 and *L. seeligeri* AC 82/4 to conventional antibiotics, nisin and peptide P34 was initially determined. The results of antimicrobial sensitivity of the strains of *L. monocytogenes* and *L. seeligeri* are summarized in **Table 2**. *L. monocytogenes* used in the study was sensitive to most antibiotics tested except nitrofurantion (MIC > 64) and ceftriaxone (MIC > 32). *L. seeligeri* was only resistant to ceftriaxone (MIC > 32), a feature found in cephalosporin-resistant *Listeria* (Poroś-Głuchowska and Markiewicz, 2003).

It was found that both bacterial strains were sensitive to vancomycin. One way to investigate the pathogenic bacteria of the genus *Listeria* is through their sensitivity to the antibiotic vancomycin. Already there are reports of the existence of mutant strains of *L. monocytogenes* and *L innocua* resistant to vancomycin, nisin and some class IIa bacteriocins, and resistance correlated with changes in wall and cell membrane (Severina et al. 1998; Gravesen et al., 2002). However, it has been suggested that the mechanism of resistance also relates to various factors such as reduced expression of genes, changes in membrane fluidity and charges on the cell surface (Dalet et al., 2001; Vadyvallo et al. 2002; Vadyvallo et al. 2004).

Table 2: MIC values for *Listeria monocytogenes* and *Listeria seeligeri* to some antibiotics tested.

	MIC	
Antibiotics	*Listeria monocytogenes*	*Listeria seeligeri*
Amoxicilin / K clavulanate	≤ 4/2	≤ 4/2
Ampicillin / Sulbactam	≤ 8/2	≤ 8/2
Ampicillin	2	≤ 0,25
Cefazolin	8	≤ 2
Ceftriaxone	> 32	> 32
Ciprofloxacin	≤ 1	2
Clindamycin	> 2	1
Erythomycin	4	2
Gatifloxacin	≤ 2	4
Gentamicin	8	2
Levofloxacin	≤ 2	4
Linezolid	> 4	> 4
Nitrofurantion	> 64	≤ 32
Norfloxacin	8	> 8
Oxacillin	> 2	1
Penicillin	2	0,12
Rifampin	≤ 1	≤ 1
Synercid	0,5	0,5
Tetracycline	≤ 4	≤ 4
Trimethoprim / Sulfamethoxazole	≤ 2/38	> 2/38
Vancomycin	≤ 2	≤ 2

The inhibition zones of peptide P34 and nisin against *L. monocytogenes* and *L. seeligeri* after the incubation time of 24 h at 37 °C and 240 h at 4 ºC are shown in **Table 3**. *L. monocytogenes* and *L seeligeri* showed similar sensitivity in activity assays when inoculated with antimicrobial peptides P34 e nisin and P34, after incubation for 24 hours at 37°C and 240 hours at 4°C, respectively.

Table 3: Antimicrobial activity of antimicrobial peptide P34 and nisin.*

	Listeria monocytogenes		*Listeria seeligeri*	
	24 h / 37°C	240 h / 4°C	24 h / 37°C	240 h / 4°C
Control	0	0	0	0
P34	9.0 ± 0,5	10.0 ± 0,7	10.0 ± 0,6	9.0 ± 0,8
Nisin	10.0 ± 1,0	12.0 ± 0,6	12.0 ± 0,5	10.0 ± 1,2

*Diameter of the inhibition zone in mm around the disk.

Based on the inhibition of *L. monocytogenes* and *L. seeligeri* by peptide P34 and nisin, the expression of three genes of *Listeria* genus*,* namely *dltA, imo1695* and *mptA,* were evaluated by real-time PCR. The values of ΔC_T and $\Delta\Delta C_T$ were used to calculate the relative expression levels ($2^{-\Delta\Delta C_T}$) of *L. monocytogenes* and *L. seelegeri*, respectively (**Table 4A** and **B**). A three-fold decrease or increase in the level of gene expression relative to control was considered significant.

A non significant increase in the levels of transcription of genes *dltA, Imo1695* and *mptA* was observed after incubation for 24 h at 37°C and 240 h at 4°C of *L. monocytogenes* exposed to peptide P34 or nisin **(Figure 1A)**.

For the levels of gene expression of *dltA* and *Imo1965* our results were consistent with those proposed by Vadyvallo et al. (2004) also found no significant changes in expression of these genes in wild-type strains of *L. monocytogenes*. The *L. monocytogenes* used in this study is a collection strain and the decrease of *mptA* gene expression has been only reported in resistant or mutant strains of this bacterium (Gravesen et al., 2000, Gravesen et al. 2002; Vadyvaloo et al., 2004).

Table 4: Relative levels transcript of genes for real-time PCR quantification after incubation of plate of *Listeria monocytogenes* ATCC7644 (**A**) and *Listeria seeligeri* AC 82/4 (**B**) for 24hs at 37ºC and 240hs at 4 ºC.

A

Gene	Time and temperature of incubation	Group	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$
dltA	24 hours at 37ºC	Control	0,12 ± 0,045	**0**	1,0
		P34	1,27 ± 0,042	**1,16 ± 0,003**	0,44
		Nisin	1,99 ± 0,142	**1,87 ± 0,097**	0,27
	240 hours at 4ºC	Control	0,54 ± 0,065	**0**	1,0
		P34	0,80 ± 0,082	**0,25 ± 0,017**	0,84
		Nisin	1,84 ± 0,097	**1,29 ± 0,032**	0,40
lmo 1695	24 hours at 37ºC	Control	1,18 ± 0,052	**0**	1,0
		P34	3,39 ± 0,187	**2,21 ± 0,135**	0,21
		Nisin	4,05 ± 0,121	**2,87 ± 0,069**	0,13
	240 hours at 4ºC	Control	1,28 ± 0,057	**0**	1,0
		P34	1,52 ± 0,071	**0,25 ± 0,014**	0,84
		Nisin	2,92 ± 0,139	**1,65 ± 0,082**	0,31
mptA	24 hours at 37ºC	Control	-0,23 ± 0,076	**0**	1,0
		P34	0,26 ± 0,057	**0,49 ± 0,019**	0,71
		Nisin	1,69 ± 0,154	**1,92 ± 0,078**	0,26
	240 hours at 4ºC	Control	-0,45 ± 0,057	**0**	1,0
		P34	0,27 ± 0,086	**0,72 ± 0,029**	0,60
		Nisin	1,65 ± 0,128	**2,10 ± 0,071**	0,23

[*] Relative gene expression considered significant for values ≤ -3 or ≥ 3.

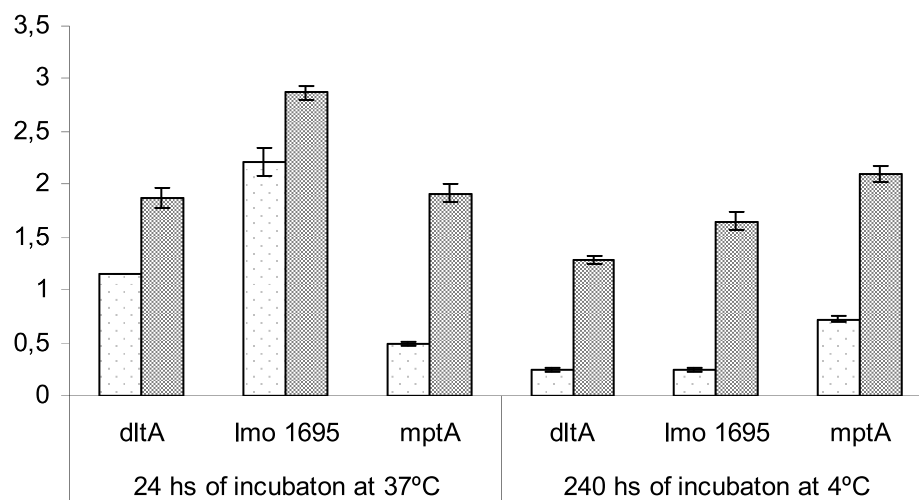
B

Gene	Time and temperature of incubation	Group	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$
dltA	24 hours at 37ºC	Control	4,90 ± 0,302	**0**	1,0
		P34	1,88 ± 0,183	**-3,02 ± 0,119**	8,11*
		Nisin	1,83 ± 0,190	**-3,07 ± 0,112**	8,39*
	240 hours at 4ºC	Control	4,99 ± 0,197	**0**	1,0
		P34	1,72 ± 0,111	**-3,27 ± 0,086**	9,64*
		Nisin	1,19 ± 0,148	**-3,80 ± 0,049**	13,92*
lmo 1695	24 hours at 37ºC	Control	3,62 ± 0,342	**0**	1,0
		P34	-7,57 ± 0,201	**-11,19 ± 0,141**	2336,2*
		Nisin	2,61 ± 0,175	**-1,00 ± 0,119**	2,0
	240 hours at 4ºC	Control	5,52 ± 0,223	**0**	1,0
		P34	1,83 ± 0,118	**-3,69 ± 0,105**	12,9*
		Nisin	3,07 ± 0,165	**-2,44 ± 0,058**	5,42*
mptA	24 hours at 37ºC	Control	-7,38 ± 0,277	**0**	1,0
		P34	-7,58 ± 0,154	**-14,96 ± 0,123**	31872,0*
		Nisin	-6,59 ± 0,132	**-13,97 ± 0,145**	16047,0*
	240 hours at 4ºC	Control	-2,70 ± 0,228	**0**	1,0
		P34	0,07 ± 0,147	**2,78 ± 0,081**	0,14
		Nisin	-0,17 ± 0,165	**2,52 ± 0,063**	0,17

* Relative gene expression considered significant for values ≤ - 3 or ≥ 3.

Therefore the result of non-significant increase in *mptA* gene expression is consistent with the literature. It has been reported that the changes in cell surface may occur after the interaction of the class IIa bacteriocin with specific receptors of bacterial cell wall and has been observed the possible increase in the level of transcription of certain genes in some strains of *L. monocytogenes* (Gravesen et al., 2002).

A



B

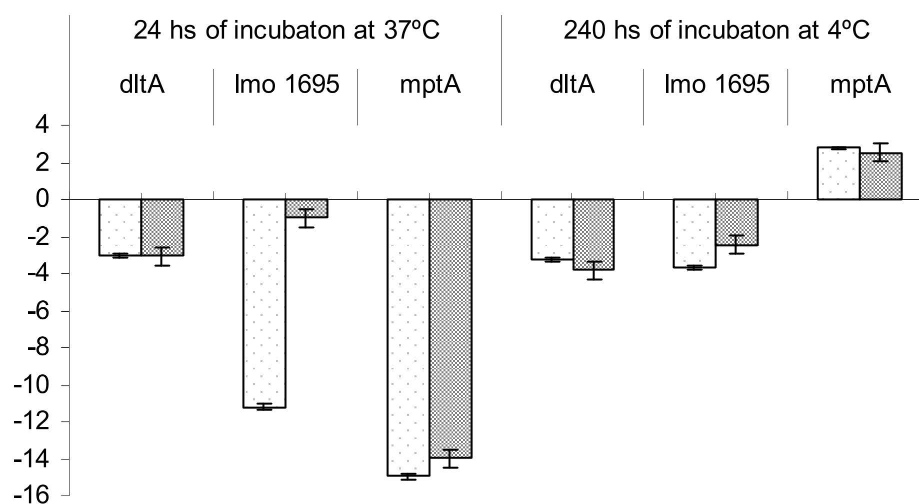


Figure 1. Expression levels of *dltA, lmo 1695* and *mptA* mRNAs in bacteriocin-treated *Listeria monocytogenes* ATCC7644 (**A**) and *Listeria seeligeri* AC 82/4 (**B**) growing on the plate 37°C and 4°C. Samples were analyzed at time 24 (white bars) and 240h (gray bars) after inoculation. All transcript levels were measured in duplicate for two independent RNA preparations. The *rpoD* gene

was used as endogeneous control. Different letters indicate significant differences at *P*<0.05.

For *L. seeligeri* **(Table 4B)** it was observed a significant decrease in the expression of *dltA*, similar to the peptide P34 and nisin after incubation for 24 h at 37°C. The decrease in the expression of this gene was enhanced after incubation of the plate for 240 h at 4°C, and that treatment with nisin resulted in lower gene expression compared with the peptide P34. The gene *lmo1695* showed a significant decrease in its expression (2000-fold) after *L. seeligeri* was exposed to the peptide P34 for 24 h at 37°C. Decreased expression of this gene was also observed for nisin, but this was not significant.

A significant decrease in the expression of gene *mptA* was also observed for *L. seeligeri* after incubation with peptide P34 and nisin for 24 h at 37ºC. As shown in **Table 4B**, the gene is 31,872-fold less expressed in the presence of peptide P34 as compared with the control. Also, the expression of this gene was significantly higher after inoculation with nisin. Interestingly, a non-significant decrease of gene expression was observed when *L. seeligeri* was incubated for 240 h at 4ºC in the plates containing peptide P34 or nisin. It is believed that the composition of membrane fatty acids of *Listeria monocytogenes* can be altered by mechanical stress that is induced under conditions slightly unfavorable for the cell, as a drop in temperature or the presence of acid in the middle, thus influencing the effectiveness of bacteriocins and their effects on target cells (van Schaik et al., 1999).

Previous studies have shown that a 1000-fold increased resistance to class IIa bacteriocins in *L. monocytogenes* and *Enterococcus faecalis* resulted from a loss of *mptA* expression (Ramnath et al., 2000; Dalet et al., 2001; Hechard et al., 2001; Gravessen et al., 2002). The cell wall of *L. monocytogenes* is composed by thick peptidoglycans containing two types of anionic polymers: teichoic acids (TA), which are covalently linked to peptidoglycan and lipoteichoic acids (LTAs), which are polyphosphoglycerols substituted with a D-alanyl (D-Ala) ester or a glycosyl residue and anchored in the membrane by their glycolipid moiety (Weidenmaier and Peschel, 2008). Some authors suggest that bacteriocin-induced lysis could be due to the release of autolytic enzymes that are usually electrostatically bound to anionic polymers (teichoic and lipoteichoic acids) of the cell wall, which are displaced by cationic bacteriocins from their binding sites (Jack et al., 1995; Maisnier-Patin et al., 1996; Vadyvaloo et al., 2002). In this study we observed non significant influence on the expression of genes of *L. monocytogenes* in agar plates containing the antimicrobial peptides P34 or nisin. However, in *L. seeligeri* both peptide P34 and nisin under the same conditions significantly influenced the expression of genes *dltA, lmo1685* and *mptA* after incubation for 24 h at 37 °C. An incubation for a period of 240 h at 4 ºC caused the more significant changes in gene expression (*dltA* and *lmo1685*) in *L. seeligeri*, and the *mptA* gene had no significant change in that condition. This fact makes clear the possible influence of these substances in such conditions, the structure cell-surface/cell-membrane. So we assume that damage to the cell surface induce a mechanism that affects the expression of genes involved in D-alanylation teichoic acid (TA)

and lipoteichoic acids (SBL) or lysination cell membrane phospholipids. Therefore, further studies should be conducted to evaluate the effect of antimicrobial peptide P34 in other strains of *Listeria* resistant to better understand the possible mechanisms that lead to an increase or decrease in expression of certain genes.

3.2.6 Acknowledgements

Authors thank Sérgio Salla Chagas from the Laboratory of Molecular Biology of LANAGRO/RS, Ministry of the Agriculture (Brazil), to facilitate the equipment for real-time PCR studies. This work received financial support of CNPq, Brazil.

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3.3 ARTIGO 3

3.3.1 Evaluation of the *in vitro* cytotoxicity of the antimicrobial peptide P34

3.3.2 Abstract

The *in vitro* cytotoxicity of the antimicrobial peptide P34 was evaluated in different eukaryotic cells. The food grade bacteriocin nisin was also analyzed for comparison. VERO cells were treated with different concentrations (0.02 - 2.5 μg ml^{-1}) of antimicrobial peptide P34 and nisin. Cell viability and plasma membrane integrity were checked by MTT, neutral red uptake (NRU) and lactate dehydrogenase (LDH) assays. The EC_{50} values to the peptide P34 in MTT and NRU assays were 0.60 and 1.25 μg ml^{-1}, respectively, while values found to nisin were 0.50 and 1.04 μg ml^{-1}. In the LDH assay, the EC_{50} values were 0.65 and 0.62 μg ml^{-1} to P34 and nisin, respectively. The peptide P34 revealed similar hemolytic activity on human erythrocytes (5.8%) when compared to nisin (4.9%). The effects on viability, motility and acrosomal exocytosis of human sperm were also evaluated. Nisin and P34 showed similar effects on sperm parameters. The evaluation of cytotoxicity of antimicrobial peptides is a critical step to guarantee their safe use.

Key words: antimicrobial peptide; *Bacillus*; bacteriocin; eukaryotic cells, MTT assay

3.3.3 Introduction

Bacteriocins are antimicrobial compounds that inhibit or inactivate microorganisms that are usually, but not always, closely related to the producer strain (Cleveland et al., 2001). In recent decades, a great interest is observed in the bacteriocins as natural preservatives and they have been extensively studied, especially those produced by lactic acid bacteria (LAB) because of their potential applications as food additive in dairy and meat systems (Sobrino-Lopez and Martin-Belloso, 2008). Antimicrobial peptides have received increasing attention, because their efficiency for the biological control of spoilage and pathogenic organisms they present potential applications in food (Deegan et al., 2006; O'Sullivan et al., 2002) and clinical (Fontana et al., 2006; Oh et a., 2006). Nisin is the first antimicrobial peptide with a "generally recognized as safe" (GRAS) status in the United States for use in processed cheese (Cleveland et al., 2001). Nisin is currently used in various food products in several countries (O'Sullivan et al., 2002; Deegan et al., 2006).

The genus *Bacillus* includes a number of bacteriocinogenic species including *B. subtilis*, *B. cereus*, *B. thuringiensis*, *B. coagulans*, among other (Bizani and Brandelli, 2002; Cherif et al., 2001). *Bacillus* species produce a large number of antimicrobial peptides representing several different basic chemical structures (von Döhren, 1995). Bacteriocins or bacteriocin-like substances (BLS) from the genus *Bacillus* have been characterized, such as thuricin 7 (Cherif et al., 2001), thuricin 439A and thuricin 439B (Ahern et al., 2003), cerein 7A and 7B (Oscáriz et al., 2007), cerein 8A (Bizani et al., 2005), and subtilosin A (Kawulka et al., 2004). Antimicrobial activity was reported

among several *Bacillus* isolated from aquatic environments of Brazilian Amazon basin (Motta et al., 2004). A novel *Bacillus* sp. strain P34 was identified and its antimicrobial activity was characterized as a bacteriocin-like substance. This antimicrobial peptide showed a broad inhibitory spectrum including *Listeria* spp., *Bacillus cereus*, *Aeromonas hydrophila*, *Pasteurella haemolytica*, among other (Motta et al., 2007a). Many strains of *Bacillus* have been safely used in food and industry (Pedersen et al., 2002) however, there is limited investigation on the cytotoxicity of specific antimicrobial peptides from *Bacillus*.

In vitro cytotoxicity assays are useful to define basal cytotoxicity, for example the intrinsic ability of a compound to cause cell death as a consequence of damage to several cellular functions (Bouziz et al., 2006). Cytotoxicity assays are also necessary to define the concentration range for further and more detailed *in vitro* testing to provide meaningful information on parameters such as genotoxicity or programmed cell death (Eisenbrand et al., 2002). A number of assays have been proposed to investigate the cytotoxicity of bioactive peptides of *Bacillus* sp., based on the determination of the hemolytic potential (hemolysis), injury to sperm cells, inhibition of cellular proliferation, and damage to energy metabolism (Andersson et al., 2004; Maher and McClean, 2006; Mikkola et al., 2000; Shin et al., 2001). The aim of this work was to investigate the *in vitro* cytotoxicity of the peptide P34, in comparison with nisin through the use of different cytotoxicity assays and eukaryotic cells.

3.3.4 Material and methods

3.3.4.1 Antimicrobial peptides

The antimicrobial peptide P34 was purified from *Bacillus* sp. strain P34, as described previously (Motta et al., 2007b). Previously, *Bacillus* sp. was cultivated aerobically in 500 ml Erlenmeyer flasks containing 200 ml of TSB broth at 30ºC, 180 rpm for 24 h. Cells were harvested by centrifugation at 10,000 x *g* for 15 min at 12ºC, and the resulting supernatant was filtered through 0.22 µm membranes (Millipore, Bedford, MA, USA). The cell free supernatant was precipitated with ammonium sulfate and subjected to sequential liquid chromatography on Sephadex G-100 and DEAE-Sepharose. Purity was checked by capillary zone electrophoresis using a 60 cm x 50 µm capillary with 20 mM TAPS buffer pH 8.4. Sample solution (5 µl) was applied for 20 s at 2 kV, the applied separation voltage was 16.0 kV (36 µA), and detection by laser fluorescence (Kist et al., 1994). Nisin (Nisaplin®; Danisco, Copenhagen, Denmark) was suspended in 20 mM HCl to obtain a 12.5 µg ml^{-1} stock solution. Peptides were further diluted in Eagle's Minimal Essential Medium (E-MEM; Gibco BRL, Rockville, MD, USA) for assays with Vero cells, phosphate buffer saline (PBS; 35 mM phosphate buffer, 150 mM NaCl, pH 7.4) for hemolysis assays, or Ham's F10 medium (Sigma, St. Louis, MO, USA).

3.3.4.2 Vero cell culture

Vero cells (Adolpho Lutz Institute, São Paulo, Brazil) from green monkey kidney were used between 20-30 passages. The cells were cultured in

96-well plates (Corning, USA) with Eagle's Minimal Essential Medium (E-MEM; Gibco BRL, Rockville, MD, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), 1% (v/v) L-glutamine (200 mM), 10 mg ml^{-1} enrofloxacin (Baytril, Bayer) and 0.025 µg ml^{-1} anphotericin B (Gibco BRL). The cells were maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere. The viability of the test cells exceeded 99% prior to cytotoxicity assay as determined by exclusion of the vital dye trypan blue.

3.3.4.3 MTT assay

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out according to Mossmann (1983). Briefly, Vero cell cultures (3 x 10^5 cells ml^{-1}) were prepared in 96-well plates and 200 µl of serial dilutions (0.02 to 2.5 µg ml^{-1}) of either peptide P34 or nisin, or 20 mg ml^{-1} SDS (positive control) were added. Fresh E-MEM was used as negative control. Plates were incubated for 24 h at 37ºC in humidified 5% CO_2 atmosphere. Later, the medium was removed by suction from all wells and 50 µl of 1 mg ml^{-1} MTT solution (USB Corporation, Cleveland, USA) prepared in E-MEM medium was added to each well and the plates were incubated for 4 h at 37ºC. The MTT solution was removed without disturbing the cells and 100 µl of DMSO was added to each well to dissolve formazan crystals. After gently shaking the plates for 5 min, whereby crystals were completely dissolved, the absorbance was read on a 680 Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules,

CA, USA) at 540 nm. The percentage of viability was calculated as A_T/A_C x 100; where A_T and A_C are the absorbances of treated and control cells, respectively.

3.3.4.4 Neutral red assay

The cell cytotoxicity was monitored by the neutral red dye uptake (NRU) assay in a modified form that described elsewhere (Borenfreund and Puerner, 1985). Vero cells were cultured in 96-well plates at a density of 3 $\times 10^5$ cells ml^{-1} for 24 h. The growing cells attached to the bottom were treated with serial dilutions (0.02 to 2.5 µg ml^{-1}) of either peptide P34 or nisin, or 20 mg ml^{-1} SDS. Fresh E-MEM was used as negative control. Plates were incubated for 24 h at 37ºC in humidified 5% CO_2 atmosphere. The solutions were removed from the plates and the cells were rinsed with 200 µl fresh media containing 10% (v/v) neutral red solution to a final concentration of 0.033% in E-MEM for 3 h at 37ºC in an atmosphere with 5% CO_2. Media was carefully removed and cells were washed with E-MEM. Incorporated dye was then solubilized by addition of 100 µl of an aqueous solution containing 50% (v/v) ethanol, 1% (v/v) acetic acid. After shaking the cultures for 10 min, absorbance values were read using a 680 Microplate Absorbance Reader (Bio-Rad Laboratories) at 540 nm. The percentage of viability was calculated as A_T/A_C x 100; where A_T and A_C are the absorbances of treated and control cells, respectively.

3.3.4.5 LDH release assay

The effect of antimicrobial peptide P34 and nisin on plasma membrane integrity of Vero cells was assessed by lactate dehydrogenase

(LDH) assay (Decker et al., 1988). The activity of LDH in the medium was determined using a commercially available kit (Labtest Diagnostic, Lagoa Santa, Brazil). Vero cell cultures were incubated with serial dilutions (0.02 to 2.5 μg ml^{-1}) of either peptide P34 or nisin, or 1% (v/v) Triton X-100 (positive control) for 24 h. The culture supernatants were removed and LDH release was determined by two-point enzymatic kinetics (Kaplan et al., 1988). The absorbance at 340 nm was monitored using a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan). The percentage of LDH release was calculated as $(A_T–A_C)/(A_X–A_C) \times 100$; where A_T is the experimental absorbance of treated cells, A_C is the control absorbance of untreated cells, and A_X is the absorbance of Triton X-100 lysed cells.

3.3.4.6 Hemolytic activity

Hemolytic activity of antimicrobial peptides was measured spectrophotometrically using a hemoglobin release assay (Shin et al., 2001), with minor modifications. Briefly, the fresh defibrinated human erythrocytes were rinsed three times with PBS, centrifuged for 15 min at 900 x g and resuspended at 4% (v/v) in PBS. Samples (300 μl) of the suspension were transferred to a fresh microcentrifuge tube. Red cells were treated with peptide P34 or nisin (0.62, 1.25 or 2.5 μg ml^{-1}) for 1h at 37ºC and then centrifuged at 1000 x g for 5 min. Aliquots of the supernatant were then transferred to a fresh microcentrifuge tube, where hemoglobin release was monitored using a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan) by measuring the

absorbance at 414 nm. Percentage of hemolysis was calculated as $(A_T–A_C)/(A_X–A_C) \times 100$; where A_T is the experimental absorbance of treated supernatants, A_C is the control absorbance of untreated cell supernatant, and A_X is the absorbance of 0.1% (v/v) Triton X-100 lysed cells.

3.3.4.7 Semen samples

Semen samples were obtained from fertile men according to WHO standards (sperm count of > 20 x 10^6/ml and total motility of > 50%). Briefly, after complete liquefaction at 37ºC, sperm were separated from seminal plasma on a 50% isotonic Percoll cushion by layering 0.5 ml of each onto 0.7 ml of Percoll and centrifuging 10 min at 900 x g. The sperm pellet was resuspended and washed twice in Ham's F10 by spinning at 900 x g. The final pellet was resuspended in Ham's F10 (1 x 10^6 cells ml^{-1}).

3.3.4.8 Sperm viability and motility

Sperm samples prepared as described above were incubated for up to 2 h at 37^oC with 1.25 µg ml^{-1} of either peptide P34 or nisin, or 10 µM calcium ionophore A23187. Fresh buffer was used as negative control. A small aliquot (0.1 ml) was removed from each sample for the assessment of viability and progressive motility on a Neubauer chamber with addition of trypan blue dye.

3.3.4.9 Acrosome reaction assay

Sperm (1 x 10^6 cells ml^{-1}) were treated with 1.25 µg ml^{-1} of either peptide P34 or nisin, or 10 µM A23187 calcium ionophore (as a full response control), or fresh medium (negative control). All incubations were carried out for 60 min at 37ºC and 5% CO_2 in air. Sperm were then washed three times in Ham's F10 and plated into polylysine-coated slides. After cells adhered to the surface, the buffer was removed and the slide submerged in 95% ethanol at 4ºC for 30 min for cell permeabilization. Acrosome reaction was evaluated using lectin staining with 50 µg ml^{-1} FITC-*Pisum sativum* agglutinin in duplicate assays (Brandelli et al., 1994). Stained cells were scored in a fluorescence microscope (Eclipse E600; Nikon, Tokyo, Japan) equipped with an epifluorescence module and Hg ultraviolet source was used to examine the slides at 1000x magnification.

3.3.4.10 Statistical analysis

Data were expressed as means ± S.E.M. for three independent determinations for each experimental point, except for acrosome reaction (duplicate assays). Statistical analysis was performed using the SAS statistical software package (SAS Institute, Cary, NC, USA). Data of MTT, NRU, LDH, hemolytic activity assay, sperm viability and motility were assessed for homogeneity and normality by method of the pondered least squares. For acrosome reaction non-parametric statistical analysis was used for determined

differences between controls and treated groups by Kruskal-Wallis test. The level of significance was of $p<0.05$ (MTT, neutral red, and LDH assays) and $p<0.01$ (hemolytic activity, sperm viability and motility and acrosome reaction), respectively. The EC_{50}, defined as the concentration that caused 50% maximum effect, was calculated by regression analysis of the dose-response curves for MTT, NRU and LDH assays.

3.3.5 Results

3.3.5.1 Purification of peptide P34

The antimicrobial peptide P34 was purified resulting in a yield of 3.8% and increased specific activity (from 76 to 20,000 AU mg $protein^{-1}$) with a purification factor of 265-fold. A single component was observed by capillary electrophoresis, confirming the homogeneity of the purified peptide (Fig. 1).

3.3.5.2 MTT and NRU assays

The cytotoxicity was initially evaluated by using MTT and NRU assays. The results of the dose-response curves for MTT and NRU assays are presented in the Fig. 2A and 2B, respectively. Increased concentrations of antimicrobial peptide P34 and nisin caused a decrease of cell viability in both assays. Analysis of variance indicates that the interaction between cell viability and peptide concentration was significant in the MTT and NRU assays

(p<0.05). When compared with nisin, P34 caused increased damage at higher concentrations in MTT assay (Fig. 2A), although this difference was not observed in NRU (Fig. 2B) and important loss of cell viability was observed in both assays with the positive control (Fig. 2A and 2B). Peptide P34 and nisin showed comparable EC_{50} values in MTT assays, however for the positive control the EC_{50} values were smaller indicating higher cytotoxicity of this treatment (Table 1). The EC_{50} for peptide P34 was about 1.3 times higher than nisin in the NRU assays.

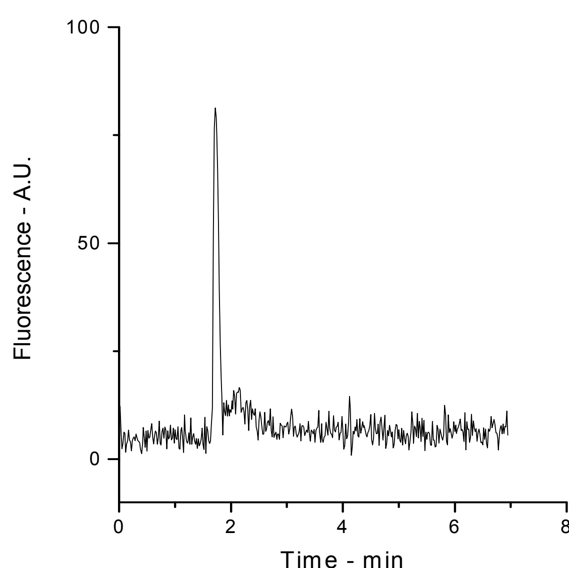
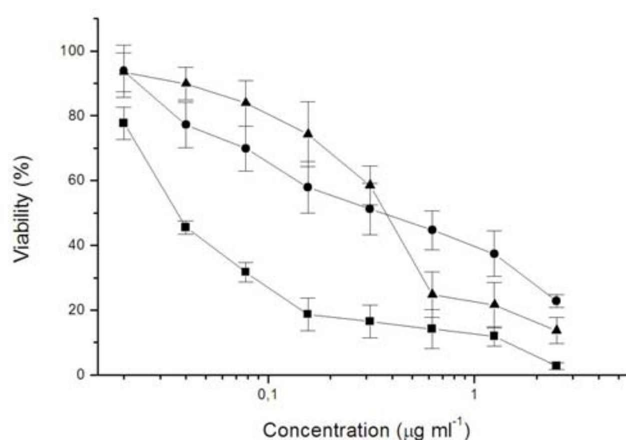


Fig. 1. Capillary zone electrophoresis analysis of the purified peptide P34. Sample solution was applied to a 60 cm x 50 μm capillary and detected by laser fluorescence.

3.3.5.3 LDH release assay

Vero cells treated with different concentrations of antimicrobial peptide P34 resulted in similar levels of LDH release when compared with nisin (Fig. 2C). Concentration-dependent increase in extracellular LDH was observed with P34 and nisin, indicating that these peptides caused some loss of plasma membrane integrity at concentrations from 0.32 μg ml^{-1}. The purified P34 presented a smaller damaging effect on the plasma membrane integrity, comparable to that observed for nisin at concentrations from 1.25 μg ml^{-1} (p<0.05). The treatment with Triton X-100 (positive control) resulted in high levels of LDH release (Fig. 2C), indicating an extensive cell damage. The EC_{50} values for the different treatments are shown in the Table 1. Similar values were observed for nisin and P34.

A



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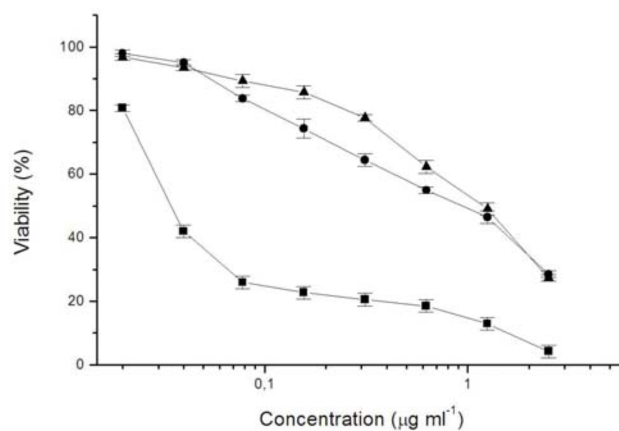

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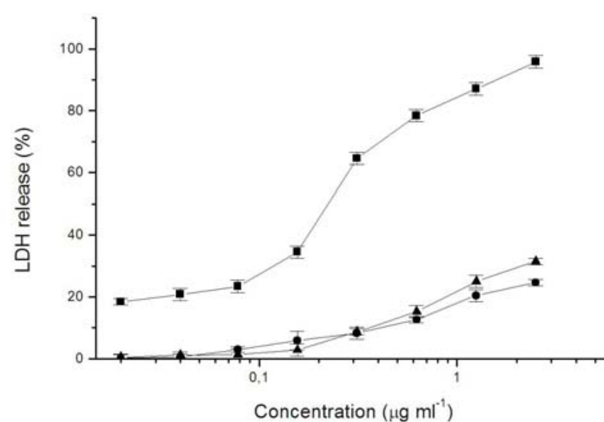


Fig. 2. Effect of antimicrobial peptides on Vero cells using (A) MTT, (B) neutral red, or (C) LDH release assays. Vero cells were cultivated for 24 h with the indicated concentrations of antimicrobial peptide P34 (triangles), nisin (circles), or 20 mg/ml SDS as positive control (squares). The results represent the mean ± S.E.M of at three independent experiments.

Table 1. EC_{50} values for Vero cells following exposure to concentration different antimicrobial peptides (0.02 to 2.5 μg ml^{-1}) for 24 h based on the dose-response curves derived from the MTT assay, neutral red assay and LDH leakage assay.

	EC_{50} (μg ml^{-1})[a]		
Assay	Peptide P34	Nisin	Control(+)[b]
MTT	0.36	0.35	0.17
Neutral red	1.05	0.79	0.04
LDH	0.65	0.62	0.18

[a] EC_{50} values were calculated by regression analysis of the dose-response curves for MTT, NRU and LDH assays.
[b] Positive control was SDS for MTT and Neutral red assay and Triton X-100 for LDH assay.

3.3.5.4 Hemolytic activity

The hemolytic activities of the antimicrobial peptide P34 and nisin were determined as the percentage lysis of human erythrocytes. According to the data obtained in this study, the purified P34 and nisin showed a hemolytic activity of 5.84% and 4.93%, respectively, at the concentration of 2.5 μg ml^{-1} (Table 2). The values observed for P34 and nisin were similar, and a decrease in the percentage of hemolysis was observed when the concentration of antimicrobial peptides was reduced to 0.62 μg ml^{-1}.

3.3.5.5 Sperm viability and motility

The percentages of sperm viability and motility after different treatments can be visualized in the Fig. 3. After 2 h of incubation a significant decrease in the number of viable sperm was verified for treatments with calcium ionophore. In both cases important differences were verified, when compared to

control cells. However, a minor decrease of the viability and motility was observed for both P34 and nisin. Significant differences were detected in the viability (Fig. 3A), but not on sperm motility (Fig. 3B), when compared to the untreated cells (p<0.01).

Table 2. Hemolytic activity of the antimicrobial peptides against human erythrocytes.

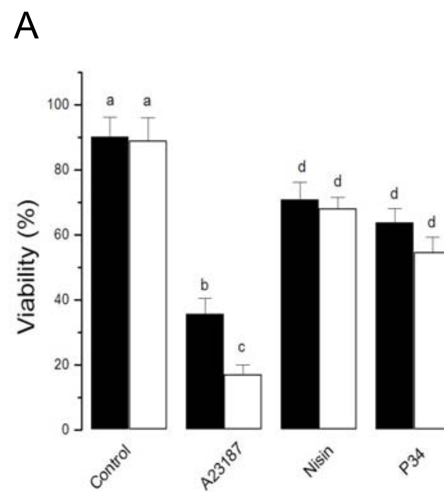
Concentration (µg ml^{-1})	Hemolysis (%) [a]		
	Peptide P34	Nisin	Triton X-100
0.62	2.8 ± 0.9 ^{a,A}	2.6 ± 1.1 ^{a,A}	100.4 ± 1.0 ^{b,A}
1.25	4.0 ± 1.0 ^{a,B}	3.2 ± 0.8 ^{a,B}	99.5 ± 0.5 ^{b,A}
2.5	5.8 ± 1.0 ^{a,B}	5.0 ± 1.0 ^{a,B}	99.0 ± 1.0 ^{b,A}

[a] Data are expressed as mean percentage hemolysis ± S.E.M for three separate experiments. Different superscript letters indicate significant differences at p< 0.01; small letters compare values within the same row; capital letters compare values within the same column.

3.3.5.6 Induction of acrosome reaction

The ability of antimicrobial peptide P34 to induce the acrosomal exocytosis of human sperm was investigated. Percentages of acrosome reaction (AR) after different treatmens are shown in Fig. 4. The percentage of AR increased by about 2-fold for peptide P34, when compared with the control cells. Induction of the AR with 10 µM calcium ionophore A23187 reached 56%. Through the specific multiple comparisons by the Kruskal-Wallis test it can be verified that the frequencies of spermatozoa that suffered acrosomal reaction (AR) by nisin and purified P34 were not significantly different than untreated cells (p<0.01).

A



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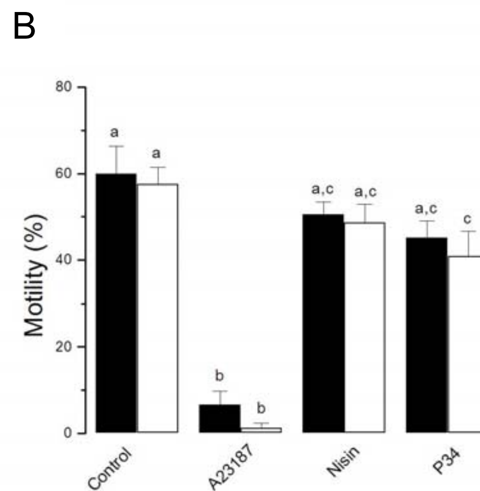

Fig. 3. Effect of antimicrobial peptides on human sperm function. Human sperm were incubated with 1.25 µg ml^{-1} of peptide P34, or nisin, 10 µM of calcium ionophore (A23187), or fresh buffer as control. The percentage of cell viability (A) and motility (B) was determined after 60 min (black bars) and 120 min (white bars). The results represent the mean percentage ± S.E.M for three independent experiments. Different letters indicate significant differences at $p<0.01$.

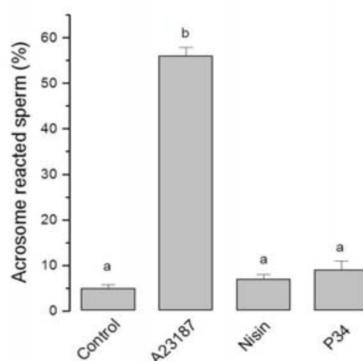

Fig. 4. Effect of antimicrobial peptides on sperm acrosomal exocytosis. Human sperm were treated with 1.25 µg ml^{-1} of peptide P34, or nisin, 10 µM of calcium ionophore (A23187), or fresh buffer as control. The percentage of acrossome reacted sperm was determined after 60 min. The results represent the mean percentage ± S.E.M for two independent experiments. Different letters indicate significant differences at $p<0.01$.

3.3.6 Discussion

In this study, the cytotoxicity of antimicrobial peptides P34 and nisin on Vero cells was investigated by different methods: MTT, NRU and LDH release assays. Vero cells presented similar sensibility after 24 h exposure to different concentrations of the peptide P34 and nisin in these assays. The MTT assay appears to be more sensitive in detecting early toxicity compared to the NRU and LDH leakage assays, as indicated by both the EC_{50} values and the dose response curves. These differences among the cytotoxicity assays may suggest intracellular effects due to exposure to antimicrobial peptides before any permanent cell membrane damage occurred. In this regard, the lantibiotics

nisin, subtilin, Pep5 and epidermin have secondary modes of action in addition to their well-stablished bacteriolytic action (McAuliffe et al., 2001). The cytotoxicity of nisin, pediocin, and colicins was tested with Vero cells using trypan blue staining viability assay (Murinda et al., 2003), but the EC_{50} values showed little correlation with the values reported here. In this regard, differential toxicity can be observed depending on differences in the membrane composition and metabolic activity of target cells, exposure time, and the cytotoxicity assay employed (Weyermann et al., 2005; Maher and McClean, 2006). Previous studies had already demonstrated that nisin presents some cytotoxicity to different cell lines and the small cytotoxicity of nisin A to vaginal, colonic and kidney, epithelial cells has been reported (Aranha et al., 2004; Murinda et al., 2003). Nisin was harmless between 0.85 and 3.4 μg ml^{-1} to Vero cells over 48 h, whereas no important toxicity was demonstrated on vaginal epithelial cells up to concentrations of 318 μg ml^{-1} (Murinda et al., 2003).

The LDH release assay indicates the loss of plasma membrane integrity in Vero cells, revealing some cytotoxicity following 24 h exposure to the higher concentrations tested of peptide P34 and nisin. This result agrees with the fact that membranes are the major target of several antimicrobial peptides, including nisin (Deegan et al., 2006) and P34 (Motta et al., 2008). However, P34 shown its antibacterial effect at lower doses (about 0.2 μg ml^{-1}), which is safe to Vero cells. In general, the composition of target membranes is related to the different sensitivity to antimicrobial peptides, where bacteria are most susceptible, lower eukaryotes (including fungi) have intermediate sensitivity, and higher organisms are mostly resistant (Mason et al., 2007).

Peptide P34 and nisin exhibited similar effect against human erythrocytes. The increase in membrane permeability and subsequent osmotic lysis of cells is thought to be due to the formation of mixed micelles in the lipid bilayer (Macián et al., 1996). *Bacillus* species produce varied antimicrobial lipopeptides, which could also act as biosurfactants (Singh and Cameotra, 2004; Dehghan-Noude et al., 2005). Despite the peptide P34 appears to posses a lipid chain in its structure (Motta et al., 2007a), its hemolytic effects was comparable with nisin.

Sperm treated with P34 and nisin showed similar motility and viability. Moreover, a significant decrease in the percentage of viable sperm in comparison with untreated cells was observed. The evaluation of sperm viability with the vital dye trypan blue has not been used for testing peptide cytotoxicity, and thus we did not find comparative data. The motility of sperm relies on ATP synthesized by oxidative phosphorylation and ceases when the mitochondria become damaged or uncoupled (Jääskaläinen et al., 2003). Loss of motility of boar spermatozoa has been suggested as a rapid bioassay for the presence of cereulide, the emetic toxin of *B. cereus* (Andersson et al., 1998). The use of this assay with strains of *B. licheniformis* involved in food poisoning also resulted in the inhibition of motility, but without the mitochondrial swelling observed in spermatozoa exposed to cereulide (Salkinoja-Salonen et al., 1999).

The acrosomal exocytosis is triggered by a complex signal transduction pathway involving G-proteins, tyrosine kinases, and calcium channels (Brandelli, 1997). Considering that the mode of action of many

antimicrobial peptides is disturbing cell membranes, it is feasible that any alteration in the membrane stability can promote increased susceptibility to acrosome reaction. Nevertheless, the effect of antimicrobial peptides on the sperm acrosome reaction has not been studied. In this work, acrosome reaction was stimulated by calcium ionophore, but nisin and P34 showed similar values than control cells, indicating that these antimicrobial peptides have no influence on acrosomal exocytosis.

Antimicrobial peptides have received increasing attention because their potential for the biological control of spoilage and pathogenic organisms. A typical example is nisin, the best-known bacteriocin that has been used as a safe food preservative and potential clinical use, owed its small cytotoxicity to human cells (Deegan et al., 2006; Reddy et al., 2004). Although the same could not be currently assumed for peptide P34, its potential as biopreservative for food use has been demonstrated.

3.3.7 Acknowledgements

Authors thank Dr. P.M. Roehe and Dr. A.C. Franco from Virology laboratories (ICBS/UFRGS) and D. Cantarelli of IPB/PUCRS for helpful support with cells assays; and Dr. R.F. Corseuil of LANAGRO/Brazil for technical support on imunofluorescence microscopy. This work received financial support from CNPq and CAPES, Brazil.

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3.4 ARTIGO 4

3.4.1 Investigation of the cytotoxicity of antimicrobial peptide P40 on eukaryotic cells

3.4.2 Abstract

The in vitro cytotoxicity of the antimicrobial peptide P40 was investigated. The food grade bacteriocin nisin was also analyzed for comparison. VERO cells were treated with different concentrations (0.02 – 2.5 $\mu g\ ml^{-1}$) of nisin and P40, and cell viability and plasma membrane integrity were checked by MTT, neutral red uptake (NRU) and lactate dehydrogenase (LDH) assays. In MTT and NRU assays the EC_{50} to the purified peptide P40 were 0.30 and 0.51 $\mu g\ ml^{-1}$, while values found to nisin were 0.35 and 0.79 $\mu g\ ml^{-1}$, respectively. In the LDH assay, the EC_{50} was 0.57 and 0.62 $\mu g\ ml^{-1}$ for P40 and nisin, respectively. The peptide P40 revealed higher hemolytical activity (19%) when compared to nisin (4.9%) at the highest concentration tested (2.5 $\mu g\ ml^{-1}$). Relatively few studies about the citotoxycity of antimicrobial peptides are available. The determination of the cytotoxicity of antimicrobial peptides is an essential step to warrant their safe use.

Key words: antimicrobial peptide, *Bacillus*, bacteriocin, MTT assay

3.4.3 Introduction

Antimicrobial peptides are widespread produced among plants, animals and microorganisms. These peptides have been classified according their chemical and structural characteristics, based on their variable molecular mass and net charge [21]. An increased interest to some antimicrobial peptides has been observed because of their potential applications as food biopreservatives [8] and as therapeutic agents against pathogenic bacteria [18].

Bacillus spp. produces a large number of antimicrobial peptides representing several different chemical structures [21], including bacteriocins and bacteriocin-like (BLS) [2]. A novel *Bacillus licheniformis* strain P40 was identified and its antimicrobial activity was characterized as a BLS [5]. This peptide also showed inhibitory activity *in planta* against *Erwinia carotovora*, a phytopathogenic bacterium that causes important agicultural losses [6]. Although many strains of *Bacillus* have been safely used in food and industry, there are relatively few specific studies on the citotoxicity of antimicrobial peptides from *Bacillus* [14].

In vitro cytoxicity assays are useful to define basal cytotoxicity, indicating the intrinsic ability of a compound to cause cell death as a consequence of damage to several cellular functions [4,22]. A number of assays and various cell types have been used with different responses to study *Bacillus* citotoxicity and none can be considered as standard [12,14,19]. Vero cells have been used with some frequency with the advantage of easy availability and fast growth [4]. The aim of this work was to investigate the *in*

vitro cytotoxicity of the antimicrobial peptide P40 on eukaryotic cells, in comparison with nisin through the use of different assays.

3.4.4 Materials and methods

3.4.4.1 Antimicrobial peptides

The antimicrobial peptide P40 was purified by ammonium sulfate and liquid chromatography on Sephadex G-100 from culture supernatant of *Bacillus licheniformis* strain P40, as described elsewhere [6]. The purification factor was 18-fold and the concentration was 800 U ml^{-1}. Nisin (Nisaplin®; Danisco, Copenhagen, Denmark) was suspended in 0.02 mol l^{-1} HCl as a 42 mg ml^{-1} stock solution. Peptides were further diluted in Eagle's Minimal Essential Medium (E-MEM; Gibco BRL, Rockville, MD, USA) for assays with Vero cells or phosphate buffered saline (PBS; 35 mmol l^{-1} phosphate buffer, 150 mmol l^{-1} NaCl, pH 7.4) for hemolysis assays.

3.4.4.2 Vero cell culture

Vero cells (Adolpho Lutz Institute, São Paulo, Brazil) from green monkey kidney were used between 20-30 passages. The cells were cultured in 96-well plates (Corning, USA) with E-MEM supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), 1% (v/v) L-glutamine (200 mmol l^{-1}), 10 mg ml^{-1} enrofloxacin (Baytril, Bayer) and 0.025 µg ml^{-1} anphotericin B (Gibco BRL). The cells were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere. The viability of the test cells exceeded 99% prior to cytotoxicity

assay as determined by exclusion of the vital dye trypan blue. Vero cell cultures (3×10^5 cells ml^{-1}) were prepared in 96-well plates and 200 μl of serial dilutions (0.02 to 2.5 μg ml^{-1}) of antimicrobial peptide P40, nisin, or SDS (positive control) were added. Fresh E-MEM was used as negative control. Plates were incubated for 24 h at 37ºC in humidified 5% CO_2 atmosphere.

3.4.4.3 MTT assay

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out according to [15]. The culture supernatants were removed and MTT solution (USB Corporation, Cleveland, USA) was added to each well and the plates were incubated for 4 h at 37ºC. The MTT solution was removed DMSO was added to dissolve formazan crystals. The absorbance at 540 nm was read on a 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). The percentage of viability was calculated as $A_T/A_C \times 100$; where A_T and A_C are the absorbances of treated and control cells, respectively.

3.4.4.4 Neutral red assay

The cell cytotoxicity was monitored by the neutral red dye uptake (NRU) assay [3]. The culture supernatants were removed and the cells were rinsed with fresh media containing 3.3 mg l^{-1} neutral red. After 3 h at 37ºC/5% CO_2, media was carefully removed and cells were washed with E-MEM. Incorporated dye was then solubilized by addition of 100 μl of an aqueous solution containing 50% (v/v) ethanol, 1% (v/v) acetic acid. The absorbance at

540 nm was read on a microplate reader. The percentage of viability was calculated as A_T/A_C x 100; where A_T and A_C are the absorbances of treated and control cells, respectively.

3.4.4.5 LDH release assay

The activity of LDH in the medium was determined using a commercially available kit (Labtest Diagnostic, Lagoa Santa, Brazil). The culture supernatants were removed and LDH release was determined by two-point enzymatic kinetics [9]. The absorbance at 340 nm was monitored using a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan). The percentage of LDH release was calculated as A_T/A_C x 100; where A_T is the experimental absorbance of treated cells, A_C is the control absorbance of untreated cells.

3.4.4.6 Hemolytic activity

Hemolytic activity of antimicrobial peptides was measured spectrophotometrically using a haemoglobin release assay [19], with modifications. Briefly, the fresh defibrinated human erythrocytes were rinsed three times with PBS centrifuged for 15 min at 900 x g and resuspended at 4% (v/v) in PBS. Cells were treated with peptide P40 or nisin (0.62, 1.25 or 2.5 μg ml^{-1}) for 1h at 37ºC and then centrifuged at 1000 x *g* for 5 min. Aliquots of the supernatant were then transferred to a fresh microcentrifuge tube, where hemoglobin release was monitored using a Hitachi U-1100 spectrophotometer (Hitachi, Tokyo, Japan) by measuring the absorbance at 414 nm. Percentage of hemolysis was calculated as $(A_T–A_C)/(A_X–A_C)$ x 100; where A_T is the

experimental absorbance of treated supernatants, A_C is the control absorbance of PBS-treated cell supernatant, and A_X is the absorbance of 0.1% (v/v) Triton X-100 lysed cells.

3.4.4.7. Statistical analysis

Data were expressed as means ± S.E.M. for three independent determinations for each experimental point. Significant differences were determined by Kruskal-Wallis test at level of significance of $P<0.05$. The EC_{50}, defined as the concentration that caused 50% maximum effect, was calculated by regression analysis of the dose-response curves for MTT, NRU and LDH assays.

3.4.5 Results

3.4.5.1 Effects on Vero cells

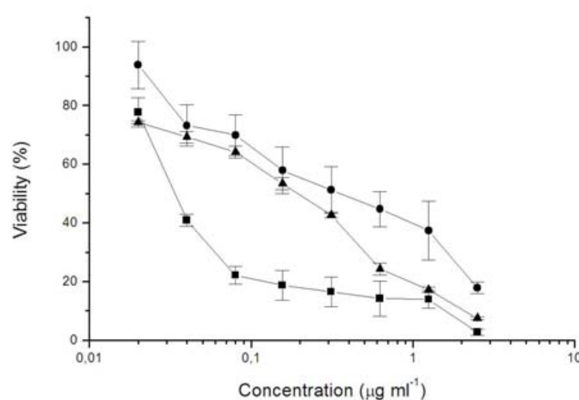
The cytotoxicity was initially evaluated by using MTT and NRU assays. The results of the dose-response curves for MTT and NRU assays are presented in the Fig. 1. Following exposure of Vero cells to antimicrobial peptides the MTT and NRU assays appear to be similar in detecting loss of viability. Peptide P40 and nisin caused a decrease of cell viability with increased concentrations in both assays. When compared with nisin, P40 caused significantly increased damage from 0.62 μg ml^{-1} concentration in MTT assay (Fig. 1A), although this difference was noted from 0.31 μg ml^{-1} in NRU (Fig. 1B).

Peptide P40 and nisin showed comparable EC_{50} values in MTT assays, however smaller EC_{50} values were observed for the positive control

indicating higher cytotoxicity of this treatment (Table 1). The EC_{50} for P40 was about 1.6 times smaller than nisin in the NRU assays.

Vero cells treated with different concentrations of antimicrobial peptide P40 resulted in higher levels of LDH release when compared with nisin (Fig. 1C). Concentration-depedent increase in extracellular LDH was observed with P40 and nisin, indicating that these peptides caused some loss of plasma membrane integrity at concentrations from 0.31 μg ml^{-1}. The peptide P40 caused LDH release higher that 50% when used at 1.25 μg ml^{-1} or beyond. The treatment with SDS (positive control) resulted in high levels of LDH release (Fig. 1C), indicating an extensive cell damage. The EC_{50} values for the different treatments are shown in the Table 1.

A



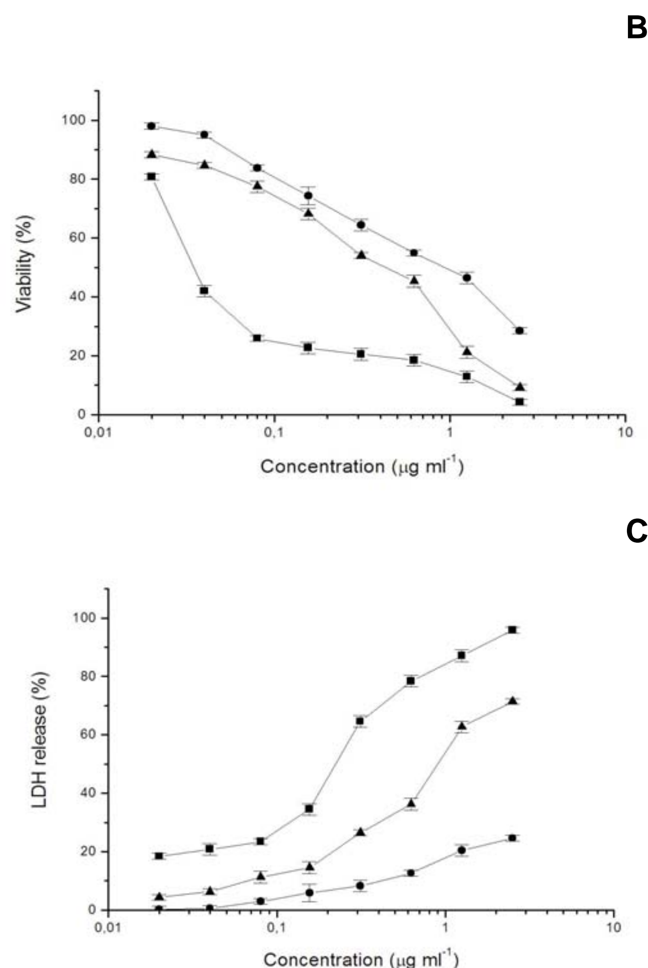


Fig. 1. Effect of antimicrobial peptides on Vero cells using (A) MTT, (B) neutral red, or (C) LDH release assays. Vero cells were cultivated for 24 h with the indicated concentrations of antimicrobial peptide P40 (triangles), nisin (circles), or SDS as positive control (squares). The results represent the mean ± S.E.M of at three independent experiments.

3.4.5.2 Activity on human erythrocytes

The hemolytic activities of the antimicrobial peptide P40 and nisin were determined as the percentage of lysis of human erythrocytes. According to the data obtined in this study, the purified P40 and nisin showed a hemolytic activity of 19.3% and 4.9%, respectively, at the concentration of 2.5 μg ml^{-1} (Table 2). The values observed for nisin and P40 were significantly different,

and a significant decrease in the percentage of hemolysis was observed when the concentration of antimicrobial peptides was reduced to 0.62 µg ml^{-1}.

Table 1. EC_{50} values for Vero cells following exposure to antimicrobial peptides for 24 h based on the dose-response curves derived from the MTT, neutral red (NRU) and LDH assays.

	EC_{50} (µg ml^{-1})		
Treatment	MTT	NRU	LDH
Control (+)	0.04	0.04	0.19
Nisin	0.35	0.79	0.62
Peptide P40	0.30	0.51	0.57

Table 2. Hemolytic activity of the antimicrobial peptides against human erythrocytes.

	Hemolysis (%) *		
Treatment	0.62 µg ml^{-1}	1.25 µg ml^{-1}	2.5 µg ml^{-1}
Nisin	$2.6 \pm 1.1^{a,A}$	$3.2 \pm 0.8^{a,B}$	$4.9 \pm 1.0^{a,B}$
Peptide P40	$10.3 \pm 0.5^{b,A}$	$15.7 \pm 0.6^{b,B}$	$19.3 \pm 0.5^{b,C}$
Crude P40	$31.1 \pm 1.6^{c,A}$	$35.6 \pm 1.2^{c,A}$	$40.7 \pm 0.7^{c,B}$

* Data are expressed as mean percentage hemolysis ± S.E.M for three separate experiments. The amount of peptide is based on protein concentration of the samples. Values for negative control (PBS) and positive control (0.1% Triton X-100) were 0.8 ± 0.04 and 99.5 ± 0.5, respectively. Different superscript letters indicate significant differences at $P<0.01$; small letters compare values within the same column; capital letters compare values within the same row.

3.4.6 Discussion

The citotoxicity of the antimicrobial peptides P40 and nisin was investigated on erythrocytes and Vero cells. Vero cells presented similar sensibility to the peptide P40 and nisin in the MTT and NRU asssays, although P40 caused an increased loss of viability at 0.62 μg ml^{-1} or higher. Besides, a dose-response effect of the antimicrobial peptides was clearly observed, since Vero cell viability gradually decreased with the increase of peptide concentrations. The small cytotoxicity of nisin A to vaginal, colonic and kidney epithelial cells has been reported [1,16]. Nisin was harmless between 2.85 and 11.4 μg ml^{-1} to Vero cells over 48 h, whereas no important toxicity to vaginal epithelial cells was observed up to concentrations of 318 μg ml^{-1} [16]. Nisin A also presented low toxicity in MTT assay using HT29 and Caco-2 cells [12]. Diferential cytotoxicity may be related to differences in plasma membrane composition and metabolic activity of target cells [17], exposure time, toxicity assay used, as well as the medium used to apply the peptides [12]. Thus, the increased cytotoxicity of nisin and other antimicrobial peptides against some cell types could be associated with differences on the cell surface hydrophobicity, which may influence the effective binding and cytotoxic action. However, the exact mechanism by which toxicity differs among different cell types is not completely elucidated.

The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage [7]. The LDH leakage assay indicates the loss of plasma membrane integrity in Vero cells, revealing some citotoxicity following 24 h exposure to nisin or peptide

P40. This fact agrees with the observation that membranes are the main target of most antimicrobial peptides, including nisin [8] and P40 [6]. However, their effects on bacteria occur at lower doses (0.3 μg ml^{-1}), which is relatively safe to Vero cells. This agrees with the observation that target membranes have different sensitivity to the disruptive effects of antimicrobial peptides, where bacteria are most susceptible, lower eukaryotes including fungi exhibit an intermediate degree of sensitivity, and higher organisms are largely resistant to antimicrobial peptides [13].

Nisin exhibited lower lytic effect against human erythrocytes than peptide P40. Comparable levels of red blood cell lysis (about 6%) were reported following 30 min incubation with 3.35 mg ml^{-1} nisin [10]. Also the increase in membrane permeability and subsequent osmotic lysis of cells is thought to be due to the formation of mixed micelles in the lipid bilayer [11]. *Bacillus* species produce varied lipopeptide antibiotics, which could also act as biosurfactants. Antimicrobial peptide P40 appears to posses a lipid chain in its structure and is an effective emulsifier [20], which agrees with its higher hemolytic effect as compared with nisin.

Antimicrobial peptides have received increasing attention because their potential for the biological control of spoilage and pathogenic organisms. A typical example is nisin, the best-known bacteriocin that has been used as a safe food preservative and potential clinical use, owed its small cytotoxicity to human cells [8,18]. Although the same could not be currently assumed for peptide P40, its relevance in crop protection [6] and as a natural biosurfactant [20] has been demonstrated.

3.4.7 Acknowledgments

Authors thank P.M. Roehe, A.C. Franco of ICBS/UFRGS and D. Cantarelli of IPB/PUCRS for technical support with Vero cells.

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3.5 ARTIGO 5

3.5.1 Evaluation of the immunogenicity and *in vivo* toxicity of the antimicrobial peptide P34 in mice

3.5.2 Abstract

Imunnogenicity and toxicity of antimicrobial peptide P34 were evaluated *in vivo*. BALB/c mice were administered intraperitoneally with 50 µg/ml peptide P34 alone and associated with the Freund's adjuvant. For acute toxicity testing, different concentrations of the peptide P34 (82.5, 165, 247.5 and 330 mg/kg) were orally administered. To evaluate the sub-chronic toxicity the tested dose of 0.825 mg/kg day of the peptide P34 and nisin were administered for 21 days. There were no hypersensitivity reactions or significant increase in antibody titer during the immunogenicity experiment or death of animals during the acute or sub-chronic toxicity tests. The LD_{50} was more the 332.3 ± 0.76 mg/kg. No significant changes in the serum biochemical parameters were observed in the animals treated with the peptide P34 unlike nisin-treated group showed a significant increase in alanine transaminase levels in comparison to controls. Signs of possible toxicity were detected in animals in the group treated with 0.825 mg/kg day of nisin, with histological changes in the spleen, skin and liver. In the group treated with peptide P34 histological changes in the spleen with the presence of megakaryocytes was observed. Few studies report the use of animal models to evaluate the *in vivo* toxicity of antimicrobial peptides and such investigation is an essential step to ensuring its safe use in foods.

Key words: antimicrobial peptide; toxicity *in vivo*; *Bacillus*; bacteriocin.

3.5.3 Introduction

Species of *Bacillus* produces a large number of antimicrobial substances including several different peptides commonly referred as bacteriocins and bacteriocin-like substances (Riley and Wertz, 2002a,b). These include those produced by *Bacillus subtilis, Bacillus thuringiensis*, *Bacillus amyloliquefaciens, Bacillus stearothermophilus, Bacillus licheniformis* and *Bacillus cereus* that have been recognized as industrially important for more than 50 years (Pedersen et al 2002; Stein, 2005). These peptides have been often classified according their chemical and structural characteristics, based on their variable molecular mass and net charge (Von Döhren, 1995). Their proteinaceous nature implies a putative degradation in the gastro-intestinal tract of man and animals, suggesting that some bacteriocins could be used as food preservatives (Eckner, 1992; Cleveland et al. 2001; Deegan et al. 2006) and as therapeutic agents against pathogenic bacteria (Reddy et al. 2004). Although many strains of *Bacillus* have been safely used in food and pharmaceutical industry, there are relatively few specific studies on the toxicity of antimicrobial peptides from *Bacillus* (Vaucher et al., 2010a, Vaucher et al., 2010b, Mikkola et al., 2000).

One of the requirements for the application of an antimicrobial peptide as preservative in food would be the evaluation of its immunogenicity and also the toxicity *in vitro* and *in vivo*. The toxicity testing of a putative food antimicrobial would consider repeated and daily administration of the substance, so this type of test should extend the time periods required (Pariza & Foster, 1983). The route of administration of a particular bacteriocin should

be the same proposal for use in humans (FDA, 1988; Post, 1996) and toxicological studies involving animals are a major component of safety assessment of bacteriocins (Moreno et al., 2000).

Bacillus sp. strain P34 produces the peptide P34, which has been characterized as a broad range antimicrobial substance that inhibits important food-borne pathogens like *Listeria monocytogenes* and *Bacillus cereus* (Motta et al. 2007a). Preliminary investigation on the *in vitro* toxicity of peptide P34 showed similar results to that obtained for nisin (Vaucher et al., 2010b). The objective of this study was to determine the acute toxicity of the antimicrobial peptide P34 and compare the effect of this peptide with the nisin by the use of subchronic toxicity tests *in vivo*.

3.5.4 Materials and methods

3.5.4.1 Microorganism

The bacterium *Bacillus* sp. strain P34, previously isolated and characterized (Motta et al., 2007a) was used for production of antimicrobial peptide. The organism was stored at -20ºC in Trypticase Soy Broth (TSB) medium containing 20% glycerol. The bacterium was propagated in fresh TSB medium before use.

3.5.4.2 Antimicrobial peptides

Bacillus sp. strain P34 was cultivated aerobically in 500 ml Erlenmeyer containing 200 ml of TSB broth at 30ºC, 180 cycles min^{-1} for 24h. The culture supernatant was obtained by centrifugation at 10,000 x g for 15 min

at 4ºC. The supernatant was filtered through 0.22 µm filter membrane (Millipore, Billerica, MA, USA) and submitted to ammonium sulfate precipitation at 20% saturation. After centrifugation for 10,000 x g at 4ºC for 15 min, the pellet was ressuspended in 10 mM sodium phosphate buffer pH 7.0 and loaded on a Sephadex G-100 column (GE Healthcare/ Pharmacia Biotech, Uppasala, Sweden). Six consecutive purifications were carried out to obtain about 180 mg of the peptide (Motta et al., 2007b). Subsequently, the peptide was lyophilized and the volume adjusted with PBS added 0.02 mol/L HCl to concentration of 17.5 mg/mL. The 0.5 g of Nisaplin® was suspended initial in PBS buffer added to 0.02 mol/L HCl to a final nisin concentration of 12.5 mg/ml. The concentrations were determined using the Folin-phenol reagent method (Lowry et al., 1951), using a calibration curve developed with bovine serum albumin as protein standard. The antimicrobial peptide P34 and nisin concentration was adjusted before the oral administration of mice with PBS with the addition of 0.02 mol/l HCl to a final concentration of 0.825 mg/Kg per day. The PBS added 0.02 mol/l HCl, was used as negative control.

3.5.4.3 Mice

For the experiments we used males mice BALB/c, weighing between 20 and 35 g, and separated by approximate weights. These animals were provided by the animal house of the Federal University of Santa Maria (UFSM/RS), Brazil and kept in plastic boxes with food and water *ad libitum*. The animals were used in experiments after a period of seven days of adaptation in captivity, with regular light-dark 12 hours and ambient temperature of 20°C with

water and food *ad libitum* (Guide for the Care and Use of Laboratory Animals, 1996). The procedures used in the assays were approved by the Ethics Committee (Protocol number P10310), and conform to international standards of animal welfare, as specified by the International Guiding Principles for Biomedical Research Involving Animals (CIMS), Geneva, 1985.

3.5.4.4 Imunnogenicity

Twenty males mice BALB/c, aged between 60 and 70 days were divided into two groups (antimicrobial peptide P34 and antimicrobial peptide P34 + Freund's complete adjuvant) each containing 10 animals. The mice were immunized intraperitoneally with 0.5 ml of the antimicrobial peptide P34 (50 µg/ml) and 0.25 ml antimicrobial peptide P34 (50 µg/ml) + 0.25 ml of Freund's complete adjuvant, respectively. After 14 and 28 days of first inoculation, the mice were again inoculated with 0.5 ml of the preparations mentioned above. To verify the presence of antibodies against the antimicrobial peptide P34, the serum collected from the tails of mice were tested at 21 and 42 days after the first inoculation by indirect Enzyme-linked immunosorbent assay (ELISA). Held titration of antigen, anti-mouse antibody labeled and serum of mouse to standardize the assay. The best results were observed with 12.5 µg/well of P34 antigen, a dilution of 1:2000 of anti-mouse antibody labeled and 1:100 serum of mouse.

3.5.4.5 Acute toxicity

We used six males BALB/c mice, with acute toxicity assessed by oral administration of increasing doses of the antimicrobial peptide P34 (82.5, 165, 247.5, 330 mg/Kg). The route of administration should be the same proposed for use in humans (FDA, 1988). After administration, the animals were kept under observation for a minimum of 48 h. The number of animals killed for each of the doses was noted and the LD_{50} calculated by the *Up and Down* method, which is one of the most used to decrease the number of animals used (Botham, 2004).

3.5.4.6 Subchronic toxicity

Eight BALB/c males were used for each of the treated groups (0.825 mg/kg day of the antimicrobial peptide P34 or nisin) and control group. This concentration was chosen based on reported ADI (Acceptable Daily Intake) for nisin of 33,000 IU or 0.825 mg/kg of body weight (Hoover & Steenson, 1993). The animals were subjected to experimental protocol, lasting 21 days, the treatments daily orally using gavage. The scheme offered food and water *ad libitum*, and body weight was recorded at days 0, 7, 14 and 21.

3.5.4.7 Laboratory blood analysis

Laboratory analysis of biochemical parameters was performed on serum samples. Serum aspartate transaminase (AST), alanine transaminase (ALT), urea and creatinine were performed in automated biochemical analyzer (LabMax 240, Labtest, Brazil).

3.5.4.8 Histopathological analysis

After 21 days of oral administration of antimicrobial peptide P34 and nisin, the animals were sacrificed by cervical dislocation, and the organs examined macroscopically. Tissue samples of stomach, intestine, spleen, liver, kidney and skin of mice were collected and fixed in 10% buffered formalin and processed for paraffin embedding. The histological sections (3-5 µm) were stained with hematoxylin-eosin (HE) (Prophet et al., 1992). The slides were coded and analyzed at the Veterinary Pathology Laboratory (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) by a veterinary pathologist who was unaware of the experimental conditions of each group.

3.5.4.9 Statistical analysis

The results were expressed as mean ± standard deviation of the groups and subjected to analysis of variance (ANOVA) and Turkey's test. The differences were considered statistically significant when $p<0.05$.

3.5.5 Results and discussion

The immunogenicity of the antimicrobial peptide P34 was assessed by intraperitoneal administration into two groups of mice. There were no deaths of animals during the experiment, or hypersensitivity reactions. A slight non-significant increase in antibody titer of group 1 mice administered only with the antimicrobial peptide P34 can be observed at day 42 compared with day zero (**Figure 1)**. In group 2 mice administered with antimicrobial peptide P34 + Freund's complete adjuvant there was a non-significant increase in antibody

titer at day 21, the increase considered significant in day 42 compared to day zero.

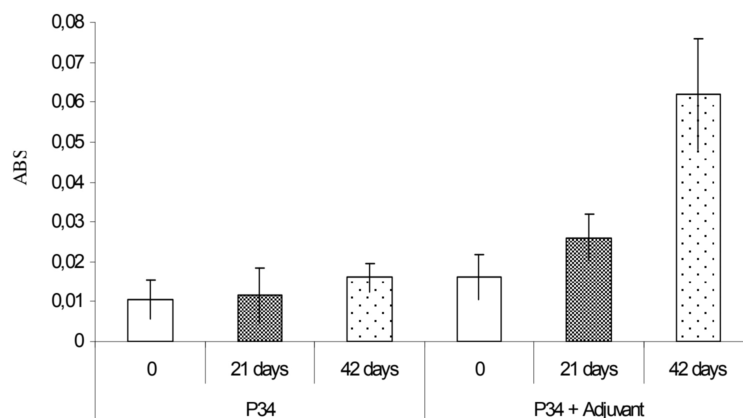

Figure 1. Determination of antibody titer of mice administered intraperitoneally with the antimicrobial peptide P34 and its association with adjuvant after 42 days. The results represent the mean ± S.E.M of absorbance of group 1 (Antimicrobial peptide P34 alone) and group 2 (Antimicrobial peptide P34 + adjuvant).

Bhunia et al. (1990) evaluated the immunogenicity of pediocin PA-1 (AcH) to mice and found that is was not immunogenic for animals. Short-term administration of diets containing nisin (Nisaplin) induced an increase of both CD4 and CD8 T-lymphocyte cell counts and also a decrease of B-lymphocyte counts. The macrophage/monocyte fraction isolated from peripheral blood became significantly increased after long-term administration (100 days) of Nisaplin-containing diets (de Pablo et al., 1999).

After the studies of immunogenicity of peptide P34, the investigation for oral acute toxicity in mice with a single dose was performed. In tests to

determine the acute toxicity and LD_{50} of peptide P34, no deaths occurred following oral administration of any of the tested concentrations (82.5, 165, 247.5, 330 mg/Kg). Thus, the LD_{50} was more the 332.3 ± 0.76 mg/kg. These tests are crucial to evaluate antimicrobial peptides with potential food preservative and the concentration used should be equivalent to at least 100 times the estimated average human exposure or at least 2000 mg/kg body weight following guidelines established by the OECD (1987). Although several antimicrobial peptides have been purified and characterized there are currently few studies on acute toxicity for comparison. Extensive toxicological studies conducted with nisin showed that intake does not cause toxic effects to the human body with a reported LD_{50} of 6950 mg/kg similar to salt, when administered orally (Jozala et al., 2007). The pediocin PA-1 is another bacteriocin that has been used for the same purpose, and studies on its toxicity have been reported (Bhunia et al., 1990; Dabour et al., 2009), although its use was not yet recommended by WHO (Drider et al., 2006). Some authors have associated high LD_{50} of bacteriocins with digestive enzymes capable of rapidly inactivating these substances, being trypsin and chymotrypsin produced in the pancreas and released into the small intestine a prime example (Hara et al., 1962, Eckner, 1992, Cleveland et al,. 2001, Deegan et al., 2006). Claypool et al. (1966) evaluated the effect of nisin in milk chocolate consumed orally and noted that only ¼ of the original concentration was detected in saliva after a minute of use. Besides this, some bacteriocins can also be sometimes sensitive to ptyalin, being not detected in human saliva 10 min after the consumption of a liquid containing bacteriocin (Chandrapati & O'Sullivan, 1998).

In tests conducted to evaluate the sub-chronic toxicity no deaths of control or treated animals were observed. There were no significant differences in body weight gain among groups (**data not shown**). Blood samples were collected from control and treated mice and processed for examination of possible changes in the biochemical parameters one day before and one after the final administrations. The values for serum aminotransferases, creatinine and urea were similar among the treatments (**Table 1**). A significant increase (p<0.05) in serum ALT levels was observed for the nisin-treated group after 22 days. These results indicate that the antimicrobial peptides may be continued ingested, although the increased level of ALT suggests a potential hepatotoxic effect of nisin.

Table 1. Biochemical parameters obtained from the serum of BALB/c mice administered for 21 days with antimicrobial peptide P34 and nisin (subchronic toxicity).

Sample		AST (U/L)	ALT (U/L)	Creatinin (mg/dL)	Urea (mg/dL)
Day 0	**Control**	143,7 ± 20,6	69,2 ± 22	0,165 ± 0,06	62,6 ± 25,5
	Nisin	155 ± 24,7	75,4 ± 14,1	0,133 ± 0,03	51,3 ± 14,9
	P34	130,3 ± 23,4	60,7 ± 14,3	0,14 ± 0,03	47 ± 15,9
Day 22	**Control**	145,6 ± 18,6	79,5 ± 13,4	0,16 ± 0,03	60 ± 11,7
	Nisin	236,5 ± 27,7*	82 ± 10,6	0,26 ± 0,04	55 ± 12,4
	P34	156,6 ± 25,3	74,5 ± 8,2	0,24 ± 0,04	47,2 ± 7,6

AST= Alanine aminotransferase, ALT= Aspartate aminotransferase. The results are presented as mean ± standard deviation. *p<0.05 (ANOVA - Test of Turkey).

Histopathological studies were performed with the stomach, intestines, liver, spleen, kidney and skin of control and treated animals. No histological changes were detected in the intestines and kidneys of any group.

Histological changes were observed in the stomach of all groups showing a glandular pattern. Signs of possible toxicity were observed in animals treated with 0.825 mg/kg day of nisin, with histological changes in the spleen, skin and liver. An enlargement in gross organ size was observed in the spleen (**Figure 2**), with a significant increase in weight (151,5 ± 2,81 mg versus 120 ± 1,71 mg of control).

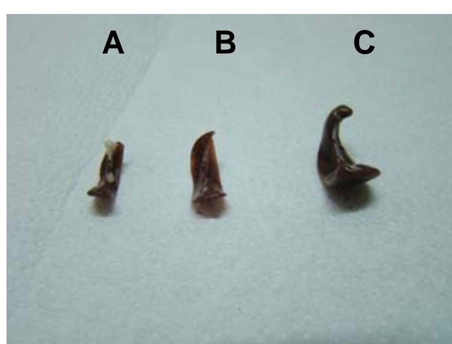

Figure 2: Comparison of spleen size observed in mice control (A), administered with the antimicrobial peptide P34 (B) and nisin (C) after 21 days (sub-chronic toxicity).

Histological analysis of the spleen showed the abundant presence of megakaryocytes (**Figure 3**). Skin lesions were found during the experiment that have increased after 21 days of oral administration of nisin, shown histological changes such as dermatitis with necrotic ulceration and presence of fibrin. In the liver of the same group, histological changes such as hepatic degeneration were observed (**Figure 4**). In the group treated with 0.825 mg/kg day of the peptide P34 it was observed histological changes in the spleen with the presence of megakaryocytes (**Figure 3**).

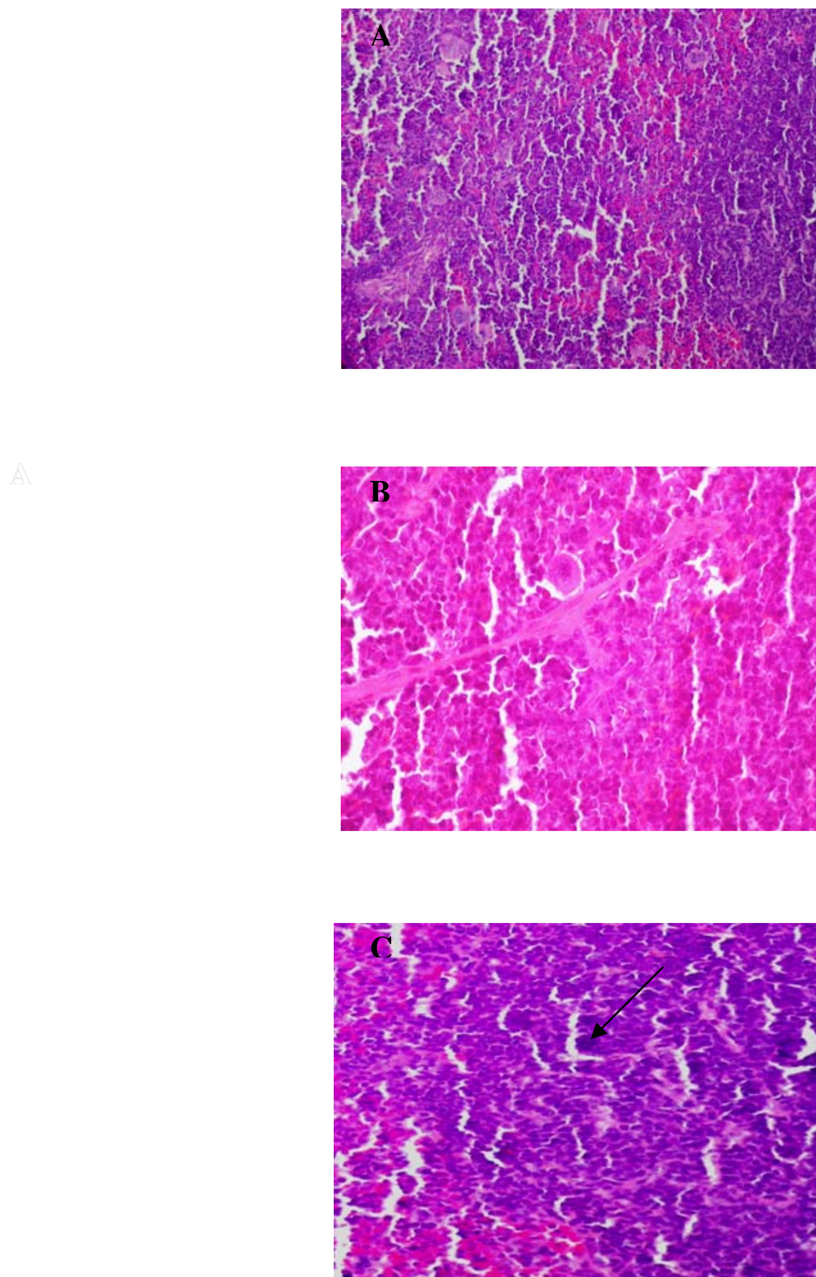

Figure 3: Histological changes of mice spleen. Spleen sections of control animals (A) and mice receiving an oral dose of 0.825 mg/kg for 21 days of peptide P34 (B) or nisin (C) with the presence of megakaryocytes (arrow). Hematoxylin-eosin (HE). Bar = 100 micron.

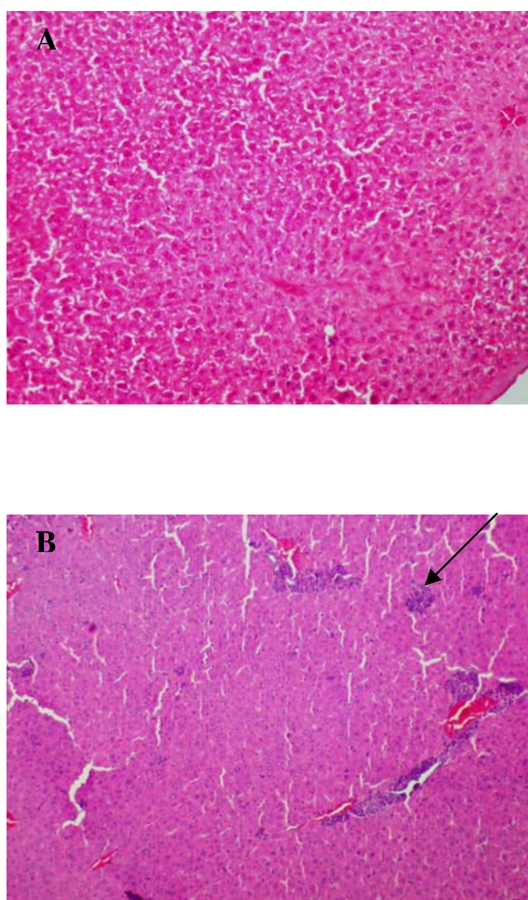

Figure 4: The liver of nisin-treated mice can be observed hepatic degeneration and presence of neutrophils (arrow). Liver sections of control animals (A) and mice receiving an oral dose of 0.825 mg/kg for 21 days with nisin (B). Hematoxylin-eosin (HE). Bar = 100 micron.

The presence of megakaryocytes indicates a possible inflammatory process. Puertollano et al. 2003 analyzed pro-inflammatory cytokines produced in spleen cells of mice in response to nisin, and reported increased levels of some cytokines involved in inflammation.

Except for a study with nisin (Frazer et al., 1962; Hoover & Steenson, 1993), few studies report the use of animal models to evaluate the *in vivo* toxicity and to assess the effects of bacteriocins in target organs (Mota-Meira et

al., 2005). Nisin was tested in pregnant rats and the treated animals and their progeny did not show any clinical signs of toxicity when compared to the control animals (Gupta et al. 2008). Recently, Dabour et al. (2009) using *in vivo* experiments showed that repeated doses of pediocin PA 1 (250 mg/day for three consecutive days) resulted in a decrease of 2 log cycles of *Listeria monocytogenes* in artificially infected animals, and promotes the disappearance the pathogen in target organs of animals (spleen and liver) within six days. In the same study, they demonstrated that consumption of feed containing the purified bacteriocin did not affect the intestinal microflora, weight change or development of the animals.

The evaluation of *in vitro* and *in vivo* toxicity of an antimicrobial peptide is an essential step before it could be considered for use in food. The results presented here agree with previous investigation on the *in vitro* toxicity of peptide P34 on erythrocytes and VERO cells, where similar results to that obtained for nisin were obtained (Vaucher et al., 2010b). The evaluation of the immunogenicity, acute and sub-chronic toxicity of the antimicrobial peptide P34 developed in this study corroborate the potential of this substance as an alternative food preservative.

3.5.6 Acknowledgements

Authors thank Dr. P.M. Roehe of Virology Laboratories (ICBS/UFRGS) for helpful support with ELISA assays and Dr. D. Driemeier of Department of Clinical Pathology and Veterinary (UFRGS) for performing the histological analysis. This work received financial support of CNPq, Brazil.

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4 DISCUSSÃO GERAL

O peptídeo antimicrobiano P34 foi previamente caracterizado (Motta *et al*., 2007a) e seu modo de ação investigado (Motta *et al*., 2008). Neste estudo, verificou-se sua influência na expressão de genes *dlt*A, *lmo*1695 e *mpt*A, em *Listeria monocytogenes e Listereia seeligeri*, além de sua citotoxicidade em diferentes linhagens de células eucarióticas.

Tendo em vista, a possibilidade de potencialização do efeito do tratamento com o peptídeo P34 em *Listeria monocytogenes* procuramos inicialmente avaliar o sinergismo dessa substância com o sobrenadante bruto de algumas LAB selecionadas.

Estes experimentos demonstraram um aumento na atividade antimicrobiana após a combinação do peptídeo P34 e do sobrenadante não neutralizado de seis diferentes cepas de LAB. Este resultado sugere que este aumento possivelmente, esta relacionado com a produção de ácidos orgânicos pelas culturas de LAB. Ficou evidente que a concentração de substâncias antimicrobianas dos sobrenadantes brutos das culturas de LAB não inibiram o crescimento de *Listeria monocytogenes*, porém intensificaram a atividade do peptídeo P34. Alguns estudos relataram que possa ocorrer um aumento da atividade antimicrobiana das bacteriocinas, em combinação a ácidos orgânicos (SOO YEON *et al*., 2001; GI-SEONG *et al*., 2002).

Foi verificada uma redução no crescimento de *Listeria monocytogenes*, após aplicação do peptídeo P34 em queijo Minas Frescal, fato que ocorreu quando adicionada a peptídeo na concentração de 6400 AU/ml. Alguns autores relataram a inibição do crescimento de *Listeria monocytogenes*

em diferentes tipos de queijo, como ricota, camembert e caseiro após a utilização de nisina (DAVIES *et al.*, 1997; MAINSNIER-PATIN *et al.*, 1992; FERREIRA e LUND, 1996). Acredita-se que altas concentrações de bacteriocinas são necessárias para reduzir as contagens de *L. monocytogenes* quando esta é adicionada diretamente ao queijo, sendo este fato associado a enzimas endógenas ou vinculação aos componentes da matriz alimentar (CLEVELAND *et al.*, 2001; AASEN *et al.*, 2003).

Em relação à influência do peptídeo P34 na expressão de genes em *Listeria monocytogenes* foi evidenciado um aumento significativo na transcrição dos genes *mptA, dltA* e *lmo 1695*, após inoculação de 6400 UA/ml do peptídeo P34 em queijo Minas Frescal e incubação de 96 horas. O gene *mptA* demonstrou um aumento na expressão de 16 vezes comparado ao controle, seguido por *dltA*, 13 vezes e *lmo 1695*, 11 vezes. Após 168 horas da inoculação, foi observada diminuição significativa nos níveis de transcrição de todos os genes, sendo o gene *lmo1695* o mais expresso se comparado ao controle. Os resultados reportados neste trabalho são contrários aos publicados por GRAVESEN *et al.* (2000; 2002b), que relataram uma diminuição na transcrição do gene *mptA*, envolvido na resistência de cepas de *Listeria monocytogenes* as bacteriocinas da classe IIa.

Os presentes resultados também diferem dos apresentados por VADYVALLO *et al.* (2004), que reportaram a não alteração na transcrição dos genes *dltA* e *lmo1695*. Portanto, como o peptídeo P34 influenciou a transcrição destes dois genes, fica claro que esta possa interagir, possivelmente, com

estruturas da superfície celular ou membrana citoplasmática em *Listeria monocytogenes*.

Segundo CLEVELAND *et al*. (2001), estudos tem demonstrado que as condições físico-químicas do alimento influenciam significantemente na atividade das bacteriocinas, sendo reportado que a gordura poderia reduzir ação das bacteriocinas na célula alvo. O queijo apresenta uma matriz rica em proteínas e gordura, fato este, que poderia explicar talvez um aumento ou redução na expressão dos genes após interação do peptídeo P34 com a bactéria e queijo.

Devido à influência da interação com o alimento acima citada, investigamos a influência do peptídeo antimicrobiano P34 na expressão dos genes, *dlt*A, *lmo*1695 e *mpt*A após os experimentos de atividade em *L. monocytogenes* e *L. seeligeri* com tempos e temperatura de incubação diferentes. Outro problema a ser observado, seria o aumento da resistência de cepas bacterianas com o uso de antimicrobianos aplicados a conservação de alimentos, incluindo a bactérias *L. monocytogenes* (RILEY e WERTZ, 2002a,b). Algumas cepas isoladas de alimentos tornaram-se resistentes a bacteriocinas convencionais, tais como a nisina, pediocina PA-1 e bavaricin A e a Antimicrobianos utilizados para o tratamento clínico como a clindamicina, linezolida, ciprofloxacina, ampicilina, rifampicina, trimetoprim, sulfametoxazol, tetraciclina e vancomicina (RASCH e KNOCHEL, 1998, VAN SCHAIK *et al*., 1999 ; CONTER *et al*. 2009)

Por este motivo, foram realizados testes de sensibilidade das cepas de *L. monocytogenes* e *L. seeligeri* para verificar a sensibilidade frente a alguns

antimicrobianos e a concentração inibitória mínima (CIM) foi determinada. A *L monocytogenes* foi sensível à maioria dos Antimicrobianos testados, exceto nitrofurantoína (MIC>64) e ceftriaxone (MIC>32). *L. seeligeri* foi resistente apenas ao ceftriaxone (MIC>32), uma característica própria deste gênero bacteriano que é resistente as cefalosporinas (POROS-GŁUCHOWSKA e MARKIEWICZ, 2003). Verificou-se que ambas as espécies bacterianas foram sensíveis à vancomicina, um fato que permite um estudo comparativo entre as duas espécies, uma vez que tem sido relatada a existência de cepas de *Listeria monocytogenes* resistente à vancomicina (CONTER *et al*., 2009).

A cepa de *L. monocytogenes* apresentou uma similar sensibilidade a *L. seeligeri* frente ao peptídeo P34 e nisina, nas condições experimentais testadas. Nossos resultados para a atividade antimicrobiana foram semelhantes a um estudo anteriormente reportado por MOTTA *et al.* (2007a).

Foi demonstrado um aumento não significativo nos níveis de transcrição dos genes *dlt*A, *lmo*1695 e *mpt*A, após incubação de 24 horas a 37 °C e 240 horas a 4 °C, das placas de *L. monocytogenes* inoculadas com o peptídeo P34 e nisina. Para os níveis de expressão gênica de *dlt*A e *lmo*1965 os resultados obtidos neste estudo foram coerentes com os reportados por VADYVALLO *et al.* (2004), que também não observaram alterações significativas na expressão destes genes em linhagens selvagens de *L. monocytogenes.* Como a cepa de *L. monocytogenes* utilizada neste estudo é uma cepa ATCC e tem sido reportado na literatura uma diminuição da expressão do gene *mptA* apenas em cepas resistentes ou mutantes de *L. monocytogenes* isoladas de alimentos (GRAVESEN *et al*., 2000; GRAVESEN

et al., 2002ab; VADYVALOO *et al.*, 2004), os resultados do aumento não significativo na expressão deste gene aqui reportados estão de acordo aos encontrados na literatura.

Além disso, tem sido relatado que as mudanças na superfície da célula podem ocorrer após a interação das bacteriocinas de classe IIa com receptores específicos da parede celular de algumas bactérias, sendo observado um possível aumento no nível de transcrição de determinados genes em algumas cepas de *L. monocytogenes* (GRAVESEN *et al.*, 2002b).

Entretanto, para a *L. seeligeri* foi observada uma redução significativa na expressão do gene *dlt*A, após incubação de 24 horas a 37°C, com o peptídeo P34 e nisina. A diminuição da expressão desse gene foi também observada após a incubação da placa por 240 horas a 4°C. O gene *lmo*1695 demonstrou uma diminuição significativa de sua expressão (2000 vezes) após a inoculação com o peptídeo P34 e incubação por 24 horas a 37°C. Para a nisina, foi observada uma diminuição não significativa da expressão deste gene.

Uma diminuição significativa na expressão do gene *mpt*A foi observada para *L. seeligeri* após a inoculação do peptídeo P34 e nisina e incubação da placa durante 24 horas a 37ºC. O gene foi (31.872 vezes) mais expresso após a inoculação com o peptídeo P34 em comparação com o controle. Também a expressão do gene foi significativamente maior após a inoculação com nisina. Curiosamente, a placa inoculada com o peptídeo P34 e

nisina e incubada por 240 horas a 4ºC não apresentou diminuição significativa da expressão deste gene.

Estudos anteriores utilizando cepas de *L. monocytogenes* e *Enterococcus faecalis* resistentes as bacteriocinas da classe IIa, tem reportado uma diminuição de mais de (1000 vezes) na expressão do gene *mptA* (RAMNATH *et al.*, 2000; DALET *et al.*, 2001; HECHARD *et al.* 2001 ; GRAVESSEN *et al.*, 2002).

Inúmeras linhagens celulares eucarióticas tem sido utilizadas com diferentes respostas para estudar a citotoxicidade de bactérias do gênero *Bacillus* (ANDERSSON *et al.*, 2004; SZABO *et al.*, 1991), entretanto nenhuma foi até agora considerada como padrão. As células Vero são comumente utilizadas por apresentarem a vantagem de pronta disponibilidade e de crescimento rápido. Eritrócitos humanos e espermatozóides são células mais sensíveis em comparação com as células Vero, no entanto, existem poucos estudos de citotoxicidade utilizando essas células (JÄÄSKELÄINEN *et al.*, 2003; BOUAZIZ *et al.*, 2006).

A citotoxicidade *in vitro* dos peptídeos antimicrobianos P34 e P40 foi investigada em diferentes células eucarióticas e comparada com a nisina. Em células Vero, utilizando os ensaios de MTT e NRU, observou-se uma maior sensibilidade das células após exposição a diferentes concentrações do peptídeo antimicrobiano P40, comparado com os valores encontrados para o peptídeo antimicrobiano P34 e nisina. Uma diminuição na viabilidade das células Vero também foi observada com o aumento das concentrações de

ambos os peptídeos e da bacteriocina nisina, resultando valores de EC_{50} comparáveis nos ensaio de MTT. Entretanto, a EC_{50} para o peptídeo antimicrobiano P40 foi 1,6 vezes menor que a da nisina nos ensaios NRU.

Estudos anteriores utilizando diferentes linhagens celulares demonstraram a baixa citotoxicidade da nisina em células eucarióticas. Foi reportado que a nisina não apresenta toxicidade para células Vero, nas concentrações entre 2,85 e 11,4 μg ml^{-1}, durante 48 horas, enquanto que nenhuma toxicidade importante em células epiteliais vaginais foi observada até a concentração de 318 μg ml^{-1} (MURINDA *et al.*, 2003). A nisina também apresentou baixa toxicidade no ensaio de MTT quando foram utilizadas células HT29 e Caco-2. Alguns parâmetros podem influenciar na ação das bacteriocinas em células eucarióticas, como o tempo de exposição, ensaio empregado, bem como o meio utilizado para aplicação dos peptídeos (MAHER & McCLEAN, 2006).

A citotoxicidade pode estar relacionada a composição da membrana plasmática e atividade metabólica dos diferentes tipos celulares. Por isso, um aumento da citotoxicidade dos peptídeos antimicrobianos e da nisina contra alguns tipos de células, poderia ser explicado pela diferença na hidrofobicidade da superfície celular, que influenciaria na ação efetiva da ligação nas células (PAPO e SHAI, 2005). No entanto, o mecanismo exato pelo qual a toxicidade difere entre os diferentes tipos de células não está ainda completamente elucidado.

Os ensaios de LDH indicaram uma perda da integridade da membrana plasmática em células Vero, revelando alguma citotoxicidade após a

exposição de 24 horas com diferentes concentrações dos peptídeos P34 e P40, sendo verificada uma liberação de LDH em mais de 50% das células quando a concentração utilizada foi de 1,25 μg ml^{-1} ou superior a este valor. O tratamento das células com Triton X-100 (controle positivo), resultou níveis elevados de LDH, indicando um dano celular extensivo. Este aumento do nível de LDH sugere que a membrana plasmática pode ser o principal alvo da maioria dos peptídeos antimicrobianos, incluindo os peptídeos P34 e P40, além da bacteriocina nisina (DEEGAN *et al.*, 2006).

Também se observou o efeito do peptídeo P34 sobre espermatozóides, este foi similar a nisina para a viabilidade e motilidade espermática, além da exocitose acrossomal duas vezes maior que o controle de células. Considerando-se que o modo de ação de muitos peptídeos antimicrobianos seria por sua ação na membrana celular, é possível que qualquer alteração na estabilidade da mesma possa promover o aumento da susceptibilidade à reação acrossômica. Recentemente, o uso de nisina como agente contraceptivo em ratas vem sendo proprosto (GUPTA *et al.*, 2009), e os estudos demonstram que a membrana das células espermáticas apresentam uma maior sensibilidade a ação da nisina que eritrócitos humanos ou células do epitélio vaginal (REDDY *et al.*, 2004b).

A imunogenicidade foi avaliada pela administração intra-peritoneal de 50 μg ml^{-1} do peptídeo P34 em camundongos. Não foram observadas mortes ou reações de hipersensibilidade nos animais durante os experimentos. Um aumento não significativo nos títulos de anticorpos dos camundongos do grupo 1 (tratados somente com o peptídeo P34) no decorrer dos 42 dias de

experimento, quando comparados ao dia zero pode ser verificado. Contrariamente, nos camundongos do grupo 2, administrados com uma mistura do peptídeo antimicrobiano P34 + adjuvante completo de Freund, verificou-se um aumento não significativo nos títulos de anticorpos no 21°dia, além de um aumento significativo no 42° dia em relação ao dia zero. Poucos estudos são disponíveis para comparação dos resultados, entretanto BHUNIA *et al.* (1990) avaliaram a imunogenicidade da pediocina PA-1 (ACH) em camundongos e reportaram que esta também não apresentou imunogenicidade aos animais. Entretanto, foi reportado que a administração de nisina (Nisaplin) na dieta dos animais pode induzir um aumento na contagem de linfócitos T CD4 e CD8 e também uma diminuição da contagem de linfócitos B (DE PABLO *et al.*, 1999).

Nos testes para determinar a toxicidade aguda e DL_{50} do peptídeo P34, não foram observadas mortes após a administração oral em nenhuma das concentrações testadas (82,5, 165, 247,5, 330 mg/kg). Assim, a DL50 foi estimada como superior a 332,3 ± 0,76 mg/kg. Estes testes são essenciais para avaliar novos peptídeos antimicrobianos com potencial de conservação de alimentos, e conforme a literatura, a concentração utilizada deve ser igual a pelo menos 100 vezes a exposição média estimada para humanos ou, pelo menos, 2000 mg/kg de peso corporal de acordo com diretrizes estabelecidas pela OCDE (1987). Apesar de vários peptídeos antimicrobianos já terem sido purificados e caracterizados, existem poucos relatos na literatura de toxicidade aguda para comparação, sendo encontrados apenas estudos com a nisina e pediocina PA-1 (CLAYPOOL *et al.*, 1966; CHANDRAPATI e O'SULLIVAN, 1998).

Nos testes para avaliar a toxicidade sub-crônica realizados durante 21 dias pela administração oral do peptídeo P34 e nisina na concentração de 0,825 mg/Kg dia, não foram observadas mortes dos animais tratados ou controles. Não ocorreram diferenças significativas no ganho de peso entre os grupos.

Um dia antes do começo dos experimentos foram coletas amostras de sangue dos camundongos com o objetivo de estudar alguns paramêtros bioquímicos, sendo uma nova coleta realizada decorridos os 21 dias da administração do peptídeo P34 e nisina. Foi verificado um aumento significativo nos níveis séricos de ALT (Aspartato aminotransferase) do grupo de animais tratados com nisina. Estes resultados indicam que peptídeos antimicrobianos podem ser continuamente ingeridos, embora o aumento do nível de ALT possa sugerir um efeito hepatotóxico da nisina.

Para confirmar estes resultados foram realizados estudos histopatológicos do estômago, intestino, fígado, baço, rins e pele dos camundongos. Não foram detectadas alterações histológicas no intestino e rins de nenhum grupo. Alterações histológicas foram observadas no estômago de todos os grupos que se encontravam com padrão aglandular.

Possíveis sinais de toxicidade foram observados nos animais tratados com 0,825 mg/kg/dia de nisina, apresentando alterações histológicas no baço, pele e fígado. A análise histológica do baço mostrou a presença abundante de megacariócitos. Lesões cutâneas foram observadas durante os experimentos, sendo potencializadas após os 21 dias de administração oral de nisina, que revelou alterações histológicas, como dermatite, necrose tecidual e

ulceração com presença de fibrina. No fígado do mesmo grupo, alterações histológicas tais como degeneração hepática também foi observada, o que justificaria o aumento da enzima ALT. No grupo de camundongos tratados com peptídeo P34, foram observadas apenas alterações histológicas no baço, com a presença de megacariócitos, evidenciando um possível processo inflamatório.

Como observado para os experimentos de toxicidade *in vitro*, existem poucos relatos do uso de modelos animais para avaliação dos efeitos de bacteriocinas em órgãos-alvo. Em um estudo recente realizado por GUPTA *et al*. (2008), a nisina foi testada em ratas grávidas, tanto os animais tratados, como seus descendentes não apresentaram sinais clínicos de toxicidade, quando comparados aos animais controle. DABOUR *et al*. (2009) avaliaram a toxicidade *in vivo* da pediocina PA1 (250 mg/dia por três dias consecutivos), em animais infectados artificialmente com *L. monocytogenes*, relatando o desaparecimento do patógeno em órgãos-alvo dos animais (baço e fígado) no prazo de seis dias.

A avaliação *in vitro* e *in vivo* da toxicidade de um peptídeo antimicrobiano é um passo essencial para sugerirmos sua utilização em alimentos. Assim, os resultados aqui apresentados estão de acordo com as investigações realizadas anteriormente, sobre a toxicidade *in vitro* do peptídeo antimicrobiano P34 em células eucarióticas, sendo estes resultados semelhantes aos reportados para a nisina. Devido a isso, acreditamos que o peptídeo P34 apresente grande potencial para aplicação em alimentos, sendo

necessários alguns estudos complementares para essa finalidade, como exemplo, um estudo da toxicidade crônica.

5 CONCLUSÕES

O peptídeo P34 em sinergismo com os sobrenadantes brutos das culturas das LAB obteve uma potencialização de sua atividade antimicrobiana, sendo verificada uma redução no crescimento de *L. monocytogenes*, de 3 ciclos logarítmicos após aplicação em queijo Minas Frescal.

Mesmo existindo a interação entre o peptídeo, bactéria e alimento, foi possível verificar influência do peptídeo P34 na parede celular de *L. monocytogenes* pelo aumento significativo na expressão dos genes *dltA, lmo 1695* and *mptA* após 96 horas de contato com este peptídeo.

Não foi demonstrada a influência significativa do peptídeo P34 na expressão de genes em *L. monocytogenes* após atividade em placa. Entretanto, em *L. seeligeri* o peptídeo P34 em determinadas condições possivelmente, influenciou a expressão dos genes (*dlt*A, *lmo*1685 e *mpt*A).

Assim, fica evidente que o dano a superfície da célula devido a ação do peptídeo P34, possa induzir um mecanismo que afeta a expressão de genes responsáveis pela constituição estrutural da parede celular em *Listeria sp.*

A citotoxicidade do peptídeo P34 e P40 foram avaliadas em células VERO tratadas com diferentes concentrações (0,02 - 2,5 μg ml^{-1}) dos mesmos. Nos ensaios de MTT, NRU e LDH os valores de EC_{50} para o peptídeo P34 foram de 0.60, 1.25, 0.65 μg ml^{-1} e do peptídeo P40 foram de 0,30, 0,51 e 0,57 μg ml^{-1}, respectivamente.

A atividade hemolítica em eritrócitos humanos foi de (5,8%) e (19%), respectivamente.

Foi verificada uma pequena diminuição da viabilidade e motilidade, tanto para P34 e nisina. O percentual de reação acrossomal provocada pelo peptídeo P34 aumentou cerca 2 vezes, quando comparado com o controle.

Após avaliação da imunogenicidade e toxicidade aguda (DL_{50} 332,3 ± 0,76 mg/kg) não foram observadas em um primeiro momento, sinais de hipersensibilidade ou toxicidade nos camundongos controles e tratados com o peptídeo P34 e nisina .

Para a toxicidade subcrônica foram verificadas sinais de toxicidade apenas para os animais tratados com 0,825 mg/Kg/dia de nisina durante os 21 dias, que apresentaram alterações em parâmetros bioquímicos hepáticos, além de histológicos como baço, fígado e pele.

6 PERSPECTIVAS

A partir dos resultados deste trabalho, e com a finalidade de explorar o potencial biotecnológico e de aplicabilidade do peptídeo P34 em alimentos é possível citar como perspectivas:

- Complementar a caracterização molecular, com o sequenciamento de aminoácidos;

- Otimizar a purificação dos peptídeos P34 e P40, pela utilização de metodologia molecular, viabilizando talvez sua produção em maior quantidade.

- Realizar estudos de toxicidade aguda, subcrônica e crônica com o peptídeo P40.

- Verificar a toxicidade crônica do peptídeo P34.

- Aplicar o peptídeo antimicrobiano P34 em nanovesículas e estudar sua possível citotoxicidade *in vitro* e toxicidade *in vivo*.

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ANEXO 1

Evaluation of the in vitro cytotoxicity of the antimicrobial peptide P34.

- **Cell Biology International** (2010) in press.

Cell Biol. Int. (2010) 000, 000–000 (Printed in Great Britain)

Cell Biology International

Research Article

Evaluation of the *in vitro* cytotoxicity of the antimicrobial peptide P34

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Abstract

The *in vitro* cytotoxicity of the antimicrobial peptide P34 was evaluated in different eukaryotic cells. The food-grade bacteriocin nisin was also analysed for comparison. Vero cells were treated with different concentrations (0.02–2.5 $\mu g \cdot ml^{-1}$) of antimicrobial peptide P34 and nisin. Cell viability and plasma membrane integrity were checked by MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide], NRU (Neutral Red dye uptake) and LDH (lactate dehydrogenase) assays. The EC_{50} values of the peptide P34 in MTT and NRU assays were 0.60 and 1.25 $\mu g \cdot ml^{-1}$ respectively, while values found of nisin were 0.50 and 1.04 $\mu g \cdot ml^{-1}$. In the LDH assay, the EC_{50} values were 0.65 and 0.62 $\mu g \cdot ml^{-1}$ to P34 and nisin, respectively. The peptide P34 revealed similar haemolytic activity on human erythrocytes (5.8%) when compared with nisin (4.9%). The effects on viability, motility and acrosomal exocytosis of human sperm were also evaluated. Nisin and P34 showed similar effects on sperm parameters. The evaluation of cytotoxicity of antimicrobial peptides is a critical step to guarantee their safe use.

Keywords: antimicrobial peptide; *Bacillus*; bacteriocin; eukaryotic cell; MTT assay

1. Introduction

Bacteriocins are antimicrobial compounds that inhibit or inactivate microorganisms that are usually, but not always, closely related to the producer strain (Cleveland et al., 2001). In recent decades, a great interest is observed in the bacteriocins as natural preservatives, and they have been extensively studied, especially those produced by LAB (lactic acid bacteria) because of their potential applications as food additive in dairy and meat systems (Sobrino-Lopez and Martin-Belloso, 2008). Antimicrobial peptides have received increasing attention because of their efficiency for the biological control of spoilage and pathogenic organisms, and they present potential applications in food (O'Sullivan et al., 2002; Deegan et al., 2006) and clinical (Fontana et al., 2006; Oh et al., 2006) aspects. Nisin is the first antimicrobial peptide with a GRAS ('generally recognized as safe') status in the United States for use in processed cheese (Cleveland et al., 2001). Nisin is currently used in various food products in several countries (O'Sullivan et al., 2002; Deegan et al., 2006).

The genus *Bacillus* includes a number of bacteriocinogenic species including *Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus coagulans*, among others (Cherif et al., 2001; Bizani and Brandelli, 2002). *Bacillus* species produce a large number of antimicrobial peptides representing several different basic chemical structures (von Döhren, 1995). Bacteriocins or BLS (bacteriocin-like substances) from the genus *Bacillus* have been characterized, such as thuricin 7 (Cherif et al., 2001), thuricin 439A and thuricin 439B (Ahern et al., 2003), cerein 7A and 7B (Oscáriz et al., 2007), cerein 8A (Bizani et al., 2005) and subtilosin A (Kawulka et al., 2004). Antimicrobial activity was reported among several *Bacillus* isolated from aquatic environments of Brazilian Amazon basin (Motta et al., 2004). A novel *Bacillus* sp. strain P34 was identified, and its antimicrobial activity was characterized as a BLS. This antimicrobial peptide showed a broad inhibitory spectrum including *Listeria* spp., *B. cereus*, *Aeromonas hydrophila*, *Pasteurella haemolytica*, among others (Motta et al., 2007a). Many strains of *Bacillus* have been safely used in food and industry (Pedersen et al., 2002); however, there is limited investigation on the cytotoxicity of specific antimicrobial peptides from *Bacillus*.

In vitro cytotoxicity assays are useful to define basal cytotoxicity, for example the intrinsic ability of a compound to cause cell death as a consequence of damage to several cellular functions (Bouaziz et al., 2006). Cytotoxicity assays are also necessary to define the concentration range for further and more detailed *in vitro* testing to provide meaningful information on parameters such as genotoxicity or programmed cell death (Eisenbrand et al., 2002). A number of assays have been proposed to investigate the cytotoxicity of bioactive peptides of *Bacillus* sp., based on the determination of the haemolytic potential (haemolysis), injury to sperm cells, inhibition of cellular proliferation and damage to energy metabolism (Mikkola et al., 2000; Shin et al., 2001; Andersson et al., 2004; Maher and McClean, 2006). The aim of this work was to investigate the *in vitro* cytotoxicity of the peptide P34, in comparison with nisin through the use of different cytotoxicity assays and eukaryotic cells.

2. Materials and methods

2.1. Antimicrobial peptides

The antimicrobial peptide P34 was purified from *Bacillus* sp. strain P34, as described previously (Motta et al., 2007b). Previously,

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Abbreviations: AR, acrosome reaction; BLS, bacteriocin-like substances; E-MEM, Eagle's minimal essential medium; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; NRU, Neutral Red dye uptake.

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ANEXO 2

Investigation of the Cytotoxicity of Antimicrobial Peptide P40 on Eukaryotic Cells.

- **Current Microbiology** (2010), v.60, p.1 - 5.

Curr Microbiol (2010) 60:1–5
DOI 10.1007/s00284-009-9490-z

Investigation of the Cytotoxicity of Antimicrobial Peptide P40 on Eukaryotic Cells

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Received: 27 June 2009 / Accepted: 17 August 2009 / Published online: 29 August 2009
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Abstract The in vitro cytotoxicity of the antimicrobial peptide P40 was investigated. The food grade bacteriocin nisin was also analyzed for comparison. VERO cells were treated with different concentrations (0.02–2.5 μg ml^{-1}) of nisin and P40, and cell viability and plasma membrane integrity were checked by MTT, neutral red uptake (NRU), and lactate dehydrogenase (LDH) assays. In MTT and NRU assays the EC_{50} to the purified peptide P40 were 0.30 and 0.51 μg ml^{-1}, while values found to nisin were 0.35 and 0.79 μg ml^{-1}, respectively. In the LDH assay, the EC_{50} was 0.57 and 0.62 μg ml^{-1} for P40 and nisin, respectively. The peptide P40 revealed higher hemolytical activity (19%) when compared to nisin (4.9%) at the highest concentration tested (2.5 μg ml^{-1}). Relatively few studies about the cytotoxicity of antimicrobial peptides are available. The determination of the cytotoxicity of antimicrobial peptides is an essential step to warrant their safe use.

Introduction

Antimicrobial peptides are widespread produced among plants, animals, and microorganisms. These peptides have been classified according their chemical and structural characteristics, based on their variable molecular mass and net charge [21]. An increased interest to some antimicrobial peptides has been observed because of their potential applications as food biopreservatives [8] and as therapeutic agents against pathogenic bacteria [18].

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Bacillus spp. produces a large number of antimicrobial peptides representing several different chemical structures [21], including bacteriocins and bacteriocin-like (BLS) [2]. A novel *Bacillus licheniformis* strain P40 was identified and its antimicrobial activity was characterized as a BLS [5]. This peptide also showed inhibitory activity *in planta* against *Erwinia carotovora*, a phytopathogenic bacterium that causes important agricultural losses [6]. Although many strains of *Bacillus* have been safely used in food and industry, there are relatively few specific studies on the cytotoxicity of antimicrobial peptides from *Bacillus* [14].

In vitro cytotoxicity assays are useful to define basal cytotoxicity, indicating the intrinsic ability of a compound to cause cell death as a consequence of damage to several cellular functions [4, 22]. A number of assays and various cell types have been used with different responses to study *Bacillus* cytotoxicity and none can be considered as standard [12, 14, 19]. Vero cells have been used with some frequency with the advantage of easy availability and fast growth [4]. The aim of this study was to investigate the in vitro cytotoxicity of the antimicrobial peptide P40 on eukaryotic cells, in comparison with nisin through the use of different assays.

Materials and Methods

Antimicrobial Peptides

The antimicrobial peptide P40 was purified by ammonium sulfate and liquid chromatography on Sephadex G-100 from culture supernatant of *Bacillus licheniformis* strain P40, as described elsewhere [6]. The purification factor was 18-fold and the concentration was 800 U ml^{-1}. Nisin (Nisaplin®; Danisco, Copenhagen, Denmark) was suspended