UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

CARACTERIZAÇÃO DE PEPTÍDEOS ANTIMICROBIANOS DE *Bacillus amyloliquefaciens* COM ATIVIDADE ANTIBACTERIANA, ANTIFÚNGICA E AMEBICIDA

LISIANNE BRITTES BENITEZ

PORTO ALEGRE, RS, Brasil

Junho, 2010

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LISIANNE BRITTES BENITEZ

Farmacêutica Bioquímica/UFSM

Mestre em Ciência e Tecnologia dos Alimentos/UFSM

Orientador: Prof. Dr. Adriano Brandelli

PORTO ALEGRE, RS, Brasil

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Autor: Lisianne Brittes Benitez

Orientador: Prof. Dr. Adriano Brandelli

[1]RESUMO

Bacteriocinas são peptídeos pequenos sintetizados por bactérias que possuem propriedades antimicrobianas. O presente estudo teve por objetivos identificar e caracterizar uma bacteriocina produzida por *Bacillus amyloliquefaciens* LBM 5006, isolado de solo da Mata Atlântica, Santa Catarina, Brasil. A produção da substância antimicrobiana tem início na fase exponencial de crescimento e sua atividade máxima ocorre na fase estacionária. O efeito do cocultivo de *B. amyloliquefaciens* com diferentes bactérias na produção do antimicrobiano foi investigado. O cultivo de células de *Escherichia coli* intactas ou inativadas pelo calor com a cepa produtora aumentou a síntese da substância antimicrobiana. A bacteriocina LBM 5006 apresentou amplo espectro de ação, produzindo atividade antagonista contra bactérias, fungos e amebas patogênicas. O modo de ação contra células de *Listeria monocytogenes* e *Paenibacillus larvae* foi bactericida e bacteriolítico. Atividade esporicida foi observada contra esporos de *P. larvae* após tratamento com 1600 UA mL^{-1}. Efeitos amebistático e amebicida contra trofozoítos de *Acanthamoeba polyphaga* com a consequente lise celular foram observados. A bacteriocina não teve efeito inibitório em células Vero nas concentrações que foram efetivas contra as amebas. A substância antimicrobiana foi isolada por precipitação com sulfato de amônio, cromatografia de gel filtração e extração com 1-butanol. O espectro ultravioleta foi típico de um polipeptídeo e o infravermelho indicou a presença de ligações peptídicas e grupamentos acil na sua estrutura. Análises por espectroscopia de massas indicaram que *B. amyloquefaciens* LBM 5006 produziu dois peptídeos antimicrobianos, com 1058 Da e 1464 Da, correspondentes a peptídeos tipo-iturina e tipo-fengicina, respectivamente.

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[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. (169 p.) Maio, 2010.

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Author: Lisianne Brittes Benitez

Advisor: Prof. Dr. Adriano Brandelli

[2]ABSTRACT

Bacteriocins are small peptides synthesized by bacteria which have antimicrobiall properties. This study aimed to identify and characterize a bacteriocin produced by *Bacillus amyloliquefaciens* LBM 5006, isolated from soil of Mata Atlântica, Santa Catarina, Brazil. It has been observed that the antimicrobial substance production starting at the exponencial grown phase and maximum activity occur at stationary phase. The effect of different bacteria on the production of antimicrobial activity by *Bacillus amyloliquefaciens* LBM 5006 was investigate. It was concluded that the presence of intact or thermally inactivated cells of *Escherichia coli* enhanced the synthesis of antimicrobial peptides by *B. amyloliquefaciens* strain. Bacteriocin LBM 5006 showed broad spectrum of action, producing antagonistic activity against bacteria, fungi and pathogenic amoebas. The mode of action against *Listeria monocytogenes* and *Paenibacillus larvae* was bactericidal and bacteriolytic. Sporicidal activity was observed against *P. larvae* spores after treatment whit 1600 AU mL^{-1}. Amoebicidal and amoebistatic effects were detected against throphozoites of *Acanthamoeba poliphaga* and cell lysis. The bacteriocin had no inhibitory effect on Vero cells at concentrations that were effective against amoebas.The antimicrobial substance was isolated by ammonium sulfate precipitation, gel filtration chromatography and 1-butanol extraction.The ultraviolet spectrum was typical of a polypeptide and the infrared spectrum indicates the presence of peptide bonds and acyl group(s) in its structure. Mass spectroscopy analysis indicated that *B. amyloliquefaciens* LBM 5006 produces two antimicrobial peptides, with main peaks at m/z 1058 Da and 1464 Da, corresponding to iturin-like and fengycin-like peptides, respectively.

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LISTA DE ABREVIATURAS E SÍMBOLOS

%: percentual
®: marca registrada
±: mais ou menos
µL: microlitro
µm: micrômetro
ºC: graus Celsius
AMPs: *Antimicrobial Peptides*
ATCC: *American Type Culture Collection*
BAL: Bactérias Ácido Lácticas
BHI: *Brain Heart Infusion*
BHA: *Brain Heart Agar*
BLS: Substância antimicrobiana tipo-bacteriocina
DNA: Ácido desoxirribonucléico
RNA: ácido ribonucléico
RNAr: ácido ribonucléico ribossômico
RNAt: ácido ribonucléico transportador
DTAs= doenças transmitidas por alimentos
FDA: *Food and Drug Administration*
g: unidade de medida da força centrífuga relativa
GRAS: *Generally Recognized as Safe*
ICTA: Instituto de Ciência e Tecnologia de Alimentos
KDa; kiloDalton
mA: miliamperagem
min: minuto
M= molar
MEV= microscopia eletrônica de varredura
mg/L= miligrama por litro
mL= mililitro
nm= nanômetro
PCR: *Polimerase Chain Reaction*
PBS= *Phosphate Buffered Saline*
rpm: rotações por minuto
SDS-PAGE: *Sodium Dodecyl Sulfate Polyacrilamide Gel Electrophoresis*
sp.= espécie
TSA: *Tryptone Soy Agar*
TSB: *Tryptone Soy Broth*
UA (AU): Unidades Arbitrárias
UFC=UnidadesFormadorasdeColônias

1 INTRODUÇÃO

A descoberta e o desenvolvimento dos antibióticos convencionais levaram a grandes avanços no combate às infecções microbianas. Apesar do uso de antibióticos ainda ser a primeira escolha no tratamento, a prevalência de resistência bacteriana é uma preocupação crescente em saúde pública. O uso indiscriminado dos antibióticos na quimioterapia levou ao desenvolvimento de mecanismos de resistência pelos microrganismos como estratégias de adaptação frente ao surgimento de condições adversas.

O fenômeno do antagonismo microbiano mediado por peptídeos impulsionou a pesquisa por novos antimicrobianos devido a sua capacidade em oferecer defesa microbiana eficiente e menor propensão para o desenvolvimento de resistência. Peptídeos antimicrobianos são antibióticos naturais codificados por genes presentes em todos os organismos. As bacteriocinas destacam-se entre a grande variedade de peptídeos antimicrobianos sintetizados por várias linhagens de bactérias Gram-positivas e Gram-negativas, cuja função primária é impedir o crescimento de espécies competidoras.

Estes compostos possuem características peculiares de estabilidade ao

calor, às variações de pH e de temperatura, sendo portanto bastante promissores para uso profilático e terapêutico em medicina humana e animal, como conservantes em sistemas alimentares e no controle de fitopatógenos.

A exigência dos consumidores por alimentos seguros, que além de apresentarem as propriedades nutricionais esperadas não causem danos à saúde, levou a indústria de alimentos a buscar alternativas que aumentassem a vida de prateleira dos produtos, como é a sua obtenção de forma minimamente processada e o uso de ingredientes e aditivos que evitam ou retardam a ação de microrganismos indesejáveis sobre os alimentos. Estas exigências, entretanto, têm implicação microbiológica porque a maioria das mudanças necessárias para obter-se um produto de composição, sabor e aspecto mais natural, como por exemplo, um menor uso de conservantes artificiais, acarretam também uma diminuição na conservação do alimento e consequentemente perdas na sua segurança.

Visando atender essa necessidade social e mercadológica pesquisadores ligados à ciência dos alimentos têm investigado as bacteriocinas como bioconservadores de alimentos, especialmente aquelas produzidas por bactérias lácticas devido a sua longa história de utilização como probióticos no processamento e conservação de alimentos.

Relativamente à agricultura sustentável, métodos de controle têm sido reestruturados e revistos nas últimas décadas. A preocupação com o uso de métodos não-nocivos ao ambiente tem enfatizado os métodos de proteção baseados em agentes de origem biológica. Assim, os agentes biológicos com propriedades antagonistas têm recebido considerável atenção no controle de

doenças de plantas, como alternativa aos pesticidas químicos.

O conhecimento do modo de atuação das bacteriocinas sobre os microrganismos-alvo permitirá o desenvolvimento de antimicrobianos mais eficazes, de amplo espectro e aplicabilidade diversa. Diferentes estudos sugerem que os peptídeos antimicrobianos teriam um papel benéfico na saúde humana e que poderiam ser utilizados no futuro como potenciais moduladores da resposta imune exercendo um efeito significativo na prevenção e tratamento das enfermidades.

Dentro deste contexto este trabalho teve por objetivo a produção, purificação e caracterização de peptídeos antimicrobianos produzidos por uma linhagem de *Bacillus amyloliquefaciens* LBM 5006 isolada de solo da Mata Atlântica.

Os objetivos específicos do estudo foram:

1. Avaliar o espectro de ação da substância antimicrobiana frente a microrganismos patogênicos e deteriorantes;

2. Purificar e caracterizar quimicamente a substância antimicrobiana;

3. Determinar o modo de ação do antimicrobiano;

4. Pesquisar os efeitos de cultivos mistos na síntese da substância antimicrobiana

2 REVISÃO BIBLIOGRÁFICA

2.1 Bacteriocinas

As bacteriocinas são definidas como proteínas ativas biologicamente contra membros da mesma espécie ou espécies muito relacionadas à linhagem produtora, porém este conceito vem sendo modificado uma vez que se tem encontrado ações bactericidas também contra cepas filogeneticamente distanciadas da cepa produtora. As bacteriocinas poderiam ser consideradas como produtos primários ou modificados extracelulares, liberados a partir da síntese proteica nos ribossomos bacterianos. A bactéria produtora não é afetada pela ação da bacteriocina por possuir um mecanismo de imunidade específica (Klaenhamer, 1988; Caplice & Fitzgerald, 1999; Sablon et al., 2000; Cotter et al., 2005).

A maioria das bacteriocinas são moléculas pequenas, com propriedades hidrofílicas e hidrofóbicas e um alto ponto isoelétrico. São citadas como características mais freqüentes das bacteriocinas a sua baixa massa molecular, a relativa estabilidade ao calor, o amplo espectro de atividade frente a bactérias Gram-positivas, sob diferentes valores de pH, e o fato de não

serem tóxicas nem antigênicas para animais por serem degradadas pelas enzimas proteolíticas do trato gastrintestinal. Estas características, essencialmente, as diferenciam de alguns antibióticos, considerados metabólitos secundários e que inibem o crescimento de bactérias quando presentes em baixas concentrações. O espectro de ação mais estreito e a síntese em ribossomos são as duas características principais que distinguem a maioria das bacteriocinas dos antibióticos clássicos (Reeves, 1965; Biswas, 1991; Giraffa, 1995; Montville & Winkoswski, 1997; Riley & Wertz, 2002b).

2.2 Classificação

Klaenhammer (1993) propôs a divisão das bacteriocinas em quatro classes: a) Classe I: caracterizada por conter os aminoácidos não-usuais dehidrobutirina (Dhb), Dehidroalanina (Dha), lantionina e beta-metilantionina. Estes resíduos incomuns são formados durante modificações pós-traducionais nas quais o aminoácido dehidro reage com a cisteína para formar anéis de tioéter de lantionina. Por este motivo, bacteriocinas que contêm estes anéis são denominadas de “lantibióticos” e são peptídeos pequenos (18 a 39 resíduos de aminoácidos) que possuem um peso molecular menor que 5 kDa. A nisina, primeira bacteriocina caracterizada e liberada para uso comercial pelo FDA (*Food and Drug Administration*), pertence a esta classe e é produzida em duas formas afins: a Nisina-A que contém uma histidina na posição 27 e a Nisina-Z que possui uma asparagina; b) Classe II: bacteriocinas formadas por moléculas pequenas de peptídeos termoestáveis. São não-lantibióticos, apresentam uma massa molecular de até 10 kDa e não estão sujeitas a extensivas modificações pós-traducionais. Este grupo subdivide-se em três outras subclasses:

subclasse IIa: constituída pelas bacteriocinas pediocina PA-1, sacacina A e P, leucocina A, bavaricina MN e curvacina A, as quais têm forte atividade contra a bactéria *Listeria monocytogenes*; subclasse IIb: contém as bacteriocinas lactococinas G, M e lactacina F, as quais necessitam de dois peptídios diferentes para sua atividade; subclasse IIc: formada pela lactacina B que requer baixas concentrações de cisteína para sua atividade; c) Classe III: inclui proteínas grandes e termolábeis de alto peso molecular (>30 kDa), como por exemplo as helveticinas, produzidas pelos *Lactobacillus helveticus*, as lactacinas A e B e as enterolisinas produzidas pelo *Enterococcus faecium*; d) Classe IV: compreendida por bacteriocinas complexas unidas a grupamentos lipídicos ou a carboidratos como são a leuconocina S, a lactocina -27 e a pediocina SJ-I. As funções das porções não-proteicas ainda não são conhecidas. Entretanto, Cleveland et al. (2001) acreditam que estes complexos são artefatos da purificação parcial do peptídeo e não uma nova classe de bacteriocinas (De Vuyst & Vandamme, 1994; Montville & Winkoswski, 1997; Mcauliffe et al., 2001; Roos et al., 2002; Cotter et al., 2005; Willey & Van der Donk, 2007).

No esquema de classificação proposto por Cotter, Hill e Ross (2005) as bacteriocinas são divididas em duas categorias distintas: as bacteriocinas contendo lantionina, os lantibióticos (classe I) e as que não contêm lantionina (classe II). As bacteriocinas da inicialmente denominada classe III são reclassificadas como “bacteriolisinas” e são proteínas antimicrobianas grandes, termolábeis e que possuem uma estrutura tipo-domínio, na qual os diferentes domínios são responsáveis pela translocação, ligação ao receptor e atividade

letal. As bacteriocinas circulares ficariam na classe II. A classe IV foi excluída da proposta por representar bacteriocinas que requerem grupamentos não-proteicos para sua atividade que ainda não foram convincentemente demonstrados. A principal mudança seria portanto a substituição da classe III por um grupo de “bacteriolisinas” tornando assim o grupo das bacteriocinas menor e melhor definido (Tabela 1).

Tabela 1: Classificação de bacteriocinas sugerida por Cotter et al. (2005)

Classificação	Grupos	Exemplos
Classe I Bacteriocinas/lantibióticos contendo lantionina	Lantibióticos simples e duplo-peptídeo	Peptídeo simples: nisina Peptídeo duplo: lacticina
Classe II Bacteriocinas contendo não-lantibióticos	Classe heterogênea de peptídeos pequenos, incluindo subclasses IIa (tipo-pediocina), IIb(duplo-peptídeo), IIc (cíclicas),IId(peptídeo linear não-pediocina)	IIa: Pediocina, Leucocina A; IIb: lactacina F; IIc: enterocina AS48, reuterina 6; IId: lactococcina A, divergicina A
Bacteriolisinas Proteínas líticas não-bacteriocinas	Proteínas grandes e sensíveis ao calor	Lisostafin, enterolisina A

Fonte: Cotter et al., 2005

Para Drider et al. (2006) as bacteriocinas seriam classificadas em três classes de acordo com suas características genéticas e bioquímicas: classe I ou lantibióticos, com peptídeos que contêm lantionina ou β-lantionina; classe II: formada por peptídeos pequenos e termoestáveis e classe III: grandes peptídeos termolábeis (>30kDa).

Devido à falta de consenso na diferenciação entre os subgrupos dos

não-lantibióticos um novo esquema de classificação foi proposto por Nes et al. (2007) o qual subdivide a classe II em quatro subclasses: classe IIa (bacteriocinas tipo-pediocina antilisteriais), IIb (bacteriocinas duplo-peptídeo), IIc (bacteriocinas que contêm um peptídeo-sinal típico (*leaderless peptide bacteriocin*) e IId (bacteriocinas circulares).

Entretanto, pesquisas recentes recomendam a criação de uma nova classe IV para incluir as bacteriocinas circulares, modificadas pós-traducionalmente (Maqueda et al., 2008).

2.3 Diversidade de bacteriocinas

Conforme Klaenhamer (1988) 99% das bactérias podem produzir pelo menos uma bacteriocina e a única razão pela qual estas ainda não foram isoladas é o fato de que são pouco investigadas. A família das bacteriocinas inclui uma diversidade de proteínas em termos de tamanho, alvo microbiano, liberação, modos de ação e mecanismos de imunidade e podem ser divididas em dois grupos principais: as produzidas por bactérias Gram-positivas e as sintetizadas por Gram-negativas (Riley & Wertz, 2002a; Riley & Wertz, 2002b).

2.3.1 Bacteriocinas de bactérias Gram-Negativas

Diferentes tipos de bacteriocinas, frequentemente maiores que 20 kDa, têm sido descritas como sendo produzidas por bactérias Gram-negativas. Desde sua descoberta, as colicinas, sintetizadas por cepas de *Escherichia coli*, tem sido as bacteriocinas de Gram-negativas mais estudadas e servem como sistemas modelo para a investigação da estrutura, função, organização genética, ecologia e evolução das bacteriocinas. A produção de colicina é

mediada pela resposta do sistema SOS e, por isto, é produzida principalmente em condições de estresse. A produção da toxina é letal para a célula produtora e para quaisquer células vizinhas reconhecidas pela colicina. Um domínio receptor na proteína colicina que une um receptor da superfície de uma célula específica determina o alvo de reconhecimento. A forma de ação das bacteriocinas varia desde a formação de poros na membrana celular até atividade da enzima nuclease contra alvos como DNA, RNAr e RNAt (Smajs et al., 1997; Riley & Wertz, 2002b; Cascales et al., 2007).

2.3.2 Bacteriocinas de bactérias Gram-Positivas

As bacteriocinas de bactérias Gram-positivas são mais abundantes e diversificadas que aquelas encontradas em bactérias Gram-negativas, além disso usualmente são menores que 8 kDa e devido as suas propriedades anfipáticas são frequentemente peptídeos permeabilizadores de membrana. Diferem das bacteriocinas de bactérias Gram-negativas em dois aspectos fundamentais: primeiro as bacteriocinas produzidas por bactérias Gram-positivas não são necessariamente letais à célula produtora devido a mecanismos de transporte específico para a liberação da bacteriocina, além disso algumas possuem um sistema específico para o transporte da bacteriocina. A síntese do antimicrobiano é autorregulada com um mecanismo de transporte que facilita sua liberação, embora algumas bactérias usem uma rota de exportação *sec*-dependente. Segundo, as bactérias Gram-positivas desenvolveram uma forma de regulação específica para as bacteriocinas, enquanto as Gram-negativas dependem de mecanismos regulatórios do hospedeiro (Tagg et al., 1976; Jack et al. 1995; Riley & Wertz, 2002b; Nes et

al., 2007).

Geralmente a produção de bacteriocinas por bactérias Gram-positivas está associada à passagem da fase *log* para a fase estacionária. A produção do antimicrobiano se inicia no meio da fase logarítimica e chega ao máximo quando as células entram na fase estacionária, como ocorre com a nisina. A regulação da expressão da bacteriocina depende da densidade do cultivo e não do ciclo celular (Breukink & De Kruijff, 1999).

As bacteriocinas de Gram-positivas, especialmente os lantibióticos, requerem normalmente uma quantidade maior de genes para sua produção do que bacteriocinas de bactérias Gram-negativas. Convencionalmente sabe-se que o espectro de ação antimicrobiana das bacteriocinas de Gram-positivas se restringe a outras bactérias Gram-positivas, porém esta extensão pode variar significativamente, de relativamente estreito, como é o caso das lactococcinas A, B e M as quais agem apenas contra *Lactococcus*, até extraordinariamente amplo como ocorre com alguns lantibióticos tipo A como a nisina e a mutacina B-Ny266 que combatem vários microrganismos incluindo *Actinomyces*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Gardnerella*, *Lactococcus*, *Listeria*, *Micrococcus*, *Mycobacterium*, *Propionibacterium*, *Streptococcus*, *Staphylococcus* e ainda bactérias Gram-negativas de interesse médico como *Campylobacter*, *Haemophilus*, *Helicobacter* e *Neisseria* (Morency et al., 2001; Riley & Wertz, 2002a; Mota-Meira et al., 2005; Nagao et. al., 2006; Martínez-Cuesta et al., 2006; Nes et al., 2007).

2.4 Biossíntese e regulação metabólica

Os genes para a produção de uma bacteriocina ativa estão geralmente organizados em operons. Estes podem estar localizados no cromossomo ou em um plasmídeo ou ainda em transposons. De uma maneira geral, os microrganismos possuem genes codificando o peptídeo estrutural, proteínas que auxiliam no processamento para a forma ativa, proteínas que auxiliam o transporte da bacteriocina através da membrana, proteínas regulatórias e proteínas que conferem imunidade ao organismo produtor. Genes que codificam proteínas de imunidade geralmente estão situados dentro ou muito próximos do operon que contém os genes requeridos para a biossíntese da bacteriocina. Produtos com funções relacionadas com a imunidade têm sido identificados como peptídeos pequenos, fortemente carregados e associados à membrana plasmática. O mecanismo de imunidade das bactérias produtoras de bacteriocinas tem a capacidade de fazer a distinção entre a bacteriocina produzida pela própria cultura e aquelas sintetizadas por outras bactérias (Cleveland et al., 2001; Hoffmann et al., 2004; Drider et al., 2006; Maqueda et al., 2008).

As bacteriocinas são geralmente sintetizadas como um pré-peptídeo inativo que inclui uma sequência líder N-terminal a qual mantém a bacteriocina na forma inativa dentro da célula produtora facilitando a interação com o transportador. Usualmente esta sequência é clivada durante a exportação da bacteriocina por um sistema dedicado de transporte ou, menos frequentemente através de uma rota de secreção geral da célula (*Sec-dependente*). A exportação da bacteriocina é usualmente mediada por um transportador ABC

que pode conter um domínio proteolítico N-terminal pertencente à família das cisteína proteases e que é responsável pela clivagem do peptídeo líder (Figura 1) (Havarstein et al., 1995; Venema et al., 1995; Diep & Nes, 2002; Cotter et al., 2005).

Relativamente à biossíntese dos lantibióticos e sua regulação, estudos recentes mostram que ocorre de uma maneira dependente da fase de crescimento ou da densidade celular (*growth-phase-dependent*) e está regulada por um sistema duplo-componente formado por um sensor histidina quinase e um regulador de resposta. Na biossíntese da nisina este sistema costuma ser denominado *quorum sensing* e inclui a própria nisina como um peptídeo feromônio (auto-indutor) e uma transdução de sinal pelo sistema regulatório duplo-componente correspondente NisRK (Klein et al., 1993; Parkinson, 1993; Ross et al., 1993; Engelke et al., 1994; Kuipers et al., 1995; Ra et al., 1996; McLaughlin, et al., 1999; Altena et al, 2000; McAuliffe et al., 2001).

A indução da expressão do gene é ativada por um acúmulo do peptídeo feromônio quando há uma baixa produção constitutiva. Quando um limiar de concentração do feromônio é atingido os peptídeos se ligam aos seus receptores (histidina proteína quinase), seguido por uma cascata de fosforilação que leva à fosforilação do regulador de resposta o qual se liga e ativa os promotores regulados levando a expressão dos genes com a consequente produção da bacteriocina (Nes et al., 1999).

A alta incidência da produção de bacteriocinas entre os diferentes grupos bacterianos reflete a sua importância na ecologia microbiana. Na

maioria dos casos, a produção de bactericinas parece ser regulada, consequentemente é produzida apenas sob condições adequadas para o crescimento do microrganismo, sendo crucial a escolha correta das condições de cultivo tais como a composição do meio, a temperatura, o pH e a atividade de água entre outros (Nes et al., 2007).

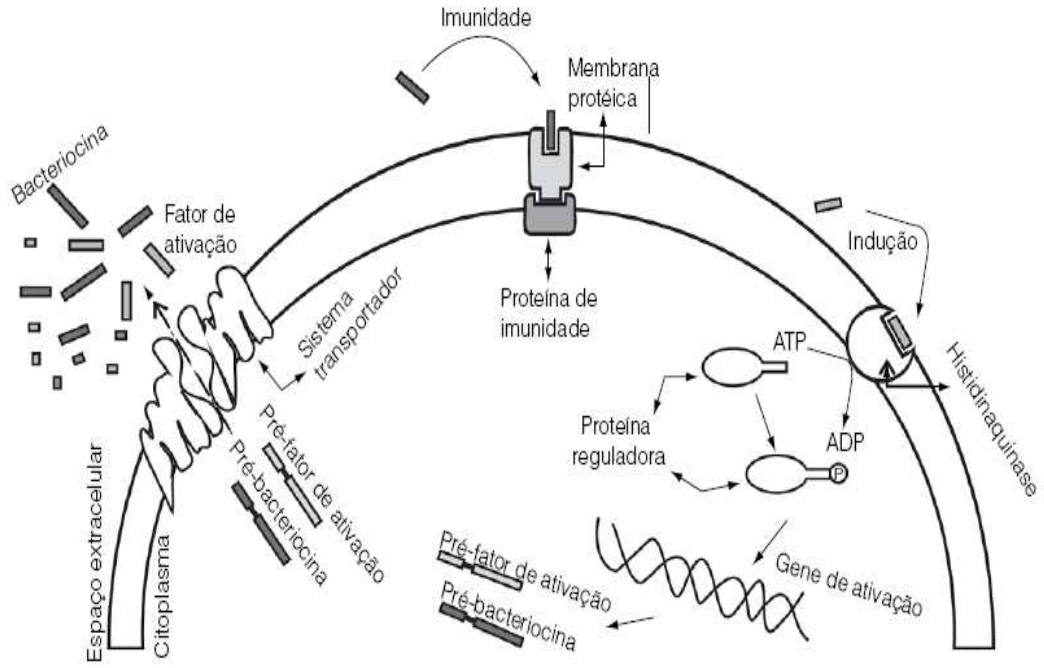

Figura 1: Mecanismo de regulação da biossíntese das bacteriocinas (Fonte: Nascimento et al., 2008).

2.5 Mecanismo de ação das bacteriocinas

Numerosos estudos têm sido realizados na tentativa de elucidar o complexo mecanismo de ação de diferentes bacteriocinas. Há um consenso de que a maioria das bacteriocinas atuam nas membranas celulares provocando sua permeabilização e eventualmente causando a morte da célula-alvo e que o arranjo estrutural anfipático destes peptídeos desempenha um papel

importante nesse mecanismo. A maioria das bacteriocinas são bactericidas com algumas exceções como a leucocina A UAL 187 que é bacteriostática. Para que o efeito bactericida ocorra, o microrganismo indicador deverá apresentar sensibilidade ao antimicrobiano que, mesmo em baixas concentrações, é capaz de provocar a morte celular rapidamente. A sensibilidade de bactérias Gram-positivas e Gram-negativas às bacteriocinas tem como base a composição química da parede celular. Estudos comprovam que as Gram-negativas tornam-se mais sensíveis às bacteriocinas quando desestabilizadas por estresse químico ou físico. O principal mecanismo bactericida destes antimicrobianos se dá pela formação de poros na membrana como ocorre com a maioria dos lantibióticos, entretanto algumas bacteriocinas atingem outros alvos celulares como a mersacidina que interage com o lipídio II, impedindo a síntese da parede celular; a microcina J25 que inibe a síntese do RNA bacteriano e a duramicina-C que inibe a fosfolipase A2. Diversas bacteriocinas atuam sobre a membrana celular ligando-se a receptores específicos. Em geral, é provável que as estruturas secundárias dos peptídios ativos tenham um papel importante na atividade biológica do microrganismo, já que as conformações em α-hélice e lâminas ß-pregueadas podem formar poros na membrana resultando na saída dos constituintes celulares e na perda do potencial de membrana. Algumas bacteriocinas usam mais de um mecanismo para matar a célula-alvo (Ennahar et al*.,* 2000; Ray et al.; 2001; Cotter et al., 2005; Nes et al., 2007; Willey & Van Der Donk, 2007; Sang & Blecha, 2008; Gautam & Sharma, 2009).

As propriedades biofísicas como a estrutura secundária, a carga total e a hidrofobicidade influenciam a interação dos peptídeos antimicrobianos com as membranas de células biológicas, assim, com base nos dados oriundos de diferentes estudos dois modelos que explicam o mecanismo de ação destes compostos são propostos:

a)Modelo *barrel stave*: Descreve a formação de canais/poros transmembrana por feixes de hélices anfipáticas, de tal forma que suas superfícies hidrofóbicas interajam com o núcleo lipídico da membrana e suas superfícies hidrofílicas insiram-se na membrana, produzindo poros aquosos. O aumento progressivo da condutividade na membrana confirma a formação de poros. O recrutamento progressivo de monômeros adicionais aumenta o tamanho dos poros levando ao extravazamento do conteúdo celular e, consequentemente, à morte da célula.

b)Modelo *carpet*: Inicialmente os monômeros do peptídeo ligam-se à cabeça do fosfolipídio e, em seguida, alinham-se na superfície da membrana, formando um tapete, de modo que sua superfície hidrofílica interage com o núcleo hidrofóbico da membrana. O peptídeo, então, desintegra a membrana por rompimento da bicamada lipídica (Matsuzaki et al., 1998; Dagan et al., 2002; Reddy et al., 2004).

As bacteriocinas cujo modo de ação foi mais estudado, até então, são a nisina (classe I) e a pediocina (classe II) que inclusive compartilham algumas características em comum. Geralmente, atuam destruindo a integridade da membrana citoplasmática pela formação de poros, o que provoca a saída de pequenos compostos ou a alteração da força protomotriz

necessária para a produção de energia e para a síntese de proteínas ou ácidos nucléicos. A nisina é uma bacteriocina péptica hidrofóbica sintetizada pelo *Lactococcus lactis* que atua contra bactérias Gram-positivas. Os organismos Gram-negativos, por possuírem membranas externas que representam uma barreira à permeabilidade, geralmente são resistentes a moléculas hidrofóbicas como a nisina (Hancock, 1984; Montville & Chein, 1998; Elliason & Tatini, 1999; Sablon, et al., 2000).

Dois modelos foram propostos para a formação de poros na membrana celular pela nisina. Inicialmente, a bacteriocina liga-se à membrana-alvo através de interação eletrostática. No primeiro modelo a nisina liga-se como um monômero, insere-se nas bicamadas lipídicas e os monômeros inseridos agregam-se lateralmente para formar os poros. No segundo modelo, os poros se formam devido a uma perturbação local na camada lipídica que ocorre pela ligação da nisina, que é então puxada para a membrana por um componente da força próton-motriz (FPM) (Ojcius & Young, 1991; Driessen et al.,1995; Montville & Chen, 1998; Moll et al., 1999).

Alguns membros da classe I das bacteriocinas (ou lantibióticos) parecem ter um modo duplo de ação, podendo ligar-se ao lipídio II, o principal transportador das subunidades de peptidoglicano do citoplasma para a parede celular, impedindo assim a síntese da parede celular ou, ainda, usando o lipídio II como uma molécula “âncora” para facilitar sua inserção na membrana celular, o que levaria à formação de poros e, consequentemente, à morte da céula. Já as bacteriolisinas atuariam diretamente na parede celular de Gram-positivas provocando a lise e a morte das células-alvo (Figura 2) (Cotter et al., 2005).

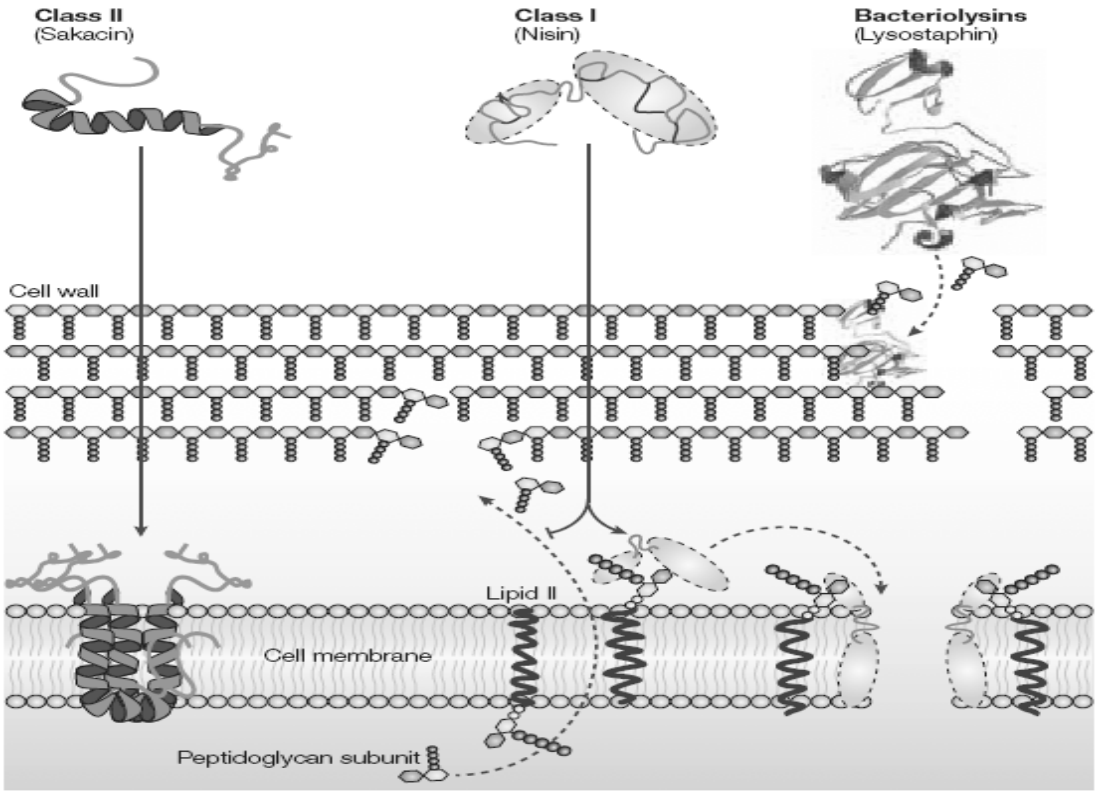

Figura 2: Modo de ação de bacteriocinas de bactérias ácido lácticas (BAL) (Fonte: Cotter et al., 2005).

2.6 Aplicações

2.6.1 Sistemas alimentares

No campo da conservação dos alimentos é interessante analisar a utilização de bacteriocinas para estender a vida útil e aumentar a segurança dos alimentos, buscando atender à demanda dos consumidores por alimentos minimamente processados que não contenham conservantes químicos. As bacteriocinas têm ganhado importância como biopreservativos naturais no controle de microrganismos deteriorantes e patogênicos em alimentos. Durante as últimas duas décadas, aplicações práticas de conservantes naturais vêm sendo investigadas e estas tecnologias demonstram inativar microrganismos e

suas enzimas sem provocar efeitos adversos significativos nas propriedades organolépticas e nutricionais dos alimentos. Pela natureza proteica das bacteriocinas assume-se que seriam seguras para consumo humano uma vez que seriam rapidamente inativadas pelas proteases do trato gastrointestinal e, consequentemente, não alterariam a microbiota do intestino. A identificação e caracterização de bacteriocinas e sua utilização potencial como controle de microrganismos indesejáveis representa um importante aspecto da segurança alimentar. A substituição parcial ou total dos conservantes químicos por biopreservativos, mais seguros e saudáveis, certamente provocará uma revolução na indústria de alimentos (Bizani & Brandelli, 2002; Cleveland et al., 2002; Martínez et al., 2003; Gautam & Sharma, 2009; Tiwari et al., 2009).

São inúmeras as aplicações das bacteriocinas em alimentos e para que possam ser empregadas devem possuir os seguintes requisitos: não oferecer riscos à saúde do consumidor; não afetar a qualidade nutricional e sensorial do alimento; apresentar termoestabilidade; a linhagem produtora deve ter *status* GRAS (Generally Recognized as Safe); o espectro de inibição sobre microrganismos patogênicos deve ser amplo. Um grande número de bacteriocinas produzidas por bactérias ácido lácticas (BAL) têm sido pesquisadas, permitindo sua caracterização química detalhada. Embora muitas bacteriocinas como subtilina, cereína, turicina, plantaricina, etc. tenham sido isoladas e caracterizadas, ainda encontram-se em processo de obtenção de *status* comercial para uso como conservantes em alimentos. As únicas bacteriocinas empregadas atualmente na preservação de alimentos são a nisina (Nisaplin®) e a pediocina PA1/AcH (ALTA®) que são sintetizadas por

BAL e, normalmente, empregadas na produção de alimentos fermentados (Holzapfel et al., 1995; Montville & Winkoswski, 1997; Enhahar et al., 2000; Cotter et al., 2005; Gautam & Sharma, 2009). A nisina, descoberta em 1928 na Inglaterra, foi a primeira bacteriocina, sintetizada pelas BAL, avaliada como antibiótico clínico e aprovada para uso na preservação de alimentos, a princípio especificamente para prevenir a germinação de esporos de *Clostridium botulinum* em queijos (Montville & Chen, 1998). A nisina tem *status* GRAS pelo FDA. Licenciada para uso em vários países, é produzida industrialmente desde 1953 (De Vuyst & Vandamme, 1994; McAuliffe et al., 2001; Cheigh & Pyu, 2005; Lubelski et al., 2008).

As bacteriocinas têm sido adicionadas, até o momento, aos alimentos de três formas diferentes: no caso de produtos fermentados, através do uso de bactérias ácido-lácticas que secretam o antimicrobiano (cultivos *starters*); como culturas adjuntas ou, ainda, como um complexo puro ou parcialmente purificado. As bacteriocinas de amplo espectro de atividade são geralmente as mais procuradas, porém outros fatores como pH ótimo, solubilidade e estabilidade devem ser levados em consideração na escolha destas substâncias como inibidoras para uso em alimentos. O isolamento e a purificação de muitas bacteriocinas potencializam o uso em uma grande variedade de alimentos, porém muitas destas substâncias têm sido difíceis de purificar, ou ainda, apresentam instabilidade na sua forma pura. Por este motivo pouco se conhece sobre a maioria das bacteriocinas, exceto a nisina (Nettles & Barefoot, 1993; Kalmokoff & Teather, 1997; Cotter et al., 2005; Nascimento et al., 2008).

O estudo de diversas bacteriocinas como bioconservadores tem gerado resultados promissores para aplicação na indústria de alimentos, conforme exemplificado na tabela 2.

Tabela 2: Aplicação de bacteriocinas na bioconservação de alimentos

Bacteriocina	Cepa produtora	Alimento	Microrganismo indicador	Referência
Cereína	*Bacillus cereus*	Queijo	*Listeria monocytogenes*	Bizani et al. (2008)
Enterocina A	*E. faecium*	Queijo	*Listeria monocytogenes*	Mirhosseini et al. (2010)
Enterocina AS-48	*E. faecalis* A 48-32	Leite	*S. aureus*	Sobrino-Lopez et al. (2009)
Pentocina 31-1	*L. pentosus* 31-1	Carne suína	*Pseudomonas* sp.; *Listeria* sp.	Zhang et al. (2010)
Nisina	*L. lactis*	Melão	*E. coli* O157:H7	Ukuku et al. (2005)

Diferentes métodos de aplicação das bacteriocinas nos alimentos são utilizados seguindo várias técnicas como: imersão direta do alimento em solução contendo o antimicrobiano; uso de filmes plásticos tipo polietileno ou filmes comestíveis a base de celulose; adsorção da bacteriocina em superfícies como polietileno, acetato de vinil etileno, polipropileno, poliéster, sílica salinizada, etc.; invólucros antimicrobianos contendo preparados de bacteriocina e culturas de BAL que podem ser usados como estratégias

tecnológicas baseadas na teoria dos obstáculos múltiplos (*Hurdle Concept*) com o objetivo de reduzir doenças transmitidas por alimentos (DTAs). A aplicação de bacteriocinas da classe IIa aos alimentos fornece apenas um obstáculo ao crescimento de microrganismos deteriorantes e patogênicos. A combinação das bacteriocinas com outros tratamentos subletais mostrou ser mais efetiva no fornecimento de barreiras antimicrobianas em sistemas alimentares. Para a obtenção de um controle eficiente de microrganismos patogênicos em alimentos as bacteriocinas devem ser utilizadas como parte de um sistema de conservação de alimentos, promovendo um efeito adicional ou sinergístico a outros fatores (Bower et al., 1995; Appendini & Hotchkiss, 2002; Drider et al., 2006; Deegan et al., 2006; Nascimento et al., 2008; Gautam & Sharma, 2009).

2.6.2 Aplicações Clínicas

O uso potencial das bacteriocinas como alternativa aos antibióticos clássicos no tratamento de infecções bacterianas é real. O rápido crescimento e a disseminação de bactérias patogênicas multirresistentes obrigam a busca por métodos alternativos de combate às infecções. Devido ao fato de que muitos peptídeos antimicrobianos (AMPs) matam as bactérias por ruptura da integridade da membrana celular, e, portanto são menos suscetíveis a induzir resistência, estão sendo amplamente estudados como potenciais drogas antimicrobianas (Riley & Wertz, 2002; Sang & Blecha, 2008).

Inúmeras pesquisas vêm investigando a potencial aplicação clínica dos lantibióticos baseadas na não-toxicidade desta classe de bacteriocinas e na sua atividade inibitória frente a patógenos Gram-positivos de humanos e

animais. A elucidação dos mecanismos de ação de alguns lantibióticos e sua atividade contra patógenos multi-droga resistentes os tornam uma opção atrativa como possíveis agentes terapêuticos (Cotter et al., 2005).

Publicações recentes têm discutido e revisado o desenvolvimento de drogas baseadas em peptídeos antimicrobianos (AMPs). A maioria dos estudos encontra-se no estágio da descoberta ou no estágio pré-clínico com alguns ensaios clínicos. A cepa de *Streptococcus salivarius*, um comensal da microbiota oral de humanos, produz várias substância tipo-bacteriocina, incluindo os lantibióticos salivaricin A2 e B que, atualmente, estão em uso ou em desenvolvimento para controle de infecções da cavidade bucal. Um exemplo disto é o produto “BLIS K12 protetor da garganta” comercializado pela empresa neozelandeza BLIS *Technologies* Ltda usado como inibidor da bactéria *Streptococcus pyogenes* e na prevenção da halitose. O lantibiótico mutacina 1140 produzido pelo *Streptococcus mutans*, o principal agente causal das cáries dentárias, é efetivo contra várias cepas da mesma espécie, portanto tem despertado grande interesse como agente preventivo desta doença infecto-contagiosa (Hillmann, 2002; Wang & Kuramitsu, 2005; Willey & Van der Donk, 2007; Tagg, 2009).

A nisina é um dos poucos exemplos de terapia antibiótica baseada em AMPs licenciada que tem sido comercializada para uso em saúde humana (como suplemento nutricional licenciado pela “*Nutrition21*” para *Biosynexus Incorporated*) e em saúde animal como antimastítico (*Pfizer Animal Health*). Este peptídeo antimicrobiano inibe os principais patógenos Gram-positivos envolvidos na mastite como cepas dos gêneros *Streptococcus* e

Staphylococcus, além disso possui uma capacidade inata para reconhecer e interagir com os fosfolipídios levando ao rompimento das membranas celulares dos microrganismos, porém não os lipídios presentes em células de mamíferos. Esta propriedade única da bacteriocina pode ser explorada no desenvolvimento de um microbiocida vaginal seguro e eficaz para a prevenção de doenças sexualmente transmissíveis, incluindo a síndrome da imunodeficência adquirida, além do uso como contraceptivo (Gordon et al., 2005; Cotter et al., 2005; Hancock & Sahl, 2006; Sang & Blecha, 2008; Gupta et al., 2009).

2.6.3 Outras aplicações

Doenças bacterianas em plantas são de difícil controle devido a falta de produtos químicos eficazes. O uso intensivo de agroquímicos levou ao surgimento de patógenos resistentes e impactos negativos ao meio ambiente. Embora os antibióticos utilizados para terapia humana possam oferecer a solução para diversas infecções são caros e seu uso não é permitido na agricultura. Assim, o controle biológico que utiliza microrganismos para suprimir doenças de plantas consiste em uma alternativa ecológica aos produtos químicos sintéticos. Há vários relatos na literatura científica sobre o uso potencial de bactérias associadas a rizosfera que estimulam o crescimento da planta além de atuarem como agentes de biocontrole (Lucy et al., 2004; Lugtenberg & Kamilova, 2009). Uma bacteriocina produzida pela bactéria *Pseudomonas syringae* pv. *ciccaronei* inibiu a multiplicação de *P. syringae* pv. *savastanoi*, o agente causal da doença conhecida como "nó da oliveira", e afetou a sobrevivência epifítica do patógeno nas folhas e galhos das plantas, levando os pesquisadores a concluírem que a bacteriocina poderia ser

efetivamente utilizada no controle do fitopatógeno (Lavermicocca et al., 2002).

Alguns AMPs apresentam características inseticidas que poderiam ser utilizadas na produção de plantas resistentes a pragas e de novos pesticidas químicos no controle de insetos e nematóides. Alguns insetos, como os pulgões, possuem células especializadas que contêm bactérias endossimbiontes que fornecem aminoácidos e outros compostos químicos ao seu hospedeiro. O uso de AMPs que agem sobre bactérias endossimbiontes seria bastante promissor no controle de pragas (Keymanesh et al., 2009). As polioxinas são uma classe de peptídeos isolados de *Streptomyces cacaoi* var. *asoensis* que tem a capacidade de inibir a enzima quitina-sintase e, portanto, impedir a síntese de quitina em insetos, tornando-os extremamente vulneráveis a infecções (Arakawa, 2003). Os efeitos antinematóides de algusn AMPs também são investigados, como é o caso do peptídeo HP (2-20) derivado da proteína ribossomal R1 (RPL1) de *Helicobacter pylori* que tem atividade nematicida contra ovos e larvas de *Caenorhabditis elegans* por rompimento da casca do ovo e membranas das larvas (Jang et al., 2004).

2.7 Bacteriocinas sintetizadas por bactérias do gênero *Bacillus*

Em resposta a condições de estresse nutricional bactérias do gênero *Bacillus* ativam uma variedade de mecanismos de defesa, incluindo esporulação, síntese de enzimas extracelulares degradativas e produção de antibióticos. A atividade antimicrobiana de *Bacillus* é bastante interessante de ser investigada uma vez que espécies deste gênero produzem um grande número de peptídios antimicrobianos com diferentes estruturas químicas. Embora a maioria dessas substâncias sejam ativas contra microrganismos

Gram-positivos, alguns componentes presentes apresentam uma larga faixa de atividade que inclui também bactérias Gram-negativas e fungos (Katz & Demain, 1977; Von Döhren, 1995; Dieckmann et al., 2001).

O solo é o habitat natural da maioria das espécies de bactérias pertencentes ao gênero *Bacillus*, porém estes microrganismos se apresentam amplamente distribuídos no ambiente. Com frequência, são submetidos a variações físico-químicas extremas do meio ambiente, como a disponibilidade de nutrientes, especialmente fontes de C, a osmolaridade e a temperatura, o que leva ao crescimento lento ou à passagem para a fase estacionária. Na transição da fase exponencial para a estacionária o microrganismo tenta adaptar-se ao meio através de diferentes respostas como a transformação genética por DNA exógeno, o desenvolvimento de motilidade, a produção de enzimas degradativas extracelulares e, até, a produção de substâncias antimicrobianas (Hyronimus et al., 1998).

Diversos estudos já foram realizados com bactérias do gênero *Bacillus* capazes de sintetizar substâncias antimicrobianas. As origens destes bacilos são bastante diversas e a resistência dos esporos permite ao gênero manter-se latente nos mais distintos ambientes. Pesquisa desenvolvida com *Bacillus licheniformis* P40, isolado do intestino de um peixe amazônico, encontrou uma substância antimicrobiana tipo-bacteriocina (BLS) capaz de inibir o crescimento de importantes bactérias patogênicas e deteriorantes de alimentos como *Listeria monocytogenes* e *Bacillus cereus*, como também de um isolado clínico de *Streptococcus* sp. e do fitopatógeno *Erwinia carotovora.* Além de apresentar atividade antimicrobiana a BLS apresentou propriedades

emulsificantes (Cladera-Olivera et al., 2004; Teixeira et al., 2009).

Uma bacteriocina denominada cereína 8A produzida por uma cepa de *Bacillus cereus* isolada de solo, foi identificada, caracterizada e apresentou um largo espectro de atividade inibitória, especialmente contra *Listeria monocytogenes*, *Clostridium perfringens*, *Escherichia coli* e *Salmonella* Enteritidis, além de um significativo efeito inibitório sobre esporos de *Bacillus cereus*. Análises por microscopia eletrônica de transmissão evidenciaram danos à parede celular das células-alvo com a consequente perda de protoplasma. Os resultados obtidos sugerem que a cereína 8A possui grande potencial para ser usada como bioconservante em alimentos (Bizani & Brandelli, 2002; Bizani et al., 2005).

Em uma investigação recente com bactérias isoladas do ambiente da Bacia Amazônica brasileira foi identificada uma nova espécie de *Bacillus* designada como cepa P34, que apresentou importante atividade antimicrobiana contra bactérias Gram-positivas como *Listeria monocytogenes* e *Bacillus cereus*. Esta substância foi purificada e caracterizada como um lipopeptídeo de 1.456 Da. Estudos de avaliação das condições ambientais para a produção da bacteriocina pela cepa P34 concluíram que a produção máxima de atividade antimicrobiana ocorria a valores de pH entre 6,0 e 8,0, temperaturas entre 25 e 37 ºC e em um meio base contendo proteína de soja. Os resultados obtidos também mostraram que não foi encontrada nenhuma relação entre o crescimento bacteriano e a produção da bacteriocina (Motta et al., 2004; Motta et al., 2007; Motta & Brandelli, 2008).

A ação de substâncias antimicrobianas naturais contra fitopatógenos

tem sido descrita para bacteriocinas produzidas por bactérias do gênero *Bacillus*. É o que ocorre por exemplo com a cepa de *Bacillus subtilis* 14B, isolada da rizosfera de amêndoas amargas, que sintetiza a bacteriocina denominada Bac 14B, uma proteína monomérica com um peso molecular de 21 kDa, que foi eficiente em reduzir as infecções causadas por *Agrobacterium tumefaciens*, o agente causal de uma doença neoplásica em diferentes plantas hospedeiras, podendo ser utilizada portanto para o biocontrole desta infecção (Hamammi et al., 2009). Membros do gênero *Bacillus* são portanto, entre as bactérias benéficas, extensamente explorados como biopesticidas microbianos. Produtos baseados em *Bacillus* representam cerca da metade dos agentes bacterianos de biocontrole disponíveis comercialmente (Favel, 2005).

2.7.1 *Bacillus amyloliquefaciens*

Welker & Campbell (1967) estudando taxonomicamente cepas de *Bacillus amyloliquefaciens* classificaram este microrganismo como bastonetes Gram-positivos, móveis por flagelos perítricos, esporogênicos e que crescem bem em caldo suplementado com 7% de NaCl. Seu perfil bioquímico se caracteriza por provocar a hidrólise do amido; produzir acetimetilcarbinol; fermentar carboidratos como glicose, sacarose e lactose com produção de gás; hidrolisar a gelatina e reduzir nitrato a nitrito. A faixa de temperatura para crescimento varia entre 30 e 40˚C. Esta bactéria possui um comportamento bioquímico bastante semelhante ao de *Bacillus subtilis,* porém pode ser diferenciada pela maior produção de α-amilase e pelo maior percentual molecular das bases Guanina + Citosina do seu DNA. A maioria das substâncias com atividade antimicrobiana produzidas por bactérias do gênero

Bacillus spp. são ativas contra organismos Gram-positivos (Slepecky & Hemphill, 1992). Entretanto, muitas pesquisas apontam cepas de *B. amyloliquefaciens* com atividade antimicrobiana de espectro amplo.

Uma cepa de *Bacillus amyloliquefaciens*, denominada RC-2, isolada de folhas de amoreira, foi capaz de exercer atividade antagonista contra o fungo *Colletotrichum dematium* causador da antracnose. O filtrado da cultura de RC-2 também inibiu o crescimento de vários outros fungos e bactérias como *Rosellinia necatrix*, *Pyricularia oryzae*, *Agrobacterium tumefaciens* e *Xanthomonas campestris*. Foram isolados do cultivo, sete tipos de compostos antimicrobianos e um deles foi identificado como sendo iturina A2, um peptídeo cíclico (Yoshida et al*.,* 2001).

A cepa de *B. amyloliquefaciens* B94 foi usada como um agente de controle biológico para suprimir *Rhizoctonia solanis* e outros fungos fitopatogênicos. Três componentes antifúngicos foram isolados da cultura do microrganismo e identificados como isômeros da iturina A. As iturinas são um grupo de lipopeptídeos cíclicos produzidos por *Bacillus subtilis* com potente atividade antifúngica. O grupo é formado por iturina A-E, bacilomicina D, F e L e micosubtilina (Yu et al., 2002).

A subtilosina, bacteriocina relatada em estudos anteriores como sendo produzida apenas *por B. subtilis* foi encontrada em uma cepa de *B. amyloliquefaciens* isolada a partir de um produto lácteo fermentado. A proteína foi ativa contra *Listeria monocytogenes* e os isolados clínicos *Gardnerella vaginalis* e *Streptococcus agalactiae* sem provocar danos à microbiota natural, levando os autores da pesquisa a propor sua utilização no tratamento de

infecções, especialmente no caso da *G. vaginalis* que tem se mostrado capaz de desenvolver resistência às drogas de escolha (Sutyak et al., 2008).

Elevada atividade inibitória *in vitro* contra múltiplos fungos e oomicetos patógenos de plantas foi encontrada para a cepa de *B. amyloliquefaciens* GA1. Quando utilizada no tratamento de sementes, apresentou diminuição de doenças nas plântulas pela ação de antibiose direta contra patógenos oriundos do solo. Além disso, reduziu a infecção pós-colheita de maçãs por *Botrytis cinerea*, o agente causal da doença do mofo cinzento. Os dados apresentados sugerem a secreção de múltiplos antibióticos e demonstram o potencial da cepa GA1 de *B. amyloliquefaciens* para ser usada como agente de biocontrole (Toure et al., 2004; Arguelles-Arias et al., 2009).

2.8 Isolamento e purificação das bacteriocinas

A detecção de cepas produtoras de bacteriocinas é o primeiro passo para o desenvolvimento de estudos de distribuição e detecção de atividade antagônica das substâncias antimicrobianas. Devido à natureza química extremamente heterogênea das bacteriocinas existe um grande número de técnicas de purificação destas moléculas. Para propósitos de purificação são necessários estudos de produção e grandes volumes de cultivo. A produção pode ser influenciada pelas condições de incubação como pH, temperatura e tempo, além da composição do meio. As condições ótimas de produção devem ser determinadas previamente para cada microrganismo produtor. Em função da grande diversidade de bacteriocinas, não existe um método único ou protocolo geral para a purificação (Rojo-Bezares et al., 2007; Muñoz-Rojas, 2008; Motta & Brandelli, 2008).

Uma vez liberadas das células produtoras as bacteriocinas ou permanecem adsorvidas à parede celular ou ficam livres no meio onde são secretadas. Em processos de purificação, as bacteriocinas são removidas das células por centrifugação e precipitação do sobrenadante líquido com uma solução saturada de sulfato de amônio. Além deste, ácido tricloroacético, etanol, metanol e acetona também podem ser usados para extração, porém a maioria dos pesquisadores faz uso do sulfato de amônio, porque precipita irreversivelmente as proteínas sem aumento considerável da temperatura do meio. A vantagem desta primeira etapa de purificação é o aumento na concentração da proteína no meio. As proteínas precipitadas são dissolvidas em água deionizada, ou tampão, e separadas pelo uso de vários métodos incluindo as cromatografias líquida de alta performance e fase reversa, de gel-filtração, de troca iônica ou, ainda, de interação hidrofóbica, utilizadas de forma isolada ou combinada. Métodos de purificação de bacteriocinas que utilizam protocolos complexos e demorados apresentam a desvantagem de obter baixo rendimento de proteínas (Carolissen-MacKay et al.,1997; Drider et al., 2006; Gautam & Sharma, 2009). A instabilidade das bacteriocinas aumenta com seu grau de pureza. O problema da instabilidade não é atribuído à proteólise mas, possivelmente, a alterações no sítio catalítico como a perda de cofatores ou modificações covalentes (Hèchard et al., 1992).

3 RESULTADOS E DISCUSSÃO

Os resultados obtidos neste estudo estão compilados no formato de artigos científicos submetidos ou a serem submetidos para publicação em periódicos especializados. Cada subtítulo corresponde a um destes artigos:

3.1 Antimicrobial activity of *Bacillus amyloliquefaciens* LBM 5006 is enhanced in the presence of *Escherichia coli* ATCC 25922. Submetido a ***Current Microbiology***

3.2 Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM 5006. Submetido a ***Microbiological Research***

3.3 Effect and mode of action of the *Bacillus amyloliquefaciens* LBM 5006 anti-listerial bacteriocin-like substance. A ser submetido a ***Food Control***

3.4 Bacteriocin-like substance from *Bacillus amyloliquefaciens* shows remarkable inhibition of *Acanthamoeba polyphaga.* A ser submetido a ***Parasitology Research***

3.5 Bacteriocin-like substance from *Bacillus amyloliquefaciens* inhibits *Paenibacillus larvae*, the causative agent of American Foulbrood. A ser submetido a ***Applied and Environmental Microbiology***

3.1 ARTIGO 1

3.1.1 Antimicrobial activity of *Bacillus amyloliquefaciens* LBM 5006 is enhanced in the presence of *Escherichia coli* ATCC 25922

3.1.2 Abstract

The effect of different bacteria on the production of antimicrobial activity by *Bacillus amyloliquefaciens* LBM 5006 was studied. Increased antimicrobial activity was observed when this strain was cultivated in the presence of thermally inactivated cells of *Escherichia coli*, but not with *Staphylococcus aureus*, *Listeria monocytogenes* or *Bacillus cereus*. *E. coli* also enhanced the antimicrobial activity when it was added to the medium in the form of living cells or as cell debris after cellular fractionation. No inducing activity was observed with addition of cell-free supernatant of *E. coli* cultures, suggesting that inducing factor is associated to the cells. Polyacrylamide gel electrophoresis revealed that additional peptide bands are secreted when *B. amyloliquefaciens* was cultivated in the presence of cell debris of *E. coli*. It was concluded that the presence of intact or thermally inactivated *E. coli* enhanced the synthesis of antimicrobial peptides by *B. amyloliquefaciens*.

Keywords: Antimicrobial activity; *Bacillus amyloliquefaciens*; Bacteriocin; Production

3.1.3 Introduction

Bacteria of the genus *Bacillus* can produce a large number of antimicrobial peptides with different chemical structures, such as bacteriocins, bacteriocin-like substances and lipopeptides [13,23]. Although most of these substances are active against Gram-positive microorganisms, some of them have a wide range of activity including Gram-negative bacteria and fungi [6,22]. These substances act as defense peptides then there is evidence of cellular signaling mechanisms involved, also the producer organisms are immune to its own bacteriocin, a property that is mediated by specific immunity proteins [4,7].

Microorganisms use considerable energy in the development of antimicrobial mechanisms and synthesis of defense peptides, but it is not yet clarified the role that these compounds exert on the microbial communities. The situations in which the biosynthesis is required are often regulated by the shortage of nutrients in the growing medium as occurs in sporulation, the synthesis of extracellular enzymes degrading and the development of genetic competence [19]. The antimicrobial peptides may play a defensive role and prevent the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells. The mediation of quorum-sensing systems is an additional role proposed for bacteriocins of Gram-positive bacteria [15,19]. The presence of certain bacteria acts as an environmental signal to activate the production of bacteriocin by *Lactobacillus plantarum* NC8 [11]. A quorum-sensing mechanism mediated by an induction factor (PLNC8IF) appears to be involved in this process. The phenomenon of induction in the production of bacteriocins has been little investigated, but several studies showing the

interference of other microorganisms in the synthesis of bacteriocins by lactic acid bacteria are reported in the literature [5,20,21]. However, the effect of competing microorganisms in the activation of bacteriocin synthesis by bacteria of the genus *Bacillus* has not been reported to date.

Bacillus amyloliquefaciens LBM 5006, originally isolated from a soil sample from the Brazilian Atlantic forest, produces a previously characterized bacteriocin-like substance (BLS) [10]. The aim of this study was to investigate the production of BLS by *B. amyloliquefaciens* LBM 5006 in the presence of other bacteria.

3.1.4 Materials and methods

3.1.4.1 Bacterial cultures, media and grow conditions

The producer strain was *B. amyloliquefaciens* LBM 5006 [10]. The strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 9634 and *Listeria monocytogenes* ATCC 7644 were used to stimulate the production of antimicrobial activity by *B. amyloliquefaciens* LBM 5006. *L. monocytogenes* ATCC 7644 was used as indicator strain for detection of antimicrobial activity. All strains were stored at -21℃ in brain-heart infusion (BHI; Oxoid, Basingstoke, UK) containing 20% (v/v) glycerol and propagated in the same medium at 37℃ before use.

3.1.4.2 Antimicrobial activity assays

The “spot-on-the-lawn” method [9] was modified as follows: plates of Trypticase Soy Agar (Mast Diagnostics, Merseyside, UK) supplemented with

0.6% yeast extract (TSAYE), were spotted with a 24 h culture of *B. amyloliquefaciens* and incubated overnight at 37ºC in aerobic conditions. After incubation, a layer of 8 mL of brain heart infusion with 0.8% agar (soft BHI agar) containing 1% of a suspension with 10^6 UFC/mL of each indicator organism was poured over the plates. After incubation under appropriate conditions for each microorganism, growth inhibition was detected by the appearance of a clear zone around the producer strain.

The bacteriocin activity was determined by the agar disc diffusion method. An aliquot of 20 μL of filtered supernatant was applied to sterile cellulose discs (6 mm) placed on BHI plates previously inoculated with a suspension of 10^8 cells/mL of the strain of *L. monocytogenes* ATCC 7644, used as indicator microorganism. The plates were incubated at 37ºC for 24 h. The bacteriocin titre was determined by the serial two-fold dilution method [16]. Activity was defined as the reciprocal of the dilution after the last serial dilution giving a zone of inhibition and expressed as activity units (AU) per mililiter.

3.1.4.3 Effect of bacteria on production of antimicrobial activity

The production of antimicrobial activity was tested in the presence of thermally inactivated cells (autoclaved at 121ºC for 30 min) of *E. coli* ATCC 25922, *S. aureus* ATCC 25923*, B. cereus* ATCC 9634 or *L. monocytogenes* ATCC 7644. Each cell pellet was suspended to 1 mL with BHI and then added to 9 mL BHI broth inoculated with *B. amyloliquefaciens* LBM 5006. The mixture was incubated at 37℃ and production of antimicrobi al activity was monitored.

For additional tests with *E. coli*, a 16 h culture was centrifuged at

10,000 x *g*, 4℃ for 15 min, the pellet of living cells (PLC) was washed three times and suspended in 5 mL of PBS and added to a 20 mL of a culture of *B. amyloliquefaciens* LBM 5006 that had just been started. Aliquots of the supernatant (SN) of *E. coli* ATCC 25922 culture were separately added to the culture of LBM 5006. The experiments were incubated at 37℃ for 24 h in an orbital shaker at 125 rpm. For the co-culture experiments, aliquots (0.5 mL) of both *E. coli* and LBM 5006 at initial concentrations of 10^8 CFU/mL and 10^7 CFU/mL, respectively, were added to 49 mL of BHI. Samples were incubated for 48 h at 37℃ with shaking, cell growth was followed by OD_{600} and antimicrobial activity was tested by the agar disc method using *L. monocytogenes* as indicator strain. Cultures of *B. amyloliquefaciens* and *E. coli* alone were included as controls.

3.1.4.4 Fractionation of *Escherichia coli* cells

Cell fractionation was carried out as described elsewhere [2], with the following modifications: Cells from a 24 h culture of *E. coli* in BHI broth were harvested by centrifugation at 10,000 x *g* for 15 min at 4ºC. Cell pellet was washed twice with 10 mmol/L phosphate buffer (pH 6.0) and resuspended in 20 mL of the same buffer. This suspension was incubated for 10 min in a boiling-water bath, sonicated for 10 min at 60 W output and centrifuged at 16,000 x *g* for 15 min at 4℃. Supernatants were filtered through a 0.22 µm membrane filter, and the filtrate was referred as intracellular fraction. The pellet was washed twice and resuspended in 20 mL of 10 mmol/L phosphate buffer (pH 6.0) and referred as cell debris fraction. These cell fractions were added (2%) to

cultures of *B. amyloliquefaciens* as putative inducing factors.

3.1.4.5 Antimicrobial activity on polyacrylamide gels

Aliquots of crude culture filtrates were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by [3]. The samples were applied to 12% polyacrilamide gels and run at 25 mA per gel, using a Mighty Smmall II apparatus (Hoefer Scientific, San Francisco, CA, USA). After running the gels were washed with sterile distilled water for removal of SDS and overlaid in a Petri dish by 10 mL semi-solid (0.75%) BHI agar containing 100 µL overnight culture of *L. monocytogenes* ATCC 7644 (10^6 CFU/mL). The Petri dishes were incubated at 37ºC for 24 h and examined for the presence of an inhibitory zone. Other gels were stained with Coomassie Brilliant blue to observe peptide bands. The kit BenchMark™ protein ladder (Invitrogen, Carlsbad, CA, USA) was used as molecular weight standard, following the instructions of manufacturer.

3.1.5 Results

The antimicrobial activity of *B. amyloliquefaciens* is shown in Table 1. Among the microorganisms tested, 72% were sensitive to the antimicrobial substances, characterizing a broad spectrum of antagonistic activity.

The control culture of *B. amyloliquefaciens* LBM 5006 produced an antimicrobial activity of 3200 AU/mL. This same activity was observed with the addition of thermally inactivated cells of *S. aureus*, *B. cereus* or *L. monocytogenes*. However, increased antimicrobial activity (12,800 AU/mL) was

obtained in the presence of thermally inactivated cells of *E. coli* ATCC 25922 (Fig. 1). This microorganism was selected for additional experiments of induction.

Cell-free supernatants of *E. coli* cultures were not able to induce an increase in the production of antimicrobial activity by *B. amyloliquefaciens* LBM 5006. Both the pellet of living cell of *E. coli* and co-culture with LBM 5006 caused an increase in antimicrobial activity, but lower than that observed with thermally inactivated cells (Fig. 1). Thus, living cells of *E. coli* are not strictly necessary to the enhancement of BLS production by strain LBM 5006.

Fig. 2 shows the production of antimicrobial activity during growth of *B. amyloliquefaciens* in the presence of *E. coli*. Similar growth curves were observed, although the lowest OD values were observed in the presence of thermally inactivated cells or co-cultivation (Fig. 2A). Antimicrobial activity was initially detected after 8 h of incubation for each treatment and maximum activity was observed at 32 h of cultivation, in the stationary growth phase, which suggests that the antimicrobial peptide is a secondary metabolite. The pure culture of *E. coli* was included as a control, and antimicrobial activity was not detected along the 48 h incubation.

B. amyloliquefaciens LBM 5006 was cultured with the addition of cell fractions of *E. coli*, and the effect on production of antimicrobial activity was examined. Intracellular fractions did not show any inducing activity in this experiment. When the cell debris fraction of *E. coli* was included the antimicrobial activity of *B. amyloliquefaciens* LBM 5006 increased to 6400 AU/mL.

The peptide profile of culture supernatant of *B. amyloliquefaciens* growing on BHI was analyzed. A profile mostly composed of polypeptides minor than 30 kDa was observed (Fig. 3, lane B). This was coinciding with the location of a broad band of antimicrobial activity observed in gels (Fig. 3, lane E). When the cell debris fraction of *E. coli* was included in the medium, additional peptide bands were observed, corresponding to molecular masses lower than 15 kDa (Fig. 3, lane C). The intensity of antimicrobial activity was increased as compared with the control (Fig. 3, lane D).

3.1.6 Discussion

In this report, *B. amyloliquefaciens* LBM 5006 produced an antimicrobial activity that inhibits several Gram-negative and Gram-positive bacteria. Those antimicrobials that have broad inhibitory spectra with high biological activity, and good stability and solubility may have great biopreservative potential [5]. This study provides evidence that the presence of *E. coli* may increase the production of antimicrobial activity by *B. amyloliquefaciens*. The cultivation with intact or thermally inactivated cells of *E. coli* increased the antimicrobial activity, suggesting that an external stimulus was able to improve the BLS production.

The cell-free supernatant of *E. coli* culture caused no increase the antimicrobial activity of *B. amyloliquefaciens* LBM 5006, suggesting that this effect was not mediated by a metabolite secreted by the inducer strain.

Bacteriocin production by *Lactobacillus plantarum* J23 was detected only when it was co-cultivated with some inducing bacteria. Induction took place

when intact or mild-heat-treated cells of the inducers were co-cultivated with J23, but not with their autoclaved cells and cell-free supernatants [20]. The increased activity of bacteriocin associated with cells of another organism, especially treated by lethal heat, was also observed in other studies. Kimchicin GJ7 production was enhanced in the presence of *Lactobacillus plantarum* KFRI 464, and the cell debris fraction of *Lactobacillus plantarum* KFRI 464 showed much higher inducing activity than the intracellular fraction [5]. Furthermore, extracellular fractions did not show any inducing activity. The maximum enhancement of bacteriocin production by *Leuconostoc citreum* GH7 was obtained in the presence of thermally inactivated cells (121℃/15 min) of *Lactobacillus plantarum* KFRI 464, probably activated by cell-to-cell contact [5]. The production of lactacin B by *Lactobacillus acidophilus* N2 increased significantly when it was grown with living, washed, or killed cells of *L. delbrueckii* subsp*. lactis* ATCC 4797, suggesting that the inducer substance is cell associated and could be located on the cell envelope [1]. Despite the investigation on lactic acid bacteria, similar studies on *Bacillus* species are very scarce. The experiments presented here suggest that the increased activity could be stimulated by component of cell surface of *E. coli*.

Although it is unknown how the inducer strain *E. coli* and *B. amyloliquefaciens* LBM 5006 interact to stimulate the synthesis of antimicrobials, bacteria must integrate extra- and intracellular signaling information to develop appropriate responses to changes in their environment.

In response to the presence of other bacteria certain strains can trigger an auto-induction mechanism, which probably is involved in regulating the

production of antimicrobial peptides [24]. Control of gene expression in mixtures of different species of bacteria showed that *E. coli* produces a small-molecule quorum-sensing autoinducer called AI-2 that stimulate nearby bacterial species to start a quorum sensing controlled behavior in response to cumulative cell number [12,17].

The competition for nutrients from the environment leads the microorganisms to develop different strategies, one of which is the production of antimicrobial substances induced by peptides, a phenomenon extensively studied in lactic acid bacteria that are competing with other bacteria [14]. The synthesis of bacteriocins can be stimulated when a producer strain recognizes a common substance (inducing factor) present in a sensitive strain [8,18].

In the case of *B. amyloliquefaciens* LBM 5006, it has been demonstrated that the presence of *E. coli* in the same environment enhances the production of antimicrobial activity. The intensity of this effect was highest when thermally inactivated cells of *E. coli* were added. During heating, some components that may act as inducers could be exposed and/or solubilized, then causing an increased inducible effect. According to SDS-PAGE analysis the inhibition zone increased and additional bands were observed in the presence of *E. coli*, suggesting that the synthesis of additional peptides was induced. The enhanced synthesis of antimicrobial peptides may be an efficient strategy for defense against competing microorganisms.

3.1.7 References

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Table 1. Antimicrobial activity of *Bacillus amyloliquefaciens* LBM 5006 against indicator bacteria

Indicator bacteria	Inhibitory activity (mm)*
Gram positive	
Bacillus cereus (ATCC 9634)	12.6 ± 1.1
Bacillus subtilis (ATCC 7972)	13.0 ± 1.0
Staphylococcus aureus (ATCC 25923)	0
Staphylococcus aureus (ATCC 9801)	7.3 ± 0.5
Listeria monocytogenes (ATCC 7644)	15.3 ± 0.5
Listeria inoccua (ATCC 33090)	6.0 ± 1.0
Leuconostoc mesenteroides (sugar cane isolate)	10.6 ± 1.1
Corynebacterium fimi ((NCTC 7547)	12.6 ± 1.5
Gram negative	
Enterococcus faecalis (clinical isolate)	0
Escherichia coli ATCC 25922	12,5 ± 0,5
Escherichia coli (food isolate)	11.0 ± 1.0
Escherichia coli (ATCC 8739)	8.0 ± 1.0
Proteus vulgaris (IAL 1016)	7.6 ± 0.5
Pseudomonas aeruginosa (clinical isolate)	0
Pseudomonas fluorescens (food isolate)	6.3 ± 0.5
Salmonella choleraesuis (food isolate)	8.0 ± 1.0
Salmonella Gallinarum (food isolate)	10.0 ± 1.0
Serratia marcescens (CDC 4120)	7.6 ± 0.5
Serratia marcescens (food isolate)	8.0 ± 1.0

* Each value is the mean of 3 replicates of the diameter (mm) of the inhibition zone in the bacterial layer. Variance is given as the standard error of the mean. ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; IAL, Instituto Adolfo Lutz.

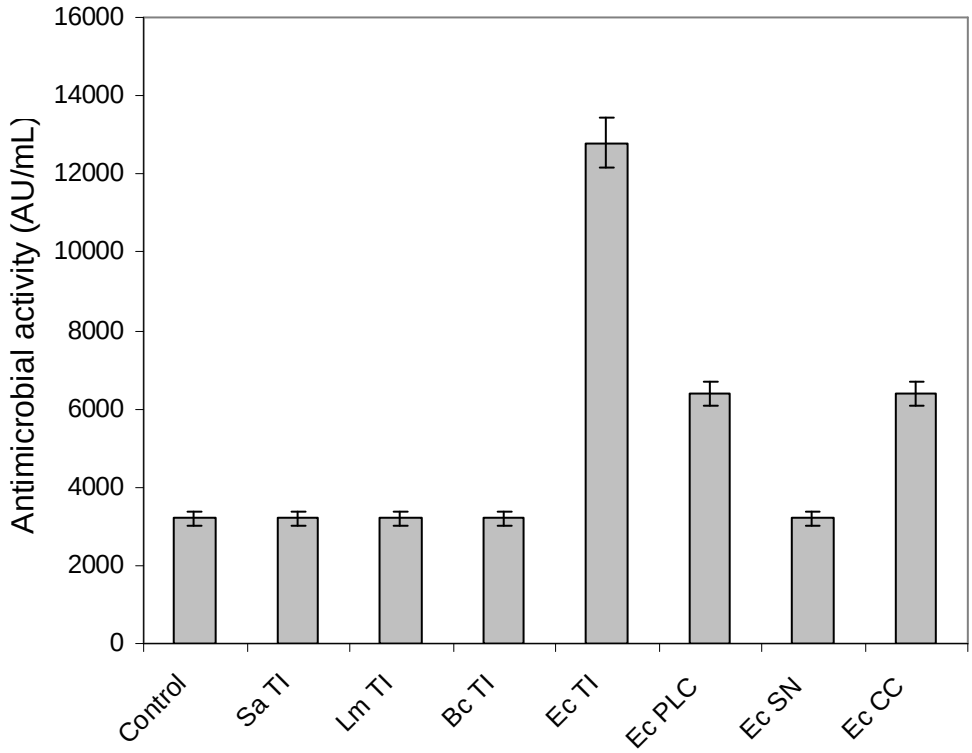

Fig. 1 Production of antimicrobial activity by *Bacillus amyloliquefaciens* LBM 5006 in the presence of thermally inactivated (TI) cells of *Staphylococcus aureus* (Sa), *Listeria monocytogenes* (Lm), *Bacillus cereus* (Bc) or *Escherichia coli* (Ec). Alternatively, *B. amyloliquefaciens* was cultivated in the presence of culture supernatant (SN), pellet of living cells (PLC), or co-cultivation (CC) with *E. coli*.

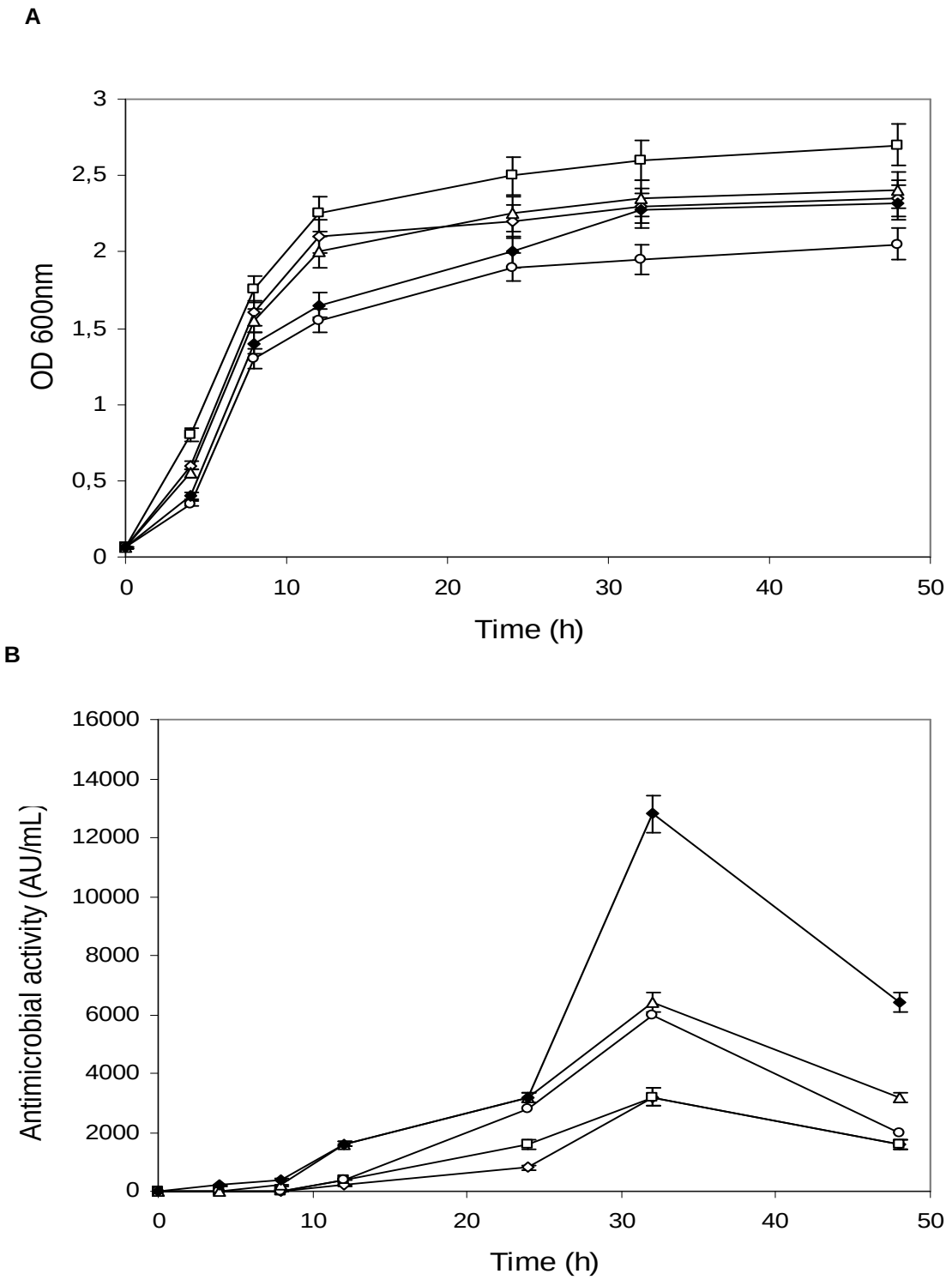

Fig. 2 Growth (**A**) and production of antimicrobial activity (**B**) by *Bacillus amyloliquefaciens* LBM 5006 in the presence of *E. coli*. Cultivation of *B. amyloliquefaciens* was performed in BHI medium alone (□, control), or in the presence of (♦) thermally inactivated cells, (◊) cell-free supernatant, (Δ) pellet of living cells, (○) co-culture of strains. Results are the means of three independent experiments.

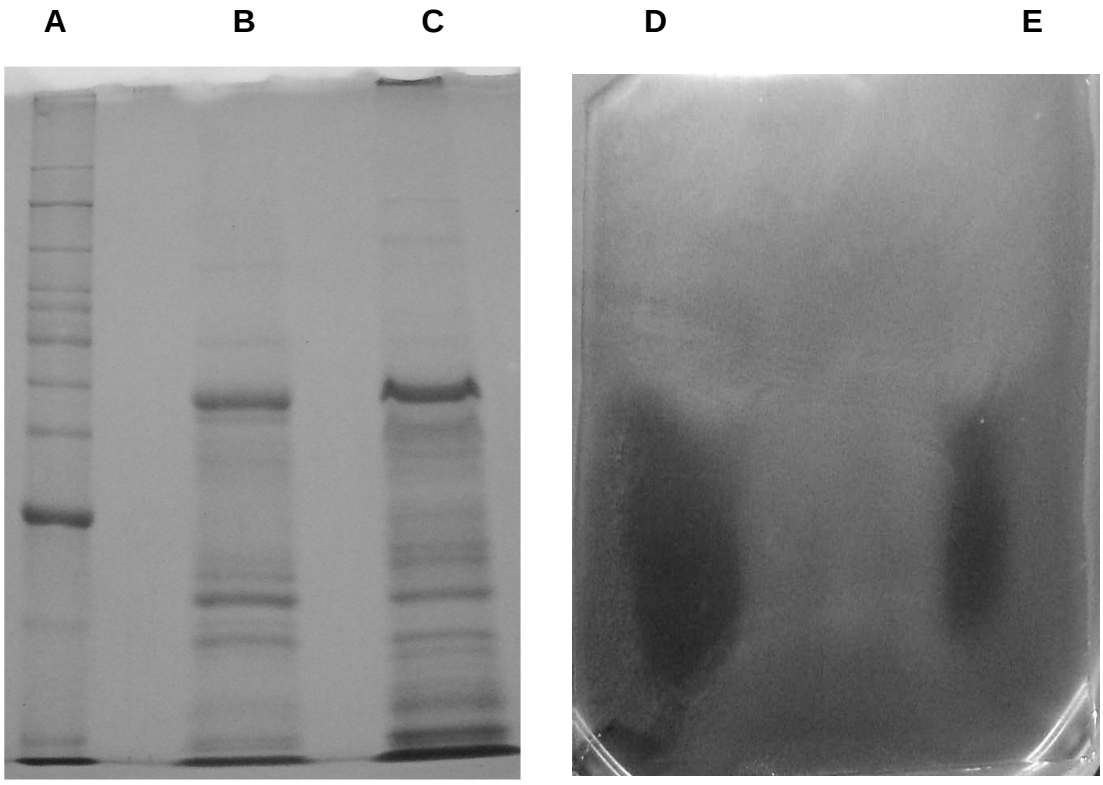

Fig. 3 Analysis of the culture supernatants of *Bacillus amyloliquefaciens* LBM 5006 by SDS-PAGE. Comassie brilliant blue staining (lanes B,C) and antimicrobial activity (lanes D,E) of culture supernatants of *B. amyloliquefaciens* in BHI (lanes B,E) or BHI containing cell debris of *E. coli* (lanes C,D). Lane A, molecular mass standards.

3.2 ARTIGO 2

3.2.1 Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM 5006

3.2.2 Abstract

Bacillus amyloliquefaciens LBM 5006 produces antagonistic activity against phytopathogenic fungi, including *Cercosporina sojina*, *Fusarium oxysporum* and *Bipolaris sorokiniana*. PCR analysis revealed the presence of *ituD*, but not *sfp* genes, coding for iturin and surfactin, respectively. The antimicrobial substance produced by this strain was isolated by ammonium sulfate precipitation, gel filtration chromatography and 1-butanol extraction. The ultraviolet spectrum was typical of a polypeptide and the infrared spectrum indicates the presence of peptide bonds and acyl group(s). The antimicrobial substance was resistant to proteolytic enzymes and heat treatment, and was reactive with ninhidrin. Mass spectroscopy analysis indicated that *B. amyloliquefaciens* LBM 5006 produces two antimicrobial peptides, with main peaks at *m/z* 1058 Da and 1464 Da, corresponding to iturin-like and fengycin-like peptides, respectively. *B. amyloliquefaciens* LBM 5006 showed significant activity against phytopatogenic fungi, showing potential for use as a biocontrol agent or production of antifungal preparations.

Keywords: *Bacillus amyloliquefaciens*; bacteriocin; antifungal activity; lipopeptide

3.2.3 Introduction

Plant diseases caused by viruses, bacteria and fungi affect crops, and are responsible for significant losses or decrease the quality and safety of agricultural products. Their control relies mainly on chemical pesticides (Agrios, 2005). Because of their rapid bactericidal and/or fungicidal activity over a broad spectrum and the low propensity of microorganims to develop resistance against them, the phenomenon of microbial antagonism mediated by peptides has gained more attention in recent years. This antimicrobial arsenal has been isolated from almost all species - microorganisms, plants, insects and other invertebrates, fish, amphibians, birds and mammals - and it has been proposed that they represent components of their immediate nonspecific defense against invading bacteria, enveloped viruses and fungi (Ajesh and Sreejith, 2009; Hancock and Diamond, 2000).

Antimicrobial peptides are widespread produced among diverse bacteria (Riley and Wertz, 2002). Although those produced by lactic acid bacteria have been extensively studied because their potential use in food industry (O'Sullivan *et al*., 2002), interesting antimicrobial peptides are produced by other classes of bacteria such as *Bacillus* spp. (Motta *et al*., 2007) and coryneform bacteria (Motta and Brandelli, 2009). The genus *Bacillus* includes varied industrially important species and has a historical of safe use. The production of antimicrobial peptides has also been described for this genus, and subtilin, subtilosin A, iturin A, bacilysin, rhizocticin and surfactin are some of these substances (Le Marrec *et al*., 2000; Pabel *et al*., 2003; Romero *et al*., 2004). Many strains of *Bacillus amyloliquefaciens* are known to suppress fungal

and bacterial growth *in vitro* by the production of several antimicrobial compounds (Yoshida *et al*., 2001; Caldeira *et al*., 2008; Sutyak *et al*., 2008).

The antifungal activity of *Bacillus subtilis* and *B. amyloliquefaciens* can be attributed, to a certain extent, to production of iturins (Tsuge *et al*., 2001). Iturins are heptapeptides linked to a β-amino fatty acid and iturin A shows a strong antibiotic activity with a broad antifungal spectrum, making it an ideal potential biological control agent with the aim of reducing the use of chemical pesticides in agriculture (Maget-Dana and Peypoux, 1994). Gene clusters involved in iturin synthesis have been investigated (Tsuge *et al*., 2001; Yao *et al*., 2003). The iturin A operon spans a region more than 38 kb long and is composed of four open reading frames: *ituD, ituA, ituB,* and *ituC*. The *ituD* gene encodes a putative malonyl coenzyme A transacylase, whose disruption results in a specific deficiency in iturin A production (Tsuge *et al*., 2001). Thus, the *ituD* gene play leading role in the production of iturin.

The environment is the main source for new microorganisms with potential industrial or commercial value. The Brazilian Atlantic forest is an ecosystem with enormous biological diversity. The forest has been reduced to less than 10% of its original size, but many efforts are now being made to conserve the remaining biodiversity of this region (Matos and Bovi, 2002). However, there are only a few studies on the microbial diversity of this region (Nascimento *et al*., 2003; Evans *et al*., 2004). The aim of this work was the purification and identification of antimicrobial peptides produced by *Bacillus amyloliquefaciens* LBM 5006 isolated from soil of Atlantic Forest and test then against plant fungi.

3.2.4 Materials and methods

3.2.4.1 Microorganisms

The producer strain *Bacillus amyloliquefaciens* LBM 5006, was isolated and characterized as described elsewhere (Lisboa *et al*., 2006). Other bacterial strains were *Bacillus subtilis* ATCC 19659 and *Listeria monocytogenes* ATCC 7644. The strains were maintained at -21ºC as frozen stocks in brain heart infusion broth (BHI; Oxoid, Basingstoke, UK) containing 20% (v/v) glycerol and propagated twice in the same medium at 37℃ bef ore use. Indicator fungal strains used in the antagonist assays are listed in Table 1. Fungi were maintained on potato dextrose agar (PDA; Biobras, Montes Claros, Brazil) slants at 4ºC.

3.2.4.2 Fungal suspension and antifungal assays

Strain LBM 5006 was tested for its ability to inhibit the growth of several fungal plant (Table 1). Fungal isolates were inoculated on plates with PDA for 7 days and 12 hours photoperiod. Aliquots of 3 mL of saline solution were poured on each of the colonies and the spores removed with the aid of a bacteriological loop. The suspension was collected and transferred to a sterile tube. The number of conidia was performed in a Neubauer chamber and the concentration adjusted for 5 x 10^3 conidia/mL. One mL of this suspension was added to 99 mL of sterile PDA at 45ºC and homogenized (Leifert *et al*., 1995). The strain *Bacillus amyloliquefaciens* LBM 5006 was inoculated with the aid of a needle in 4 points for plate previously prepared with the fungal inoculum. The inhibition zones were measured after five days of incubation to 25 ± 2ºC and 12

h photoperiod. The isolates that showed inhibition zones were selected to additional tests with the extracts obtained from *B. amyloliquefaciens* LBM 5006.

The assay for antifungal activity with culture supernatants and purified substance was carried out on PDA plates (Ye *et al*., 1999). Around the central disk (0.5 cm in diameter) sterile paper disks were placed at 1 cm from it. Aliquots (10 µL) of the test samples were added to the disks. The plates were incubated at 30ºC for 48 h until mycelial growth from the central disk had enveloped peripheral disks containing the negative control (phosphate buffered saline) and had formed a crescent-shaped zone of inhibition around disks containing samples with antifungal activity.

3.2.4.3 PCR Analysis

DNA was extracted from overnight cultures of *B. amyloliquefacies* LBM 5006 and *B. subtilis* ATCC 19659 using the Promega Wizard SV Genomic DNA kit (Promega Corp., Madison, WI, USA). Specific primers for the functional genes of the bacteriocins iturin A and surfactin are listed in Table 2. PCR was conducted using an Mastercycler® Personal Eppendorf (Eppendorf AG, Hamburg, Germany) under the following parameters: denaturation for 1 min at 94℃, annealing for 1 min at 50℃, and elongation f or 1.5 min at 72℃ for a total of 30 cycles for iturin A and denaturation for 1 s at 94℃, annealing for 30 s at 46℃, and elongation for 1 min at 72℃ for a total of 25 cycles for surfactin.

3.2.4.4 Sequencing methods

PCRs products were sequenced in the ACTGene Laboratory (*Centro de Biotecnologia*, UFRGS, Porto Alegre, Brazil) using the automatic sequencer ABI-PRISM 100 Genetic Analyzer armed with 50 cm capillaries and POP6 polymer (Applied Biosystems). Sequencing data were collected using the software Data Collection v1.0.1 (Applied Biosystems). The BLAST algorithm was used to retrieve for homologous sequences in GenBank (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) using the software CLUSTAL W, version 1.8 (Thompson *et al*., 1994). The 16S rDNA sequence obtained for *B. amyloliquefaciens* LBM 5006 (1499 bp) has been submitted to GenBank under the accession number GU368920.

3.2.4.5 Production, isolation and purification of antimicrobial substance

For the production of antimicrobial substance, the strain LBM 5006 was grown in 200 mL of BHI medium at 37℃ in a rotary shaker at 125 cycles min^{-1} for 48 hours. After cultivation the cells were harvested by centrifugation at 10,000 x *g* for 15 min, the culture supernatant was sterilized by filtration with 0.22 µm membranes (Millipore, Bedford, USA). The filtrate was precipitated with ammonium sulfate at 20% (w/v) saturation and the resulting pellet was dissolved in 10 mM phosphate buffer pH 6.0. This solution was further purified by gel filtration chromatography on Sephadex G-100 column eluted with this same buffer and the fractions showing antimicrobial activity were pooled and freeze-dried. Fractions were also monitored for A280 nm using a spectrophotometer (UV-mini 1240, Shimadzu, Tokyo, Japan). This material was

dissolved in 10 mM phosphate buffer pH 6.0 and then extracted twice with 1-butanol. The organic phases were combined and evaporated under reduced pressure. The resulting powder was suspended in 10 mM phosphate buffer pH 6.0. During purification the antimicrobial activity (AU/mL) was quantified as described elsewhere using *L. monocytogenes* ATCC 7644 as indicator organism (Motta and Brandelli 2002), and antifungal activity was confirmed against *Fusarium oxysporum.*

3.2.4.6 Analytical methods

Soluble protein concentration was determined by the Folin phenol reagent method (Lowry *et al*., 1951). Concentration of free amino groups was determined colorimetrically by the ninhidrin method (Moore and Stein, 1957).

3.2.4.7 Effects of enzymes and heat on antimicrobial activity

Samples of the antimicrobial substance were treated at 37℃ for 1 h with 2 mg ml^{-1} final concentration of the following enzymes: papain, trypsin, proteinase K and pronase E. Samples were then boiled for 2 min to inactivate the enzyme. To analyze thermal stability, samples were exposed to temperatures ranging 25 to 90℃ for 30 min, and 121 ℃/141 kPa for 15 min. After the treatments, the samples were tested for antimicrobial activity against *L. monocytogene*s ATCC 7644 and *F. oxysporum*.

3.2.4.8 Spectroscopy

The ultraviolet absorbance spectrum was recorded using a Shimadzu UV1601PC double bean spectrophotometer (Tokyo, Japan), from 190 to 600 nm at 0.5 nm resolution. The infrared spectrum was measured as a potassium bromide pellet. Four scans of the sample were taken using a Mattson 3020 FITR spectrophotometer (Madison, USA).

Mass spectrometry analysis was performed after the peptides were eluted from a C18 chromatographic resin (Vydac, USA). The column was eluted with 80% acetonitrile 0.046% TFA and concentrated in a vacuum centrifuge (SpeedVac SC100, Savant, USA). The sample was analyzed in a MALDI-TOF mass spectrometer (Ettan MALDI-TOF ProSystem, Amersham Biosciences, Sweden) operating in reflection mode and using a matrix of α-ciano-4-hydroxycinnamic acid.

3.2.5 Results

3.2.5.1 Antifungal activity of *Bacillus amyloliquefaciens* LBM 5006

Spectrum of antifungal activity of LBM 5006 strain was determined based on the degree of growth inhibition of plant fungi by direct antagonism on agar plates. In this experiment, LBM 5006 showed inhibition against nine of the twelve tested fungi. There was no activity against *Phomopsis*, *Rhizoctonia* and *Verticillium* sp. (Table 1).

The antifungal activity of culture supernatants of *B. amyloliquefaciens* LBM 5006 was also tested against the indicator fungi. The inhibitory activity of

the filter-sterilized crude supernatant was observed on 60% of the strains tested (Table 1).

3.2.5.2 Identification of genes related to antimicrobial peptides

PCR analysis of the *Bacillus amyloliquefaciens* LBM 5006 showed that the strain exhibited potential for the functional gene-encoding malonyl Coa transacylase (*ituD)* but not for the putative transcription terminator gene (*sfp*) (Fig. 1). The DNA sequence of the amplified PCR product was determined. Sequences of 1203 bp fragments showed elevated similarity (minimum identity 98%) with the gene-encoding malonyl CoA transacylase (*ituD*), and only point mutations were observed.

3.2.5.3 Isolation and characterization of antimicrobial substance

The antimicrobial activity produced by *B. amyloliquefaciens* was purified from the culture supernatant. The purification steps and the recovery values are summarized in Table 3. The final specific activity of the antimicrobial substance was increased approximately 18-fold compared to that in the culture supernatant and the recovery was 4%. The purified substance inhibited growth of all tested fungal plant pathogens (Table 1), as illustrated in Figure 2 for *Fusarium oxysporum*.

The antimicrobial activity was stable in the pH range from 2 to 11, was resistant to heating at temperatures higher than 80°C for 30 min, and by treatment with all the proteolytic enzymes tested. The substance was reactive with ninhidrin.

The UV absorption spectrum of the purified substance was examined

between 190 and 340 nm. The purified peptide showed maxima absorbance at 198 and 210 nm, and there is no appreciable absorbance above 300 nm (Fig. 3A). Minor absorbance peak was also observed at 270 nm. Intense signal was observed at lower than 210 nm, typical of protein absorption. A shoulder at 220 nm is corresponding to characteristic absorption of peptide bonds.

The infrared spectrum is shown in Fig. 3B. Characteristic absorption bands at 1553, 1653 and 3250 cm^{-1} indicate that the substance contains peptide bonds. Bands that result from C-H stretching (2950, 2850, 1454, 1428 cm^{-1}) indicate the presence of aliphatic chain(s). A lactone ring is suggested by the absorption at 1740 cm^{-1}.

In order to determine accurately the molecular mass of the antimicrobial peptide, mass spectroscopy analysis was carried. The mass spectrum revealed two clusters with major peaks at *m/z* 1058 Da and 1464 (Fig. 4). The cluster of 6 peaks that were observed at *m/z* 1506, 1492, 1477, 1464, 1450 and 1436, differed by 14 Da each other. The other cluster showed peaks at *m/z* 1090, 1074, 1058, 1044, 1036 and 1022.

3.2.6 Discussion

The soil isolate *B. amyloliquefaciens* LBM 5006 produces an antimicrobial substance which inhibits several pathogenic and food-spoilage bacteria, such as *Listeria monocytogenes*, *Bacillus cereus*, *Serratia marcescens*, and *Pasteurella haemolytica* (Lisboa *et al*., 2006). In this study the antimicrobial activity of strain LBM 5006 was observed against most fungi tested, implying it has great potential for biological control of many fungal plant

diseases. The potential of some strains of *Bacillus* spp. to synthesize a wide variety of metabolites with antifungal and/or antibacterial activity has been described (Souto *et al*., 2004; Chen *et al*. 2008).

Antimicrobial activity from *B. amyloliquefaciens* LBM 5006 was isolated by gel filtration chromatography and 1-butanol extraction. The substance eluted near the void volume of Sephadex G-100 column, contrasting with the lower MW observed by mass spectroscopy. Discrepancy in MW determination has been shown for some bacteriocins and has been associated to the strong hydrophobic nature of the peptides (Oscáriz and Pisabarro, 2000). This behaviour is similar to linocin M18 (Valdés-Stauber and Scherer, 1994) and iturin A_2 (Yu *et al*., 2002).

The ultraviolet and FITR spectra of the purified antimicrobial substance offer valuable information about its structure. The ultraviolet spectrum is compatible with a polypeptide. In agreement, most peptide antibiotics produce the characteristic absorbance pattern of absorbance maxima at 210-230 and 270-280 nm (Kurusu and Ohba, 1987).

Analysis of the FTIR spectrum shown typical absorption bands corresponding to N-H stretching of proteins and peptide bonds (Maquelin *et al*., 2002), concrete evidence that the substance contained peptides in its structure. The presence of an absorption band at 1653 cm^{-1} associated with absorption between 3500 and 3200 cm^{-1} indicate amide functional group. The band at 1653 cm^{-1} associated also with the band at 2925 cm^{-1} indicate the carboxyl group (Maquelin *et al*., 2002). The presence of carboxyl and amino groups indicate the existence of amino acids, suggesting a polypeptide structure. Two

prominent spectral bands in the region between 1800 and 1500 cm^{-1} corresponded to amide I (1653 cm^{-1}) and amide II (1553 cm^{-1}) (Fabian et al., 1993). The first band arose from the vibrational stretching of the chain of C=O group and the second doubt to the vibration of the bond N-H strongly linked to the stretching of C-N bond. The absorption in the region between 1600 and 1700 cm^{-1} appears, mainly, doubt to the vibrational stretching of carbonyl group of peptide bonds (Gaussier *et al*., 2003). Additional absorption valleys indicating aliphatic chains may be related with predominance of hydrophobic amino acids such as Val, Leu, Ile, or it contains a fatty acid in their structure. Antimicrobial activity of lipopeptides produced by *Bacillus* spp. has been described (Yakimov *et al*., 1995; Souto *et al*., 2004). Such peptides belong to a subclass of peptolides, cyclic peptides that contain fatty acids, often presenting less than 10 amino acids and molecular mass as lower as 1500 Da (Vater *et al*. 2002). In addition, there is evidence that a lactone ring is present in this case, typical of fengycin families of lipopeptides.

MALDI-TOF has been used as an efficient tool for identification of antimicrobial peptides, including hydrophobic lipopeptides in the range of 1-1.5 kDa (Stein, 2008). The analysis of the mass spectrum of antimicrobial peptides of *B. amyloliquefaciens* LBM 5006 indicated that the peak at *m/z* 1464 correspond to C16-fengycin A (Hu *et al*., 2007). These peaks differ by 14 Da, suggesting a series of homologous molecules or fragments having different length of fatty acid chain (CH_2 = 14 Da). The peak at *m/z* 1506 matches with C17-fengycin B, which has the amino acid Val instead Ala at position 6 (Hu *et al*., 2007). The main peak at *m/z* 1058 Da agrees with iturin E (Besson and

Michel, 1987) and isomers of iturin A described for *B. amyloliquefaciens* RC-2 (Hiradate *et al*., 2002) and *B. amyloliquefaciens* CCMI 1051 (Caldeira *et al*., 2008), and the surround peaks were deduced to be isoforms with different fatty acid side chains. This *m/z* is also close to C15-bacillomycin L $[M+Na]^+$ and C15-surfactin $[M+Na]^+$ (Stein, 2008), although the strain LBM 5006 lacks the functional *sfp* gene to produce surfactin. Positive reaction with ninhidrin also indicate free amino groups in the constituent amino acids, like Asn and Gln that are present in iturin A and fengycin, respectively. These results suggest that antifungal activity of strain LBM 5006 is associated with the lipopeptides iturin and fengycin. The co-production of different lipopeptides by *Bacillus* spp. difficult the purification, and the structural variability makes it complicated to definitively identify different families of lipopeptides and their homologues (Akpa *et al*. 2001; Caldeira *et al*., 2008; Chen *et al*. 2008).

The presence of gene *ituD*, related to production of iturin, and absence of the surfactin-related gene *sfp*, was observed in *B. amyloliquefaciens* LBM 5006. Both peptides are synthesized by the action of large multienzyme complex, and these genes are essential to their respective production (Schneider *et al*., 1998; Stein, 2005). *B. amyloliquefaciens* is known to produce iturins, a family of cyclic lipopeptide antibiotics (Hiridate *et al*. 2002). *B. amyloliquefaciens* and *B. subtilis* are closely related and a diversity of lipopeptide antibiotics, such as surfactins, iturins, bacillomycins and mycosubtilin, are produced by *B. subtilis* (Stein, 2005), whose production is thought to be under complex regulation (Duitman *et al*., 1999; Yao *et al*., 2003).

Our results indicate that *B. amyloliquefaciens* LBM5006 produces a

mixture of antimicrobial peptides during cultivation in BHI broth as well.

Iturin A production is often an important factor conferring antifungal activity to the *Bacillus* strains. Iturin and fengycin lipopeptides have been assigned as key factors in antagonism of *B. subtilis* toward *Podosphaera fusca*, an ectoparasite causing powdery mildew in many cucurbits (Romero *et al*., 2007). In our study, *B. amyloliquefaciens* LBM 5006 showed inhibitory activity against *Aspergillus phoenicis, Bipolaris sorokiniana, Fusarium oxysporum f. licopersici*, among others, suggesting that the broad inhibitory activity observed may be associated to the action of iturin-like and fengycin-like peptides.

The development of fungicide-resistant strains of pathogens, the detection of undesirable chemical residues in the food chain and deregistration of some of the most effective fungicides have intensified the search for safer approaches to efficiently control microbial infections (Winteringham, 2008). Among the alternatives, biological control through the use of natural antagonistic microorganisms has been extensively studied and some *Bacillus* strains have been shown to be effective against various pathogens (Yu *et al*., 2002). Antimicrobial peptides produced by *Bacillus* spp. have been proposed as biocontrol agents against several phytopathogenic microorganisms (Bais *et al*., 2004; Cladera-Olivera *et al*., 2006). To sum up, this study identified a novel isolate showing significant activity against phytopatogenic fungi, showing potential use for the production of antifungal preparations.

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Table 1. Antifungal activity of *Bacillus amyloliquefaciens* LBM 5006

Indicator fungi	Antifungal activity [a]		
	Direct antagonism	Crude supernatant	Purified
Aspergillus niger ATCC 16404	7.0 ± 0.8	10.5 ± 0.7	10.0 ± 0
Aspergillus phoenicis	13.5 ± 1.7	0.0	16.5 ± 0.7
Aspergillus flavus	13.5 ± 2.3	13.5 ± 2.1	14.0 ± 2.8
Apiosordaria sp. [b]	16.0 ± 0.8	13.5 ± 2.1	13.0 ± 1.4
Bipolaris sorokiniana	22.2 ± 1.7	13.5 ± 2.1	15.0 ± 1.4
Cercosporina sojina	21.0 ± 1.1	21.0 ± 1.4	17.5 ± 3.5
Diplodia sp.	6.0 ± 0.8	15.5 ± 0.7	15.0 ± 1.4
Fusarium oxysporum f. licopersici	20.5 ± 2.9	0.0	15.5 ± 0.7
Fusarium graminearum	24.5 ± 1.3	0.0	12.5 ± 0.7
Phomopsis sp.	0.0	ND	ND
Rhizoctonia sp.	0.0	ND	ND
Verticillium albatrum	0.0	ND	ND

[a] Values are the means ± s.e.m. of 3 independent determinations of the inhibition zone (mm). ND, not determined.
[b] *Apiosordaria* sp., *Diplodia* sp., *Phomopsis* sp., *C. sojina*, *F. graminearum*, and *V. albatrum* were gently given by MSc. Nelson Neto, UNICRUZ (Universidade de Cruz Alta, Brazil); other strains were from our culture collection.

Table 2. Primers for the functional genes of iturin A and surfactin.

Bacteriocin	Primer	Sequence (5’ - 3’)	Reference
Iturin A	*ituD-f*	ATGAACAATCTTGCCTTTTTA	Hsieh *et al.,* 2008
	ituD-r	TTATTTTAAAATCCGCAATT	
Surfactin	*sfp-f*	ATGAAGATTTACGGAATTTA	Hsieh *et al.,* 2004
	sfp-r	TTATAAAAGCTCTTCGTACG	

Table 3. Purification of antimicrobial activity of *B. amyloliquefaciens*.

Purification step	Total protein (mg)	Total activity (AU)	Specific activity (AU mg^{-1})	Purification fold	Yield (%)
Crude supernatant	320	80,000	250	1.0	100
$(NH_4)_2SO_4$ precipitation	3.7	12,800	3459	13.8	16
Sephadex G-100 chromatography	3.6	12,800	3555	14.2	16
1-Butanol extraction	0.7	3,200	4571	18.3	4

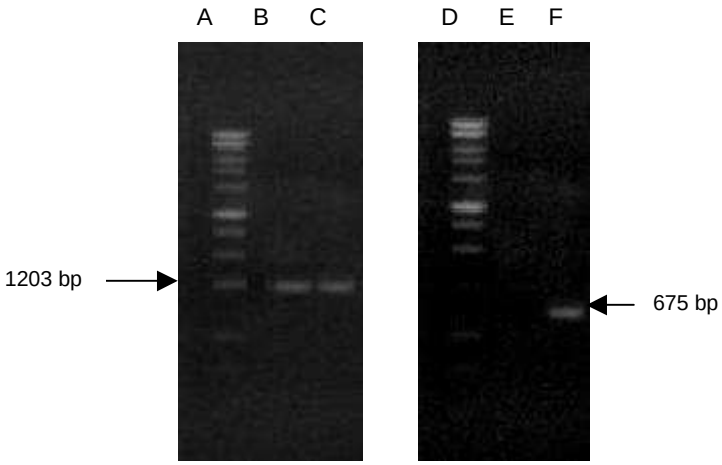

Fig. 1 PCR product profiles of representative samples tested. Molecular weight marker (lanes A and D); gene *ituD* for *B. amyloliquefaciens* LBM 5006 (lane B) and *B. subtilis* ATCC 19659 (lane C); gene *sfp* for *B. amyloliquefaciens* LBM 5006 (lane E) and *B. subtilis* ATCC 19659 (lane F).

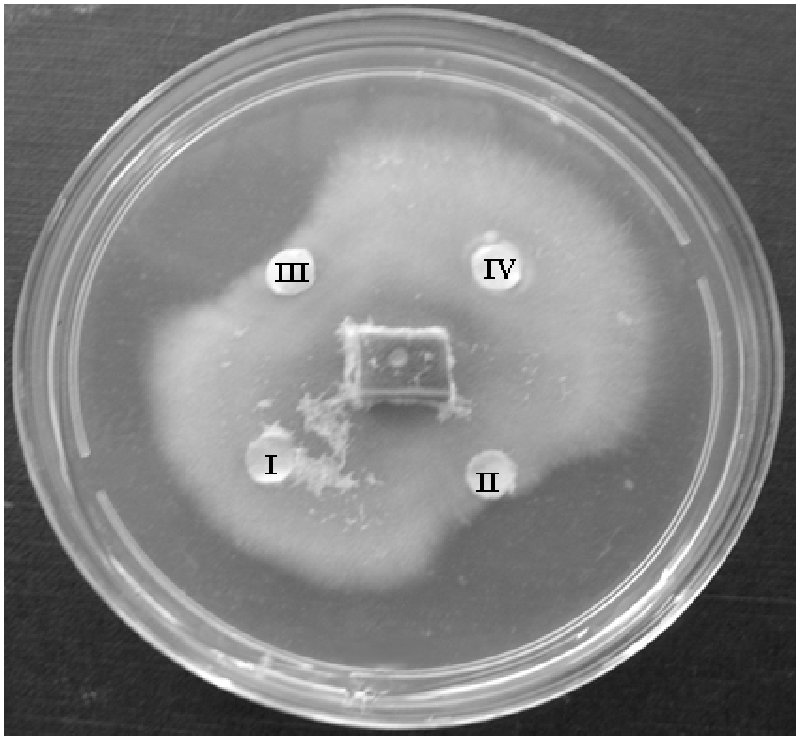

Fig. 2 Inhibitory activity of *Bacillus amyloliquefaciens* LBM 5006 against *Fusarium oxysporum f. licopersici*. (I) Crude supernatant, (II) partially purified, (III) purified, and (IV) negative control (phosphate buffered saline).

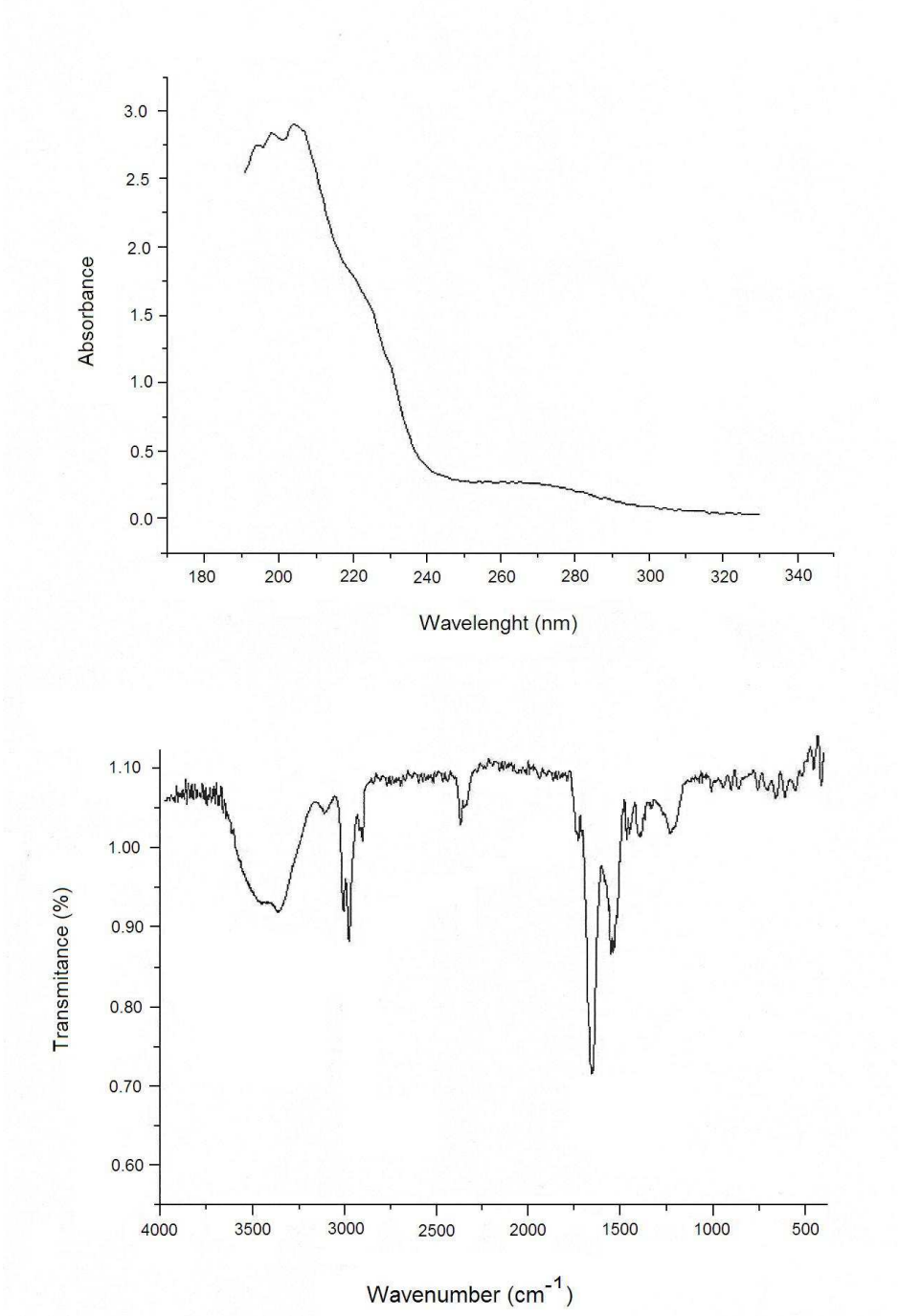

Fig. 3 Ultraviolet (A) and FTIR (B) spectra of purified antimicrobial substance produced by *B. amyloliquefaciens* LBM 5006.

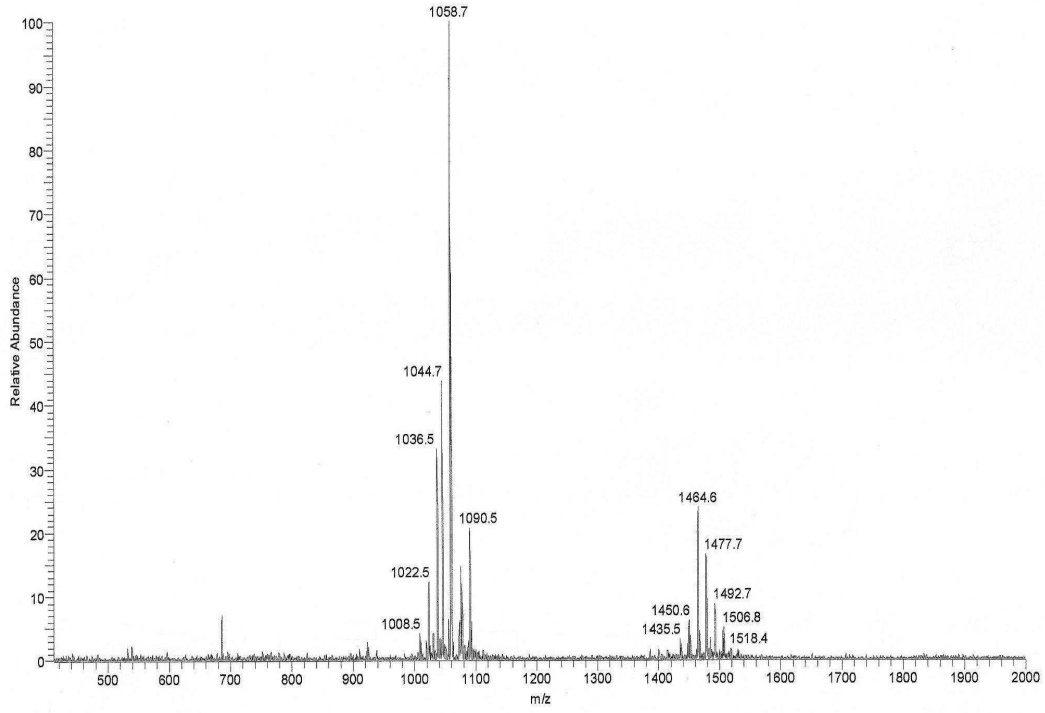

Fig. 4 MALDI-TOF spectrum of purified antimicrobial substance produced by *B. amyloliquefaciens* LBM 5006.

3.3 ARTIGO 3

3.3.1Effect and mode of action of the *Bacillus amyloliquefaciens* LBM 5006 anti-listerial bacteriocin-like substance

3.3.2 Abstract

Bacillus amyloliquefaciens LBM 5006 is the producer of a bacteriocin-like substance (BLS) with inhibitory activity against pathogenic and food spoilage bacteria. In this paper, the effect and the mode of action of this antimicrobial peptide was investigated against *Listeria monocytogenes* ATCC 7644. Exposure of cell suspensions of *L. monocytogenes* to the BLS caused extensive cell viability loss and reduction in optical density (OD) associated with cell lysis. Scanning electron microrscopy showed changes in cell morphology and topography associated with the damages inflicted in the cell envelope. The BLS was bacteriolytic and bactericidal to *L. monocytogenes.* The understanding of the mode of action of the bacteriocin would be important to allow an effective utilization of this antimicrobial substance.

Keywords: Mode of action; Bacteriocin; *Bacillus amyloliquefaciens*; *Listeria monocytogenes.*

3.3.3 Introduction

Production of antimicrobial peptides is found as a widespread strategy used by plants, animals and microorganisms to combat pathogenic microorganism. These peptides are divided in several groups according their molecular masses, secondary and tertiary structures, presence or absence of disulfide bridges (Hwang & Vogel, 1998; Reddy et al., 2004; Cotter et al., 2005). The mode of action of antimicrobial peptides is diverse, and many studies have demonstrated that antimicrobial peptides kill target cells by membrane permeabilization through peptide-lipid interactions. Various mechanisms have been proposed, including the formation of the channels that dissipate ion gradients across the membrane, phase separation due to specific peptide- lipid interaction, disturbance of the lipid bilayer as a result of carpet- like peptide binding, and detergent- like solubilization of the membrane (Bauer & Dicks, 2005; Ladokhin & White, 2001; Shay, 1999; Matsuzaki, 1998).

The genus *Bacillus* includes several industrially important species and has a historical of safe use in foods and industry. Enzymes, antibiotics, amino acids and antifungal peptides are part of the many commercially available products from *Bacillus* spp. The production of bacteriocins or bacteriocins-like substances (BLS) has been also described for this genus (Romero et al., 2004; Marrec et al., 2000; Klein et al., 1993).

The Brazilian Atlantic forest contains an enormous biological diversity (Matos & Bovi, 2002). There are only a few studies on the microbial diversity of this region (Evans et al., 2006; Nascimento et al., 2003) but no specific reports on screening for biotechnologically relevant microorganisms and theyr mode of

action. *Bacillus amyloliquefaciens* LBM 5006, isolated from the soil of the Atlantic Forest, produces a BLS that inhibits several pathogenic and food spoilage microorganisms (Lisboa et al., 2006). In this paper, the effect and the mode of action for this BLS has been studied with *Listeria monocytogenes*.

3.3.4 Materials and methods

3.3.4.1 Bacterial strains

The microorganism *B. amyloliquefaciens* LBM 5006 was used for peptide LBM 5006 production (Lisboa et al., 2006). The indicator strain was *L. monocytogenes* ATCC 7644. Other strains were obtained from ATCC collections and our own culture collections (UFRGS, Porto Alegre, Brazil) (Table 1). The cultures of the test microorganisms were stored at –20 ºC in BHI broth (Oxoid, Basingstore, UK) containing 20% (v/v) glycerol and propagated twice in the same medium at 37℃ before use.

3.3.4.2. Production of antimicrobial substance

The strain LBM 5006 was grown in 200 mL of TSB medium at 37℃ in a rotary shaker (Cientec, Piracicaba, Brazil) at 125 cycles min^{-1} for 48 hours. The culture was centrifuged for 15 min at 10,000 x *g* and the resulting culture supernatant fluid was sterilized by filtration with 0.22 µm cellulose membranes (Millipore, Bedford, MA, USA). The filter-sterilized crude supernatant was used to evaluate the inhibitory effect on growth of *L. monocytogenes*. Antimicrobial substance was further partially purified by precipitation with ammonium sulfate and gel filtration chromatography as described elsewhere (Lisboa et. al, 2006).

3.3.4.3. Antimicrobial activity

The antimicrobial activity against all indicator strains was detected by agar disc diffusion assay (Motta & Brandelli, 2002). Aliquots of 20 µL were applied into cellulose discs on plates of TSA previously inoculated with a suspension of 10^8 cells mL^{-1} of *L. monocytogenes* ATCC 7644. Plates were incubated at 37 ºC for 24 h. The amount of antibacterial activity was determined by the serial two-fold dilution method previously described (Mayr-Harting et al., 1972). The reciprocal value of the highest dilution where an inhibition was observed was taken as activity units mL^{-1} (AU mL^{-1}).

3.3.4.4. Screening for hemolytic activity on blood agar plates

The method used by Hsieh et al. (2004) was modified as follows. Colonies of *Bacillus amyloliquefaciens* strain were grown for 24 h in BHI broth at 37℃. The strain was inoculated in Columbia shee p blood agar plates (Difco). The plates were incubated for 24 – 48 h at 37℃. Th e positive reactions produced a hemolysis zone around the colony. *Staphylococcus aureus* ATCC 9801 with known hemolytic activity was used as positive control.

3.3.4.5. Effect on the growth of *L. monocytogenes*

An overnight culture of *Listeria monocytogenes* ATCC 7644 was obtained in BHI at 37 ºC for 18-24 h. A 500 µL of this culture containing 10^8 CFU mL^{-1} was inoculated in tubes containing 16 mL of BHI and incubated at 37 ºC. Sterile BHI medium was added to control tubes. The growth was monitored at 2 h intervals by optical density (OD) at 600 nm and by viable cells counts

(CFU mL^{-1}). Crude LBM 5006 preparation (final concentration 1600 AU mL^{-1}) was added to culture of indicator strain after 4.5 h of cultivation, and the effect of the antimicrobial substance on turbidity and on the number of viable cells was determined at 2 h intervals.

3.3.4.6. Determination of the MIC and the MBC

The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of LBM 5006 were determined as described previously (Sirtori *et al*., 2008). Sterile 96-well microplates (Corning, New York, NY, USA) were filled with 100 µL of serial dilutions of LBM 5006 (concentrations ranging from 1600 AU ml^{-1} at 25 AU ml^{-1}) and then a standardized number of bacteria (100 µL of a 10^6 CFU mL^{-1} suspension from *L. monocytogenes*) was added into each well. A positive control was done using sterile NaCl solution instead LBM 5006. Negative controls were developed with sterile BHI plus different concentrations of LBM 5006. Microplates were incubated at 37 ºC for 24 h and then the MIC was determined as the last dilution at which no increase in turbidity was observed. MBC was determined inoculating an aliquot of 20 µL from the wells presenting negative growth to another microplates containing 100 µL of sterile BHI. Controls were also reinoculated. Microplates were incubated at 37 ºC for 24 h. The last dilution where no growth was observed was considered as MBC.

3.3.4.7. Dose-response curve

A dose response curve was determined using different concentration of LBM 5006 partially purified (between 50 and 3200 AU mL^{-1}) and a initial inoculum of 10^6 CFU mL^{-1} *L. monocytogenes*. Viable counts were determined after incubation at 37 ºC for 120 minutes. Kinetics of the LBM 5006 effect on *L. monocytogenes* was determined at 37 ºC with a LBM 5006 concentration of 3200 AU mL^{-1} and an initial inoculum of 10^6 CFU mL^{-1} from *L. monocytogenes*. The viable cells counts (CFU mL^{-1}) and OD 600 nm were determined after 2, 4, 10, 20, 30, 40, 50, 60, 75, 90 and 105 min of incubation.

3.3.4.8. Scanning electron microscopy

The bacteriocin treated and non-treated cells of *L. monocytogenes* ATCC 7644 were prepared by the method described by Kalchayanand et al. (2004) with slight modification. Exponentially cultures of *L. monocytogenes* incubated at 37 ºC for 24 h, were centrifuged (3000 g, 15 min at 20℃). The cells were suspended in BHI broth (controls) or bacteriocin (3200 UA mL^{-1}) and reincubated for 2 h at 37 ºC. To scanning electron microscopy the cell suspensions were fixed with 2% glutaraldehyde in Na-cacodylate buffer (100 mM, pH 7.1). There, the cells were pelleted, washed to remove gluteraldehyde and resuspended in the same buffer. A drop from each suspension was transferred to a poly-L-lysine-treated silicon wafer chips, which were kept for 30 min in a hydrated chamber for the cells to adhere. The attached cells were post fixed by immersing the chips in 1% osmium tetroxide (OsO4) in cacodylate buffer for 30 min, rinsed in the same buffer and dehydrated in ethanol in

ascending concentrations (%) of: 50, 70, 95 (2x) and 100 (2x), for 10 min each. The chips were mounted on aluminum stubs and coated with gold in a sputter coater (Emitech K550, Ashford, Kent, England). The chips were viewed at 15 kV accelerating voltage in a scanning electron microscope (Jeol® JSM-6060).

3.3.5 Results

3.3.5.1 Effect of the peptide LBM 5006 on indicator strain

The results of the inhibitory substance on the growth of *L. monocytogenes* ATCC 7644 are shown in Fig. 1. When the crude supernatant was added to culture of *L. monocytogenes* after 4.5 h of cultivation a reduction in the growth rate was noticed compared to the control culture without the bacteriocin, followed by a decrease in the cell number (final count of about 10^2 cell mL^{-1}).

The antimicrobial substance (1600 AU mL^{-1} final concentration) added to about 10^8 CFU mL^{-1} of *L. monocytogenes* resulted in a decrease (4 log cycles) in the number of viable cells and optical density (OD_{600}) during incubation time, suggesting that the effect of LBM 5006 is both bactericidal and bacteriolytic. The reduction of cell viability and OD_{600} were simultaneous.

3.3.5.2 MIC, MBC and Hemolytic Activity

The Minimal Inhibitory Concentration (MIC) was determined as 400 AU mL^{-1}, whereas a dose of 3200 AU mL^{-1} was needed to achieve a bactericidal effect (MBC). MIC values for other strains of *Listeria* are listed in the Table 1.

The hemolytic activity was assayed on sheep blood agar plates and

negative reactions were observed with crude and purified preparations of the antimicrobial substance (data not shown).

3.3.5.3 Dose-response curve

The effect of different concentration of the antimicrobial substance on the survival of *L. monocytogenes* ATCC 7644 is shown in Fig. 2. The decrease in the cell counts depend on the bacteriocin concentration expressed in AU mL^{-1}. The OD and CFU/mL were determined initially and after the end of incubation time and compared with the respective control. The OD and CFU in the control increased during 120 min of incubation, suggesting growth of *L. monocytogenes*. During incubation with the bacteriocin the indicator microorganism had a reduction in both CFU/mL (from 6.74 to 4.62 log cycles) and OD (from 0.08 to 0.02), indicating viability loss and cell lysis, respectively.

Treatment of the target cells with higher levels of bacteriocin LBM 5006 (3200 AU mL^{-1}) resulted in a earlier reduction of viable cell numbers.

The kinetics of LBM 5006 effect on growth of *L. monocytogenes* was investigated. The addition of bacteriocin (3200 AU mL^{-1}) to a cell suspension of *L. monocytogenes* resulted in a decrease in viable counts of 4 log cycles related to the control within 30 min of incubation (Fig. 3). Thus, this antimicrobial substance displayed a bactericidal mode of action.

3.3.5.4 Scanning electron microscopy of the indicator microorganism

Listeria monocytogenes cells were also examined by scanning electron microscopy to visualize the changes in morphology following treatment with the bacteriocin LBM 5006. The SEM-generated photomicrographs of the control and treated cells of the microorganism presented in Figure 4, show the changes in cell morphology and topography. The control cells are typical rods, have normal granular texture and are intact (A). The bacteriocin-treated cells appeared collapsed, with alterations in morphology as as rod shaped cells were changed to elongated (B) and ovoid cells (C); it could be associated with the damage in the cell wall and cell membrane and subsequent lysis and reduction in OD.

3.3.6 Discussion

Interest in natural antimicrobials has expanded in recent years in response to consumer demand for greener additives. Natural preservatives that inactive microorganims and enzymes without significant adverse effects on organoleptic or nutritional properties have been investigated for practical applications. Bacteriocin production can be exploited by food processors to provide an additional barrier to undesirable bacterial growth in foods (Tiwari et al., 2009).

In this work the effect and the mode of action of bacteriocin LBM 5006 on indicator microorganism were addressed, particularly using *L. monocytogenes*. Bacteriocin LBM 5006 is an antimicrobial peptide that inhibit

the growth of many Gram-negative and Gram-positive bacteria including important food pathogenic microorganisms such as *Bacillus cereus* and *L. monocytogenes* (Lisboa et al., 2006). This study obtained evidence that LBM 5006 showed bactericidal and bacteriolytic effects toward *L. monocytogenes* which indicate that the bacteriocin induces autolysis of susceptible cells. The viability loss of *L. monocytogenes* following treatment with the bacteriocin LBM 5006 is associated with the damages inflicted in the cell envelope (wall and membrane) resulted in viability loss as well as cell lysis. The bacteriolytic and bactericidal mode of action has also been described for the antimicrobial substances produced by *Bacillus* sp. P45 (Sirtori et al., 2008), *Bacillus thuringiensis* subsp. *entomocidus* HD110 (Cherif et al., 2008) and *Lactobacillus paracasei* subsp. *paracasei* (Bendali et al., 2008). These observations were confirmed by sccaning electron micrographs which showed that the cell lysis had occurred after treatment with the bacteriocin. The mechanisms of bacteriocin-induced cell lysis in some microorganisms have an active autolysin system. The bacteriocins cause the death of target cells by permeabilizing the cell membrane leading to depletion of cellular energy. There is then an imbalance in the control mechanism that regulate the action of an active autolysin system and promotes an uncontrolled degradation of cell wall and lysis of cells (Kalchayanand et al., 2002; Martínez-Cuesta et al., 2000). Our results suggested that the bacteriocin caused lysis and cell death and probably occurred by a similar mechanism.

The foodborne pathogen *L. monocytogenes* is a major concern in the food industry. A variety of food products have been involved in listeriosis

outbreaks that have occurred in the last years. Because of bacteriocin's antimicrobial activity, many studies have evaluated the use of bacteriocins as natural food bio-preservatives to control or kill pathogens including *L. monocytogenes* (Mirhosseini et al., 2010; Zhou et al., 2008; Motta et al., 2008; Lisboa et al., 2006). Based on the above results, the bacteriocin LBM 5006 produced by *B. amyloliquefaciens* may represent a novel antimicrobial compound with potential applications in food safety, as natural food bio-preservatives to control spoilage and pathogenic bacteria.

3.3.7 References

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Table 1. Minimal inhibitory concentration (MIC) of antimicrobial peptide LBM 5006 for selected *Listeria* strains

Indicator bacteria	MIC (AU mL^{-1})
Listeria monocytogenes ATCC 7644	400
Listeria monocytogenes ATCC 19115	400
Listeria monocytogenes ATCC 19112	400
Listeria monocytogenes ATCC 15313	400
Listeria monocytogenes 78/03	200
Listeria monocytogenes 4C	200
Listeria inoccua ATCC 33090	400
Listeria inoccua (food isolate)	400
Listeria inoccua 1572	200
Listeria seeligeri AC 82/4	200
Listeria sp. (clinical isolate)	400

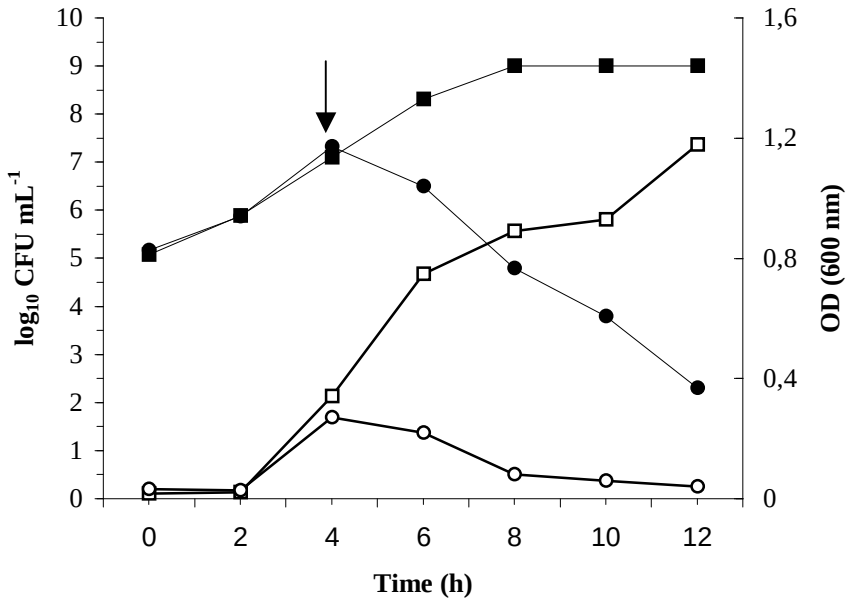

Figure 1. Effect of crude supernatant of *Bacillus amyloliquefaciens* LBM 5006 on growth of *Listeria monocytogenes* ATCC 7644. Turbidity (open symbols) and viability (black symbols) were monitored in control (squares) and treated (circles) cells with a final concentration of 1600 AU $mL^{-1.}$ The arrow indicates the time of antimicrobial substance addition. Each point represents the mean of three independent experiments.

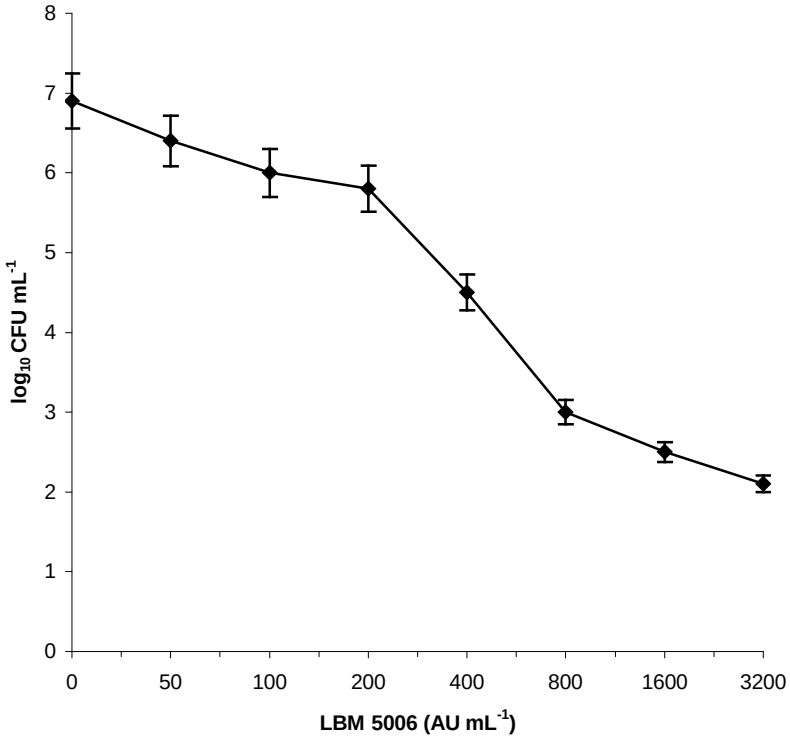

Figure 2: Effect of bacteriocin concentration on cell viability of *L. monocytogenes* ATCC 7644. Viable cell counts were determined after treatment with different concentrations of the antimicrobial substance during 120 min at 37 ^{o}C. The initial inoculum was 10^{6} CFU mL^{-1}. Each point represents the mean ± SEM of three independent experiments.

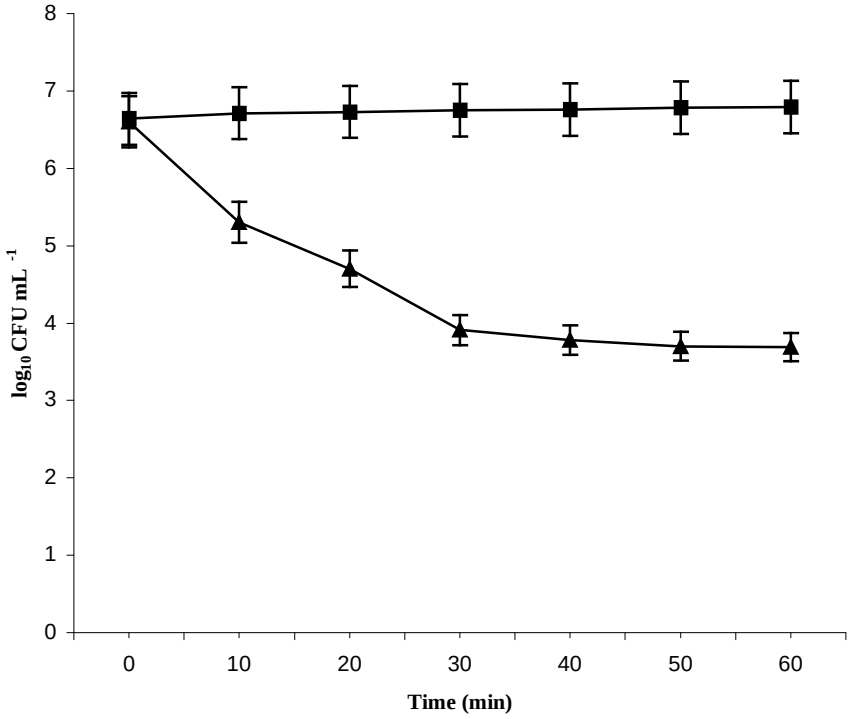

Figure 3: Kinetics of the LBM 5006 effect on *L. monocytogenes* ATCC 7644. Viability was monitored in control (■) and treated (▲) cells with a LBM 5006 concentration of 3200 AU mL^{-1} . Each point represents the mean of three independent experiments.

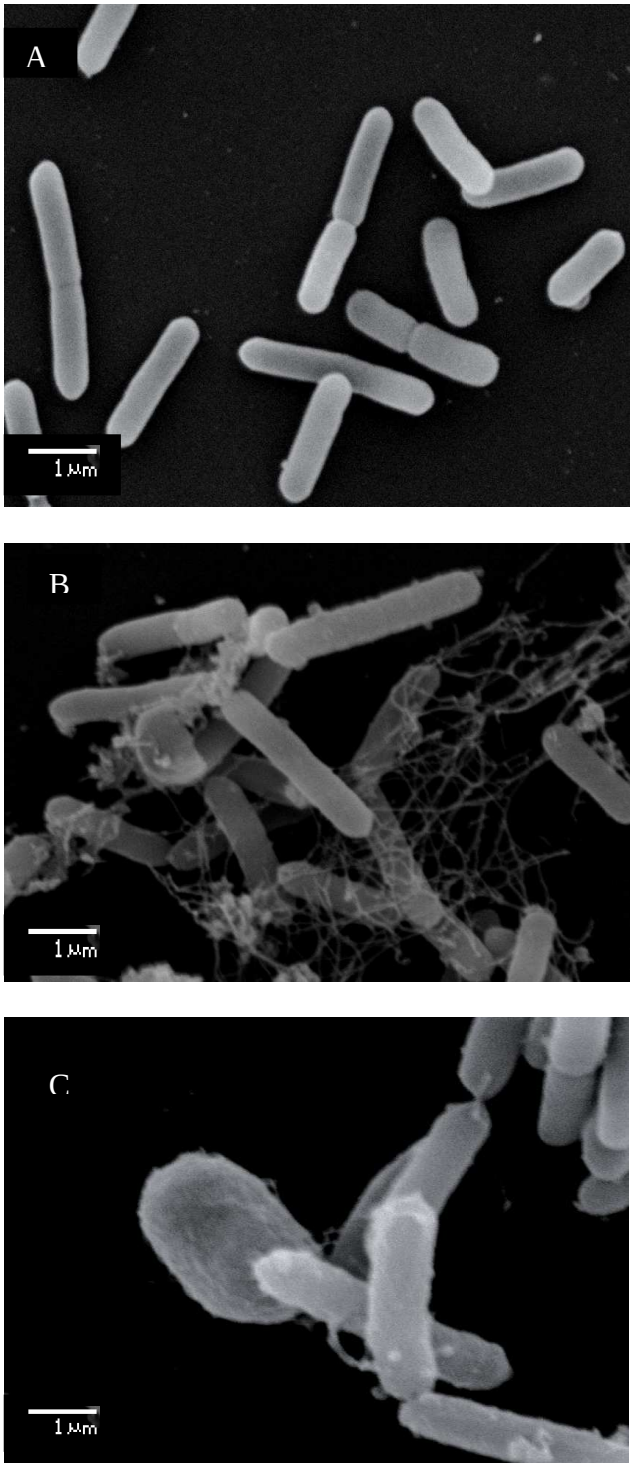

Figure 4: Scanning electron micrographs of *Listeria monocytogenes* ATCC 7644. Cells were left untreated (A; 12000x) or were treated for 60 min at 37 ºC with 3200 AU mL^{-1} of the bacteriocin (B; 12000x and C; 14000x).

3.4 ARTIGO 4

3.4.1Bacteriocin-like substance from *Bacillus amyloliquefaciens* shows remarkable inhibition of *Acanthamoeba polyphaga*

3.4.2 Abstract

The effectiveness of a bacteriocin-like substance (BLS) produced by *Bacillus amyloliquefaciens* was tested against *Acanthamoeba polyphaga* strains and its cytotoxic potential on Vero cells was investigated. Amoebicidal activity of the purified BLS was tested by plate bioassays with concentrations ranging from 12.5 to 6400 AU ml^{-1}. Damage to *A. pholyphaga* cells was monitored using an inverted microscope and counted in a Fuchs-Rosenthal chamber after 24, 48 and 72 h. According to the results obtained, the BLS showed remarkable amoebicidal and amoebostatic effect on *A. polyphaga* and showed no cytotoxicity on the Vero cells. These results may have great relevance in the development of new acanthamoebicidal compounds.

Keywords: *Acanthamoeba*, keratitis, antimicrobial peptide, *Bacillus amyloliquefaciens*.

3.4.3 Introduction

Acanthamoeba are small, ubiquitous, free-living protozoa that can exist as motile trophozoites and double-walled cysts (Ma et al. 1990; Saragoza 1994). Occasionally, *Acanthamoeba* cause human infections: granulomatous amoebic encephalitis (GAE), a chronic fatal brain infection that occurs mostly in immunodeficient individuals (Marciano-Cabral & Cabral 2003); amoebic keratitis (AK), a sight-threatening corneal infection related to contact lens misuse (Awwad et al. 2007; Illingworth and Cook 1998); and cutaneous, nasopharyngeal, and systemic infections (Schuster and Visvesvara 2004). In developed countries roughly 83% of the AK cases are diagnosed in contact lens wearers (Carvalho et al. 2009). Poor hygiene and insufficient disinfection may be determinants for the establishment of the disease. In contrast, in non developed countries the majority of cases are related with agricultural-based activities and/or ocular trauma (Clarke and Niederkorn 2006; Gopinathan et al. 2009).

Eradication of these protozoa from the infection sites is difficult because, under adverse conditions, the amoebas encyst and the medical therapy is often less effective against cysts than against trophozoites (Lloyd et al. 2001). Although drug therapy for *Acanthamoeba* keratitis has been revolutionized with the introduction of combination chemotherapy, (Martinez et al. 2000; Kumar & Lloyd 2002; Mills 2003) failures can still occasionally occur. Thus, further research is needed to exploit new therapeutic agents and strategies (Schuster and Visvesvara 1998; Ródio et al. 2008; Goze et al. 2009).

A wide variety of antimicrobial peptides (AMPs) are synthesized by bacteria. Bacterial ribosomally synthesized antimicrobial peptides are generally referred to as bacteriocins. The AMPs are an important component of the natural defences of most living organisms against invading pathogens. These are relatively small (<10 kDa), cationic and amphipathic peptides of variable length, sequence and structure. Some peptides may exhibit broad-spectrum activity against a wide range of microorganisms including Gram-positive and Gram-negative bacteria, protozoa, yeast, fungi and viruses (Papagianni 2003; Reddy et al. 2004). Bacteriocins or bacteriocin-like substances (BLS) are antimicrobial peptides widespread produced among bacteria of the genus *Bacillus*. The *Bacillus amyloliquefaciens* LBM 5006 strain, originally isolated from a soil sample from the Brazilian Atlantic forest, produces a previously characterized bacteriocin-like substance (BLS) with broad antibacterial activity (Lisboa et al. 2006).

The aim of the present study was to evaluate the amebicidal activity of the bacteriocin-like substance LBM 5006 against *Acanthamoeba polyphaga* strains and their cytotoxic potentials on Vero cells.

3.4.4 Materials and methods

3.4.4.1 Microorganisms, culture media and grown conditions

The bacteriocin-producer strain *Bacillus amyloliquefaciens* LBM 5006 was originally isolated from soil of Atlantic Forest (Lisboa et al. 2006). The strain was maintained at –21ºC as frozen stocks in brain-heart broth (BHI; Oxoid, Basingstoke, UK) containing 20% (v/v) glycerol and propagated twice in the

same medium at 37℃ before use.

Indicator strains were two isolates of *A. polyphaga*. The first (ATCC 30461) was isolated from a cornea of a keratitis patient and the second (ATCC 30872) is an environmental, freshwater isolate. The *Acanthamoeba* isolates were cultured axenically in PYG medium [Proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v), and glucose 1.5 % (w/v)] at 30 ℃. For the assays, trophozoites in exponential growth (72-96 h) were washed twice in phosphate-buffered saline (PBS) buffer (pH 7.2) and used immediately for testing.

3.4.4.2 Production, isolation and purification of BLS

B. amyloliquefaciens LBM 5006 strain was cultivated aerobically in 500 mL erlenmeyer flasks containing 200 mL of TSB broth at 37 ºC, 125 rpm for 48 h. Cells were harvested by centrifugation at 10,000 ×*g* for 15 min at 4ºC, and the resulting supernatant was filtered through 0.22 µm membranes (Millipore, Bedford, USA). The cell free supernatant (CFS) was submitted to precipitation with ammonium sulfate to 20% saturation. The resulting pellet was resuspended in 10 mM sodium phosphate buffer (pH 6.0), and applied to gel filtration column (Sephadex G-100, Pharmacia Biotech, Uppsala, Sweden) and eluted with 10 mM sodium phosphate buffer, pH 6,0. Fractions positive for antimicrobial activity were pooled and freeze-dried. Fractions were also monitored for A280 nm using a spectrophotometer (UV-mini 1240, Shimadzu, Tokyo, Japan). This material was dissolved in 10 mM phosphate buffer pH 6.0 and then extracted twice with l-butanol. The organic phases were combined and evaporated under reduced pressure. The resulting powder was suspended in 10 mM phosphate

buffer pH 6.0. During purification the antimicrobial activity (AU mL^{-1}) was quantified as described elsewhere using *L. monocytogenes* ATCC 7644 as indicator organism (Motta and Brandelli 2002), and amebicidal activity was confirmed against *Acanthamoeba polyphaga.* The determination of soluble protein was carried out by the Folin phenol reagent method (Lowry et al. 1951) with bovine serum albumin as standard.

3.4.4.3 Amoebicidal activity

In order to study the effectiveness of the amoebicidal activity of the bacteriocin LBM 5006 the purified fraction was tested by plate bioassays. Experiments were performed in sterile 96-well plates. Samples (100 µL) of *A. polyphaga* culture and 100 µL of each test solution were inoculated into each well. Serial dilutions (concentrations ranging from 6400 AU ml^{-1} to 12.5 AU ml^{-1}) of the BLS were prepared in PYG medium. The amoebas were washed twice in phosphate-buffered saline (PBS) buffer (pH 7.2) and suspended in PYG medium at a concentration of $1x10^4$ cells/100 µL. One hundred microliters of the calibrated trophozoite suspension was added to each well, and then the plates were sealed and incubated at 30℃. Negative control wells received 100 µL PBS and 100 µL of culture of *A. polyphaga* and positive control received amphotericin B with an IC_{50} at concentration 10 µg/mL with 100 µL of culture of *A. polyphaga*. The plates were sealed and incubated at 30℃, monit ored by means of an inverted microscope, and counted in a Fuchs-Rosenthal counting chamber after 24, 48 and 72 h. Tests were performed in duplicate and were repeated at least three times.

3.4.4.4 Cytotoxicity assay

The BLS was tested at a range of concentrations (12.5 to 3200 AU mL^{-1}) against African green monkey kidney (Vero) cell cultures (De Jonckheere 1980) to determine possible cytotoxicity. Cells were cultured in Eagle minimal essential medium (E-MEM), supplemented with 10% fetal calf serum, 2.5 µg of amphotericin B and 10 µg of enrofloxacin. The cytotoxic assays were performed by incubating the samples in triplicate onto Vero cell monolayers cultured in 96-well plate. Serial dilutions of the BLS were prepared, and 100 µL of each dilution was added to the cell monolayer in wells. Plates were incubated at 37℃ with 5% CO_2 for 24 and 48 h. The morphological alterations of the treated cells were observed by means of an inverted optical microscope after 24 and 48 h. After 48 h was made fixing (70% ethanol) and staining the cell monolayer with Gram crystal violet (Protocols, Fisher Scientific, Kalamazoo, MI) for visualization of monolayer integrity (Schuster and Visvesvara 1998).

3.4.5 Results and discussion

AMPs have been tested against various protozoa. Reports of antiparasitic activities of natural AMPs are mainly related to *Plasmodium* and *Leishmania*, two of the most widely distributed parasites worldwide. Antimalarial activities have been described for two classes of cationic AMPs, the linear amphiphilic peptides, and the cysteine-rich open-ended peptides (Papagianni, 2003). The hybrid peptides Vida-1, 2 and 3 showed activity against the sporogenic-stage of the rodent malarial parasites *Plasmodium berghei* and *Plasmodium yoelii nigeirensis* (Arrighi et al., 2002). Sacramento et al. (2009)

showed that AMPs from distinct structural groups are capable of interfering with growth and cellular permeability of *A. castellanii* trophozoites. Gomesin, a ß-hairpin amphipathic peptide, of the spider *Acanthoscurria gomesiana* haemocytes, known to be resistant to proteases is quite effective in permeabilizing trophozoites, compared to mammalian cells.

In this work, we show for the first time that a bacteriocin-like substance isolated from the bacterium *Bacillus amyloliquefaciens*, have potent amoebicidal activity against *Acanthamoeba polyphaga* trophozoites and are able to kill the protozoan. Figure 1 (A and B) show viable trophozoite numbers of the cultures exposed to serial dilutions of the BLS (concentrations ranging from 12.5 to 6400 AU mL^{-1}) at 24, 48, and 72 h. The trophozoites viability showed a direct relationship with the extract concentration and amoebicidal and amoebistatic activities were detected. At the concentration of 6400 AU mL^{-1} no viable trophozoites were detected at the end of any incubation time for both *A. polyphaga* ATCC 30461 and *A. polyphaga* ATCC 30872.

The morphology of *A. polyphaga* strains was evaluated using an inverted light microscope over 72h of incubation with the antimicrobial substance. The protozoan cells showed morphological changes such as elongation, roundness, encystment and cellular lysis in various concentrations (Fig. 2A and B). According to the results obtained from the tests, the BLS showed remarkable amoebicidal effect on *A. polyphaga* strains.

Medical therapy for *Acanthamoeba* infections is still difficult because antiprotozoal drugs are not completely effective in some anatomical sites. Moreover, some anti-amoebic drugs show only an amoebistatic effect,

molecules used for keratitis treatment are toxic for the host cells and some eye drop preparations are not well tolerated when used for prolonged periods (Fiori et al. 2006). More recently, some natural agents from plant origin showing amoebicidal effect on *Acanthamoeba* trophozoites have been described (Goze et al. 2009; Ródio et al. 2008; Polat et al. 2007).

Sensitivity of tissue culture cells to purified BLS was tested. The bacteriocin was added to 96-well plates with monkey kidney cell monolayers. The bacteriocin had no inhibitory effect on Vero cells at the highest concentration tested after 24 and 48 h. Although the cells are in direct contact with the BLS in the culture media, they did not show any signs of injury and kept their morphological characteristics and wholeness similar to negative control. Thus, we conclude that the BLS produced by the strain LBM 5006 has little or no toxicity to mammalian cells at a concentration that was effective against amoebas.

From a clinical perspective, the emergence of drug-resistant pathogens makes the identification of novel antimicrobials even more important. Biotechnology companies worldwide are developing novel antimicrobial drugs, based on natural peptides, which are directed mainly against bacterial and fungal infections. However, the reported in vitro activities of some antimicrobial peptides against parasites make them a powerful source of antiparasitic compounds. Their use for the development of a new generation of drugs for topical or systemic treatment of important parasitic diseases is a promising hope for the new century. Human parasite infections cause millions of deaths around the world every year. New antiparasitic drugs are needed to be used

alone or as complement for existing products, and to overcome problems such as antimicrobial resistance (Papagianni 2003; Reddy et al. 2004).

In conclusion, the bacteriocin LBM 5006 shows important amebicidal activity against trophozoites of *A. polyphaga*. Thus, the results presented in this paper are encouraging the development of new agents for the prevention and therapy of *Acanthamoeba* infections, although further *in vitro* and *in vivo* studies are needed to define efficacy and the toxicity of the acanthamoebicidal peptide.

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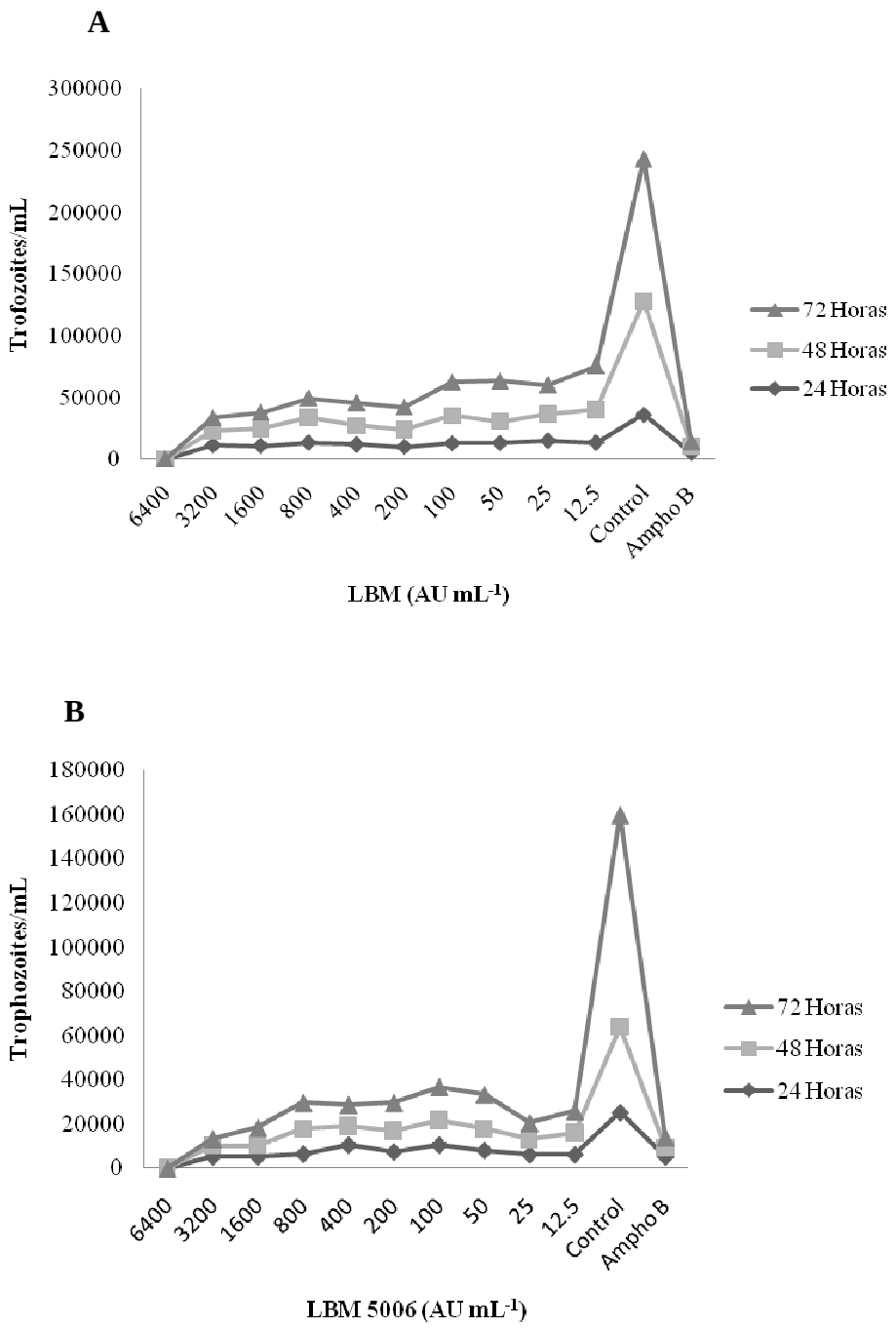
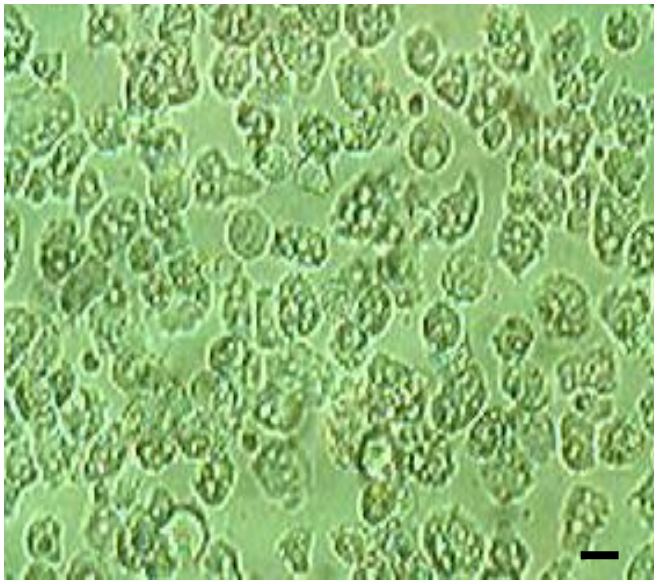


Fig. 1 A, B. Effect of bacteriocin concentration on proliferation of *Acanthamoeba polyphaga* trophozoites, **A** for ATCC 30461 and **B** for ATCC 30872. Viable cell counts were determined after treatment with different concentrations of the purified bacteriocin for 24, 48, and 72 h. The initial inoculum was 1,0 x10^4 cells. The control group represents the amebae grown in the absence of bacteriocin. Positive control received amphotericin B at concentration 10 µg mL^{-1}. Values are the means of at least three different experiments.

A



B

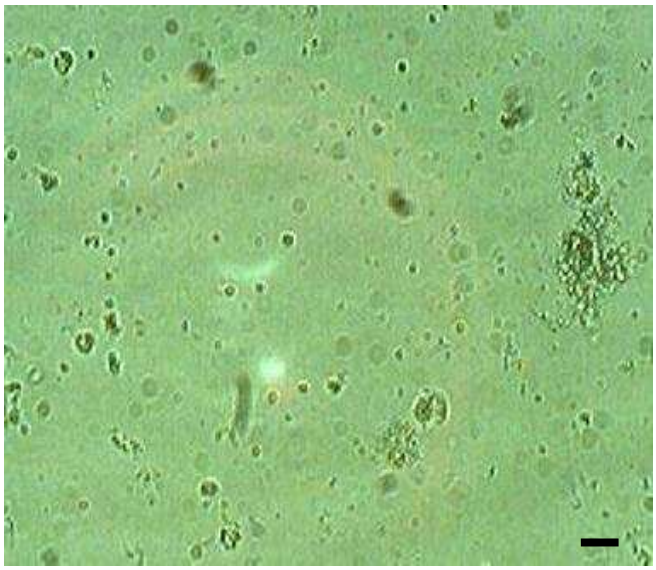


Fig. 2. Representative pictures of cellular viability using an inverted microscope, after 24 h of incubation of *Acanthamoeba polyphaga* trophozoites with (A) only PYG medium (negative control), (B) purified BLS fraction (6400 AU mL^{-1}). Scale bar 20 µm.

3.5 ARTIGO 5

3.5 Bacteriocin-like substance from *Bacillus amyloliquefaciens* inhibits *Paenibacillus larvae*, the causative agent of American Foulbrood

3.5.2 Abstract

Bacillus amyloliquefaciens LBM 5006 produces a bacteriocin-like substance (BLS) active against *Paenibacillus larvae*, a major honeybee pathogen. The antimicrobial effect and the mode of action of the antimicrobial peptide was investigated. The antibacterial activity was produced starting at mid-logarithmic growth phase, reaching its maximum at the early and during stationary phase. Exposure of cell suspensions of *P. larvae* to this antimicrobial resulted in loss of cell viability and reduction in optical density (OD) associated with cell lysis. Scanning electron microscopy showed damaged cell envelope and loss of protoplasmic material. The antimicrobial peptide from *B. amyloliquefaciens* LBM 5006 showed a bactericidal effect against *P. larvae* cells and spores. This BLS presents potential for use in the biological control of foulbrood in honey bees.

Keywords: bacteriocin; *Bacillus*; mode of action; honeybee pathogen; *Paenibacillus larvae*

3.5.3 Introduction

Bacteriocins or bacteriocin-like substances (BLS) are antimicrobial peptides widespread produced among bacteria from the genus *Bacillus*. In particular, different *Bacillus amyloliquefaciens* strains are producer of antimicrobial substances with potential applications for the biological control of spoilage and pathogenic microorganisms in food and as a biocontrol agent to suppress plant pathogens (Caldeira et al. 2008; Sutyak et al., 2008; Sang-Cheol Lee et al., 2007; Lisboa et al., 2006; Yu et al., 2002; Yoshida et al., 2001).

Paenibacillus larvae is the causative agent of American Foulbrood (AFB), the most severe bacterial disease that affects larvae of the honey bee *Apis mellifera*. AFB presents a worldwide distribution, causing a significant decrease in honeybee populations and production (honey, pollen, propolis, royal jelly and beeswax) (Hansen and Brodsgaard, 1999; Sabaté et al., 2009; Genersch et al., 2006). Besides their importance for the beekeeping industry, honey bees play an essential role in the ecology of different environments throughout pollination, being essential for the production of agricultural systems and conservation of natural ecosystems (Antunéz et al., 2009).

AFB is a serious bacterial disease of honey bee brood, not only able to kill infected individuals but also potentially lethal to infected colonies (Ashiralieva & Genersch, 2006), and has unique problems for prevention and control because the spores can remain viable for long periods of time (Hrabak and Martinek, 2007; De Graaf et al., 2006) and survive environmental adversities (Hansen and Brodsgaard, 1999; Yue et al., 2008). A control method is to burn the diseased bee colonies (Matheson and Reid, 1992; Ratnieks,

1992) and is in use in many countries. In Argentina, where disease incidence is high (Alippi et al., 2004), the use of antibiotics appear as an alternative to the burning of infected bee hives. Currently, the only antibiotic approved for prevention and control of AFB in honey bee colonies is oxytetracycline, however, there is evidence of oxytetracycline-resistant isolates of *P. larvae* in certain areas of the USA, Canada, and Argentina (Alippi, 2000; Colter, 2000; Evans, 2003). The widespread use of antibiotics favors the natural selection of resistant bacterial strains, diminishes the expectation of half life of honeybees, and causes disequilibria in the normal microbiota of the beehive (Charbonneau et al., 1992) with the risks of contamination of honey. For these reasons, the search for alternative non-contaminating natural biocides for the control of AFB is a great challenge that will improve the quality of honeys avoiding the presence of undesirable residues. Therefore, the aim of this work was to investigate the effect and the mode of action of the antimicrobial peptide LBM 5006, and to evaluate its potential use as antimicrobial to prevent the proliferation of *P. larvae*.

3.5.4 Materials and methods

3.5.4.1 Bacterial strains and culture conditions

The microorganism *Bacillus amyloliquefaciens* LBM 5006, isolated from the soil of the Atlantic Forest, Santa Catarina, Brazil, was used for peptide LBM 5006 production. Bacterial identification was based on 16S rRNA gene sequencing analysis and biochemical analysis using API 50 CHB test kit with automated interpretation (Lisboa et al., 2006). *Paenibacillus larvae* 1655 and

Paenibacillus larvae 165B, isolated from honey bee brood with symptoms of AFB (field strains), were chosen as the indicator strains to demonstrate and measure anti-bacterial activity. For long term storage, the bacteria were kept at -21ºC in 20% glycerol in their cultural broths. Before the experiments, each bacterial strain was sub cultured at least two times (1% v/v) at 24 h intervals.

3.5.4.2 Production of antimicrobial activity by *B. amyloliquefaciens* LBM 5006

Culture of *B. amyloliquefaciens* was carried out at 37 ºC in 20 mL TSB for 24 h. Medium was inoculated at 10% with 2 mL 24 h pre-culture (approximately 10^6 cells mL^{-1}) of *B. amyloliquefaciens*. The growth (OD 600 nm) and the anti-bacterial activity were monitored at time intervals of 0, 4, 8, 12, 24, 32 and 48 h. The antimicrobial activity against *P. larvae* 1655 strain was detected by agar disc diffusion assay (Motta & Brandelli 2002). Aliquots of 20 µL were applied into 6 mm cellulose discs on TSA plates previously inoculated with a suspension of 10^6 cells of *P. larvae* 1655. Plates were incubated at 37 ºC for 24 h. The amount of antibacterial activity was determined by the serial two-fold dilution method. The reciprocal value of the highest dilution where an inhibition was observed was taken as activity units mL^{-1} (AU mL^{-1}).

3.5.4.3 Mode of action

i. Isolation of antimicrobial substance

For the production of BLS, *B. amyloliquefaciens* LBM 5006 was grown in 200 mL TSB-medium at 37℃ in a rotary shak er (Cientec, Piracicaba,

Brazil) at 125 cycles min^{-1} for 48 hours. The cells were harvested by centrifugation at 10,000 x *g* for 15 min at 4 ºC and the resulting culture supernatant fluid was sterilized by filtration with 0.22 µm membranes (Millipore, Bedford, MA, USA). Antimicrobial substance was further purified by precipitation with ammonium sulfate at 20% saturation. After centrifugation at 10,000 x *g* for 15 min the pellet was suspended in 10 mmol L^{-1} sodium phosphate buffer pH 6.0 and submitted to gel filtration chromatography using a Sephadex G-100 column (Pharmacia Biotech, Uppsala), eluted with the same buffer (Motta et al., 2008).

ii. Effect on the growth of *P. larvae* 165B and 1655

Overnight cultures of *P. larvae* 165B and 1655 were obtained in BHI at 37ºC for 18-24 h. 500 µL of these cultures containing 10^6 CFU mL^{-1} were inoculated in tubes containing 16 mL of BHI and incubated at 37ºC. Sterile BHI medium was added to control tubes. The growth was monitored at 2 h intervals by optical density (OD) at 600 nm and by viable cells counts (CFU ml^{-1}). Crude LBM 5006 preparation (final concentration 1600 AU mL^{-1}) was added to culture of indicator strain after 4.5 h of cultivation, and the effect of the antimicrobial substance on turbidity and on the number of viable cells was determined at 2 h intervals.

iii. Dose-response curve and MIC determination

A dose response curve was determined using different concentration of LBM 5006 (between 50 and 3200 AU mL^{-1}) and an initial inoculum of 10^5

CFU mL^{-1} from *P. larvae* 1655. Viable counts were determined after incubation at 37 ºC for 120 minutes. The minimal inhibitory concentration (MIC) values were determined by a standard microbroth dilution method as described previously (Sirtori *et al*., 2008). Sterile 96-well microplates (Corning, New York, NY, USA) were filled with 100 µL of serial dilutions of LBM 5006 (concentrations ranging from 3200 AU ml^{-1} at 0 AU ml^{-1}) and then a standardized number of bacteria (100 µL of a 10^5 CFU mL^{-1} suspension from *P. larvae* 165B and 1655 were added into each well. Microplates were incubated at 37 ºC for 24 h. The MIC was determined by taking into account the higher dilution at which no growth of the test organism was visible.

iv. Measurement of UV-absorbing materials

UV-absorbing materials release was measured as an index of cell lysis of *P. larvae* 1655 and *P. larvae* 165B cell suspensions, which corresponded to a 0.5 McFarland turbidity standard solution in 10 $mmol^{-1}$ phosphate buffer pH 6.0, were mixed (1:1, v/v) with antimicrobial substance (final concentration 1600 AU mL^{-1}) and incubated at 37 ºC. Samples were removed after 3 h and filtered through 0.22 µm membranes. The absorbance of the filtrates was measured at 260 and 280 nm using a spectrophotometer UV-mini 1240 (Shimadzu, Tokyo, Japan). Cultures of the indicators microorganisms without antimicrobial substance were included as controls.

3.5.4.4 Effect on *P. larvae* spores

Spore production by *P. larvae* 165B and *P. larvae* 1655 was carried out in TSB agar. After incubation at 37 ºC for 7 days, samples were harvested, suspended in sterile MilliQ water and treated at 80 ºC for 10 minutes to kill vegetative cells. Aliquots were diluted with sterile MilliQ water to the initial concentration of 10^5-10^6 spores mL^{-1}. Spores were incubated in BHI for 120 min at 37 ºC with BLS at a concentration of 1600 AU mL^{-1} and then plated to determine viable cell counts (Abriouel et al., 2002). Plates were incubated at 37 ºC for up to 5 days before counting.

3.5.4.5 Scanning electron microscopy

The bacteriocin treated and non-treated cells of *P. larvae* 1655 were prepared by the method described by Kalchayanand et al. (2004) with slight modification. Exponentially cultures of *P. larvae* 1655 incubated at 37 ºC for 24 h, was centrifuged (3000g, 15 min at 20℃). The cells were suspended in BHI broth (controls) or bacteriocin (3200 UA mL^{-1}) and incubated for 120 min at 37 ºC. To scanning electron microscopy the cell suspensions were fixed with 2% glutaraldehyde in Na-cacodylate buffer (100 mM, pH 7.1). There, the cells were pelleted, washed to remove gluteraldehyde and suspended in the same buffer. A drop from each suspension was transferred to a poly-L-lysine-treated silicon wafer chips, which were kept for 30 min in a hydrated chamber for the cells to adhere. The attached cells were post fixed by immersing the chips in 1% osmium tetroxide (OsO_4) in cacodylate buffer for 30 min, rinsed in the same buffer and dehydrated in ethanol in ascending concentrations (%) of: 50, 70, 95

(2x) and 100 (2x), for 10 min each. The chips were mounted on aluminum stubs and coated with gold in a sputter coater (Emitech K550, Ashford, Kent, England). The chips were viewed at 15 kV accelerating voltage in a scanning electron microscope (Jeol® JSM-6060).

3.5.5 Results and Discussion

3.5.5.1 Kinetics of BLS production

The culture of the strain *B. amyloliquefaciens* LBM 5006 was carried out at 37ºC in TSB medium. Maximum bacteriocin production was detected after 32 h of cultivation (Fig.1), corresponding to the stationary growth phase. The activity was equal to 6400 AU mL^{-1}. The production of the active substance was dependent on the bacterial growth phase. The strain LBM 5006 started to produce bacteriocin in TSB medium (800 AU mL^{-1}) during the early logarithmic growth phase.

3.5.5.2 Effect on *P. larvae* 165B and 1655

The effect of bacteriocin (1600 AU mL^{-1}) on the sensitive strains (*P. larvae* 165B and *P. larvae* 1655) was examined to establish the mode of action of the antimicrobial substance. This concentration led to marked decrease (about 4 log units) in the number of viable cells, initially 10^8 cells mL^{-1}, during incubation time. The decrease in cell counts of *P. larvae* occurred simultaneously with the decrease in optical density (OD_{600}), indicating a bactericidal effect with simultaneous cell lysis (Fig. 2a and b).

3.5.5.3 Efflux of UV-absorbing materials

To determine whether the bacteriocin-like substance has an effect on the integrity of cell membranes of *P. larvae* 1655 and *P. larvae* 165B the efflux of cytoplasmic content was measured by optical density (260 and 280 nm) in the culture supernatant of control cells and cells treated for 3 h with the active substance. Treatment of *P. larvae* 1655 and *P. larvae* 165B cells whit 1600 AU mL^{-1} of LBM 5006 bacteriocin caused a leakage of UV-absorbing materials measured at both 260 and 280 nm (Table 1).

3.5.5.4 Dose-response curve and MIC

When *P. larvae* 1655 and 165B cells were incubated with different concentrations of BLS, a dose-response curve was obtained (Fig. 3). The CFU/mL were determined initially and after the end of incubation time and compared with the respective control. Reducing the number of viable cells, in both indicators strains, with increasing concentration of antimicrobial substance reinforces the theory that the BLS has a bactericidal activity. MIC values were determined as 200 AU mL^{-1} (*P. larvae* 1655) and 800 AU mL^{-1} (*P. larvae* 165B), respectively.

3.5.5.5 Effect on spore outgrowth

The effect of BLS on spores of *P. larvae* 1655 and 165B was investigated. An approximately 2 $\log_{10}$ reduction (from 5.2 to 3.2 and 5.0 to 3.4 log cycles, respectively) was observed in cell counts when spores were treated with the antimicrobial substance in the tested concentration of 1600 AU mL^{-1}.

3.5.5.6 Scanning electron micrographs of BLS-treated *P. larvae* cells

P. larvae cells were examined by scanning electron microscopy to visualize the changes in morphology following treatment with BLS. Representative photomicrographs at 20,000x (10 kV) magnifications are presented in Figure 4 (A-C). The control cells (A) showed the characteristic rods, intact with normal texture. Exposure to BLS for 120 min at 37ºC caused extensive lysis and degradation of the cells, apparently due to changes in the structure of the cell wall and cell membrane. The disruption of cells in various places has caused the leakage of cellular content. The bactericidal effect of the bacteriocin was accompanied by bacterial lysis.

3.5.6 Discussion

Innocuous bacterial strains that may produce antimicrobial substances are of great interest to confine or inhibit different pathogens or spoilage microorganisms by natural resources. The non-toxigenic *Bacillus* species do not harbor drug resistance genes and are non-pathogenic, so they are considered safe and could be used for agricultural, human and veterinary purposes (Sabaté et al., 2009; Duc et al., 2004).

Within this context, the effect and the mode of action of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from soil, on indicator microorganism *P. larvae* were investigated.

The maximum antimicrobial activity was determined to coincide with the stationary growth phase. Barboza-Corona et al. (2007) reported the

production of bacteriocin-like inhibitor substances by five Mexican strains of *Bacillus thuringiensis* (Bt-BLIS) with high levels of activity against *Bacillus cereus* and other gram-positive bacteria. Differences were observed in the kinetics of the Bt-BLIS production that grouped the strains in two categories (A and B). The strains of the group A produced the antimicrobial substance at the beginning of the stationary phase with maximum levels occurring at the end of that stage as determined by the relative levels of bactericidal activity. In the group B, Bt-BLIS bactericidal activity was observed in samples collected at middle of the logarithmic phase and achieved the highest levels at the start of the stationary period.

This report obtained evidence that the BLS showed bactericidal and bacteriolytic effects toward *P. larvae* cells. The view that BLS was bactericidal and bacteriolytic is consistent with the fact that damaged the cell envelope (wall and membrane) of the sensitive bacteria. Indeed, release of UV-absorbing materials was detected after treatment of cells with the antimicrobial substance. It appears from these results that, following treatment of bacterial cells by the BLS, the cells were injured, which was confirmed by scanning electron microscopy. Bacteriocins exert their bactericidal mode of action by destabilization and permeabilization of sensitive cell membranes and may be accompanied by lysis of sensitive cells. Binding of these bacteriocins to teichoic, lipoteichoic and teichuronic acids in the cell wall of sensitive bacteria lead to release, and therefore activation, of autolytic enzymes, which under normal conditions are electrostatically bound to these polymers (Cintas et al., 2001; Jack et al., 1995). The bacteriocins, like pediocin AcH, specifically

destabilize three-dimensional structures of cell walls and cell membranes leading to loss of normal functions including controlling the action of autolysin. This, in turn, triggers autolysin resulting in uncontrolled degradation of the cell wall and eventual cell lysis (Kalchayanand et al., 2002).

AFB is a cosmopolitan disease and one of the major threats to beekeeping, since it is highly contagious and able to kill affected colonies (Genersch, et al. 2005). Only honeybee larvae are susceptible to infection (Wilson, 1971) and only the spores of *P. larvae* are infective (Tarr, 1937; Hornitzky, 1998). Larvae become infected by ingestion of spore-contaminated larval food (glandular secretions and processed honey). The susceptibility to infection depends on the larval age, the spore dose necessary for successful infection of a larva increases with increasing larval age (Brodsgaard et al., 1998). An interesting finding from this study was the bactericidal activity observed against *P. larvae* spores treated with BLS. The ability to make spores more sensitive to antimicrobials agents has been investigated by other researchers. Similar results were obtained on *Bacillus* spores with cerein 8A, a peptide of *Bacillus cereus* (Bizani et al., 2005) and enterocin EJ97 produced by *Enterococcus faecalis* (Viedma et al., 2010).

Some *Bacillus* species have been reported to inhibit *P. larvae* but the chemical nature of the metabolites involved in the inhibitory activity was not determined (Allipi & Reynaldi, 2006). Only the study of Sabaté et al. (2009) related three *Bacillus* strains isolated from honey samples and bee gut that inhibit *P. larvae* by surfactin synthesis.

Hence, the BLS from *B. amyloliquefaciens* may have a valuable

potential as biological control agent, because of its antimicrobial activity against *P. larvae* vegetative cells and germinating spores.

3.5.7 References

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Table 1 Release of intracellular UV-absorbing materials of *P. larvae* 1655 and 165B

	Untreated cells		**Bacteriocin-treated cells**	
	Nucleic acids ($A_{260\ nm}$)	**Proteins ($A_{280\ nm}$)**	**Nucleic acids ($A_{260\ nm}$)**	**Proteins ($A_{280\ nm}$)**
***P. larvae* 1655**	0.10 ± 0.02	0.14 ± 0.04	0.31 ± 0.03	0.45 ± 0.01
***P.larvae* 165B**	0.09 ± 0.03	0.12 ± 0.01	0.42 ± 0.09	0.44 ±0.02

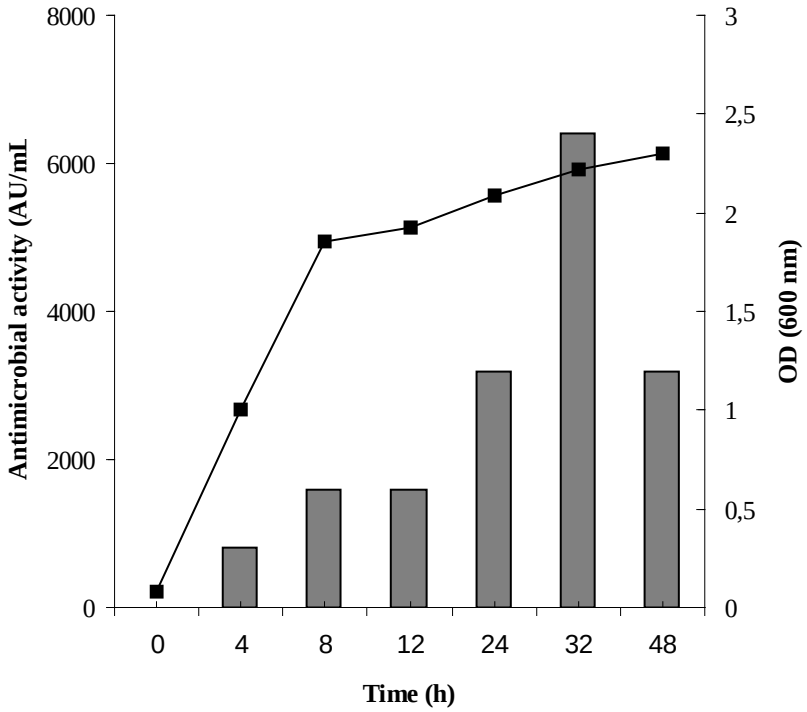

FIG. 1. Growth pattern (OD 600 nm, ■) of the strain *Bacillus amyloliquefaciens* LBM 5006 and kinetic of the bacteriocin production (AU mL^{-1} ▮). Results are mean of three independent experiments

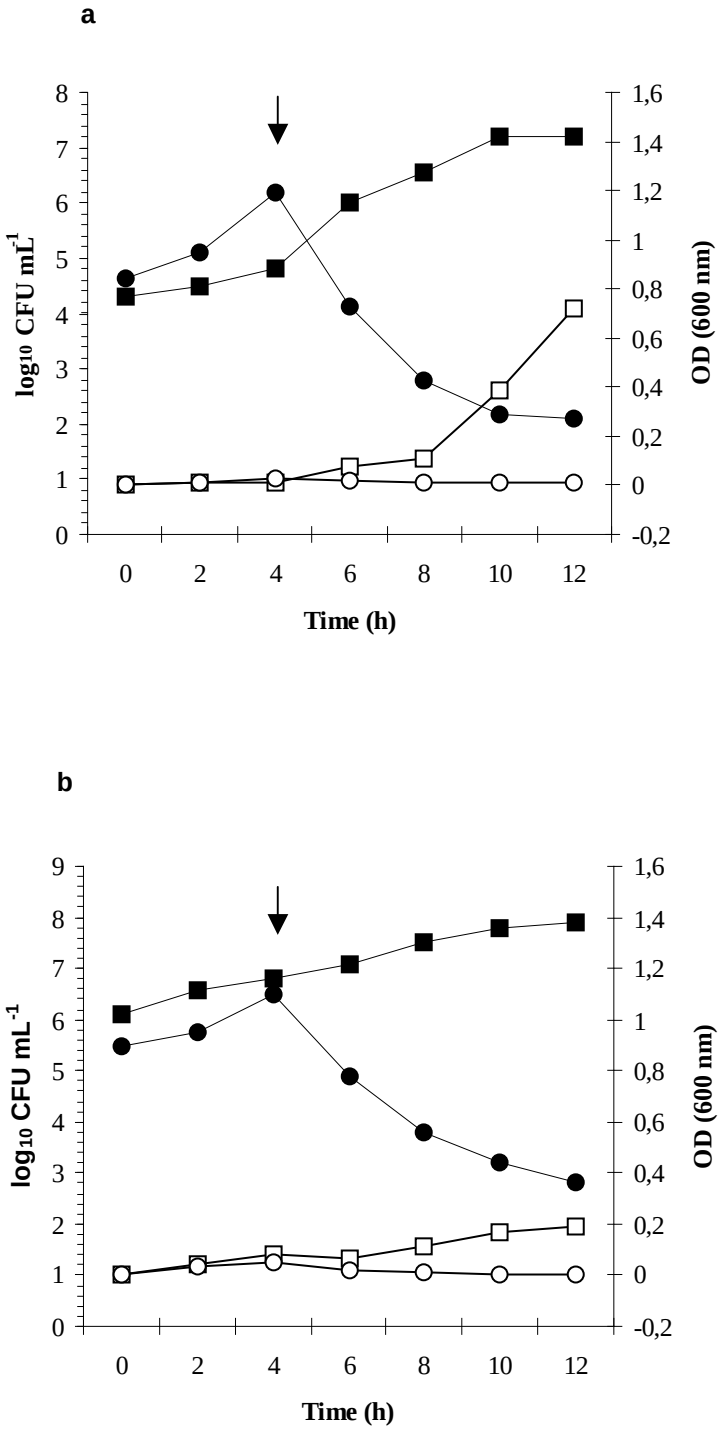

FIG. 2. Effect of crude supernatant of *Bacillus amyloliquefaciens* LBM 5006 on growth of *Paenibacillus larvae* 165B (a) and *Paenibacillus larvae* 1655 (b). Turbidity (open symbols) and viability (black symbols) were monitored in control (squares) and treated (circles) cells with a final concentration of 50 AU $mL^{-1.}$ The arrow indicates the time of antimicrobial substance addition. Each point represents the mean of three independent experiments.

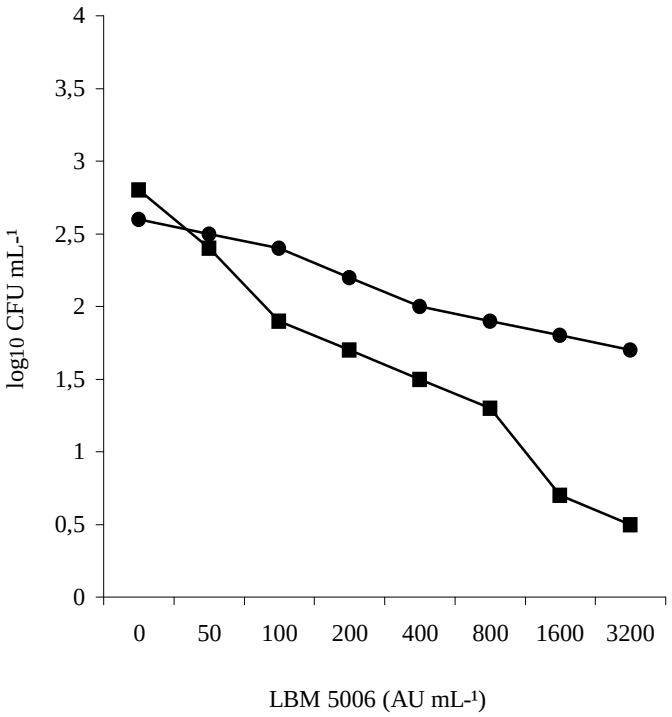

FIG. 3. Effect of LBM 5006 concentration on cell viability of *P. larvae* 1655 (circles) and *P. larvae* 165B (squares). Viable cell counts were determined after treatament with different concentrations of the antimicrobial substance during 120 min at 37 ºC. The initial inoculum was 10^6 CFU mL^{-1}. Each point represents the mean of three independent experiments.

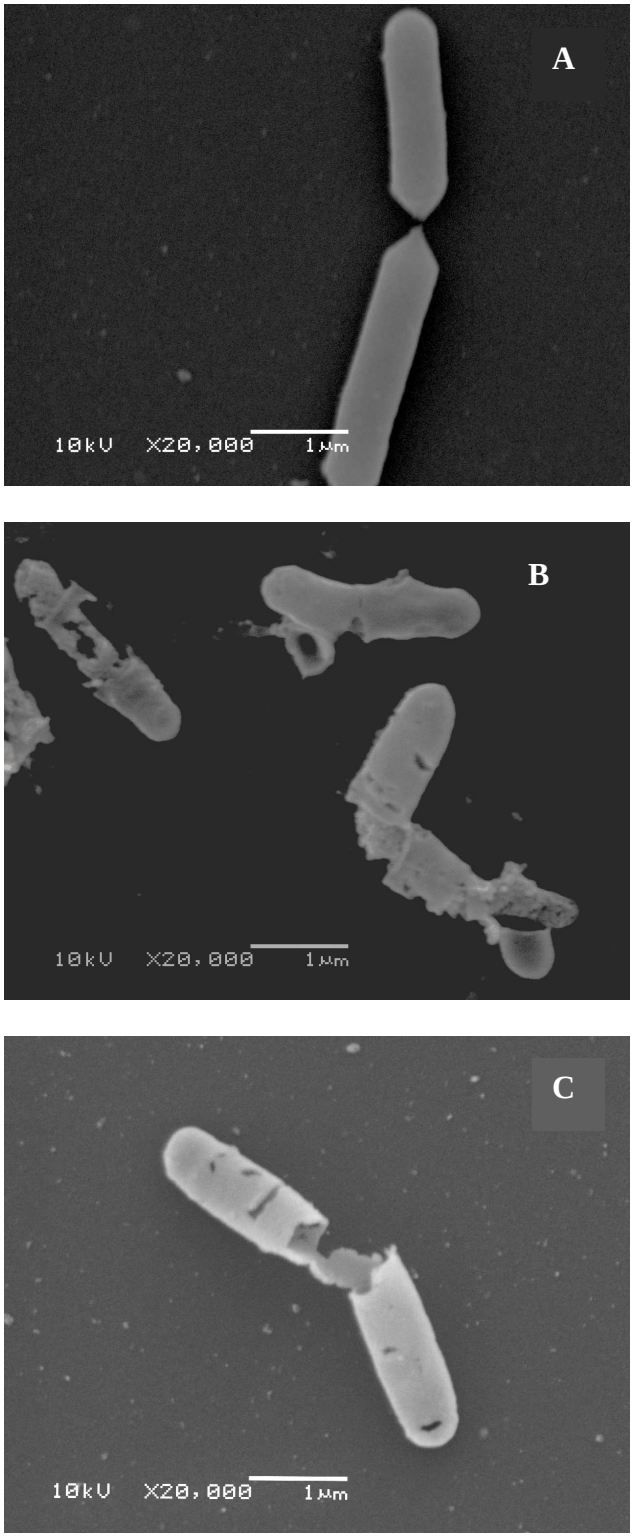

FIG. 4. Scanning electron micrographs of *Paenibacillus larvae*. Untreated cells (A) and treated cells for 120 min at 37 ºC with 3200 AU mL^{-1} of the bacteriocin (B and C).

4 DISCUSSÃO GERAL

Peptídeos antimicrobianos, como as bacteriocinas, auxiliam os microrganismos na sua sobrevivência. No entanto, as propriedades destes compostos vêm sendo investigadas para uso em diversas aplicações, da agricultura à medicina, como ferramenta para combater doenças e ainda como bioconservantes em sistemas alimentares com o objetivo de aumentar a segurança e estender a vida útil. Com o crescente desenvolvimento de resistência das bactérias aos antibióticos um significativo interesse nas bacteriocinas vem acontecendo. Estas proteínas são consideradas como candidatas ideais para os mais diversos usos devido ao fato de que seu especto de ação pode ser amplo e, enquanto atinge um patógeno específico, teoricamente não teria efeitos prejudiciais na microbiota normal dos seres humanos.

Neste estudo a substância antimicrobiana produzida pela cepa de *Bacillus amyloliquefaciens* LBM 5006, isolada de solo da Mata Atlântica, foi purificada, caracterizada e denominada bacteriocina LBM 5006.

A substância antimicrobiana foi produzida quando o isolado foi cultivado tanto em BHI como em TSB. A síntese teve início na fase logarítimica

e a produção máxima foi detectada após 32 h de cultivo, o que corresponde à fase estacionária de crescimento e sugere que o peptídeo antimicrobiano é secretado como um metabólito secundário.

A bacteriocina purificada apresentou manutenção da atividade frente a tratamentos térmicos, quando submetida à liofilização e em valores de pH entre 2 e 11. O tratamento com enzimas, nas concentrações testadas, não afetou a atividade da substância purificada. Algumas espécies de *Bacillus* sintetizam peptídeos antimicrobianos que apresentam estruturas cíclicas com constituintes não usuais, como D-aminoácidos, geralmente resistentes à hidrólise por peptidases e proteases (Stein, 2005).

Espectroscopias de infravermelho (FTIR) e de ultravioleta da substância purificada forneceram informações adicionais sobre sua estrutura que foi compatível com a de um polipeptídeo formado por cadeias alifáticas que podem estar associadas à predominância de aminoácidos hidrofóbicos ou à presença de um ácido graxo. Análises por espectrometria de massas indicaram a produção de dois peptídeos antimicrobianos com picos de 1058 Da e 1464 Da correspondentes a lipopeptídeos tipo-iturina e tipo-fengicina, respectivamente.

A análise de genes de *B. amyloliquefaciens* LBM 5006, através da Reação em Cadeia da Polimerase (PCR), constatou a presença do gene *ituD* relacionado a produção do peptídeo antimicrobiano iturina A e ausência de genes responsáveis pela síntese de surfactina. A síntese de iturina A por cepas de *Bacillus* é um fator considerado importante para conferir atividade antimicótica a este gênero microbiano.

Várias espécies de *Bacillus* são conhecidas como produtoras de metabólitos com atividade antimicrobiana. Geralmente três classes diferentes de peptídeos bioativos podem ser distinguidos: peptídeos antifúngicos, como bacilomicina e rizhoctina; lipopeptídeos antifúngicos como as surfactinas, iturinas e fengicinas e polipeptídeos como a subtilina (Pabel et al., 2003). Inúmeras cepas de *B. amyloliquefaciens* têm sido relacionadas à produção de lipopeptídeos com atividade antifúngica. Arguelles-Arias et al. (2009) demonstraram que a cepa de *B. amyloliquefaciens* GAI produz os lipopeptídeos cíclicos iturina A, fengicina A e B e surfactina que possuem grande potencial de uso no biocontrole de patógenos associados a plantas. Hiradate et al. (2002) isolando compostos bioativos da cepa RC-2 de *B. amyloliquefaciens* relacionaram sua atividade antimicrobiana a produção das iturinas A2 a A8. No estudo de Caldeira et al. (2008) *B. amyloliquefaciens* CCMI 1051 produziu vários compostos antifúngicos comparáveis a iturina e surfactina.

As bacteriocinas podem desempenhar um papel de defesa e impedir a invasão de outras cepas ou espécies em uma comunidade microbiana pré-estabelecida ou ainda limitar o avanço de células adjacentes. A mediação do *quorum sensing* é um papel adicional proposto para as bacteriocinas de bactérias Gram-positivas (Miller & Bassler, 2001; Riley & Wertz, 2002).

A avaliação dos efeitos de cultivos mistos (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923*, Bacillus cereus* ATCC 9634 e *Listeria monocytogenes* ATCC 7644) na síntese de bacteriocina por *Bacillus amyloliquefaciens* LBM 5006 indicou que o crescimento em associação com outros microrganismos pode induzir a um aumento na produção de

bacteriocinas por bactérias do gênero *Bacillus*. O cultivo com células de *Escherichia coli* intactas ou desnaturadas pelo calor (autoclavadas a 121ºC por 30 min) aumentou a atividade antimicrobiana da bacteriocina produzida pela cepa LBM 5006 o que sugere que um estímulo externo foi capaz de induzir a síntese do antimicrobiano. Como nos experimentos envolvendo as células da cepa indutora observou-se aumento da atividade da bacteriocina pode-se presumir que a produção é ativada pelo contato entre as células das cepas produtora e indutora.

O espectro de ação das bacteriocinas varia de acordo com o tipo de microrganismo produtor. A bacteriocina LBM 5006 apresentou um espectro de ação relativamente amplo pois foi capaz de inibir diversos microrganismos Gram-negativos e Gram-positivos, incluindo várias linhagens de *Listeria*, especialmente *Listeria monocytogenes*, que é o agente etiológico da listeriose, doença adquirida principalmente pelo consumo de alimentos contaminados, e que pode levar a uma taxa de mortalidade em torno de 30% em populações de risco como idosos, crianças, mulheres grávidas e indivíduos imunodeprimidos (Cleveland et al., 2001).

Esta bactéria encontra-se amplamente distribuída na natureza e há evidências de que cepas deste microrganismo isoladas de alimentos altamente processados poderíam ter origem no ambiente de processamento e não na matéria-prima utilizada. Estudos mostram que a *Listeria monocytogenes* pode adaptar-se muito bem ao ambiente de processamento passando a ser uma fonte de listeriose, além de gerar problemas de ordem social e econômica (Norrug & Kovgaard, 1993; Felon et al., 1996).

Atualmente muitos estudos se baseiam na produção das bacteriocinas e no uso como bioconservantes naturais para alimentos com o objetivo principal de controlar o desenvolvimento de bactérias deteriorantes e patogênicas. No entanto, antes de uma bacteriocina ser considerada para aplicação em alimentos, informações como características genéticas e bioquímicas, espectro antimicrobiano e modo de ação devem ser conhecidos. O uso efetivo das bacteriocinas em conservação de alimentos requer o entendimento do seu modo de ação e do efeito de sua ação inibitória nas diferentes condições bioquímicas que naturalmente ocorrem nos alimentos (Atrih et al., 2001; Zhou et al., 2008).

Foi avaliado o efeito provocado pela bacteriocina LBM 5006 em células de *Listeria monocytogenes*. Cultivos deste microrganismo, em fase exponencial de crescimento, foram tratados com a bacteriocina e observou-se um modo de ação tanto bacteriolítico quanto bactericida do peptídeo, com perda da viabilidade celular e diminuição da densidade óptica simultâneas. A captação de imagens por Microscopia Eletrônica de Varredura evidenciou alterações na morfologia e na topografia das células associadas aos danos possivelmente provocados pela bacteriocina nas paredes e membranas celulares do microrganismo indicador.

Bacteriocinas podem ter um modo de ação bactericida ou bacteriostático sobre células sensíveis. Esta distinção é influenciada por diversos fatores como a dose de bacteriocina e o seu grau de purificação, o estado fisiológico das células indicadoras (por exemplo, fase de crescimento) e as condições experimentais (por exemplo, pH, temperatura, presença de

agentes que afetam a integridade da parede celular e outros compostos antimicrobianos). A maioria das bacteriocinas exerce um efeito bactericida sobre microrganismos sensíveis, embora algumas atuem de forma bacteriostática. A atividade bactericida das bacteriocinas pode ser acompanhada por lise das células sensíveis (bacteriocinas bacteriolíticas) (Cintas et al., 2001).

A atividade antimicrobiana da bacteriocina LBM 5006 também foi observada sobre *Paenibacillus larvae*, uma bactéria esporogênica considerada como um dos patógenos mais importantes que afeta abelhas provocando uma doença altamente contagiosa e virulenta denominada *American Foulbrood* que é frequentemente fatal para as larvas de abelhas (Genersch et al., 2006).

A bacteriocina apresentou um mecanismo de ação bactericida sobre células vegetativas e esporos de *P. larvae*. Considerando que as larvas das abelhas se infectam através dos esporos da bactéria a ação de compostos antimicrobianos sobre estas estruturas é relevante. Também foram observados danos à estrutura do envelope celular (parede e membrana) com consequente efluxo de conteúdo celular o que caracteriza o peptídeo como uma bacteriocina bacteriolítica.

Compostos antimicrobianos de origem natural despertam particular interesse como agentes de biocontrole contra doenças de plantas por não agredirem o meio ambiente. Diversos estudos utilizando microrganismos produtores de bacteriocinas ou preparações a base de bacteriocinas vêm sendo abordados como uma alternativa de grande potencial para o controle de várias enfermidades e promoção de crescimento de plantas (Cladera-Olivera et

al., 2006; Guo et al, 2007; Trias et al., 2008; Lee et al., 2009). Inibição no crescimento dos fungos de plantas *Aspergillus phoencis*, *Aspergillus niger*, *Aspergillus flavus*, *Apiosordaria* sp., *Bipolaris sorokiniana*, *Cercosporina* sp., *Diplodia* sp., *Fusarium oxysporum* f. *lycopersici* e *Fusarium graminearum* caracteriza a atividade antimicótica da bacteriocina LBM 5006.

Membros do gênero *Acanthamoeba* são amebas de vida livre e estão presentes em diversos ambientes, da água doce ao solo, e também em seres humanos causando graves danos cerebrais e infecções da córnea. Seu ciclo de vida apresenta dois estágios: de trofozoítos ativos e de cistos dormentes (Lemgruber et al., 2010). Um grande número de agentes terapêuticos e extratos de plantas têm sido testados *in vitro* para atividade amebicida frente a espécies patogênicas de *Acanthamoeba* spp, entretanto resultados conflitantes são relatados. Em infecções humanas as terapias combinadas vêm apresentando melhores resultados que aquelas que utilizam uma única droga, porque algumas substâncias têm efeito amebostático mas não amebicida, além disso nenhuma droga simples mostrou ser efetiva contra trofozoítos e cistos simultaneamente (Marciano-Cabral, 1988; Marciano-Cabral & Cabral, 2003). Inúmeros peptídeos antimicrobianos sintetizados por bactérias apresentam amplo espectro de atividade, incluindo inibição de protozoários (Papagianni, 2003; Reddy et al, 2004). Neste estudo o composto antimicrobiano secretado por *B. amyloliquefaciens* LBM 5006 apresentou destacada atividade amebicida contra trofozoítos de cepas de *Acanthamoeba polyphaga*.

O surgimento na década de 80 de bactérias multirresistentes

estimulou as pesquisas por novos antimicrobianos baseados em peptídeos (AMPs). No desenvolvimento de AMPs para uso em terapêutica humana peptídeos oriundos de diferentes organismos e seus análogos sintéticos representam um papel crucial. Relatos de estudos da atividade *in vitro* de alguns AMPs contra parasitos torna-os candidatos em potencial para a produção de novos compostos antiparasitários usados de forma tópica ou sistêmica no combate a importantes doenças parasitárias (Papagianni, 2003). Neste sentido a bacteriocina LBM 5006 por ter apresentado atividade amebicida significativa encoraja o desenvolvimento de novos agentes baseados em AMPs para prevenção e tratamento de infecções causadas por *Acanthamoeba.*

A substância tipo-bacteriocina LBM 5006, produzida por *B. amyloliquefaciens*, foi purificada e caracterizada. Através do conhecimento de suas propriedades podemos inferir sobre seu potencial de uso como um agente de controle biológico, para a produção de preparações visando o controle de fungos fitopatogênicos e de *P. larvae* responsável pela AFB, patologia grave que acomete larvas de abelhas provocando grandes perdas econômicas para os apicultores. Além disso, a atividade inibitória da LBM 5006 frente à bactéria *L. monocytogenes*, patógeno de veiculação alimentar, sugere que o uso desta substância como bioconservante é viável e merece estudos adicionais de toxicidade, de possíveis interações com as demais moléculas presentes nos alimentos e de sinergismo com outros fatores de conservação.

As bacteriocinas representam um dos principais sistemas de defesa utilizados pelos microrganimos. Apesar do conhecimento limitado que se tem

sobre seu papel ecológico e suas relações evolutivas, devido a abundância e diversidade destes peptídeos, muitos estudos vêm sendo feitos para sua utilização como bioconservantes em alimentos, como antimicrobianos alternativos em saúde humana e animal e no controle biológico de patógenos na agricultura evitando os resíduos de produtos químicos e a consequente agressão ao meio ambiente.

5 CONCLUSÕES

A substância antimicrobiana purificada, produzida pela cepa LBM 5006 de *Bacillus amyloliquefaciens*, caracterizou-se quimicamente por ser uma mistura de dois peptídeos com massas moleculares de 1058 Da e 1464 Da correspondentes a lipopeptídeos tipo-iturina e tipo-fengicina, respectivamente.

A bacteriocina apresentou amplo espectro de ação, sendo inibitória para várias linhagens de *Listeria* spp., especialmente *Listeria monocytogenes*, um patógeno de veiculação alimentar.

O modo de ação da bacteriocina LBM 5006 frente a *Listeria monocytogenes* e *Paenibacillus larvae* foi bactericida e bacteriolítico, promovendo a morte das células por danos ao envelope celular e consequente efluxo do conteúdo celular.

Acentuada atividade amebicida da bacteriocina frente a *Acanthamoeba polyphaga* promoveu mudanças na morfologia das células, encistamento e lise celular.

O cultivo de *Bacillus amyloliquefaciens* LBM 5006 em presença de células de *Escherichia coli* inativadas termicamente estimulou a síntese da substância antimicrobiana pela cepa produtora.

Na biossíntese da bacteriocina LBM 5006 a atividade máxima foi observada na fase estacionária de crescimento, sugerindo uma cinética de metabólito secundário.

Características como natureza proteica, termoestabilidade, manutenção da atividade em ampla faixa de pH e amplo espectro de ação indicam que a bacteriocina LBM 5006 tem potencial para uso como bioconservante em alimentos, no controle biológico de fungos fitopatogênicos e de *Paenibacillus larvae* e na prevenção e terapia de infecções causadas por *Acanthamoeba*.

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