UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

PURIFICAÇÃO, CARACTERIZAÇÃO E AVALIAÇÃO DE TOXICIDADE DE UM PEPTÍDEO PRODUZIDO POR *Bacillus subtilis* subsp. *spizezinii* COM ATIVIDADE ANTIMICROBIANA FRENTE A *Haemophilus parasuis*

Mário Lettieri Teixeira

Porto Alegre, RS, Brasil
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Orientador: Dr. Adriano Brandelli

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do Grau de Doutor em Ciências.

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SUMÁRIO

LISTA DE ABREVIATURAS

AMP: peptideo antimicrobiano

ATCC: *American Type Culture Collection*

AU: Unidades arbitrárias

BLS: Substância tipo-bacteriocina

CBiot: Centro de Biotecnologia do Estado do Rio Grande do Sul

FDA: *Food and Drug Administration*

GRAS: *Generally Regarded as Safe*

Hb: hemoglobina

LDH: lactato desidrogenase

MFC: concentração fungicida mínima

MIC: concentração inibitória mínima

Purificação, caracterização e avaliação de toxicidade de um peptídeo produzido por *Bacillus subtilis* subsp. *spizezinii* com atividade antimicrobiana frente a *Haemophilus parasuis*

Autor: Mário Lettieri Teixeira

Orientador: Adriano Brandelli

RESUMO

Haemophilus parasuis é uma das primeiras e mais prevalentes bactérias colonizadoras de leitões, que afetam a população de suínos, entre 2 semanas a 4 meses de idade, causando a doença de Glässer. O objetivo deste trabalho foi purificar, caracterizar e avaliar a toxicidade de um peptídeo com atividade antimicrobiana frente a *H. parasuis* produzido por *Bacillus subtilis* subsp. *spizezinii*. A substância antimicrobiana foi purificada por precipitação com sulfato de amônio, cromatografia de filtração em gel (Sephadex G-50) e cromatografia de troca iônica (DEAE-celulose). A taxa de purificação foi cerca de 100 vezes com um rendimento de 0,33%. O peptídeo apresentou massa molecular de 1.083 Da e sua sequência foi determinada por espectrometria de massa (NRWCFAGDD). A inibição completa do crescimento de *H. parasuis* foi observada quando testada com concentração de 20 µg/mL do peptídeo antimicrobiano (AMP) após 20 min de exposição. O peptídeo foi testado para determinar a concentração inibitória mínima (MIC) e concentração fungicida mínima (MFC) contra diferentes isolados clínicos de leveduras susceptíveis. O peptídeo inibiu *Candida glabrata, C. tropicalis, C. parapsilosis* e *C. krusei*, mas não foi capaz de inibir *Trichosporon asahii* e *Cryptococcus neoformans*. O AMP foi testado em relação ao potencial citotóxico em tecido de suínos, sendo que o resultado da avaliação histopatológica do músculo esquelético, fígado, coração, rim e cérebro incubados com esta substância e também incubados com nisina não mostraram lesões microscópicas. Células suínas tratadas com as diferentes frações purificadas resultaram em aumento dos níveis de liberação de lactato desidrogenase e hemoglobina em comparação com a nisina. Tendo em vista a relevância desta pesquisa, estudos complementares devem ser realizados para avaliar o potencial biotecnológico deste composto químico.

Purification, characterization and evaluation of toxicity of a peptide produced by *Bacillus subtilis* subsp. *spizezinii* with antimicrobial activity against *Haemophilus parasuis*

Author: Mário Lettieri Teixeira

Advisor: Adriano Brandelli

ABSTRACT

Haemophilus parasuis is one of the earliest and most prevalent bacteria colonizing piglets, affecting the population of pigs from 2 weeks to 4 months of age, causing disease Glasser. The aim of this study was to purify, characterize and evaluate the toxicity of a peptide with antimicrobial activity against *H. parasuis* produced by *Bacillus subtilis* subsp. *spizezinii*. The antimicrobial substance was purified by ammonium sulfate precipitation, gel filtration chromatography (Sephadex G-50) and ion exchange chromatography (DEAE-cellulose). The purification rate was about 100 fold with a yield of 0.33%. The peptide had a molecular mass of 1,083 Da and its sequence was determined by mass spectrometry (NRWCFAGDD). The complete inhibition of *H. parasuis* growth was observed when tested at 20 µg/mL concentration of the antimicrobial peptide (AMP) after 20 min of exposure. The peptide was tested to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against different yeast strains susceptible. The peptide inhibited *Candida glabrata, C. tropicalis, C. krusei* and *C. parapsilosis*, but was not able to inhibit *Trichosporon asahii* and *Cryptococcus neoformans*. The AMP was tested against the cytotoxic potential in tissue of pigs, and the result of the histopathological evaluation of skeletal muscle, liver, heart, kidney and brain incubated with this substance and also incubated with nisin showed no microscopic lesions. Pig cells treated with the different purified fractions resulted in increased levels of lactate dehydrogenase release and hemoglobin and compared to nisin. Given the relevance of this research, further studies should be conducted to evaluate the biotechnological potential of this chemical compound.

1. INTRODUÇÃO

O uso inadequado de medicamentos e agentes terapêuticos no combate a microrganismos patogênicos tem sido um grande problema nos dias atuais. Esta conjuntura tem levado indústrias farmacêuticas a investirem uma grande soma de recursos financeiros em pesquisas que busquem novas substâncias que apresentem este potencial biotecnológico.

Uma das ferramentas mais utilizadas é a pesquisa de peptídeos produzidos por bactérias com atividade antimicrobiana, os quais podem ser sintetizados por via ribossomal ou não-ribossomal. Estes compostos são produzidos durante a fase *log* de crescimento microbiano e podem apresentar características químicas diversas e amplo espectro de ação.

Uma das possíveis aplicações deste tipo de composto químico pode ser na área da suinocultura. Esta atividade econômica é uma das principais fontes de renda para a região sul do Brasil, pois aproximadamente 50% do rebanho suíno está concentrado nesta área territorial (IBGE, 2010). Neste sentido, o aumento desta atividade econômica está baseado no aumento do número de animais, fato este que corrobora com o aumento da probabilidade da disseminação de patógenos, entre as quais, pode-se citar o *Haemophilus parasuis.*

Esta bactéria é responsável por um aumento na mortalidade de leitões na fase de creche em granjas, pois acomete o sistema respiratório superior destes animais causando dificuldade para respirar, inapetência e anorexia. O tratamento desta doença é feito de forma profilática através de programas de vacinação em massa ou pelo tratamento terapêutico a base de antibióticos de última geração, fato este que pode selecionar estirpes resistentes deste patógeno a estes compostos químicos, dificultando assim, o combate a esta enfermidade (MARTINDALE, 2009).

Dentro deste panorama, esta pesquisa se justifica pela busca de uma nova substância com atividade contra *H. parasuis* produzida por *Bacillus subtilis*, bem como pela investigação de uma metodologia de produção e purificação deste novo peptídeo antimicrobiano. Buscou-se, deste modo, caracterizar quimicamente este composto, elucidando a sua sequência de aminoácidos e sua massa

molecular, determinando o modo de ação e sua implicação toxicológica quando em contato com células de determinados tecidos de suínos. Além disso, também foi testada a sua atividade frente a algumas leveduras que causam doenças que apresentam implicações importantes à saúde do ser humano.

2. OBJETIVOS

2.1. Objetivo Geral

Expressar, purificar e caracterizar um peptídeo antibacteriano sintetizado por *Bacillus subtilis* subespécie *spizizenii* com atividade contra *Haemophilus parasuis.*

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2.2. Objetivos Específicos

1. Obter um composto com atividade antimicrobiana a partir de uma cultura de *B. subtilis* subespécie *spizizenii* ATCC 6633.
2. Purificar esta substância através de métodos cromatográficos.
3. Determinar a estabilidade do peptídeo frente a enzimas, pH, temperatura, compostos químicos.
4. Caracterizar a atividade antimicrobiana, avaliando qual a concentração inibitória e cinética de ação contra *Haemophilus parasuis*.
5. Avaliar a toxicidade deste peptídeo frente a células periféricas de suínos (hemácias, células musculares, hepatócitos, células renais, células cardíacas e tecido cerebral).
6. Avaliar a absorção do peptídeo antimicrobiano no intestino de suínos.
7. Avaliar a atividade antifúngica contra leveduras (*Candida glabrata, Candida krusei, Candida parapsilosis* e *Candida tropicalis*).

3. REVISÃO BIBLIOGRÁFICA

3.1. Antibióticos e Quimioterápicos

Antibióticos e quimioterápicos são compostos químicos capazes de inibir o crescimento e destruir determinadas espécies microbianas, de forma específica, a baixas concentrações e sem toxicidade (ou muito baixa) para o organismo humano. Os antibióticos são obtidos a partir do cultivo de microrganismos e os quimioterápicos são obtidos por síntese química. O cultivo microbiano é muito mais vantajoso que a síntese, uma vez que os antibióticos apresentam grande diversidade e complexidade química e, além disso, o gasto na realização da síntese é muito elevado (MARTINDALE, 2009).

Segundo a *American Society of Health-System* (2007), os antibióticos podem ser classificados de acordo com vários critérios, que são:

- quanto à origem:

1. Biológicos: são produzidos por microrganismos e estão subdivididos em:

a) Produzidos por bactérias: são sintetizados por bactérias, como por exemplo, a polimixina que é produzido pelo *Bacillus polymyxa*.

b) Produzidos por actinomicetos: Exemplo: cloranfenicol, produzido por *Streptomyces venezuelae*.

c) Produzidos por fungos: Exemplo: penicilina, produzida pelo *Penicillium notatum*.

2. Sintéticos: são produzidos exclusivamente por síntese química, como exemplo, pode-se citar os nitrofuranos e as sulfonamidas.

3. Semisintéticos: constituem o grupo mais numeroso e importante. Ao núcleo básico desses antibióticos, que são produzidos por microrganismos, adicionam-se radicais sintéticos na posição mais adequada, o que lhes confere melhor desempenho, redução da toxicidade, dentre outros.

- Quanto ao espectro de ação:

1. Amplo espectro: interferem no crescimento de numerosas espécies bacterianas. Exemplo: cloranfenicol e tetraciclinas.

2. Espectro intermediário: atuam frente a um número limitado de espécies. Exemplo: Penicilina.

3. Espectro curto: são eficazes apenas em relação a um número reduzido de espécies. Exemplo: Polimixinas.

- Quanto à forma e ação:

1. Bacteriostático: bloqueiam o desenvolvimento e multiplicação das bactérias, mas não promovem sua lise. Exemplo: cloranfenicol.

2. Bactericida: provocam a morte da bactéria. Exemplo: β-lactaminas.

- Quanto ao mecanismo de ação:

Antimicrobianos: atuam inibição da síntese da parede celular, da membrana citoplasmática e síntese protéica, de ácidos nucléicos e de metabólitos essenciais.

- Quanto à estrutura química:

Os antibióticos estão agrupados em famílias que, além de terem características químicas comuns, apresentam mecanismos de ação e espectro semelhante. As famílias mais importantes são β-lactâmicos, polipeptídicos, tetraciclinas, cloranfenicol, macrolideos, lincosamidas, rifamicinas, fosfinas, sulfamidas, nitrofurantoína, quinolonas e derivados nitro-heterocíclicos (AMERICAN SOCIETY OF HEALTH-SYSTEM*,* 2007; MARTINDALE*,* 2009).

Antibióticos são descobertos através da análise de microrganismos que ainda não foram examinados e principalmente, pelas técnicas de engenharia genética. Para que um antibiótico seja produzido comercialmente, primeiro ele deve apresentar uma satisfatória produção em larga escala. Um dos maiores problemas está no desenvolvimento de eficientes métodos de purificação. Se um antibiótico é solúvel num solvente orgânico, o qual é imiscível em água, a purificação do antibiótico pode ser relativamente simples. Se o antibiótico não é solúvel, então ele pode ser separado do líquido por adsorção ou precipitação química, em todos os casos, a meta é obter um produto cristalino, de elevada pureza. Mas, alguns antibióticos não cristalizam e são de difícil purificação. Um problema que surge é que algumas culturas frequentemente produzem outros produtos finais, incluindo outros antibióticos, e é essencial que no final exista um único produto consistindo de um único antibiótico. A seleção de estirpes envolve

mutação da cultura inicial, cultivando tipos mutantes, e testando-os para a produção de antibióticos. Em muitos casos, seres mutantes produzem menor quantidade de antibiótico (AMERICAN SOCIETY OF HEALTH-SYSTEM*,* 2007; MARTINDALE*,* 2009).

3.2. Peptídeos Antimicrobianos: Função e Distribuição

A descoberta de microrganismos resistentes aos antibióticos convencionais gerou um crescente interesse no estudo de peptídeos naturais com atividades antibióticas. Tais peptídeos, denominados peptídeos antimicrobianos, participam do sistema inato de defesa de animais e plantas. Atualmente, existem mais de 500 peptídeos antimicrobianos isolados e estudados. A produção de peptídeos antimicrobianos faz parte dos mecanismos de defesa do hospedeiro durante as etapas iniciais de infecção e sua importância na proteção contra patógenos tem sido descrita nas últimas décadas (ZASLOFF, 2002; GILLOR *et al.,* 2009; MEYER *et al.,* 2010).

3.2.1. Peptídeos antimicrobianos produzidos por bactérias

As bacteriocinas são definidas como substâncias bactericidas de natureza proteica ou, ainda, complexos de proteínas, sintetizados por algumas linhagens bacterianas com ação contra outras populações da mesma espécie, ou sobre outros organismos (TAGG *et al.*, 1976; RILEY, 1998; MARINGONI & KUROZAWA, 2002; VAUCHER *et al.*, 2010). A diferença de espectro de ação entre as bacteriocinas e os antibióticos tradicionais é que as primeiras apresentam uma ação direcionada a uma determinada bactéria, ou a algumas bactérias, portanto, possuem um espectro de ação curto, fato este que não ocorre com os antibióticos. Dessa forma, a quase totalidade das bactérias são produtoras de algum tipo de bacteriocina (TAGG *et al.*, 1976; RILEY, 1998; MARINGONI & KUROZAWA, 2002; NAGAO *et al.,* 2006; TEIXEIRA *et al.,* 2009).

As bacteriocinas foram caracterizadas, em primeiro lugar, nas bactérias Gram-negativas. Entre elas, destaca-se a família das colicinas (originadas de estirpes de *Escherichia coli*). As colicinas constituem um grupo bastante heterogêneo de proteínas antimicrobianas, pois atuam através de diversos mecanismos de ação, como, por exemplo, aumento da permeabilidade da membrana externa, inibição da síntese da parede celular e ainda podendo atuar na diminuição da atividade de enzimas como a RNA polimerase e DNA polimerase (JAMES *et al*., 1996; GOUAUX, 1997, TEIXEIRA, *et al.,* 2009; PAPAGIANNI & ANASTASIADOU, 2009).

A maior parte dos estudos na literatura que estão relacionados com a produção de bacteriocinas se refere às produzidas por bactérias Gram-positivas do que às produzidas por Gram-negativas. Esta afirmação pode ser confirmada quando se refere à aplicação tecnológica destes compostos protéicos, pois o maior exemplo disso são as bactérias ácido-lácticas (BAL), as quais são extensivamente utilizadas em processos de fabricação de produtos lácteos fermentados. A bactéria envolvida em diversos destes processos, *Lactococcus lactis*, é responsável pela produção da nisina (JACK *et al.,* 1995), a única bacteriocina liberada pela Agência Nacional de Vigilância Sanitária (ANVISA) para uso como conservante em alimentos (ANVISA, 2010).

Muitas bactérias ácido-lácticas têm papéis importantes na produção de alimentos fermentados, e algumas destas bactérias podem ser capazes de inibir o crescimento de uma ampla variedade de organismos presentes nos alimentos (CLEVELAND *et al*., 2001). Quanto à utilização das bacteriocinas na indústria para biocontrole, podem-se seguir três caminhos para sua introdução no alimento: Primeiro, nos alimentos fermentados, podem ser produzidos *in situ* pela adição de culturas lácticas bacteriocinogênicas no lugar das tradicionais culturas iniciadoras. Segundo, a adição destas culturas como adjuvantes. E terceiro, pela adição direta de preparações de bacteriocinas, sintética ou purificada de um sobrenadante de cultura da estirpe produtora. A produção *in situ* da bacteriocina pode acarretar o aumento da competitividade da estirpe produtora na matriz do alimento e contribuir para a prevenção da degradação do alimento (HUGAS *et al.,* 2003).

3.3. Características e classificação das bacteriocinas produzidas por bactérias Gram-positivas

Atualmente, as bacteriocinas sintetizadas por bactérias Gram-positvas são divididas nas seguintes classes (KLAENHAMMER, 1993; McAULIFFE *et al.*, 2001; EILSINK *et al.*, 2002, MEYER *et al.,* 2010):

- Classe I – lantibióticos (5 kDa)

Tipo Ia: moléculas de forma alongada

Tipo Ib: moléculas globulares

- Classe II

Subclasse IIa: bacteriocinas tipo-pediocina

Subclasse IIb: bacteriocinas com dois peptídeos

Subclasse IIc: bacteriocinas cíclicas (porção N-terminal e C-terminal ligadas convalentemente)

Subclasse IId: bacteriocinas não tipo-pediocina com estrutura linear

- Classe III

Bacteriocinas compostas por proteínas de elevada massa molecular.

Os lantibióticos (classe I) são peptídeos termoestáveis de baixa massa molecular (≤5 kDa), diferenciados dos demais pela presença de lantionina e derivados, resultante da associação de duas alaninas ligadas por uma ponte dissulfeto, ou alfa-metilantionina, resultante de um ácido aminobutírico ligado a uma alanina por uma ponte dissulfeto (JACK *et al*., 1995; NAGAO *et al.*, 2006). São sintetizados ribossomalmente, e sofrem modificações após a tradução e contêm 19-50 resíduos de aminoácidos, para adquirir forma ativa (McAULIFFE *et al.,* 2001). Neste grupo encontra-se a nisina, uma bacteriocina produzida por *Lactococcus lactis* (KAWAMOTO *et al.*, 2002).

Os lantibióticos são subdivididos em dois tipos de acordo com sua estrutura: Tipo Ia, são moléculas flexiveis e alongadas que têm peptídeos catiônicos e hidrofóbicos, que têm como mecanismo de ação a formação de poros na membrana. Apresentam uma massa molecular entre 2 e 5 kDa. O tipo Ib comprrende peptídeos globulares, com carga neutra ou negativa, e massa

molecular de 2 kDa. Estes peptídeos atuam interferindo nas reações enzimáticas celulares (SMITH & HILLMAN, 2008; PAPAGIANNI & ANASTASIADOU, 2009).

A classe II (≤10 kDa) é formada por peptídeos que apresentam estabilidade térmica, possuem uma estrutura helicoidal anfifílica, e esta facilita sua inserção na membrana citoplasmática da célula alvo. Este fato promove a despolarização da membrana e, consequente, morte celular. Esta classe pode ser subdividida em quatro subgrupos. A classe IIa é considerada como o maior subgrupo de bacteriocinas produzidas por BAL, pela sua atividade e seu potencial de aplicação. São constituidas por uma porção N-terminal com configuração de folha pregueada e uma porção C-terminal contendo uma ou duas alfa-hélices (FIMLAND *et al.*, 2005; GILLOR *et al.,* 2008). As bacteriocinas desta classe se inserem na membrana celular do microrganismo alvo pela porção C-terminal, promovendo a formação de poros e consequente dissipação da força próton motriz (DRIDER *et al*., 2006; GILLOR *et al*., 2008). Na tentativa de manter ou restaurar a força próton motriz, ocorre uma aceleração no consumo do ATP e, consequentemente, morte celular. Este subgrupo é classificado com base na similaridade na sequência de aminoácidos da parte N-terminal e sua alta especificidade na atividade anti-*Listeria*, consequentemente sendo considerado um potencial bioconservante em alimentos (ENNAHAR *et al.*, 2000, MEYER *et al.*, 2010).

A classe IIb é formada por bacteriocinas, cuja atividade depende da ação complementar de dois peptídeos diferentes. O mecanismo de ação também envolve a dissipação do potencial de membrana e diminuição da concentração intracelular de ATP (GARNEAU *et al.*, 2002, MEYER *et al.,* 2010).

A clase IIc contém todos as bacteriocinas não lantibióticas e que não pertencem as classes IIa e IIb e representam uma diversidade de bacteriocinas de várias BAL. Apresenta uma união covalente das terminações C e N, resultando em uma estrutura cíclica (KAWAI *et al.*, 2004).

As bacteriocinas da classe III são proteínas de elevada massa molecular (>30 kDa) e termolábeis. É a classe menos conhecida (ENNAHAR *et al.,* 2000; NIELSE *et al.*, 2003). Seu mecanismo de ação se diferencia das demais bacteriocinas, promovendo a morte celular através da lise da parede celular do

microrganismo alvo. Estas apresentam uma porção N-terminal similar a uma endopeptidase, responsável pela catálise da parede celular, e uma porção C-terminal responsável pelo reconhecimento da célula alvo (CHANG *et al.,* 2009).

3.4. Peptídeos Antimicrobianos: Mecanismo e Espectro de Ação

Apesar das diferenças, os modos de ação dos diversos peptídeos antimicrobianos envolvem, em geral, associação com os lipídios de membrana plasmática microbiana provocando aumento de sua permeabilidade. Em um primeiro momento ocorre uma atração eletrostática entre as moléculas de peptídeo (que geralmente possuem carga positiva) e lipídios aniônicos presentes na membrana. Em seguida, a estrutura anfipática dos peptídeos antimicrobianos desempenha o seu papel, promovendo a interação dos peptídeos antimicrobianos com a interface hidrofílica/hidrofóbica presente na superfície das biomembranas (MAGET-DIANA, 1999; NAGHMOUCHI, *et al.*, 2007).

Os diversos mecanismos propostos para o aumento na permeabilidade de biomembranas devido à interação com peptídeos antimicrobianos foram revistos por SCHREIER *et al*., (2000); LADOKHIN & WHITE, (2001); LOHNER, (2001); ZASLOFF (2002). Estes mecanismos podem ser resumidos em três modelos:

1- Formação de poros organizados compostos por múltiplas unidades de peptídeo organizadas em forma de um barril (*barrel stave model*) ou toróide (*wormhole model*) que atravessa a bicamada lipídica.

2- Os peptídeos comportam-se como detergentes, provocando alterações estruturais na membrana, deslocando lipídios e causando a formação de poros temporários ou mesmo lisando completamente a membrana (*carpet like model*).

3- A interação dos peptídeos com a superfície das biomembranas provoca distúrbios físico-químicos. O acúmulo de moléculas de peptídeos (muitas vezes envolvendo a formação de agregados) ocasiona flutuações e instabilidades estruturais locais na bicamada lipídica que resultam na formação de poros transientes.

Existem duas hipóteses para explicar como a permeabilização da membrana afeta a viabilidade dos microrganismos. Uma baseia-se na perda de energia provocada pelo desequilíbrio e consequente destruição do potencial eletroquímico devido à formação de poros. A outra hipótese seria de que o peptídeo, após danificar a membrana, pode atravessá-la e interferir com funções intracelulares, através de interação com macromoléculas como proteínas e ácidos nucléicos. Em alguns casos ainda, foi demonstrada a ligação do peptídeo antimicrobiano com receptores de membrana (ANDREU & RIVAS, 1998; WU, *et al*., 1999; GANZ & LEHNER, 2001). Entretanto, ainda existe muita discussão sobre o mecanismo de ação dos peptídeos antimicrobianos.

Apesar das discordâncias sobre o modo de ação dos peptídeos antimicrobianos, existe um consenso de que, na grande maioria dos casos, a membrana está diretamente envolvida. Os peptídeos antimicrobianos são ativos contra alvos que possuem membrana plasmática, como bactérias, fungos, protozoários e células tumorais (HANCOCK & DIAMOND, 2000).

O efeito generalizado destes peptídeos sobre a membrana dificulta a seleção de linhagens resistentes destes patógenos, tendo em vista a improbabilidade da ocorrência de mutações capazes de produzir grandes alterações no componente lipídico da membrana dos microrganismos (ANDREU & RIVAS, 1998; ZASLOFF, 2002).

3.5. Bacteriocinas Produzidas pelo Gênero *Bacillus*

O gênero *Bacillus* é reconhecido por apresentar muitas espécies que produzem peptídeos com ação bactericida e fungicida. Estes são classificados como metabólitos secundários, sendo secretadas para o meio extracelular, onde exercem a ação antimicrobiana (KATZ & DEMAIN, 1977; KENNEY & MORAN, 1991; ZHENG, *et al*., 1999).

B. subtilis tem sido alvo de estudos bioquímicos durante várias décadas, e é considerado como um marco na pesquisa de bactérias produtoras de peptídeos

antibacterianos que apresentam um amplo espectro de atividade (MOSZER *et al*., 2002; SONENSHEIN *et al*., 2003).

Algumas espécies de *Bacillus* têm uma história de produção de substâncias utilizadas na indústria alimentícia devido a sua inocuidade, inclusive como aditivos alimentícios (DE BÔER & DIDERICHSEN, 1991; PEDERSEN *et al*., 2002). Este gênero apresenta uma grande variedade de espécies que produzem bacteriocinas ou substâncias do tipo-bacteriocinas ("bacteriocin-like substance, BLS"), que apresentam atividade antimicrobiana contra microrganismos deteriorantes e patogênicos, tais como, *Listeria monocytogenes*, *Staphylococcus aureus* e *Erwinia carotovora* (CLADERA-OLIVERA, *et al*., 2006; NAGHMOUCHI *et al*., 2007). Estes incluem *B. subtilis* (ZHENG *et al*., 1999), *B. thuringiensis* (KAMOUN *et al*., 2005), *B. amyloliquefaciens* (LISBOA *et al*., 2006), *B. cereus* (BIZANI & BRANDELLI, 2002) e *B. licheniformis* (TEIXEIRA *et al*., 2009)

3.6. Bacteriocinas Produzidas por *Bacillus subtilis*

Bacillus subtilis tem sido alvo de muitos estudos sobre bacteriocinas ao longo das últimas décadas devido à grande produção de peptídeos e moléculas com ação antibacteriana. Os peptídeos antimicrobianos exibem propriedades químicas especificas como elevada rigidez, estruturas hidrofóbicas e/ou cíclicas com componentes atípicos como D-aminoácidos e são geralmente resistentes à hidrólise por peptidases e proteases. Um exemplo desta classe de compostos químicos é a subtilina, um lantibiótico pentacíclico de 32 aminoácidos, amplamente utilizado em associação com a nisina como biopreservativo (ROSS *et al.* 2002). Estudos relatam a produção de ericina por estirpes de *B. subtilis* A1/3. Este peptídeo apresenta duas isoformas (A e S), que são codificadas pelos genes estruturais *eriA* e *eriS.* A ericina S e subtilina diferem quimicamente apenas em quatro resíduos de aminoácidos, mas apresentam propriedades antimicrobianas semelhantes. No entanto, a ericina A tem uma organização química diferente (16 substituições de aminoácidos em comparação com ericina S) e apresenta uma atividade antibacteriana um pouco diferente (STEIN, 2005).

Outro lantibiótico produzido por esta espécie é a mersacidina, a qual é representante de lantibiótico do tipo B, esta molécula apresenta uma estrutura mais globular e atua por inibição da síntese da parede celular (BROTZ *et al*., 1997).

A literatura ainda cita a produção de lantiobióticos não-usuais como a sublancina, um peptídeo com 168 resíduos de aminoácidos que apresenta duas pontes dissulfeto na sua composição e que apresenta ação inibitória contra bactérias Gram-positivas (PAIK *et al.,* 1998; WESTERS *et al.*, 2003). Outro representante desta classe é a subtilosina A, produzida por várias estirpes de *B. subtilis*. Esta molécula apresenta uma estrutura macrocíclica, com três ligações inter-residuais, tem o seu efeito bactericida contra bactérias Gram-positivas, incluindo *L. monocytogenes* (ZHENG et al., 1999;. MARX *et al.*, 2001; KAWULKA *et al*., 2004; STEIN, 2005),

Estudos também demonstraram a produção de peptideos antibacterianos não ribossomais. Estes compostos estão amplamente difundidos entre bactérias e fungos e apresentam as mais variadas rotas metabólicas de síntese, envolvendo β-hidróxi ou β-aminoácidos na sua composição, além de variações de comprimento e ramificações das cadeias de ácidos graxos e substituições de aminoácidos (SIEBER & MARAHIEL, 2003; FINKING & MARAHIEL, 2004; WALSH *et al.*, 2011). Um exemplo desta classe é o lipoheptapeptideo surfactina, que é considerado um dos mais potentes biotensoativos conhecidos, podendo exercer uma ação detergente nas membranas biológicas (CARRILLO *et al*., 2003.), além de uma alta atividade emulsificante, antiviral e antimicoplasma (PEYPOUX *et al*., 1999). Outro representante é a família da iturina, a qual engloba os lipoheptapeptideos micosubtilina, iturina e bacillomicina, os quais apresentam uma semelhança estrutural além de um espectro de atividade semelhante, atuando contra bactérias, fungos e por serem agentes com ação hemolítica (DUITMAN *et al.,* 1999; TSUGE *et al*., 2001; MOYNE *et al*., 2004).

Uma série de novos antibióticos têm sido recentemente isolados a partir de estirpes de *B. subtilis.* A bacilisocina, que se caracteriza por ser um fosfolípido derivado do fosfatidilglicerol, foi isolada de estirpes de *B. subtilis* 168 (TAMEHIRO *et al.*, 2002). Amicoumacina é também um exemplo de um novo composto isolado

de estirpes de *B. subtilis*, mas não é produzida pela estirpe de *B. subtilis* 168. Esta substância apresenta atividades antibacterianas e ação anti-inflamatória (PINCHUK *et al.*, 2001; PINCHUK *et al.*, 2002). Da mesma forma, a produção de neotrehalosadiamina (NTD), um potente antimicrobiano, foi reportado em estirpes selvagens de *B.subtilis* após a indução de resistência a rifampicina (INAOKA *et al*., 2004).

3.7. Toxicidade de Peptídeos Produzidos por *Bacillus subtilis*

Embora exista ampla informação sobre as características químicas e estruturais de diversas bacteriocinas, relativamente poucos estudos descrevem seu potencial toxigênico, e cujas comparações são difíceis porque existe uma grande discrepância de metodologias utilizadas na verificação da mesma. Mais recentemente alguns estudos têm comparado a relação atividade antimicrobiana *versus* citotoxicidade para algumas espécies de bactérias (EIJSINK, *et al*., 1998; CLEVELAND *et al.,* 2001; SMITH & HILLMAN, 2008).

Algumas bactérias já foram descritas na literatura como produtoras de bacteriocinas que apresentaram ação tóxica, como *B. licheniformis,* associado a quadros patológicos, como gastroenterites, septicemia, peritonite, e à deterioração de alimentos. Ainda, existem relatos na literatura que *B. thuringiensis*, *B. mycoides* e *B. cereus* produzem toxinas com propriedades hemolíticas e citotóxicas, causando distúrbios do trato gastrintestinal. Além disso, estirpes de *B. licheniformis* podem produzir toxinas que causam inibição da motilidade de espermatozóides, apresentando um limiar tóxico superior inclusive de estirpes de *B. cereus,* reconhecidamente um agente extremamente tóxico (SALKINOJA-SALONEN, *et al*., 1999; MIKKOLA, *et al*., 2000; NIEMINEN, *et al*., 2007; TEIXEIRA *et al*., 2009).

A literatura reporta a produção de precursores isoprenóides com atividade tóxica por *B. subtilis*, esta toxicidade está relacionada ao acúmulo destes compostos o que compromete a biossíntese de derivados do ciclopentanofenantreno (SIVY *et al.,* 2011). A listeriolisina O, produzida por

estirpes de *B. subtilis* BR1-S, apresenta atividade citotóxica em células eucarióticas, inclusive em células de seres humanos (STACHOWIAK *et al.,* 2011).

3.8. *Haemophilus parasuis*

Haemophilus parasuis é uma bactéria Gram-negativa, comensal do trato respiratório superior de suínos. Este organismo é particularmente interessante por causa da sua habilidade de invadir o hospedeiro e causar lesões graves, as quais são caracterizadas por poliserosite, poliartrite e meningite fibrinosas. Até o presente momento, pouco é conhecido sobre a patogenia, fatores de virulência e imunogenicidade de *H. parasuis,* o que dificulta o controle de infecções sistêmicas causadas por este organismo (OLIVEIRA & PIJOAN, 2002). O genoma completo desta bactéria foi completamente sequenciado em 2009, esta sequência está depositada no *GenBank* como *H. parasuis* (estirpe SH0165) (código CP 001321). Esta estirpe foi isolada de animais que apresentavam a doença de Glässer em uma fazenda localizada na China. Este genoma apresenta 2.269.156 nucleotídeos, que codificam 2.299 genes (NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION, 2010).

O diagnóstico de *H. parasuis* como um agente primário responsável pela mortalidade no rebanho requer a associação entre o isolamento deste organismo e a presença de sinais clínicos e lesões características da enfermidade de Glässer. O controle da enfermidade, por outro lado, depende da caracterização das diferentes estirpes envolvidas em doença sistêmica e do estudo da epidemiologia do agente dentro e entre os rebanhos afetados (SEGALES *et al.,* 2004; SACK & BALTES, 2009; OLVERA *et al.*, 2010).

O *H. parasuis* apresenta 15 sorotipos diferentes, mas em estudos epidemiológicos realizados a sorologia não tem apresentado resultados confiáveis, pois uma parte das estirpes de *H. parasuis* não reagem, sendo consideradas como estirpes não-tipadas (KIELSTEIN & RAPP-GABRIELSON, 1992; OLIVEIRA & PIJOAN, 2002). A presença de um perfil protéico celular e virulência do *H. parasuis* está associada ao tipo de sorovar, enquanto a produção

de oligopolissacarídeos podem ocorrer em estirpes virulentas e avirulentas (ZUCKER *et al.*, 1996). Os sorovares que apresentam maior virulência são os 1, 5, 10, 12, 13 e 14, enquanto os sorovares 2, 4 e 15 são classificados como moderadamente virulentos e os sorotipos restantes 3, 6, 7, 8, 9 e 11 são considerados não virulentos. A classificação dos sorotipos está relacionada com o índice de mortalidade de cada estirpe (KIELSTEIN & RAPP-GABRIELSON, 1992; AMANO *et al.,* 1994).

3.8.1. Fatores de virulência do Haemophilus parasuis

Estudos sobre os fatores de virulência do *H. parasuis* ainda não estão completamente elucidados, mas existem pesquisas sendo desenvolvidas neste sentido, revelando que alguns compostos químicos participam no processo de instalação da doença causada por esta bactéria. As várias linhagens de *H. parasuis* expressam em comum, por exemplo, determinadas proteínas, como as que compõem a membrana externa, as quais podem estar associadas aos perfis de virulência. Pesquisas atuais estão focadas na expressão de genes *in vivo*, em que já está sendo identificada uma variedade de genes potencialmente relacionados a uma maior virulência. Entre estes genes, podem-se citar os que codificam proteínas transportadoras de membrana, enzimas e proteínas de superfície. Ainda, neste contexto, a presença de compostos químicos do tipo lipo-oligossacarídeos aumenta consideravelmente a adesão desta bactéria ao tecido do animal, especialmente nas células da traquéia, fato este que contribui para o aumento da gravidade da doença causada por este agente etiológico (RUIZ *et al.*, 2001; METCALF & MACINNES, 2007; BOUCHET *et al.,* 2009).

A resposta imunológica do organismo vivo que está infectado por esta bactéria está relacionada ao grande número de proteínas do tipo adesinas e proteases que são expressas pelo *H. parasuis*. Entre estas proteínas, as mais bem caracterizadas são as proteínas de membrana externa P2 e P5. A proteína P2 é codificada pelo gene *ompP2*, é uma porina de expressão dominante, e entre as linhagens de *H. parasuis* apresenta-se de forma heterogênea. Esta proteína

está associada na difusão e transporte de substratos específicos de derivados de nicotinamida-dinucleotideo, extremamente necessários ao desenvolvimento deste organismo. Desta maneira, alguns tipos de antibióticos atuam neste local, alterando a permeabilidade da membrana, causando, consequentemente, a morte desta bactéria (HILTKE *et al*., 2002; YUE *et al.*, 2009).

Outra proteína bastante estudada é a proteína de membrana P5, codificada pelo gene *ompP5*, a qual está presente na grande maioria de bactérias Gram-negativas. Esta proteína apresenta oito regiões transmembrana e quatro regiões expostas na superfície da membrana, e isto está relacionado ao desenvolvimento de uma resposta imune por parte do hospedeiro. Além destas proteínas, a presença dos genes *hhdA* (*putative hemolysin precursor*), *fimB* (*fimbrial assembly chaperone*), e *hsdR* (*type I site-specific restriction modification system, R subunit*) também podem ser considerados como fatores de virulência em isolados clínicos de *H. parasuis* (HILL *et al.,* 2001; DAVIES & LEE, 2004; MCVICKER & TABATABAJ, 2006; ZHANG *et al.*, 2013).

3.9. Purificação de Peptídeos Antimicrobianos

Um dos esquemas mais bem sucedidos para a purificação de bacteriocinas tipo-lantionina tem a vantagem de relacionar a natureza catiônica e a hidrofobicidade destas moléculas e foi planejado inicialmente por Sahl & Brandis (1981) para a purificação do Pep5 (lantibiótico produzido por estirpes de *Staphylococcus epidermidis*). O método, que envolve etapas sequenciais de adsorção, de cromatografia de troca-iônica e de cromatografia em gel-filtração, com modificações incluindo cromatografia de fase reversa e líquida de alta pressão (HPLC), foi usado subseqüentemente para purificar uma variedade de lantibióticos incluindo subtilina (SCHÜLLER *et al.,* 1989), a SA-FF22 (JACK *et al*., 1992, 1994) e a salivaricina A (ROSS *et al*., 1993).

Um grande número de bacteriocinas não-lantioninas são purificadas de acordo com métodos que consistem no crescimento de estirpes produtoras em um meio nutriente apropriado (preferivelmente líquido) sob condições ótimas para

a produção de bacteriocina, formando um precipitado de células bacterianas e um sobrenadante (após centrifugação), contendo as proteínas de interesse. Após este processo, ocorre a precipitação das proteínas do sobrenadante por meio da adição de sulfato de amônio. As proteínas precipitadas são dissolvidas subseqüentemente em água deionizada ou em um tampão de baixa força iônica, e as moléculas de bacteriocina são separadas pelo uso de vários procedimentos incluindo cromatografia de interação hidrofóbica, de troca-iônica, e de gel-filtração (BERRIDGE *et al*., 1952; BHUNIA *et al*., 1988, 1991; HASTINGS *et al*., 1991; HOLO *et al*., 1991; MURIANA *et al*., 1991; VAN BELKUM *et al*., 1991; HECHARD *et al*., 1992; HENDERSON *et al*., 1992; HOLCK *et al*., 1992; TAHARA *et al*., 1992; TICHACZEK *et al*., 1993; VENEMA *et al*., 1993).

4. CAPÍTULO I

ISOLATION OF *Haemophilus parasuis* FROM DIAGNOSTIC SAMPLES IN THE SOUTH OF BRAZIL.

Nesse capítulo será discutido o isolamento de *Haemophilus parasuis* isolados de suínos de propriedades localizadas na região sul do Brasil. O estudo contempla o aumento de casos desta bactéria no período de 2007 a 2010.

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Short Communication

Isolation of *Haemophilus parasuis* from Diagnostic Samples in the South of Brazil

Mario L. Teixeira[1], Suzana S. Kuchiishi[2], Adriano Brandelli[3]

[1]Curso de Medicina Veterinária, Instituto Federal Catarinense - Campus Concórdia, Concórdia, Brazil
[2]Centro de Diagnóstico em Sanidade Animal (CEDISA), Concórdia, Brazil
[3]Laboratório de Bioquímica e Microbiologia Aplicada, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
Corresponding author: Adriano Brandelli, Laboratório de Bioquímica e Microbiologia Aplicada, Universidade Federal do Rio Grande do Sul. Av. Bento Gonçalves, 9500 - ICTA, 91501-970 Porto Alegre, Brazil. fax: +55-51-33087048
E-mail: abrand@ufrgs.br

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Abstract

Haemophilus parasuis is one of the first and most prevalent colonizers of piglets, affecting the swine population from 2 weeks to 4 months of age. In this work, the relative incidence of *H. parasuis* isolated from swine in the west of Santa Catarina from 2007 to 2010 was investigated. White, flat and non-hemolytic colonies (satellitism growth with *Staphylococcus aureus*) of approximately 1 mm in length were identified by biochemical tests. Out of 384 samples examined during this period, 32 (8.33%) tested positive to isolation of *H. parasuis*. In 2007 and 2008 only 1 case of *H. parasuis* was isolated per year from a total of 62 and 60 samples, respectively. In 2009, 19 cases from 126 (15.08%) and in 2010, 11 cases of *H. parasuis* were isolated from a total of 136 samples (8.09%). These results suggest a growing incidence of this bacterium in the swine flock in this area.

Key Words: *Haemophilus parasuis*, swine, respiratory system, microbiology

Pig breeding in farms is an activity of high economical importance in Southern Brazil. The pig flock in this region increased from 15,984,115 to 17,798,250 in the last 3 years. In this same period, the flock in the western of Santa Catarina State increased from 5,491,599 to 5,952,862. This area is responsible for 75.87% of the swine flock in the state and 16.17% of the swine flock in the country (3). Therefore, the slightest problem in animal sanitation may significantly impact the economy of both the state and country. This clearly indicates the need of appropriate methods to control major pathogens such as *Haemophilus parasuis* (9).

The bacterium *H. parasuis* is the etiological agent of Glässer's disease, which is characterized by polyserositis and fibrinopurulent polyarthritis (14, 15). *H. parasuis* is one of the earliest and most prevalent colonizers of piglets and this microorganism can affect the swine population from 2 weeks to 4 months of age. However, it is more generally observed in pigs from 5 to 8 weeks (9, 14). This disease is currently considered an economical threat to the swine industry due to the high cost of antibiotic treatments and the discarding of affected animals since it also occurs in farms with high sanitary standards (8).

H. parasuis infections have been traditionally diagnosed through clinical signs, presence of lesions during necropsy, and bacteriologic culture (20). Bacterial isolation is difficult and the best method to achieve this is to collect exudates of certain organs. The most commonly used cultural medium is tryptose blood agar because *H. parasuis* is a fastidious microorganism (14). Alternative techniques for the diagnosis of *H. parasuis* infections have been proposed, like immunohistochemistry, PCR-based assays, restrictive endonuclease fingerprinting, enzyme immunoassays (ELISA), among other (10, 12, 16, 17).

Considering the importance of diagnosing *H. parasuis* in swine and clean sanitation practices in pig breeding, the objective of this work was to study the relative incidence of *H. parasuis* isolated from swine in the west of Santa Catarina from 2007 to 2010.

H. parasuis strains were isolated from samples of clinical cases and sent to Center of Diagnosis of Animal Sanitation from 2007 to 2010. A total of 384 cases were analyzed during this period.

The samples used to isolate the microorganism were lungs, and pericardial and cerebrospinal fluids. Blood agar (5% defibrinated sheep blood) plates with a streak of *Staphylococcus aureus*, which provides the required Factor V, were used. Plates were incubated for 48 h at 37°C in microaerophilic condition (5% CO_2 in air). If satellitism growth was observed, the colonies were analyzed. White, flat and non-hemolytic colonies approximately 1mm in length were identified using biochemical tests and gram staining. These colonies were replicated in Nicolet agar in microaerophilic condition (5% CO_2) at 37°C for 48h. All isolates were subsequently characterized biochemically by fermentation of glucose and sucrose and non-fermentation of lactose, mannitol, xylose, arabinose, threalose, raffinose, esculin (negative hydrolysis), negative for urease production and non decomposition of tryptophan in indole and catalase positive (6). The gram stain showed gram negative coccobacilli. Samples presenting this profile were considered positive for *H. parasuis*.

Between January 2007 and December 2010, 32 *H. parasuis* strains were isolated from 384 samples examined (8.33%). In 2007, only 1 strain of *H. parasuis* was isolated from 62 samples (1.61%). One strain was also isolated in 2008 (1.66%), but an increase in positive cases for *H. parasuis* was observed in 2009, since 19 strains were isolated from 126 samples (15.08%). In 2010, 11 cases of *H. parasuis* were isolated from a total of 136 samples (8.09%) (Fig. 1).

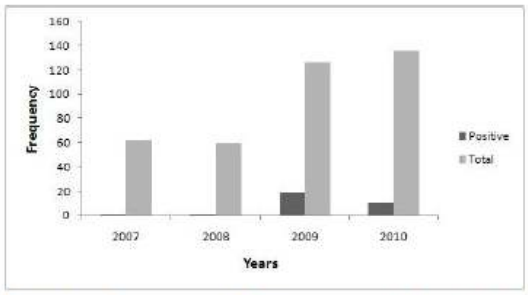

Figure 1. Frequency of the cases of *Haemophilus parasuis* in the period from January 2007 to December 2010, in the South of Brazil.

A total of 103 of *H. parasuis* were isolated from diseased pigs in association with routine diagnostics in Denmark, and most isolates were from cases of animals with bronchopneumonia without systemic disease (1). In the period of June 2003 to December 2004, 828 cases of clinically affected pigs in China were investigated for *H. parasuis*, which was isolated from 183 cases (2). For those cases, co-infection with other species of swine bacterial pathogens was also observed.

The number of positive cases of *H. parasuis* was classified according to the exudate type (Table 1). In the years of 2007 and 2008, strains were isolated from the lung. Although in 2009 and 2010 some strains were also obtained from pericardial and cerebrospinal fluids, most samples positive for *H. parasuis* were also from lungs. The percentage of *H. parasuis* strains isolated from lungs in 2009 and 2010 corresponded to 78.95% and 72.73%, respectively.

Table 1. Number of positive cases for *Haemophilus parasuis* according to the exudate source in the period from 2007 to 2010 in the South of Brazil.

Exudate source	Years			
	2007	2008	2009	2010
Lung	1	1	15	8
Pericardial fluid	0	0	3	3
Cerebrospinal fluid	0	0	1	1
Total of cases	1	1	19	11

The results of this work indicate an important increase in isolation of *H. parasuis* in this area of Brazil. This increase was nearly 2,000% (from 1 annual case to 19 cases from 2008 to 2009). The number of exams also increased but not in the same proportion, growing from an average of 61 annual tests (2007-2008) to 126 exams in the year of 2009, and 136 exams in 2010. These numbers could be underestimated since small farms possess limited financial resources to maintain efficient sanitary control. In other words, the number of cases of *H. parasuis* affected piglets may be higher and this etiological agent could be contaminating more animals causing an increased number of deaths. A fact that can be associated with the increased number of *H. parasuis* isolation in the period of 2009 is that the laboratory observed a larger demand of samples, which were positive in the diagnosis of viral pneumonia. This clinical picture might have contributed to a larger spread of this pathogen, because the immunodepression caused by the pneumonia facilitates the installation of this bacterium, resulting in a secondary infection. In 2010, this situation repeated but in a smaller number of diagnosed cases.

The number of *H. parasuis* cases has been increasing worldwide, mainly in the countries that export pork meat because the fattening process is done as fast as possible and in some case without sufficient

sanitary standards. In a study done in Australia, 19 of 20 researched farms showed the presence of *H. parasuis*, even in 2 areas that were considered free from this pathogen for many years (19). In another study, it was confirmed that several genotypes of *H. parasuis* are thoroughly distributed in the southeast of China (4). In this context, the feeding is optimized for the production of fast weight gain and the use of antibiotics is thoroughly diffused. The consequences are the production of weak animals and the development of antibiotic resistance. Another important factor is the presence of *H. parasuis* with other pathogens related to the respiratory system, such as *Pasteurella multocida*, *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, *Actinobacillus suis* and *Bordetella bronchiseptica*. The association of more than one of these microorganisms complicates the function of the immune system of the animal making them easy targets of respiratory diseases. Consequently, the mortality rate increases considerably (5, 7, 18).

Based on these results, a larger sanitary control would be desirable to protect the flock from the spread of *H. parasuis*. Therefore, the adoption of safety regulations to avoid the spread of this pathogen could be: (a) better handling and maintenance of the maternity and daycare (followed by a sanitary emptiness for around six days), (b) the improvement of hygiene in the facilities, (c) frequent disinfections and avoiding infected animals from entering the facilities, (d) to eliminate the factors of predisposition that can act negatively on the pig increasing its sensibility to infection (such as the cut on a tooth/ teeth, allowing lesions in the gum and/or tongue) (10). A more severe sanitary control should be implemented, since other diseases can reduce the immunity of the animal and may facilitate the proliferation of *H. parasuis*, which belongs to the natural microbiota of the upper respiratory tract of healthy swine (11). Finally, the use of prophylactic treatment, such as vaccines, a tool utilized in countries like USA and Spain (13), could be used to control the disease in a certain area.

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4.1. Conclusões

- A bactéria *Haemophilus parasuis* está presente no país e o número de casos aumentou consideravelmente no biênio 2009-2010 em relação ao biênio 2007-2008 na região produtora de suínos;
- É de suma importância a implantação de medidas de controle sanitário para diminuir o número de casos de infecções causadas por *H. parasuis*;
- A utilização de medidas profiláticas, como vacinas, pode ser uma ferramenta útil neste processo.

5. CAPÍTULO II

CHARACTERIZATION OF AN ANTIMICROBIAL PEPTIDE PRODUCED BY *Bacillus subtilis* subsp. *spizezinii* SHOWING INHIBITORY ACTIVITY TOWARDS *Haemophilus parasuis*

Nesse capítulo serão discutidas as etapas de purificação do peptídeo antimicrobiano produzido por *B. subtilis* subespécie *spizizenii.* O estudo contempla, ainda, a caracterização química, a sequência de resíduos de aminoácidos do peptídeo antimicrobiano, bem como o modo de ação do mesmo.

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Characterization of an antimicrobial peptide produced by *Bacillus subtilis* subsp. *spizezinii* showing inhibitory activity towards *Haemophilus parasuis*

Mário Lettieri Teixeira, Andréia Dalla Rosa and Adriano Brandelli

Correspondence
Adriano Brandelli
abrand@ufrgs.br

Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos, ICTA, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, Brasil

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Haemophilus parasuis is the pathogen that causes Glässer's disease, a major illness affecting young pigs. The aim of this work was to investigate the antagonistic activity of antimicrobial substances produced by *Bacillus* species against *H. parasuis*. Among the tested strains, only *Bacillus subtilis* ATCC 6633 inhibited *H. parasuis* growth. The antibacterial substance was purified by ammonium sulfate precipitation, gel filtration chromatography on Sephadex G-50 and ion-exchange chromatography on DEAE-cellulose. The purification was about 100-fold with a yield of 0.33 %. The purified substance was resistant up to 80 °C and pH ranging 3–7, but the substance lost its activity when it was treated with proteases. The peptide had a molecular mass of 1083 Da and its sequence was determined by MS as NRWCFAGDD, which showed no homology with other known antimicrobial peptides. The complete inhibition of *H. parasuis* growth was observed at 20 μg peptide ml^{-1} after 20 min of exposure. The peptide obtained by chemical synthesis also showed antimicrobial activity on *H. parasuis*. The identification of antimicrobial substances that can be effective against *H. parasuis* is very relevant to combat this pathogen that causes important losses in swine production.

INTRODUCTION

Antimicrobial peptides (AMPs) are an important group of antimicrobial agents, including some relevant and currently used antibiotics such as vancomycin and teicoplanin (Jeya *et al.*, 2011). AMPs are widespread synthesized by bacteria of the genus *Bacillus*, showing activity against several pathogenic and spoilage micro-organisms (Riley & Wertz, 2002; Stein, 2005). Among *Bacillus*, the production of AMPs has been recognized for *B. amyloliquefaciens*, *B. subtilis*, *B. thuringiensis*, *B. cereus* and *B. licheniformis*, but they were also reported in many other *Bacillus* species (Lisboa *et al.*, 2006; Begley *et al.*, 2009; Halimi *et al.*, 2010).

A large diversity of chemical structures has been associated with AMPs from *Bacillus*, including bacteriocins and lipopeptides. *Bacillus* species may produce bacteriocins that resemble those produced by lactic acid bacteria, such as lantibiotics and pediocin-like bacteriocins (Abriouel *et al.*, 2011). In addition, a variety of lipopeptide antibiotics consisting of a hydrophobic fatty acid linked to a hydrophilic peptide chain are produced by *Bacillus* species via non-ribosomal peptide synthetases (Roongsawang *et al.*, 2011). This complex structural diversity warrants the broad spectra of inhibition of some *Bacillus* strains, which includes not only Gram-positive and Gram-negative bacteria, but fungi and amoeba as well (Benitez *et al.*, 2011). Some AMPs like subtilosin A, subtilin, iturin A and surfactin, produced by *B. subtilis* and closely related species, have been characterized in detail (Stein, 2008; Abriouel *et al.*, 2011).

Haemophilus parasuis is one of the first and most prevalent colonizers of piglets, affecting the swine population from 2 weeks to 4 months of age, although it is more commonly found in piglets between 5 and 8 weeks. *H. parasuis* is the pathogen causing Glässer's disease, a systemic illness characterized by polyserositis and fibrinopurulent polyarthritis (Rapp-Gabrielson *et al.*, 2006). This disease has been emerging as a main bacterial infection affecting the swine population worldwide, causing important economical losses to animal production (Nedbalcova *et al.*, 2006; Teixeira *et al.*, 2011). Many efforts have been targeted to advance in the diagnosis and characterization of virulent *H. parasuis* strains (Olvera *et al.*, 2007; Xu *et al.*, 2011), but an adequate treatment is not currently available. Therefore, the research for alternatives to combat this pathogen is of utmost relevance.

The objective of this work was to investigate the effect of AMPs from *Bacillus* species against *H. parasuis*. A novel peptide produced by *B. subtilis* subsp. *spizezinii*

Abbreviation: AMP, antimicrobial peptide.

showing antagonistic effects against this pathogen was characterized.

METHODS

Micro-organisms and initial screening. *H. parasuis* ATCC 19417 and *H. parasuis* strains isolated from 11 samples of clinical cases (eight from lungs, two from pericardial fluid and one from liquor; Teixeira *et al.*, 2011), were tested as indicator strains. The antimicrobial activity was also checked against *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. The initial screening to determine the production of antimicrobial activity was performed using *Bacillus cereus* ATCC 11778, *Bacillus circulans* ATCC 21783, *Bacillus mycoides* ATCC 6462 and *Bacillus subtilis* subsp. *spizezinii* ATCC 6633. The organisms were stored at −20 °C in Brain Heart Infusion broth (BHI) medium (Acumedia Manufacturers) containing 20 % (v/v) glycerol. The bacteria were propagated twice in fresh Trypticase Soy Broth (TSB) medium (Acumedia Manufacturers) before use. *Bacillus* strains were grown in TSB broth for 72 h at 35 °C and then the culture supernatant was obtained by centrifugation at 5000 *g* for 30 min at 15 °C. The supernatant was filtered through a 0.22 µm membrane (Millipore), and the filtered liquid was considered as crude antimicrobial preparation.

Assay of antimicrobial activity. Antimicrobial activity was monitored as described elsewhere (Teixeira *et al.*, 2009). Plates were inoculated with a swab which was submerged in indicator strain suspension containing about 10^7 c.f.u. ml^{-1}; 6 mm cellulose discs were placed onto the agar surface and then 20 µl antimicrobial preparation was applied. The plates were incubated at 37 °C for 24 h (5 % CO_2 in air for *H. parasuis* strains) and the clearing zones were measured around the discs. The antimicrobial titre (AU ml^{-1}) was determined by using the serial twofold dilution method (Motta & Brandelli, 2002), using *H. parasuis* ATCC 19417 as indicator strain.

Purification of antimicrobial peptide. The crude antimicrobial preparation was submitted to ammonium sulfate precipitation at 20 % (w/v) saturation. After centrifugation at 5000 *g* for 30 min at 15 °C, the pellet was suspended in 10 mmol phosphate buffer l^{-1}, pH 7.0, and loaded on a Sephadex G-50 column (GE Healthcare/Pharmacia Biotech). The column was eluted with 10 mmol phosphate buffer l^{-1}, pH 7.0, as mobile phase. Fractions of 1 ml were collected and those that presented antimicrobial activity were pooled and sterilized through a 0.22 µm filter membrane (Millipore). These fractions were loaded on a DEAE-cellulose column (GE Healthcare/Pharmacia Biotech) and eluted by a step gradient (10 ml of 0, 0.5, 1.0, 2.0 mol NaCl l^{-1} in 10 mmol phosphate buffer l^{-1}, pH 7.0) and fractioned by 1 ml. The fractions positive for antimicrobial activity were pooled and stored at 4 °C until needed.

Effect of enzymes, temperature, pH and time on antimicrobial activity. The effect of enzymes, temperature and pH on antimicrobial activity was determined as described elsewhere (Bizani & Brandelli, 2002). The proteolytic enzymes tested were pronase E (Sigma) and proteinase K (Merck) at working concentrations of 2, 5 and 10 mg ml^{-1} for 60 min at 37 °C. The effect of different pH values between 3 and 7 was investigated. For analysis of thermal stability, the antimicrobial substance was exposed to temperatures ranging from 50 to 120 °C. The residual antimicrobial activity during the storage time was determined for samples that were kept at three different temperatures (−20, 8 and 22 °C). After the treatment, residual activity was determined against *H. parasuis* ATCC 19417. Each experiment was done in triplicate.

Mode of action. A culture of *H. parasuis* was obtained in BHI medium at 37 °C for 24 h. A dose–response curve was determined using different concentrations of purified peptide (between 0.625 and 20 µg ml^{-1}, which corresponds to 25–800 AU ml^{-1}) and an initial inoculum of 10^7 c.f.u. ml^{-1}. The antimicrobial tulathromycin was used as a positive control in the range of 31.25 to 1000 µg ml^{-1}. Additionally, the antimicrobial peptides nisin, surfactin and iturin A (Sigma) were tested in the range of 31.25–1000 µg ml^{-1}. Viable cell counts were determined after incubation at 37 °C for 120 min in microaerophilia (5 % CO_2 in air). Kinetics of the antimicrobial effect on *H. parasuis* was determined at 37 °C with a peptide concentration of 20 µg ml^{-1} and an initial inoculum of 10^7 c.f.u. ml^{-1}. The viable cell count was determined at different time intervals up to 120 min incubation at 37 °C and 5 % CO_2 in air. The control was taken with addition of 10 mmol phosphate buffer l^{-1}, pH 7.0, and the positive control with 1 mg tulathromycin ml^{-1}. Aliquots of untreated and peptide-treated cells were removed at 0 and 30 min to be observed in a bright-field microscope at 1000x magnification. Each experiment was done in triplicate.

Electron microscopy. The cells of *H. parasuis* were prepared by the method described by Kalchayanand *et al.* (2004) with slight modifications. Cultures of *H. parasuis* ATCC 19417 were treated with 20 µg ml^{-1} AMP for 120 min at 37 °C and 5 % CO_2 in air. The cell suspensions were fixed with 12 % (v/v) glutaraldehyde in 0.2 mol Na-phosphate buffer l^{-1}, pH 7.4. Then, the cells were washed to remove glutaraldehyde 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 cell adhesion. The attached cells were post-fixed by immersing the chips in 10 mg osmium tetroxide (OsO_4) ml^{-1} in cacodylate buffer for 30 min, rinsed in the same buffer and dehydrated in ascending ethanol concentrations (%, v/v) of 50, 70, 95 (2x) and 100 (2x), for 10 min each. The chips were mounted on aluminium stubs and coated with gold in a sputter coater (Emitech K550). The chips were viewed at 10 kV accelerating voltage in a scanning electron microscope (JEOL JSM-6060).

Spectrometry. The UV-visible spectrum of the antimicrobial peptide was obtained using a Shimadzu UVmini 1240 spectrophotometer (Columbia).

The peptide was applied to reverse-phase HPLC system using a C18 column µBondapak (10 µm, 125 Å, 25 × 100 mm), eluted with a water/acetonitrile gradient. The gradient was started with 0 % acetonitrile for 5 min and then steadily increased to 90 % acetonitrile, with flow rate of 5 ml min^{-1}. The collected peak was analysed by MALDI-TOF/TOF mass spectrometer (Ultraflex III TOF-TOF MS) and using a matrix of α-cyano-4-hydroxycinnamic acid. The spectrum was acquired in the positive ion reflectron mode. The selection of the mass range was set between 50 and 1500 Da, considering signal-to-noise ratio (Sparbier *et al.*, 2007).

Peptide sequence analysis. The result of the fragment ion spectra was processed using MASCOT distiller and the software MASCOT 2.2 (Matrix Science) and the BLAST algorithm (http://www.ncbi.nlm.nih.gov) were used to database search in the NCBI sequence database (NCBInr 20120318; 17574240 sequences; 6033299959 residues) restricted to *Bacillus subtilis* (27630 sequences). Only peptides with $P<0.05$ were considered significant hits. The search for homologous sequences was also performed on the Antimicrobial Peptide Database (www.aps.unmc.edu/AP). The modelling of peptide structure was performed using the mobile server of the PEP FOLD algorithm (Thévenet *et al.*, 2012).

Peptide synthesis. Based on the sequence obtained by MS, the peptide was synthesized by the F-moc chemical synthesis technique by

a custom service (China Peptides). The synthesized peptide has a purity degree of 99.25 as determined by HPLC analysis and the molecular mass was confirmed by ESI-MS as 1083.15.

RESULTS

The cell-free culture supernatant of *B. subtilis* subsp. *spizezinii* showed antimicrobial activity against the *H. parasuis* strains. This crude antimicrobial preparation was also able to inhibit *L. monocytogenes*, *S. aureus* and *E. coli*. The crude extracts obtained from cultures of *B. cereus*, *B. circulans* and *B. mycoides* strains inhibited *L. monocytogenes* and *S. aureus* but did not present antimicrobial activity on *H. parasuis* strains or *E. coli*.

The antimicrobial substance produced by *B. subtilis* subsp. *spizezinii* was secreted to the culture medium and it was purified. The purification results are summarized in Table 1. The protocol resulted in a purification of 100-fold and a yield of 0.33 %. The gel filtration chromatography resulted in an important step of this purification, although the activity eluted did not coincide with the main peak of protein (Fig. 1a). The antimicrobial substance was further purified through the chromatographic column DEAE-cellulose and only three fractions showed antimicrobial activity, coinciding with a unique peak of protein (Fig. 1b).

The antimicrobial activity was affected by the action of the proteases pronase E and proteinase K, but the absence of the inhibition zone against *H. parasuis* was only observed when the enzymes were used at 10 mg ml^{-1} (Table 2). The antimicrobial activity remained in a pH range of 3–7, although only 70 % of the maximum activity was observed at pH values lower than 5 (data not shown). The antimicrobial substance showed heat resistance up to 80 °C, but when it was exposed to higher temperatures (100 and 120 °C) a total loss of antimicrobial activity was observed after 30 min. The antimicrobial was stored at different temperatures and 100 % of its initial activity was maintained up to 10, 30 and 60 days at 22, 8 and −20 °C, respectively.

The UV–visible spectrum indicated the presence of aromatic amino acids and typical absorbance of peptide bonds at 220 nm was also observed (data not shown). The peptide eluted in a C18 reversed phase column resulted in a thin peak and its molecular mass was determined by MS. The mass spectrum showed major $[M+H]^+$ ions at *m/z* 1084 and 626 (Fig. 2a). The $[M+H]^+$ ion at *m/z* 1084 was fragmented by MS/MS, and these data correspond to –y9 fragment ions (Fig. 2b). The –y1 corresponds to a residue of aspartate (*m/z* 134), the –y2 dipeptide (*m/z* 249) DD, the –y3 tripeptide (*m/z* 306) GDD, the –y4 tetrapeptide (*m/z* 377) AGDD, the –y5 pentapeptide (*m/z* 524) FAGDD, the –y6 hexapeptide (*m/z* 627) CFAGDD, the –y7 refers to the sequence WCFAGDD (*m/z* 813), the –y8 corresponds to the sequence RWCFAGDD (*m/z* 813) and the –y9 refers to the peptide NRWCFAGDD (*m/z* 1084). The research in the database did not show identity or homology with other proteins synthesized by *B. subtilis*. Besides, the sequence was not homologous to other AMPs available at the Antimicrobial Peptide Database.

The peptide structure was modelled *in silico* using the PEP FOLD software. The model that presented the best score is shown in Fig. 3(a). The predicted values for TM score, GDT_TS and Q mean score were 0.249, 0.768 and 0.527, respectively. The coarse grained energy (sOPEP) was −5.08 (Zhang & Skolnick, 2004; Wang *et al.* 2011). The structure appears as 'U' shape and a β-bend involving the residues Arg, Trp and Cys was predicted. It is expected that the large R groups of Trp and Phe cause a spatial hindrance that induces the peptide curvature and forces the placement of these residues to the external part of the structure. The hydropathy graphic indicates that the peptide is amphiphatic, with hydrophilic residues in the extremities and hydrophobic residues in the middle of the sequence (Fig. 3b).

The purified AMP was tested against the clinical isolates of *H. parasuis* in agar diffusion assays. Six isolates showed similar inhibition haloes to those observed with the strain ATCC 19417, while the other five isolates were not inhibited by the AMP. The effect of AMP concentration on *H. parasuis* survival was investigated. The number of viable cell counts decreased as the concentration of AMP increased (Fig. 4a). Complete growth inhibition was observed at 20 µg ml^{-1}. The positive control tulathromycin caused a complete inhibition of *H. parasuis* at 250 µg ml^{-1}. These results were similar for both *H. parasuis* ATCC 19417 and clinical isolates (Fig. 4a). The synthetic and the purified AMP showed similar inhibitory activity at 2.5 and 10 µg ml^{-1}, respectively (Fig. 4b). AMP obtained by chemical synthesis caused complete inhibition of *H. parasuis* at 10 µg ml^{-1}. Nisin, surfactin and iturin A were also tested, but none of these antimicrobial peptides were inhibitory to *H. parasuis* (Fig. 4b), even at the higher

Table 1. Purification of AMP from *B. subtilis* subsp. *spizezinii*

Step	Total protein (mg)	Total activity (AU)	Specific activity (AU mg^{-1})	Purification fold	Recovery
Crude filtrate	2343	960000	409.71	1	100
Pellet	32.23	57600	1787.09	4.36	6.0
Sephadex G-50	0.39	6400	16558.86	40.42	0.67
DEAE-cellulose	0.08	3200	40894.57	99.81	0.33

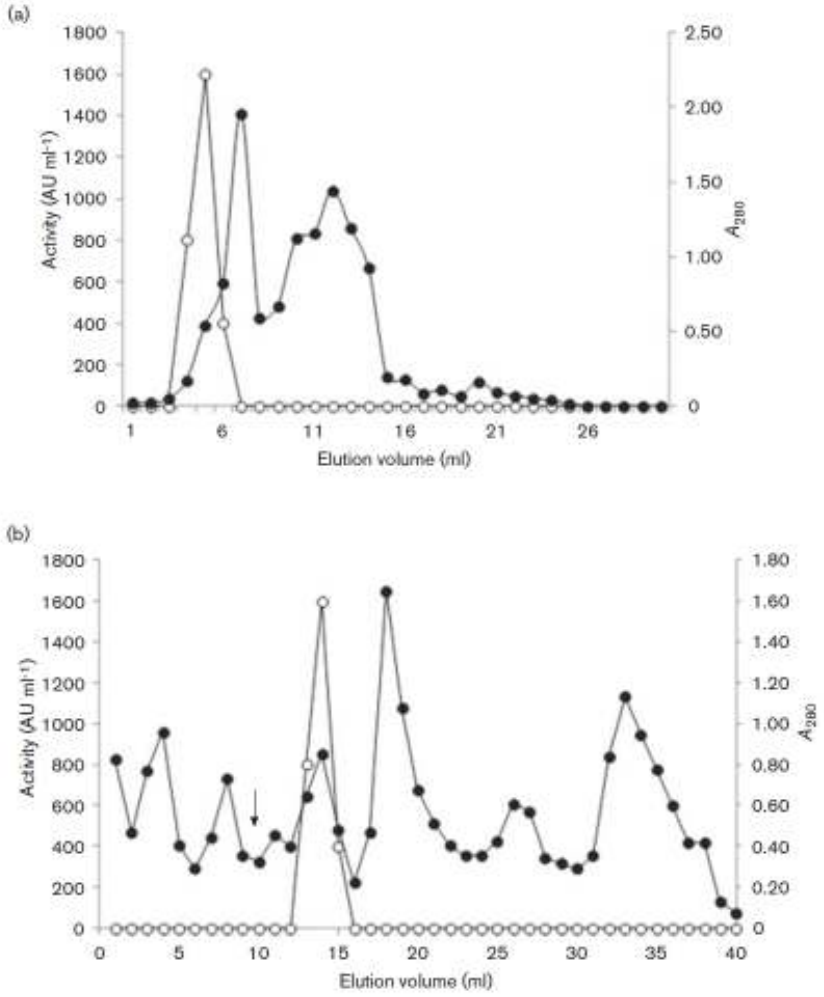

Fig. 1. Purification of antimicrobial peptide from *B. subtilis* subsp. *spizezinii*. The pellet obtained after precipitation with ammonium sulfate was applied to a Sephadex G-50 column (a) and the active fractions were pooled and loaded on a DEAE-cellulose column (b). Fractions were monitored for antimicrobial activity (open symbols, AU ml^{-1}) and absorbance at 280 nm (black symbols). Arrow indicates the start of NaCl gradient.

Table 2. Effect of proteolytic enzymes on the AMP produced by *B. subtilis* subsp. *Spizezinii*

Enzyme	Final concentration (mg ml^{-1})	Residual activity (%)
Pronase E	2	92±6
	5	47±8
	10	0
Proteinase K	2	84±12
	5	36±9
	10	0

*Data are means ± SEM.

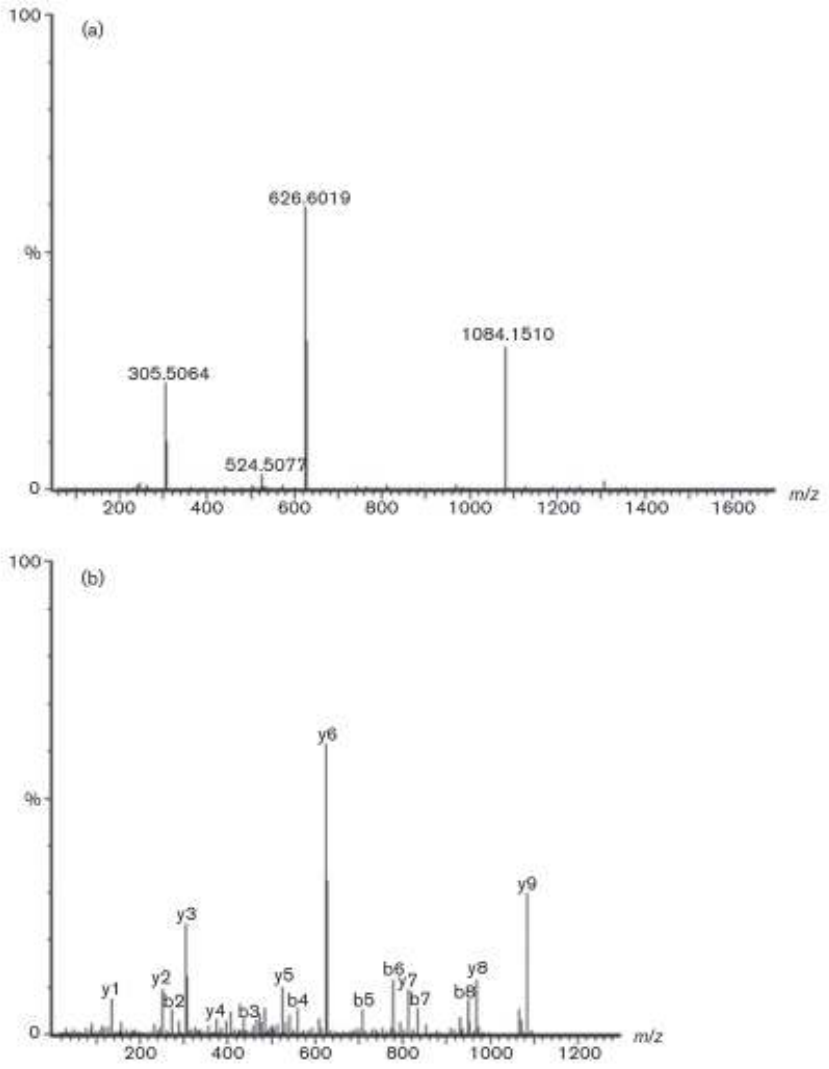

Fig. 2. MS analysis of AMP produced by *B. subtilis* subsp. *spizezinii*. The purified peptide was eluted from a C18 reversed phase column and the mass spectrum was determined (a). (b) MS/MS spectrum of the *m/z* 1084 fragment.

concentrations tested. The kinetics of the peptide effect on growth of *H. parasuis* is demonstrated (Fig. 5). When the peptide was tested at 20 μg ml^{-1}, complete growth inhibition of *H. parasuis* was observed in 20 min, and this inhibition was maintained for at least 90 min of incubation. Throughout the incubation time, the reduction of *H. parasuis* viable counts resulted in simultaneous reduction of OD_{600} (data not shown). The electron microscopy images of the *H. parasuis* cells exposed to the AMP for 120 min are shown in Fig. 6. The cells showed a rough appearance and irregular surfaces, but cell lysis was not noticeable. Similar results were observed for both AMP and synthetic AMP.

DISCUSSION

An antimicrobial peptide with inhibitory activity against *H. parasuis* was purified from *B. subtilis* subsp. *spizezinii* culture. The inhibition of *H. parasuis* is very relevant since this bacterium is responsible for the increasing mortality of piglets in nursery phase, by affecting the upper respiratory system of these animals causing breathing difficulties, loss of appetite and anorexia (Teixeira *et al.*, 2011). Previous reports on AMPs inhibiting this pathogen have not been found in the current literature.

B. subtilis is already described as an AMP producer, including a diversity of lipopeptides like iturins, fengycins

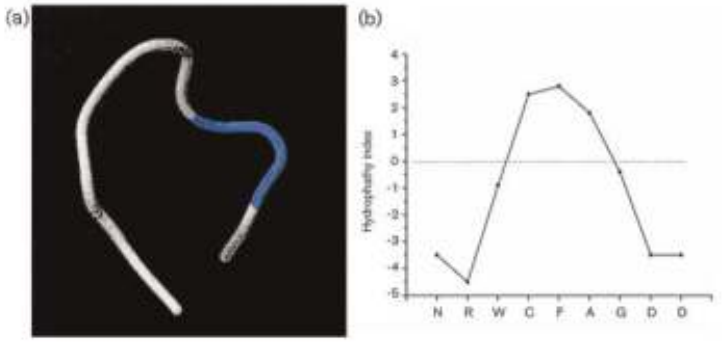

Fig. 3. Structural modelling of AMP produced by *B. subtilis* subsp. *spizezinii*. (a) Model generated by the PEP FOLD algorithm, showing a predicted β-bend motif involving the residues RWC (highlighted). (b) Hydropathy index of amino acid R groups, measuring the tendency to seek an aqueous environment (− values) or a hydrophobic environment (+ values) (Kyte & Doolittle, 1982).

and surfactin (Stein, 2005), and a number of bacteriocins (Abriouel *et al.*, 2011). Specifically, strain ATCC 6633 used in this work is known because it produces subtilosin A and subtilin (Heinzmann *et al.*, 2006). The fact that no other known AMPs of *B. subtilis* were detected in this work may be associated with the fact that the screening for antimicrobial activity was performed on *H. parasuis* as an indicator strain. Thus, the production of different antimicrobials by *Bacillus* species is recognized to be under complex regulation, and the type of antimicrobial compound(s) that is effectively synthesized is really influenced by cultivation conditions (Lisboa *et al.*, 2006; Anthony *et al.*, 2009).

The rates of recovery and purification fold obtained for the AMP produced by *B. subtilis* subsp. *spizezinii* were similar to those described for some bacteriocins (Martínez & Rodríguez, 2005; Papagianni & Papamichael, 2011). The purification of the peptide demonstrated a very successful step in the gel filtration, where the antibacterial activity eluted was free of a large amount of proteins. The use of a low saturation of ammonium sulfate to precipitate the peptide combined with gel filtration chromatography, and the elution in the first fractions suggest that the peptide is eluted as aggregates of elevated molecular mass, a fact also observed for other AMPs (Bizani *et al.*, 2005; Kamoun *et al.*, 2005; Hammami *et al.*, 2009). The presence of amino acids with a polar side chain may be associated with the formation of aggregates. The elution pattern from the cation exchange column indicates that the AMP presents a weak residual negative charge, because it was eluted with a low ionic strength (0.5 mol NaCl l^{-1}). This fact also suggests the presence of a polar amino acid side chain(s) containing a carboxyl group.

As additional characteristics, typical absorbance of peptide bonds and aromatic amino acids were revealed by UV spectroscopy and the antimicrobial activity was sensitive to the treatment with a broad range of proteases like pronase E and proteinase K. In addition, the peptide displays a lower activity at acidic pH, suggesting that the COOH groups should be in the ionized form for its maximum antimicrobial activity. Decreased activity at low pH values was also described for other AMPs from *Bacillus* (Bizani *et al.*, 2005; Teixeira *et al.*, 2009).

The molecular mass of about 1083 Da is lower than typical antimicrobial peptides of *Bacillus*, such as subtilin and subtilosin A (3319 and 3340 Da, respectively), but comparable to lipopeptides like surfactins and iturins (1000–1100 Da). A similar molecular mass (*m/z* 1083.7) was associated with the potassium adduct of the peptide C15-bacillomycin D (Ramarathnam *et al.*, 2007). However, the sequence obtained from the fragment at *m/z* 1084 indicates that a different peptide was isolated, since no significant homology with other previously described peptides from *Bacillus* or other AMPs was observed. The AMP obtained by chemical synthesis was also inhibitory to *H. parasuis*, confirming that the activity was due to the purified peptide.

The negative net charge of the peptide may suggest a difficult interaction with the outer membrane of *Haemophilus*, since the lipopolysaccharides (LPSs) are also negatively charged. However, anionic peptides can interact through chelation with Zn^{2+} or Mg^{2+} (Brogden *et al.*, 1996). Relatively few anionic AMPs have been well characterized. Anionic AMPs rich in aspartic acid, originally isolated from ovine pulmonary material, are present in the airway surface liquid (Brogden *et al.*, 1996). These peptides are thought to be produced by the respiratory epithelium and showed inhibition of Gram-negative bacteria like *Pasteurella haemolytica* and *Pseudomonas aeruginosa* (Kalfa & Brogden, 1999). In this way, it seems very interesting that the respiratory pathogen *H. parasuis* could be inhibited by an anionic peptide as well.

More recently, some anionic defence peptides have also been characterized in invertebrates. The tick *Amblyomma hebraeum* produces a defensin-like peptide (pI 4.44) which exhibits activity against *E. coli* and *S. aureus* (Lai *et al.*, 2004). An anionic AMP with predicted pI 4.12 was described in the lepidopteran insect *Bombyx mori* (Wen *et al.*, 2009), and the wax moth *Galleria mellonella* produces an AMP (pI 4.79) that induces surface alterations in *E. coli* and kills this bacterium in combination with lysozyme (Zdybicka-Barabas *et al.*, 2012).

The effect and mode of action of the *B. subtilis* AMP was studied using *H. parasuis* as indicator bacteria, due to the

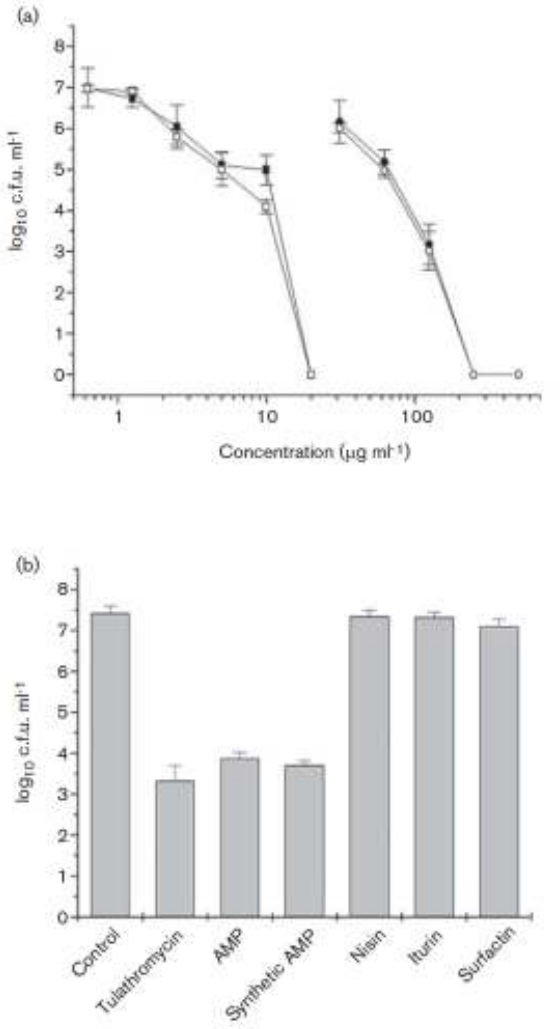

Fig. 4. Effect of antimicrobial peptides on *H. parasuis*. (a) Dose–response effect of AMP produced by *B. subtilis* subsp. *spizezinii* on the survival of *H. parasuis*. Cells were incubated in the presence of increasing concentrations of AMP (squares) or tulathromycin (circles), used as a positive control. Viable cell counts of *H. parasuis* ATCC19417 (black symbols) and a clinical isolate of *H. parasuis* (open symbols) were determined after 120 min incubation at 37 °C and 5 % CO_2 in air. (b) Viable counts of *H. parasuis* ATCC 19147 after 120 min incubation with 125 μg tulathromycin ml^{-1}, 10 μg AMP ml^{-1}, 2.5 μg synthetic AMP ml^{-1} or 125 μg ml^{-1} of either nisin, iturin A or surfactin.

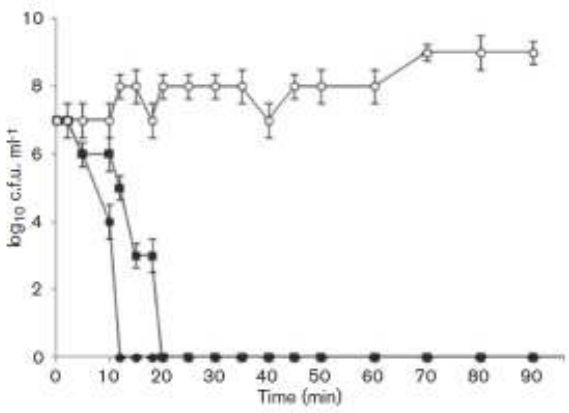

Fig. 5. Effect of AMP produced by *B. subtilis* subsp. *spizezinii* on the growth of *H. parasuis*. Cells were treated with 20 μg AMP ml^{-1} (black squares), 1 mg tulathromycin ml^{-1} (positive control, black circles) or in the absence of antimicrobials (negative control, open circles). Viable cell counts were determined after incubation at 37 °C and 5 % CO_2 in air for the indicated time.

restricted research literature on the antimicrobial activity of peptides against *Haemophilus* species. The fact that some clinical isolates were not inhibited by the AMP could represent a drawback to its practical utilization, but also encourages further investigation into the mechanism of action of this peptide and resistance strategies of this bacterium. *Haemophilus ducreyi* is resistant to several cationic AMPs, such as α- and β-defensins and cathelicidin, but is susceptible to killing by the porcine AMP protegrin 1 (Mount *et al.*, 2007). Current research indicates that the Sap protein mediates the import of AMPs, as a strategy to reduce periplasmic and inner membrane accumulation of these peptides in *H. influenzae* (Shelton *et al.*, 2011). The multiple transferable resistance transporter in *H. ducreyi* promotes resistance to LL-37 and human β-defensins in a proton motive force-dependent manner (Rinker *et al.*, 2011). The antimicrobial activity of four human neuropeptides, namely somatostatin, calcitonin gene-related peptide, neuropeptide Y and substance P, was recently demonstrated on *H. influenzae* (Augustyniak *et al.*, 2012). In this work, the results on the mode of inhibition of *H. parasuis* indicate a bactericidal effect and established a noticed decline in the number of viable cells of indicator strain after the addition of the AMP. The AMP produced by *B. subtilis* subsp. *spizezinii* effectively inhibited the *H. parasuis* growth, which was observed after 20 min of treatment. The rapid cell death caused by this peptide suggests that the target is the cell envelope, similar to what was observed for many bacteriocins (Dupuy & Morero, 2011; Soliman *et al.*, 2011). This action is often dependent on the concentration of the antibacterial substance present in the medium. In the case of most cationic peptides, cell lysis is induced by the association of AMPs with anionic compounds of the bacterial cell surface, such as LPS, teichoic and lipoteichoic acids. Cationic AMPs displace native divalent cations from the outer membrane of Gram-negative bacteria since they have a higher affinity for LPS, leading to membrane perturbation (Powers & Hancock, 2003). Although the mechanism of action of anionic AMPs has not been

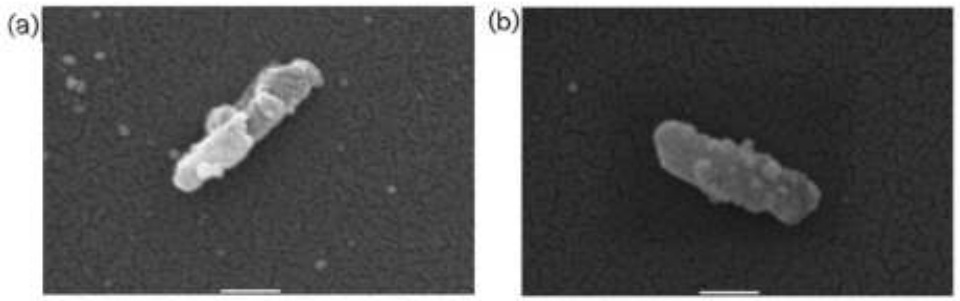

Fig. 6. Scanning electron microscopy of *H. parasuis* cells treated with (a) AMP and (b) synthetic AMP for 120 min at 37 °C and 5% CO_2 in air. Bar, 0.5 μm.

completely elucidated, it has been suggested that the negative charge and amphiphilic characteristics of such peptides are essential for their antimicrobial activity. An asymmetrical distribution of hydrophobicity along an oblique α-helical architecture may cause a peptide to penetrate membranes at a shallow angle, thereby disturbing membrane lipid organization and compromising bilayer integrity (Dennison *et al.*, 2005).

AMPs have gained importance as alternative agents for controlling spoilage and pathogenic micro-organisms. This study indicates for the first time to our knowledge that an antimicrobial peptide can effectively inhibit *H. parasuis*, and may represent a potential alternative to combat this pathogen. These results also reinforce the importance of *Bacillus* species as a source of a variety of antimicrobial substances. Despite the fact that several AMPs are recognized by their low toxicity, an evaluation of the harmful effects on mammalian cells must be conducted before this peptide could be used in field experiments. The identification and characterization of novel AMPs and their potential use in the control of microbial infections are topics of greatest relevance.

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5.1. Conclusões

- O peptídeo antimicrobiano (AMP) produzido por *B. subtilis* subespécie *spizezinii* ATCC 6633 foi purificado por protocolo de precipitação com sulfato de amônio, cromatografia de gel filtração e cromatografia de troca-iônica;
- O AMP apresentou atividade frente a *Haemophilus parasuis*;
- O AMP possui massa molecular de aproximadamente 1083 Da, apresentando a sequência de aminoácidos NRWCFAGDD;
- O AMP foi totalmente inativado por pronase E, proteinase K em concentrações de 10 mg/mL, manteve a atividade antimicrobiana quando exposta a temperaturas inferiores a 80°C e na faixa de pH entre 3,0 e 7,0;
- O AMP tem o seu mecanismo de ação danificando a membrana celular e/ou membrana externa da bactéria *Haemophilus parasuis*;

6. CAPÍTULO III

Anti-*Candida* activity of bacteriocin produced by *Bacillus subtilis* subsp. *spizezinii*

Nesse capítulo será discutida a atividade antifúngica do peptídeo antimicrobiano produzido por *B. subtilis* subespécie *spizizenii.* O estudo contempla a concentração inibitória mínima e a concentração fungicida mínima do peptídeo antimicrobiano contra estirpes de leveduras causadoras de enfermidades de grande impacto social.

Artigo a ser submetido ao periódico *Journal of Antimicrobial and Chemotherapy*

Anti-*Candida* activity of an antimicrobial peptide produced by *Bacillus subtilis* subsp. *spizezinii*

Mário Lettieri Teixeira[1,3], Alexandre Meneghello Funtefria[2] and Adriano Brandelli[1]*

[1]Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos, ICTA, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, Brasil

[2]Laboratório de Micologia, Departamento de Análises, Faculdade de Famácia, Universidade Federal do Rio Grande do Sul, 90610-000 Porto Alegre, Brazil

[3]Laboratório de Bioquímica, Curso de Medicina Veterinária, Instituto Federal Catarinense – Campus Concórdia, 89700-000 Concórdia, Brasil

*Correspondence: ICTA-UFRGS, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, Brasil; e-mail: abrand@ufrgs.br

Short running title: Anti-*Candida* activity of an antimicrobial peptide

Keywords: *Candida, Bacillus subtilis,* antimicrobial peptide

Synopsis

Objectives: Antimicrobial peptides (AMPs) are widely distributed in nature. The objective of this work was to investigate the antimicrobial activity of a novel AMP from *Bacillus subtilis* subsp. *spizezinii* against some yeasts of emerging clinical relevance.

Methods: The AMP produced by *B. subtilis* subsp. s*pizezinii* was secreted to the culture medium and purified. The peptide was tested to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) against different strains of the susceptible yeasts.

Results: The peptide inhibited *Candida glabrata, Candida tropicalis, Candida parapsilosis* and *Candida krusei*, but it was not able to inhibit *Trichosporon asahii* and *Cryptococcus neoformans.* The most sensitive species were *C. glabrata*, *C. tropicalis* and *C. krusei.* However, the most sensitive of all tested strains was *C. parapsilosis* 07 with a MIC of 8 mg/L. The MIC values varied in the range of 16-64 mg/L for *C. glabrata*, *C. tropicalis* and *C. krusei.* Moreover, the strains of *C. albicans* had higher MFC values (512 mg/L), while the CPA 07, CT 750 and CG 06 strain exhibited MFC values of 32, 64 and 64 mg/L, respectively.

Conclusions: These results suggest that the AMP present a selective anti-*Candid*a activity. Therefore, the peptide produced by *B. subtilis* subsp. *spizezinii* might be a potential source of new antimicrobial drug.

Introduction

Antimicrobial peptides (AMPs) are widely distributed in nature as a universal defense mechanism of all forms of life, with examples in organisms ranging from bacteria, plants, and animals. Among prokaryotes, AMPs can be synthesized by both gram negative and gram positive bacteria[1,2]. These compounds are active against other bacteria, either of the same species (narrow spectrum), or across genera (broad spectrum). AMPs are widespread produced by bacteria of the genus *Bacillus*, showing activity against several pathogenic and spoilage microorganisms[3,4]. Among *Bacillus*, AMP production was recognized for *B. amyloliquefaciens, B. subtilis, B. thuringiensis, B. cereus* and *B. licheniformis,* but they were also reported in many other *Bacillus* species[5-7].

Candida species are responsible for a wide variety of infections ranging from superficial to systemic candidiasis. Presence of some predisposing factors, such as immunosuppression, broad-spectrum antibiotics, and cytotoxic therapies, presence of intravenous catheters, diabetes, neutropenia, malnutrition and AIDS, can facilitate the emergence of candidiasis. In this context, the indiscriminate use of antifungal agents has reduced the number of active drugs against this kind of yeast, reducing the chances of success of chemotherapy[8-10]. The antifungal activity of AMPs produced by *Bacillus* species have been reported in the literature, and the filamentous fungi *Aspergillus*, *Fusarium, Penicillium* and *Rhizopus* are the most frequently genera sensitive to these AMPs [11,12].

A novel AMP produced by *Bacillus subtilis* subsp. *spizezinii* was recently characterized[13]. This AMP is an unusual anionic peptide that inhibits the swine pathogen *Haemophilus parasuis*. The objective of this work was to investigate the

antimicrobial activity of this AMP from *Bacillus subtilis* subsp. *spizezinii* against some pathogenic yeast.

Methods

Antimicrobial peptide

Bacillus subtilis subsp. *spizezinii* ATCC 6633 was sowed twice in fresh Trypticase Soy Broth (TSB) medium (Acumedia Manufacturers, Lansing, MI, USA) before using it. *Bacillus* strain grew in TSB for 72 h at 35°C and then the supernatant was obtained by centrifugation at 5,000 *g* for 30 min at 15°C. The supernatant was filtered through a 0.22 µm membrane (Millipore, Billerica, MA, USA), and the filtrate was considered as a crude antimicrobial preparation.

The crude antimicrobial preparation was submitted to ammonium sulfate precipitation at 20% (w/v) of saturation. After centrifugation at 5,000 *g* for 30 min at 15°C, the pellet was suspended in 10 mmol/L phosphate buffer pH 7.0 and loaded on a Sephadex G-50 column (GE Healthcare/Pharmacia Biotech, Uppsala, Sweden). The column was eluted with 10 mmol/L phosphate buffer pH 7.0 as mobile phase. Fractions of 1 mL were collected and these ones that presented antimicrobial activity were pooled and sterilized through a 0.22 µm filter membrane (Millipore, Billerica, MA, USA). These fractions were loaded on a DEAE-cellulose column (GE Healthcare/Pharmacia Biotech, Uppsala, Sweden), and eluted by a gradient step (10 mL of each 0, 0.5, 1.0, 2.0 mol/L NaCl in 10 mmol/L phosphate buffer pH 7.0) and all of them were fractioned by 1 mL. The positive fractions for antimicrobial activity were pooled, and stored at 4°C until used[13].

Antifungal activity assay

A total of 19 strains (clinical isolates derived from the Yeast Clinical Library of the Faculty of Pharmacy, University Federal of Rio Grande do Sul) of opportunistic yeast were tested for the susceptibility to AMP from *B. subtilis: Candida albicans* (CA 02, CA04 e CA05), *C. glabrata* (CG 04, CG 05, CG 06), *C. krusei* (CK 02, CK 03, CK 04), *C. parapsilosis* (CPA 07, CPA 08, CPA 134), *C. tropicalis* (CT 08, CT 06, CT 750). *Trichosporon asahii* (TRA 01) and *Cryptococcus neoformans* (CRY 02, CRY 14, CRY 17).

Minimal inhibitory concentration (MIC) of the AMP was determined by the broth microdilution method according to M27-A3 document determined by the Clinical Laboratory and Standards Institute[14], with RPMI-MOPS (RPMI 1640 medium containing L-glutamine, without sodium bicarbonate - Sigma-Aldrich Co., St Louis, USA - buffered to pH 7.0 with 0.165 mol/L MOPS buffer – Sigma). The concentrations of the peptide ranged from 1.9 to 500 mg/L and 100 μL-aliquots were inoculated on a flat-bottom 96-well microtiter plate. The MIC was defined as the lowest concentration of compounds that is able to stop the growth of the tested microorganism. Fluconazole (FLC) kindly supplied by Cristália® (Brazil) was used as a positive control. The experiments were carried out in triplicate.

The minimal fungicidal concentration (MFC) was determined by sub-culturing volumes of 10 μL from wells without visible growth in Sabouraud dextrose agar (SDA) with cloramphenicol (Difco, Detroit, USA) and incubated at 35°C for 48 h. Minimum fungicidal concentration (MFC) was defined as the lowest concentration yielding negative subcultures.

Results

The antifungal substance produced by *B. subtilis* subsp. *spizezinii* was secreted to the culture medium and was purified[13]. The peptide was tested to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against different strains of the susceptible yeasts. The peptide inhibited *Candida glabrata, C. tropicalis, C. parapsilosis* and *Candida krusei*, but it was not able to inhibit *Trichosporon asahii* and *Cryptococcus neoformans* (Table 1). According to Table 2, although the most sensitive species were *C. glabrata*, *C. tropicalis* and *C. krusei,* the most sensitive among all strains tested was *C. parapsilosis* (CPA 07) with an MIC of 8 mg/L. The MIC values varied in the range of 16-64 mg/L (for *C. glabrata*, *C. tropicalis* and *C. krusei,*), while CPA 134 strain showed a MIC value of 256 mg/L and the strains of *C. albicans* showed a MIC range 64-128 mg/L. Moreover, the strains of *C. albicans* had MFC values are higher (512 mg/L), while the CPA 07, CT 750 and CG 06 strain exhibited MFC values of 32, 64 and 64 mg/L, respectively.

Discussion

The activity of *B. subtilis* subsp. *spizezinii* was previously demonstrated against *L. monocytogenes, S. aureus, E. coli* and *H. parasuis* strains. Furthermore, the antimicrobial substance active against *H. parasuis* was chemically characterized as a peptide and its amino acid sequence was identified[13]. The presence of antifungal activity associated with peptides synthesized by this genus has been reported in other studies, as in the case of zwittermicin A (ZmA), an aminopolyol antibiotic encoded by the *zma* gene in

138 *Bacillus* species[15,16.] Thus, both the N-terminal region of the peptide as ZmA may
139 have the function to interact with yeast cell membranes, allowing the anchoring of
140 the remainder of the molecule, whereas the C-terminal region can be related to
141 pore formation. This mechanism of action is based on the behavior of the
142 hydrophobic and amphiphatic peptide according to the amino acids
143 sequence[13,17,18].

144 The antifungal activity of *Bacillus* spp. has been associated to cyclic
145 lipopeptides like iturin and fengycin, and is often demonstrated against filamentous
146 fungi[11]. The inhibitory activity of lipopeptides produced by *B. subtilis* and *B.*
147 *amyloliquefaciens* against *Candida albicans* was recently reported[19,20]. Although
148 the production of such lipopeptides can not be ruled out, expression of lipopeptide
149 synthetases is low in *B. subtilis* ATCC 6633 as compared to other strains[21].

150 The increasing emergence of microorganisms resistant to conventional
151 antibiotics has generated a growing interest in the study of natural peptides with
152 antibiotic activity. These peptides, called antimicrobial peptides, participate in the
153 innate defense of animals and plants. Currently, there are over 500 antimicrobial
154 peptides isolated and studied. The production of antimicrobial peptides is part of
155 the mechanisms of host defense during the early stages of infection and its
156 importance in protecting against pathogens has been described in recent
157 decades[22]. According to the results presented in Table 1, the peptide produced by
158 *B. subtilis* subsp. *spizezinii* exhibited a broad action spectrum. Similar results were
159 also obtained with bacteriocins produced by the genus *Bacillus*[23,24]*.* The AMP was
160 more effective than antifungal reference (FLC) in inhibiting strains of *C. krusei*. *C.*

161 *krusei* strains were resistant to FLC. The fungicidal activity of AMP front strains of
162 *C. krusei* tested refer to the prospect of a new application of this substance.

163 Considering the MIC (Table 2), it is noteworthy that the peptide showed
164 higher potency against *C. krusei, C. glabrata* and *C. tropicalis*. In this case, the
165 MIC range is comparable to many clinically used antifungal agents[25]. In other
166 studies, strains of clinical isolates of *C. albicans* exhibited MIC values from 0.25-
167 0.5 mg/L (Amphotericin B (AMB)), 0.008-0.016 mg/L (Voriconazole (VRC)), 64-128
168 (FLC), 4-8 (Itraconazole (ITR)); *C. glabrata* MIC values from 0.25-1.0 mg/L (AMB),
169 0.25-0.5 mg/L (VRC); *C. tropicalis* MIC values from 0.5-1.5 mg/L (AMB), 0.016-
170 0.06 mg/L (VRC) and *C. parapsilosis* MIC values 0.25-1.0 mg/L (AMB), 0.008-0.06
171 mg/L (VRC)[26,27]. Some studies have also shown that the previous administration of
172 broad spectrum antibiotics represents a risk factor for such infections as well as
173 resistance to antifungal agents[28,29]. Thus, the peptide produced by *B. subtilis*
174 subsp. *spizezinii* may represent an alternative for the treatment of these emerging
175 pathogenic fungi.

176 Antimicrobial activity is promising when concentrations between 16 and 32
177 mg/L are achieved for isolated compounds[30]. In this range of desirable values, the
178 peptide demonstrated inhibitory activity only on some strains of *C. krusei, C.*
179 *glabrata, C. parapsilosis* and *C. tropicalis*. In general, the MIC for these yeasts
180 varied widely, which could indicate only a limited activity of this peptide. Such
181 results could be explained by various virulence factors commonly reported for
182 these microorganisms such as adherence, biofilm formation, secretion of
183 hydrolytic enzymes (proteases, phospholipases and haemolysins) and filamentous
184 growth[31].

In conclusion, the peptide produced by *B. subtilis* subsp. *spizezinii* confirmed its potential as source of a new antimicrobial drug. In general, the AMP has demonstrated a broad spectrum of fungicidal action and is effective against emerging yeast as *C. krusei, C. glabrata* and *C. tropicalis.* The biological activity of the peptide should be further investigated to confirm the results of this study, as well as to determine its mechanism of action.

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Transparency declarations

None to declare.

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Table 1. Antifungal screening of AMP produced by *Bacillus subtilis* subsp. *spizezinii*.

Yeast	***Peptide (AMP)[a]***	***Fluconazole susceptibility***
Candida albicans CA 02	FtA	S
Candida albicans CA 04	FtA	S
Candida albicans CA 05	FtA	S
Candida glabrata CG 04	FgA	S
Candida glabrata CG 05	FgA	S
Candida glabrata CG 06	FgA	S
Candida krusei CK 02	FgA	R
Candida krusei CK 03	FgA	R
Candida krusei CK 04	FgA	R
Candida parapsilosis CPA 07	FgA	S
Candida parapsilosis CPA 08	FgA	S
Candida parapsilosis CPA 134	FtA	S
Candida tropicalis CT 08	FgA	S
Candida tropicalis CT 750	FgA	S
Candida tropicalis CT 06	FgA	S
Trichosporon asahii TRA 01	R	S
Cryptococcus neoformans CRY 02	R	S
Cryptococcus neoformans CRY 14	R	S
Cryptococcus neoformans CRY 17	R	S

[a]Tested concentration = 500 mg/L; Resistance (R); Fungistatic activity (FtA); Fungicidal activity (FgA); Sensible (S)

292 **Table 2.** Minimum inhibitory concentration (MIC) and minimum
293 fungicidal concentration (MFC) of AMP produced by *Bacillus subtilis*
294 subsp. *spizezinii* against emerging yeasts pathogens.

Yeast	***Peptide (AMP)***		
	MIC	**MIC range**	**MFC**
Candida albicans			
CA 02	128[a]		
CA 04	64	64 - 128	512
CA 05	128		
Candida glabrata			
CG 04	64		128
CG 05	64	32 - 64	256
CG 06	32		64
Candida krusei			
CK 02	32		
CK 03	64	16 - 64	128
CK 04	16		
Candida parapsilosis			
CPA 07	8		32
CPA 08	128	8 - 256	256
CPA 134	256		500
Candida tropicalis			
CT 08	64		256
CT 750	32	32 - 64	64
CT 06	64		128

295 [a] Data antifungal susceptibility in mg/L;

6.1. Conclusões

- O AMP produzido por *Baciilus subtilis* subesp. *spizezinii* apresentou atividade antifúngica contra *Candida glabrata, C. tropicalis, C. parapsilosis* e *C. krusei;*
- O AMP não inibiu o crescimento de *Trichosporon asahii* e *Cryptococcus neoformans;*
- O AMP inibiu estirpes de *Candida albicans,* mas apresentou valores de MFC bem superiores quando comparado com as estirpes sensíveis de *Candida glabrata, C. tropicalis, C. parapsilosis* e *Candida krusei;*
- Este AMP apresenta uma potencial aplicação como agente antifúngico.

7. CAPÍTULO IV

Investigation of the cytotoxicity of antimicrobial peptide produced by *Bacillus subtilis* subsp. *spizezinii*

Nesse capítulo será discutida a atividade citotóxica do peptídeo antimicrobiano produzido por *B. subtilis* subespécie *spizizenii.* O estudo contempla a avaliação histopatológica de tecidos de suínos submetidos à ação do peptídeo antimicrobiano, além de ensaios de dosagem de lactato desidrogenase e hemoglobina. Também foi realizado o teste de simulação de absorção intestinal do mesmo.

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Investigation of the cytotoxicity of antimicrobial peptide produced by *Bacillus subtilis* subsp. *spizezinii*

Mário Lettieri Teixeira[1,5], Alexandre Meneghello Funtefria[2], Francielli Cordeiro Zimermann[3], Renata Assis Casagrande[4] and Adriano Brandelli[1]*

[1]Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos, ICTA, Universidade Federal do Rio Grande do Sul, 91501-970 Porto Alegre, Brazil

[2]Laboratório de Micologia, Departamento de Análises, Faculdade de Famácia, Universidade Federal do Rio Grande do Sul, 90610-000 Porto Alegre, Brazil

[3]Laboratório de Histologia, Curso de Medicina Veterinária, Universidade Federal de Santa Catarina – Campus Curitibanos, 89520-000 Curitibanos, Brazil

[4]Laboratório de Patologia, Curso de Medicina Veterinária, Instituto Federal Catarinense – Campus Concórdia, 89700-000 Concórdia, Brazil

[5]Laboratório de Bioquímica, Curso de Medicina Veterinária, Instituto Federal Catarinense – Campus Concórdia, 89700-000 Concórdia, Brazil

*Correspondence: ICTA-UFRGS, Av. Bento Gonçalves 9500, 91501-970 Porto Alegre, Brasil; e-mail: abrand@ufrgs.br

SUMMARY

Microorganisms, plants and animals naturally can produce a wide variety of chemical compounds that may have antibacterial activity. These molecules may differ chemically and structurally to each other, and are classified according to these characteristics. A number of assays and various cell types have been used with different responses to study *Bacillus* cytotoxicity and none can be considered as standard. The aim of this study was to investigate the *in vitro* cytotoxicity of the antimicrobial peptide (AMP) produced by *Bacillus subtilis* subsp. *spizezinii* on eukaryotic cells, in comparison with nisin. Histopathological evaluation, lactate dehydrogenase (LDH) release, hemolytic activity and intestinal absorption tests were performed. The result of the histopathological evaluation of skeletal muscle, liver, heart, kidney and brain incubated with AMP and nisin showed no microscopic lesions. Cells treated with the different purified fractions resulted in increasing levels of LDH release when compared to nisin, as the percentage of cells lysed. Regarding the percentage of cells lysed, the purified AMP and nisin showed a hemolytic activity of 26.91 and 21.12%, respectively, at the concentration of 20 $\mu g.ml^{-1.}$ Intestinal absorption test was conducted demonstrating antimicrobial activity between 400 and 800 $AU.ml^{-1}$ against *Haemophilus parasuis*. These results suggest that the AMP is a little more cytotoxic that nisin. Thus, despite the great potential of AMP produced by *B. subtilis* subsp. *spizezinii* as an antimicrobial agent against *H. parasuis*, more studies should be done to be able to distinguish this AMP as generally recognized as safe (GRAS).

Key words: antimicrobial; bioactive peptide; bacteriocin; *Bacillus*; cytotoxicity activity.

INTRODUCTION

Microorganisms, plants and animals naturally can produce a wide variety of chemical compounds that may have antibacterial activity. These molecules may differ chemically and structurally each other, and are classified according to these characteristics. Due to this large chemical diversity, antimicrobial peptides can be used in various fields of industry such as, for example, food biopreservatives and as therapeutic agents against pathogenic bacteria (Reddy et al., 2004; Deegan et al., 2006; Pasupuleti et al., 2012).

Bacillus spp. synthetizes a extensive number of antimicrobial peptides, with different chemical structures, including bacteriocins and bacteriocin-like (BLS) (Bizani et al., 2005; Stein, 2008). A novel antimicrobial peptide from *Bacillus subtilis* subsp *spizezinii* was identified and characterized as a BLS. This peptide showed inhibitory activity against *Haemophilus parasuis, Listeria monocytogenes, Staphylococcus aureus* and *Escherichia coli* (Teixeira et al., 2013). However, even with the great use of *Bacillus* species for scientific research and the biotechnological potential of these antimicrobial peptides there are scarce specific studies on the cytotoxicity of these compounds (Vaucher et al., 2010).

In vitro cytotoxicity assays are effective and efficient to specify basal cytotoxicity, designating the intrinsic ability of a substance to cause cell death as a consequence of damage to several cellular functions (Weyermann et al., 2005; Bouaziz et al., 2006). A number of assays and various cell types have been used

with different responses to study *Bacillus* cytotoxicity and none can be considered as standard (Mikkola et al., 2000; Maher and McClean, 2006). The aim of this study was to investigate the *in vitro* cytotoxicity of the antimicrobial peptide (AMP) produced by *Bacillus subtilis* subsp. *spizezinii* on eukaryotic cells, in comparison with nisin, through the use of different assays.

MATERIALS AND METHODS

Purification of antimicrobial peptide

Bacillus subtilis subsp. *spizezinii* ATCC 6633 was sowed twice in fresh Trypticase Soy Broth (TSB) (Acumedia Manufacturers, Lansing, MI, USA) before using it. *Bacillus* strains grew in 400 mL TSB for 72 h at 35^{o}C and then the supernatant was obtained by centrifugation at 5,000 *g* for 30 min at 15^{o}C. The supernatant was filtered through a 0.22 µm membrane (Millipore, Billerica, MA, USA), and the filtrate was considered as a crude antimicrobial preparation.

The crude antimicrobial preparation (Fraction I) was submitted to ammonium sulfate precipitation at 20% (w/v) of saturation (Fraction II). After centrifugation at 5,000 *g* for 30 min at 15^{o}C, the pellet was suspended in 10 mmol l^{-1} phosphate buffer pH 7.0 and loaded on a Sephadex G-50 column (GE Healthcare/Pharmacia Biotech, Uppsala, Sweden). The column was eluted with 10 mmol l^{-1} phosphate buffer pH 7.0 as mobile phase. Fractions of 1 ml were collected and these ones that presented antimicrobial activity were pooled and sterilized through a 0.22 µm filter membrane (Millipore, Billerica, MA, USA). These fractions (Fraction III) were loaded on a DEAE-cellulose column (GE Healthcare/ Pharmacia Biotech, Uppsala, Sweden), and eluted by a gradient step (10 ml of

each 0, 0.5, 1.0, 2.0 mol l^{-1} NaCl in 10 mmol l^{-1} phosphate buffer pH 7.0) and all of them were fractioned by 1 ml. The positive fractions for antimicrobial activity were pooled (Fraction IV), and stored at 4^{o}C until used (Teixeira et al., 2013).

Histopathological evaluation

Tissue samples from adult male pigs, freshly slaughtered at the Institute Federal of Santa Catarina – campus Concordia, were used to evaluate the formation of tissue damage due to the action of the AMP produced by *Bacillus subtilis* subsp. *spizezinii* ATCC 6633. Pigs were slaughtered according to the rules of the Brazilian Ministry of Agriculture, respecting animal welfare (Brasil, 2013). The study was approved by the regional ethics committee for animal care (process number 302/2011). Muscle, liver, cardiac, kidney and brain tissues were chosen for this study. All tissues were removed within a period of 5 minutes after the slaughter and transported to the laboratory in ice-cold Krebs-Hepes buffer. All tissues were incubated with AMP (concentration 0.05 mg.ml^{-1}) for 30 minutes at 37°C. The control group was treated with nisin at a concentration of 0.05 mg.ml^{-1}. The negative control was performed with saline solution 0.85% (Obatomi et al., 1998). Fragments of these tissues were harvested, fixed in 10% neutral-buffered formalin, processed routinely and stained with hematoxylin and eosin (HE), and examined under light microscopy. The experiments were carried out in triplicate.

LDH Release Assay

The activity of LDH in the medium was determined using a commercially available kit (Labtest Diagnostic, Lagoa Santa, Brazil). Porcine red blood cells

were treated with antimicrobial peptide or nisin (between 0.625 and 20 μg.ml^{-1}) for 1 h at 37°C. Supernatants were removed and LDH release was determined by two-point enzymatic kinetics (Kaplan et al., 1988). The absorbance at 340 nm was monitored using a BTS 370 plus spectrophotometer (Biotecnica, Varginha, Brazil). The percentage of LDH release was calculated as A_T/A_C x 100; where A_T is the experimental absorbance of treated cells, A_C is the control absorbance of untreated cells. The experiments were carried out in triplicate.

Hemolytic Activity

Hemolytic activity of antimicrobial peptides was measured spectrophotometrically using a hemoglobin release assay (Shin et al., 2001), with modifications. Briefly, fresh defibrinated porcine red blood cells were rinsed three times with PBS, centrifuged for 10 min at 1,000 *g* and resuspended at 4% (v/v) in PBS. Cells were treated with antimicrobial peptide or nisin (between 0.625 and 20 μg.ml^{-1}) for 1 h at 37°C and then centrifuged at 1,000 *g* for 5 min. Aliquots of the supernatant were then transferred to a fresh microcentrifuge tube, where hemoglobin release was monitored using a BTS 370 plus spectrophotometer (Biotecnica, Varginha, Brazil) by measuring the absorbance at 414 nm. Percentage of hemolysis was calculated as $(A_T - A_C)/(A_X - A_C)$ x 100; where A_T is the experimental absorbance of treated supernatants, A_C is the control absorbance of PBS-treated cell supernatant, and A_X is the absorbance of 0.01% (v/v) KCN lysed cells. The experiments were carried out in triplicate.

Intestinal absorption test

143 Samples of the small intestine (jejunum) of adult male pigs freshly
144 slaughtered of the Institute Federal Catarinense – campus Concordia, were used
145 to evaluate the absorption capacity for of the AMP produced by *Bacillus subtilis*
146 subsp. *spizezinii* ATCC 6633. Pigs were slaughtered according to the rules of the
147 Brazilian Ministry of Agriculture, respecting animal welfare. The technique
148 employed was adapted from Galvao et al (1993) in which the exposure time of the
149 tissue with AMP (concentration between 12.5 and 800 $AU.ml^{-1}$) was 30 minutes at
150 37°C. After this period, were collected 100 µL content internal bag and applied to
151 test the antimicrobial activity against *Haemophilus parasuis* as described by Motta
152 and Brandelli (2002). The experiments were carried out in triplicate.
153
154 **RESULTS**
155 The antimicrobial peptide produced by *B. subtilis* subsp. *spizezinii* was
156 secreted to the culture medium and was purified (Teixeira et al., 2013). The result
157 of the histopathological evaluation of skeletal muscle, liver, heart, kidney and brain
158 incubated with AMP and nisin showed no microscopic lesions (Figure 1).
159 Cells treated with the different purified fractions resulted in increasing levels
160 of LDH release (Figure 2a) when compared to nisin. The percentage of cells lysed
161 injured decreased according to the higher level of AMP purification (Figure 2b).
162 The results of fraction IV are similar to the results for cells treated with nisin. AMP
163 behaves similarly to nisin in a dose-response curve, but with a level of cell injury
164 slightly higher when compared to nisin at the same concentration at 0.625 $\mu g.ml^{-1}$
165 of AMP and nisin, the concentration of extracellular LDH was 11.69% and 8.37%,

166 respectively (Figure 2c). The loss of cell membrane integrity is closely related to
167 the concentration of AMP and nisin.

168 The hemolytic activities of the antimicrobial peptide and nisin were
169 determined as the percentage of lysis of erythrocytes. According to this study, the
170 fraction IV showed a concentration of 3.40 $g.dl^{-1}$ released hemoglobin, while the
171 fractions III, II and I showed 4.80, 5.77 and 7.20 $g.dl^{-1}$, respectively. Nisin had a
172 concentration of 2.8 g.dL hemoglobin released (Figure 3a). Regarding the
173 percentage of cells lysed, the purified AMP and nisin showed a hemolytic activity
174 of 26.91 and 21.12%, respectively, at the concentration of 20 $\mu g.ml^{-1}$ (Figure 3b).

175 Intestinal absorption test was conducted and the solution contained within
176 the bag was tested against *Haemophilus parasuis* ATCC 19417 demonstrating
177 antimicrobial activity between 400 and 800 $AU.ml^{-1}$.

178

179 **DISCUSSION**

180 The cytotoxicity of the AMP produced by *B. subtilis* subsp. *spizezinii* was
181 investigated in erythrocytes and tissues of freshly slaughtered pigs. The
182 histopathological observation of tissues treated with AMP and nisin showed no
183 significant changes. The absence of histopathological abnormalities in tissues
184 treated with nisin was expected, since according to Aranha et. al. (2004), nisin
185 does not cause histopathological abnormalities in tissues treated with this
186 bacteriocin. The low cytotoxicity of nisin A to kidney epithelial cells has been
187 reported (Murinda et al., 2003). Cells treated with AMP and nisin showed similar
188 sensitivity assays developed in this study, noting that the cell damage was
189 stronger in cells treated with AMP, causing an increased loss of cell viability in the

concentration range tested (0.625 to 20 μg.ml^{-1}). Therefore, cell viability decreased gradually with increasing concentration of peptide. Maher and McClean (2006) conducted tests on HT29 and Caco-2 cells in which the nisin also showed a low cytotoxicity. Different levels of cytotoxicity may be related to biochemical parameters of the cells involved, as the plasma membrane composition and metabolic activity, time of exposure to the toxic agent, toxicity assay used, the medium used to apply the peptides, and also the degree of purification of the peptides (Papo and Shai, 2005; Maher and McClean, 2006).

Therefore, the increased cytotoxicity of nisin and other antimicrobial peptides against some cell types may be associated with the physical-chemical properties, which can interfere with the interaction of AMP with the target cell, due to differences in cell surface hydrophobicity, interaction membrane transport protein, porins, integrins, cellular receptors, thus influencing a greater or lesser cytotoxic effect, although this type of mechanism of action of AMPs in different cell types is not fully elucidated (Murinda et al., 2003).

One of the indicators of cellular death is the release of intracellular content into the extracellular medium, and quantification of LDH in the supernatant of the cell culture medium, indicates cell membrane damage and consequent cell death (Decker and Lohmann-Matthes, 1988). The LDH assay thus indicates loss of integrity of the plasma membrane of treated cells, showing some cytotoxicity after 24 h of exposure to nisin or AMP. This result agrees with the results of other literature that the mechanism of action of antimicrobial peptides is mostly associated with the membrane, for example, destabilization, formation of membrane pores and the action biosurfactants, including nisin and AMP produced

214 by *B . subtilis* subsp. *spizezinii* (Deegan et al., 2006; Teixeira et al., 2009; Teixeira
215 et al., 2013).

216 The antimicrobial effect of AMP occurs at very low doses (Teixeira et al.,
217 2013) so that the concentration used in the cytotoxicity assay presented a low
218 level of toxicity to cells treated with AMP (20 $\mu g.ml^{-1}$). This fact confirms the
219 observation that the target membranes have different sensitivity to the action of
220 the AMP, in which the sensitivity decreases according to the complexity of the
221 organism involved, and are more susceptible bacteria, lower eukaryotes, including
222 fungi and higher organisms are largely resistant to AMP (Mason et al., 2007).

223 The simulation of the intestinal absorption of AMP and, consequently,
224 antibacterial activity, suggests great potential in the use of this substance as a
225 therapeutic agent, since enterocytes are directly involved in the transfer of
226 oligopeptides and peptide-derived drugs across the enterocyte brush border
227 membrane, the essential step for intestinal absorption (Ziv and Bendayan, 2000).
228 Thus, more studies should be considered in order to understand the complexity of
229 this transport mechanism, as well as the structure-activity relationship of AMP, so
230 that the AMP can be better absorbed. These studies should involve molecular
231 characterization and interaction AMP/membrane receptor.

232 The wide range of use of AMP has provided a series of studies involving
233 the types of mechanism of action, biological control, use as biopreservative as
234 biosurfactant agent, and also on their cytotoxicity. Thus, as nisin has been
235 extensively studied and cleared for use as a safe food preservative, and also
236 suggesting to potential clinical use due to their small toxicity to human cells
237 (Reddy et al., 2004; Deegan et al., 2006), other AMP are being investigated.

238 Therefore, despite the great potential of AMP produced by *B. subtilis* subsp.
239 *spizezinii* as an antimicrobial agent against *H. parasuis*, more studies should be
240 done to be able to recognize this AMP as generally recognized as safe GRAS.
241
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245
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314

315 **FIGURE LEGENDS**

316

317 **Figure 1.** Histopathological evaluation of porcine cells treated with AMP, nisin and
318 negative control 100 times magnification. 1a. hepatocytes treated with AMP
319 (concentration 0.05 $mg.ml^{-1}$). 1b. hepatocytes treated with nisin (concentration
320 0.05 $mg.ml^{-1}$). 1c. hepatocytes treated with saline (0.85%). 2a. kidney cells treated
321 with PMA (concentration 0.05 $mg.ml^{-1}$). 2b. kidney cells treated with nisin
322 (concentration 0.05 $mg.ml^{-1}$). 2c. kidney cells treated with saline (0.85%).

323

324 **Figure 2.** Evaluation of cytotoxicity of the antimicrobial peptide (AMP) produced by
325 *B*. *subtilis* subsp. *spizezinii* in porcine red blood cells, according to the
326 methodology proposed by this study. a. LDH activity. b. Lysed cells. c. LDH
327 release. AMP (triangles), Nisin (circles) and the SDS positive control (squares).

328

329 **Figure 3.** Evaluation of cytotoxicity of the antimicrobial peptide (AMP) produced by
330 *B*. *subtilis* subsp. *spizezinii* in porcine red blood cells, according to the
331 methodology proposed by this study. a. Hb concentration. b. Lysed cells.

332

333

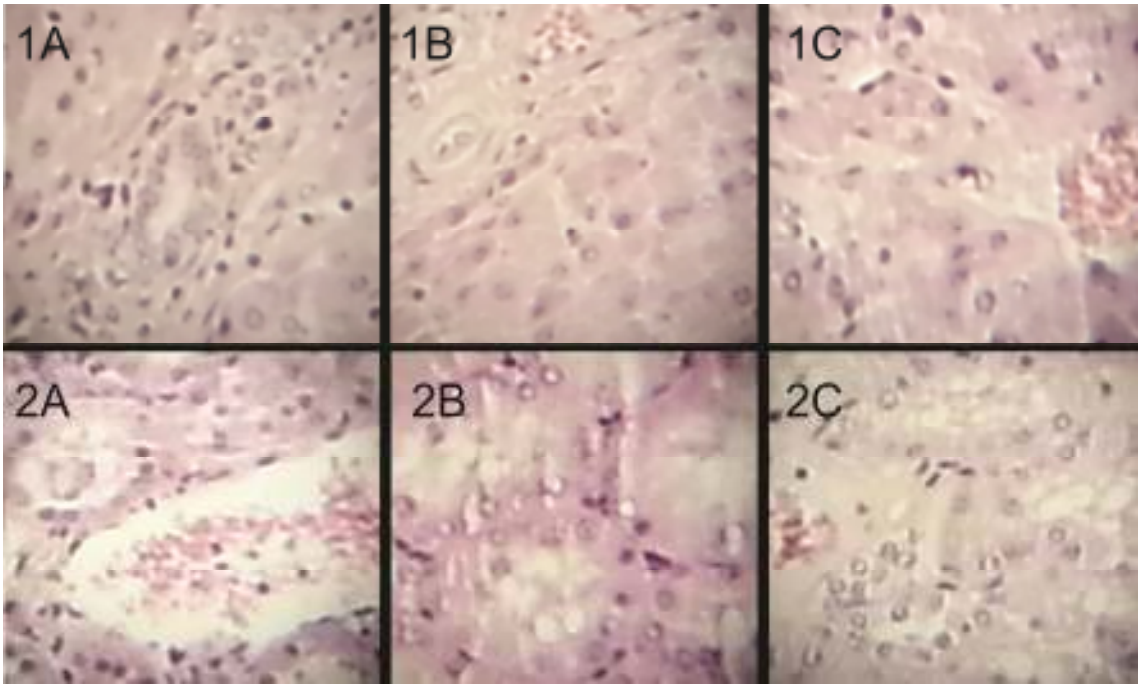
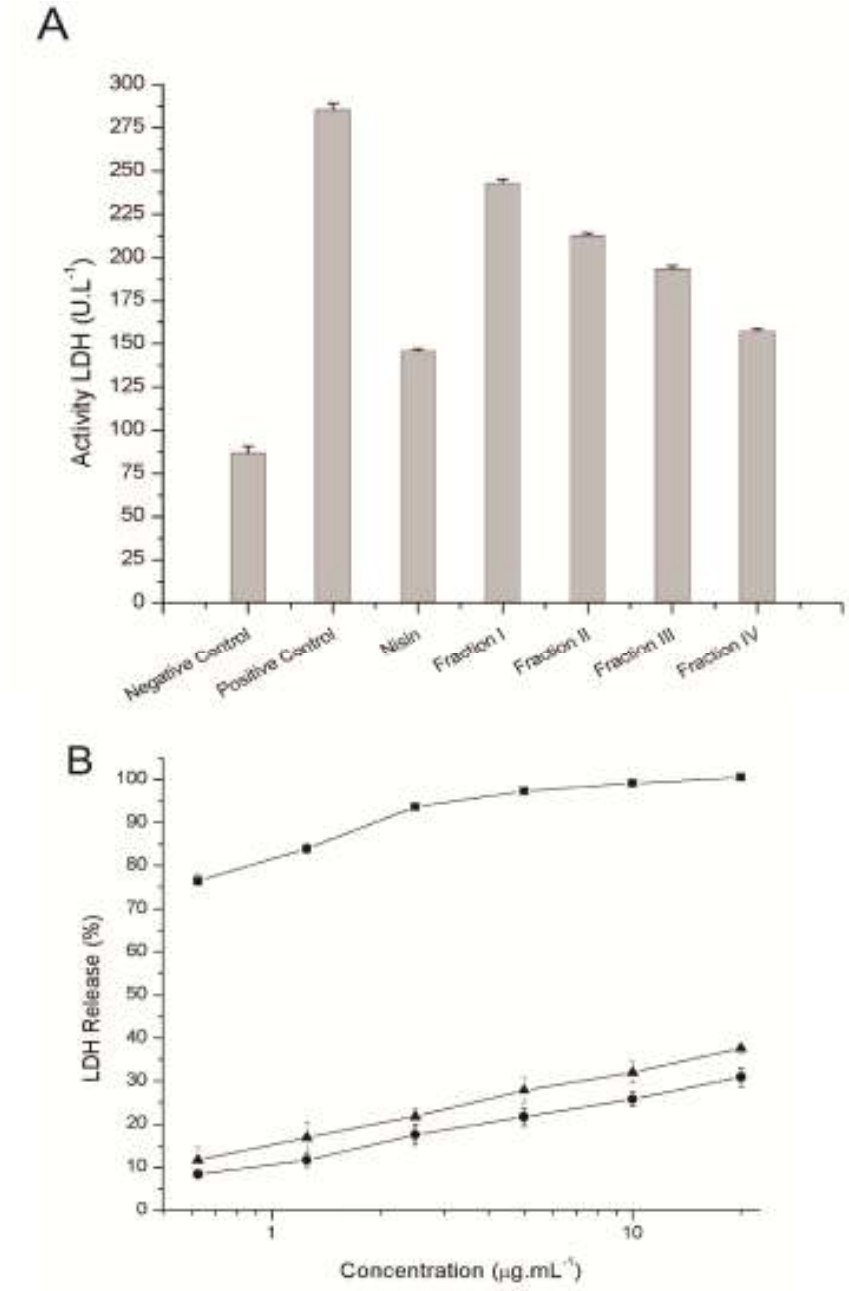
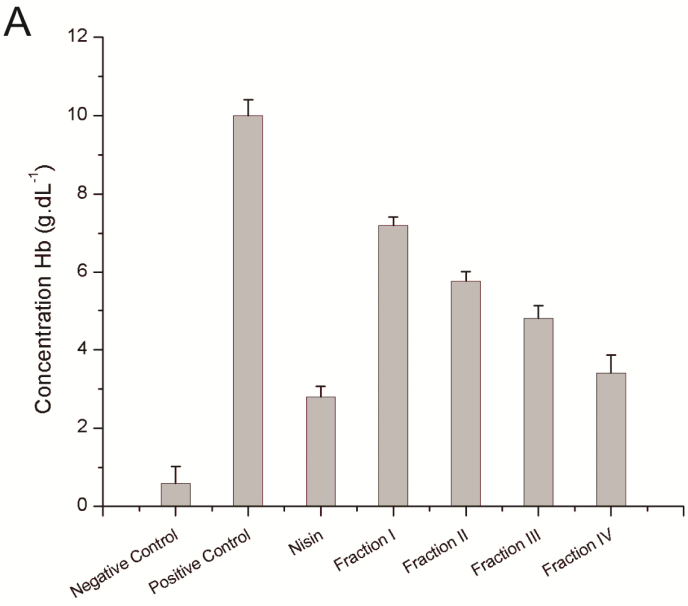


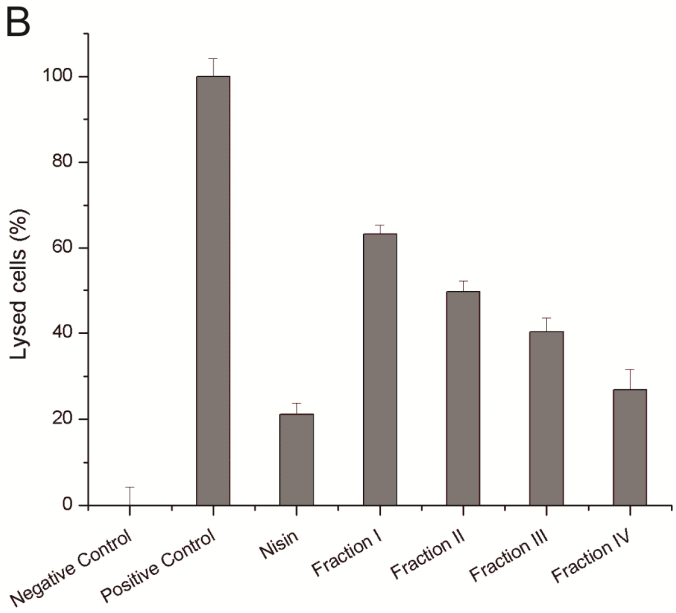
Figure 1. Histopathological evaluation of porcine cells treated with AMP, nisin and negative control at 100 times magnification. 1A. hepatocytes treated with AMP (concentration 0.05 $mg.ml^{-1}$). 1B. hepatocytes treated with nisin (concentration 0.05 $mg.ml^{-1}$). 1C. hepatocytes treated with saline (0.85%). 2A. kidney cells treated with PMA (concentration 0.05 $mg.ml^{-1}$). 2B. kidney cells treated with nisin (concentration 0.05 $mg.ml^{-1}$). 2C. kidney cells treated with saline (0.85%).



341

342 **Figure 2.** Evaluation of cytotoxicity of the antimicrobial peptide (AMP) produced by
343 *B*. *subtilis* subsp. *spizezinii* in porcine red blood cells, according to the
344 methodology proposed by this study. A. Lactate dehydrogenase (LDH) activity. B.
345 LDH release. AMP (triangles), Nisin (circles) and the SDS positive control
346 (squares).





347

348 **Figure 3.** Evaluation of cytotoxicity of the antimicrobial peptide (AMP) produced by
349 *B*. *subtilis* subsp. *spizezinii* in porcine red blood cells, according to the
350 methodology proposed by this study. A. Hemoglobin (Hb) concentration. B. Lysed
351 cells.

7.1. Conclusões

- O AMP produzido por *Baciilus subtilis* subsp. *spizezinii* não apresentou alterações histopatológicas nos tecidos de suínos testados quando comparado a nisina*;*
- O AMP apresentou diferentes níveis de lise celular de acordo com a fração purificada, sendo que a fração IV apresentou os menores níveis de citotoxicidade nos ensaios de LDH e hemólise*;*
- O AMP, comparado com as mesmas concentrações de nisina, apresentou níveis maiores de citotoxicidade nos ensaios de LDH e hemólise;
- O AMP foi absorvido pela mucosa intestinal e apresentou atividade antimicrobiana contra *Haemophilus parasuis* ATCC 19417.

8. DISCUSSÃO E CONSIDERAÇÕES FINAIS

Os resultados deste trabalho indicam um importante aumento no isolamento de *H. parasuis* no período de 2007 a 2010 no sul do país. O aumento no número de exames pode ser um dos responsáveis por este aumento no número de casos, mas a proporção de exames positivos para *H. parasuis* cresceu mais que a proporção de exames, sugerindo a disseminação deste patógeno na região. A maior incidência de casos de pneumonia viral pode ter contribuído para a propagação deste agente, devido à imunodepressão causada por esta doença.

De acordo com esta situação, algumas medidas devem ser tomadas para melhorar o controle sanitário do rebanho de suínos da região. Tais medidas compreendem: (a) um melhor manuseio e manutenção da maternidade e creche (seguido por um sanitário vazio), (b) melhoria das condições de higiene nas instalações da propriedade rural, (c) desinfecções freqüentes e proibição do contato de animais infectados com as instalações, (d) eliminação dos fatores de predisposição (tais como o corte de um dente/dentes, permitindo lesões na gengiva e/ou língua) e (e) uso de tratamento profilático, como vacinas (RAPP-GABRIELSON & GABRIELSON, 1992; OLIVEIRA, 2007).

Em relação à prospecção de uma bactéria produtora de peptídeo antimicrobiano com ação contra *H. parasuis*, o gênero *Bacillus* apresenta um grande potencial biotecnológico. A utilização da estirpe de *Bacillus subtilis* subesp*. spizezinii* satisfez o objetivo desta pesquisa. O AMP mostrou resistência a vários solventes orgânicos, mantendo sua atividade antimicrobiana. A atividade bactericida deste AMP foi determinada através da observação da diminuição no número de células viáveis de *H. parasuis* em um período de aproximadamente 20 minutos após a adição do AMP contendo 800 $UA.mL^{-1}$. Esse tempo por ser bastante reduzido, sugere que o mecanismo de ação contra este patógeno seja pelo rompimento da parede celular e membrana plasmática (BIZANI & BRANDELLI, 2002). O AMP, por apresentar características emulsificantes, pode interagir com a parede celular e membrana citoplasmática, permitindo a abertura de poros nas mesmas, dessa forma, ocorre o extravasamento do conteúdo citoplasmático da bactéria, e consequente, morte celular. O AMP produzido por *B.*

subtilis subsp*. spizezinii* foi purificado e elucidada a sua sequência de aminoácidos (NRWCFADD). Este peptídeo também demonstrou um amplo espectro de ação, apresentando atividade bactericida frente a bactérias patogênicas, tais como, *Listeria monocytogenes, Escherichia coli* e *Staphylococcus aureus* (TEIXEIRA, *et al*., 2013).

De posse do espectro de massas (MS-MS), pode-se verificar que este peptídeo apresenta uma massa molecular próximo ao de outras bacteriocinas já descritas na literatura, informações que sugerem que existe relação com outros peptídeos antimicrobianos. A prevalência de aminoácidos com características apolares, responsáveis pelo comportamento em meio aquoso, é característico de outros peptídeos antimicrobianos já isolados e identificados, como é o caso da liquenisina, surfactina, esperina, entre outros (THOMAS & ITO, 1990; BAUMGART, *et al*., 1991; KLAENHAMMER, 1993; KONZ, *et al*., 1999).

O estudo também contribuiu para elucidar o tipo de mecanismo que este AMP apresenta, sugerindo que seja baseado na lise da parede celular e membrana celular de bactérias Gram-positivas e Gram-negativas, as quais possuem representantes que causam a deterioração de alimentos e podem causar doenças em seres humanos (KISS, *et al*., 2006).

A descoberta da atividade antifúngica deste AMP teve como base outros estudos na literatura que já descreveram este tipo de atividade em outros AMP`s sintetizados por outras espécies de *Bacillus* (RAFFEL *et al.*, 1996;. CHERIF *et al.*, 2003). De posse das características químicas, se pode sugerir que o mecanismo de ação do AMP deste estudo está relacionado com o composição de aminoácidos com características hidrofóbicas e também com características anfipáticas. Desta forma, a região N-terminal do peptídeo tem a função de interagir com as membranas das células de levedura, permitindo a ancoragem do restante da molécula, enquanto que a região C-terminal pode estar relacionada com a formação de poros (DRIDER *et al.,* 2006, TOSSI, 2011; TEIXEIRA *et al.,* 2013). O grande potencial deste AMP em ser utilizado como antifúngico deve ser considerado, pois com concentrações que variaram entre 16 e 32 µg/mL houve inibição de estirpes de *Candida krusei, Candida glabrata, Candida parapsilosis* e *Candida tropicalis* (LÜDERS *et al*., 2003).

A citotoxicidade deste AMP produzido por *B. subtilis* subsp. *spizezinii* foi investigada utilizando-se células provenientes de tecidos de suínos recentemente abatidos. A análise microscópica destes tecidos não apresentou alterações histopatológicas, fato este semelhante ao grupo controle tratado com nisina. Esta ausência de alterações histopatológicas em tecidos tratados com nisina era esperada, pois de acordo com Aranha e colaboradores (2004), a nisina não causa alterações histopatológicas em tecidos e células. Nos outros ensaios citotóxicos realizados, o AMP e a nisina mostraram o mesmo comportamento, mas o grau de lesão celular sempre foi um pouco maior nas células tratadas com o AMP do que nas células tratadas com nisina, independente da concentração testada (0,625 a 20 µg/mL). Este comportamento citotóxico destas moléculas está associado às propriedades físico-químicas. Estas podem interferir com a interação do AMP com a célula alvo, devido a diferenças de hidrofobicidade da superfície celular, a interação com proteínas de transporte de membrana, porinas, integrinas, além dos receptores celulares, influenciando, assim, um maior ou menor efeito citotóxico. Embora, se deva ressaltar que este tipo de mecanismo de ação do AMP em diferentes tipos de células não está ainda completamente elucidado.

Portanto, a ampla gama de utilização destes peptídeos antimicrobianos está alavancando uma série de estudos que envolvem os tipos de mecanismo de ação, o controle biológico, aplicabilidade (biopreservativo, biotensioativo), além de estudos toxicológicos. Assim, como a nisina tem sido extensivamente estudada, já é reconhecida como *Generally Recognized As Safe (*GRAS), e possui uma pequena toxicidade para as células humanas (REDDY *et al.,* 2004; DEEGAN *et al.*, 2006), outros peptídeos antimicrobianos estão seguindo os mesmos encaminhamentos. Assim, apesar do grande potencial do AMP produzido por *B. subtilis* subesp. *spizezinii* como um agente antimicrobiano contra *H. parasuis*, mais estudos devem ser feitos para possibilitar o reconhecimento deste AMP como GRAS.

9. PERSPECTIVAS

- Ensaios toxicológicos *in vivo* para avaliar o uso potencial na terapêutica veterinária .
- Ensaios imunogênicos para avaliar a capacidade do AMP em sensibilizar o organismo.
- Clonar o gene e tentar aumentar a sua expressão, para possibilitar o aumento da síntese do AMP.
- Utilizar a atividade antifúngica para desenvolver um medicamento sintético com atividade anti-*Candida*.

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