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

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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 NRWCFA\[G\]D, 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. *spizezini*...
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. *spizizenii* 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 10⁷ 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% CO₂ 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 1⁻¹, pH 7.0, and loaded on a Sephadex G-50 column (GE Healthcare/Pharmacia Biotech). The column was eluted with 10 mmol phosphate buffer 1⁻¹, pH 7.0, as mobile phase. Fractions of 1 ml were collected without deproteinization. The fractions positive for antimicrobial activity were pooled and sterilized through a 0.22 μm filter membrane (Millipore), and 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 10⁶ 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% CO₂ 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 10⁶ 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% CO₂ in air. The control was taken with addition of 10 mmol phosphate buffer 1⁻¹, 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% CO₂ in air. The cell suspensions were fixed with 12% (v/v) glutaraldehyde in 0.2 mol Na-phosphate buffer 1⁻¹, 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 (OsO₄) ml⁻¹ in cacaoodylate 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 (20 μm, 125 A, 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 x-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 (Schreiber 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 (NCBIdb 20120318; 17574 240 sequences; 6033 299 959 residues) restricted to *Bacillus subtilis* (27 630 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

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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Final concentration (mg ml(^{-1}))</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronase E</td>
<td>2</td>
<td>92 ± 6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>47 ± 8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>2</td>
<td>84 ± 12</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>36 ± 9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

*Data are means ± SEM.*
concentrations tested. The kinetics of the peptide effect on growth of *H. parasuis* is demonstrated (Fig. 5). When the peptide was tested at $20 \mu g \, ml^{-1}$, 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.
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 & Rodriguez, 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 those described for some bacteriocins (Martínez & Rodriguez, 2005; Papagianni & Papamichael, 2011). 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.

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).

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 Zn²⁺ or Mg²⁺ (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 & Brodgen, 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
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...
Antimicrobial peptide inhibiting H. parasuis

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% CO₂ 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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