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Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by *Bacillus cereus*

Delmar Bizani[1,3]
Amanda S. Motta[1]
Juliana A.C. Morrissy[1]
Renata M.S. Terra[2]
André A. Souto[2]
Adriano Brandelli[1*]

[1]Department of Food Sciences, ICTA, Rio Grande do Sul Federal University, Porto Alegre, Brazil
[2]Department of Chemistry, Rio Grande do Sul Catholic University, Porto Alegre, Brazil
[3]Centro Universitário La Salle, Canoas, Brazil

*Corresponding author:
A. Brandelli
Laboratório de Bioquímica e Microbiologia Aplicada
Departamento de Ciência de Alimentos, ICTA
Universidade Federal do Rio Grande do Sul
91501-970 Porto Alegre, Brasil
Fax +55-5133167048
E-mail: abrand@ufrgs.br

Summary. The mode of action of cerein 8A, a bacteriocin produced by the soil bacterium *Bacillus cereus* 8A, was investigated. The effect of cerein 8A was tested against *Listeria monocytogenes* and a bactericidal effect at 400 arbitrary units (AU)/ml was observed. In addition, cerein 8A was bactericidal against *Bacillus cereus* at 200 AU/ml, and inhibited the growth of *Escherichia coli* and *Salmonella* Enteritidis. Stronger inhibition of these gram-negative bacteria was achieved when the chelating agent EDTA was added together with bacteriocin. The effect of cerein 8A on *B. cereus* and *L. monocytogenes* was also investigated by Fourier transform infrared spectroscopy (FTIR). Treated cells had an important frequency increase at 2920 cm^{-1} and a decrease at 1400 cm^{-1}, corresponding to assignments of fatty acids. Transmission electron microscopy showed damaged cell walls and loss of protoplasmic material. These results suggest that the mode of action of cerein 8A is to interfere with cell membranes and the cell wall. [**Int Microbiol** 2005; 8(2):125-131]

Key words: *Bacillus cereus* · *Listeria monocytogenes* · antimicrobial peptides · bacteriocins · infrared spectroscopy

Introduction

Many bacterial species produce peptide antibiotics, called bacteriocins, that often have an antimicrobial effect on closely related organisms [17,26,35]. These compounds have been extensively studied because of their potential applications in the food industry as natural biopreservatives and in pharmaceuticals as antimicrobials [18,23]. Bacteriocins produced by lactic-acid bacteria have been the focus of many investigations because of their particular importance in the dairy industry [15,30]. Based on their chemical structures, stability, and mode of action, bacteriocins have been classified as: (i) lantibiotics; (ii) small heat-stable peptides; (iii) large heat-labile proteins; and (iv) complex proteins that require carbohydrate or lipid moieties for activity [17].

The mechanisms of action of peptide antibiotics are diverse, but the bacterial membrane is the target for most bacteriocins [17]. The type-A lantibiotics nisin, subtilin, Pep5, and epidermin have novel secondary modes of action in addition to their well-established lytic activity [34]. This dual antibiotic activity of a single molecule no doubt plays an important role in the antimicrobial mechanism of some bacteriocins [22]. Several bacteriocins or bacteriocin-like molecules have been described for *Bacillus* spp., including coagulin [14], cerein 7 [28], subtilosin [36], and thuricin 7 [8], and some of them have a broad spectrum of antibacterial activity. *Bacillus cereus* 8A, isolated from soils of native woodlands of southern Brazil, has antagonistic action against several pathogenic and food-spoilage microorganisms, such as *Listeria monocytogenes*, *B. cereus*, and pathogenic bacteria involved in bovine mastitis [4]. Treatment with proteases and trichloroacetic acid inhibits *B. cereus* 8A antimicrobial activity [4], suggesting that it produces a bacteriocin-like substance.

The aim of this work was to investigate the mode of action of cerein 8A and to explore its potential use as an antimicrobial to prevent the proliferation of *L. monocytogenes* and other pathogenic and food-spoilage microorganisms.

Materials and methods

Bacterial strains. *Bacillus cereus* 8A isolation and characterization have been described elsewhere [4]. The indicator strains *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 25922 and *Salmonella* Enteritidis ATCC 13076 were stored in 20% glycerol at –21°C, and propagated twice in BHI broth (Becton & Dickinson, Cockeysville, MD) before use.

Bacteriocin preparation and activity. *B. cereus* 8A was grown in 200 ml BHI broth at 30°C in a shaker at 125 cycles/min for 32 h. Cells were harvested by centrifugation at 10,000 ×*g* for 15 min and the culture supernatant was filtered through a 0.22-μm membrane (Millipore, Bedford, MA). Bacteriocin activity was purified by precipitation with ammonium sulfate at 65% saturation. The pellet was resuspended in phosphate buffered saline (PBS) and extracted with 1-butanol (0.7 volumes). After butanol evaporation under reduced pressure, the bacteriocin was stored at 4°C until its use for antimicrobial assays.

The antimicrobial activity of the purified bacteriocin was assessed by agar disc diffusion assay [25]. Aliquots (20 μl) of the bacteriocin preparation were applied onto 6-mm cellulose discs on agar plates previously inoculated with a 0.5 McFarland suspension of the indicator strain. Inhibition zones were measured after incubation at 37°C for 24 h. The bacteriocin titer was calculated by the serial dilution method [25].

Growth determination. Viable cell counts, expressed as colony-forming units (cfu/ml), were determined as described previously [25]. Bacterial suspensions were diluted to 10^{-8} in 8.75 g NaCl/l; the samples were homogenized and then loaded (20 ml) in triplicate onto nutrient agar (Becton Dickinson) plates. The plates were incubated at 37°C for 24 h, and 30–100 colonies were counted. In parallel, the optical density of the cultures at 600 nm (OD_{600})was measured.

Effect of cerein 8A against the indicator organisms. An overnight culture of each indicator organism was obtained by growing the respective bacteria in BHI at 37°C. Samples (1 ml) of each culture were inoculated into Erlenmeyer flasks containing 99 ml BHI, which were then incubated at 37°C. Growth was monitored at different intervals by determining the OD_{600} and by viable cell counts (cfu/ml). Bacteriocin, at a final concentration of 120 or 400 arbitrary units (AU)/ml, was added to cultures of *L. monocytogene*s and *B. cereus*, and its effects on turbidity and the number of viable cells were assessed at 2-h intervals. In addition, the effects of cerein 8A on gram-negative bacteria (*E. coli* and *Salmonella* Enteritidis) was evaluated at a concentration of 400 AU/ml. To evaluate the effect of a chelator on bacteriocin activity, 20 mM disodium EDTA (Riedel, Hannover) was added. Samples treated with EDTA alone served as controls.

Hemolysis and hemagglutination. The hemolytic and hemagglutination properties of the bacteriocin were verified using human and sheep erythrocytes. Tests were carried out essentially as described in [3]. Hemolysis was observed by visual inspection of sheep-blood agar plates. A clinical isolate of *Aeromonas* sp. with known hemolytic activity was used as positive control. For hemagglutination tests, 50 μl of the bacteriocin was mixed with the same volume of a 6% (v/v) washed erythrocyte suspension on a white porcelain tile. The negative control consisted of erythrocytes suspension and PBS. The reactions were visualized using a bright-field microscope, and considered positive if agglutination occurred within 10–15 min of incubation.

Hydrolytic activities. Murein hydrolytic activity was assayed on peptidoglycan agar plates [2]. Commercial lysozyme (Merck, Darmstadt, Germany) was used as positive control. To check whether the compound had phospholipase activity, the bacteriocin was spotted onto egg-yolk plates [13], using commercial phospholipase (Sigma, St. Louis, MO) as the control. Proteolytic activity was investigated using either skimmed-milk agar plates or the substrate azocasein [32], with pronase E (Sigma) as positive control. Bacteriocin dilutions were spotted onto the plates and the appearance of clear zones was monitored.

Fourier transform infrared (FTIR) spectroscopy. Bacteriocin was added (400 AU/ml) to cell suspensions of about 10^6 cfu/ml of either *L. monocytogenes* or *B. cereus*. After incubation for 60 min, both treated and control cells were washed three times with MilliQ water. Twenty microliters of each bacterial sample was evenly applied onto a ZnSe optical plate, dried for approximately 15 min under vacuum, and then analyzed by FTIR spectroscopy. The curves represent the average of three individual measurements of the same experiment. All IR spectra (4000–650 cm^{-1}) were obtained by the attenuated total reflection (ATR) technique, using a Perkin-Elmer Spectrum One spectrometer (Perkin Elmer, Uberlingen, Germany) with a horizontal ATR device (Se, 45°). Fifty scans were taken with 4 cm^{-1} resolution. Reproducibility of the normalized spectra was ± 2%.

Transmission electron microscopy. Samples were taken from exponentially growing cultures (10^8 cfu/ml) of either *L. monocytogenes* or *B. cereus* treated or not with cerein 8A (320 AU/ml). Cells were harvested by centrifugation and washed twice with 0.1 M phosphate buffer (pH 7.3). The cells were fixed with 2.5% (v/v) glutaraldehyde, 2.0% (v/v) formaldehyde in 0.12 M phosphate buffer for 10 days and then postfixed in 2% (w/v) osmium tetroxide in the same buffer for 45 min. The samples were dehydrated in a graded acetone series (30–100%) and embedding in Araldite-Durcupan for 72 h at 60°C. Thin sections (microtome UPC-20, Leica) were mounted on grids, covered with collodion film, and poststained with 2% uranyl acetate in Reynold's lead citrate. All preparations were observed with a JEOL JEM 1200ExII electron microscope (JEOL, Tokyo) operating at 120 kV.

Results

Effect on *Listeria monocytogenes* and *Bacillus cereus*. Figure 1 shows the effect of bacteriocin concentration on the survival of *L. monocytogenes* and *B. cereus*. The number of viable cells of both bacteria decreased as the bacteriocin concentration increased. Complete inhibition of the growth of *L. monocytogenes* was observed with 400 AU bacteriocin/ml, corresponding to an EC_{50} ("effective concentration", defined as the drug concentration producing 50% maximum effect) of approximately 120 AU/ml. In the case of *B. cereus*, growth was completely inhibited at a dose of 200 AU cerein 8A/ml, and the EC_{50} was approximately 40 AU/ml.

The kinetics of the bacteriocin effect on the growth of *L. monocytogenes* and *B. cereus* are shown in Fig. 2. Bacteriocin (120 AU/ml final concentration) added to a cell suspension of *L. monocytogenes* resulted in a difference in viable counts corresponding to 3 exponential-growth cycles compared to the control (Fig. 2A). The inhibition of *L. monocytogenes* growth was immediate and resulted in a decrease in OD_{600} during the incubation (Fig. 2A), suggesting that cell lysis is

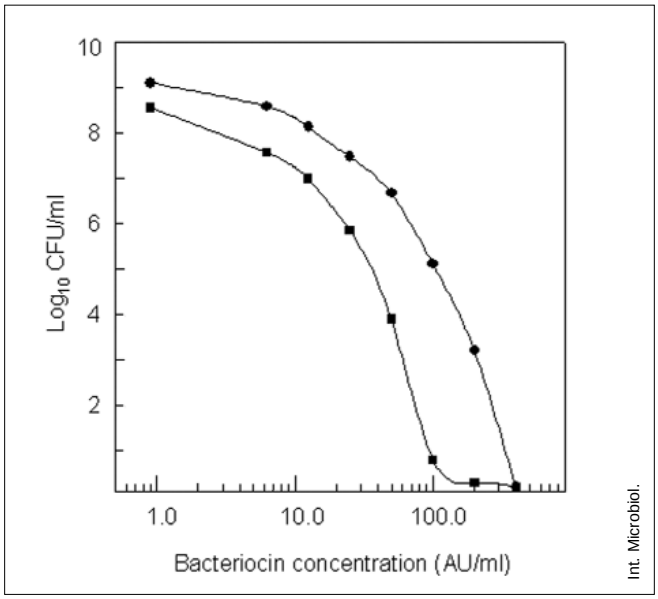

Fig. 1. Effect of cerein 8A concentration on the survival of *Listeria monocytogenes* (circles) and *Bacillus cereus* (squares). Bacterial cells were incubated for 60 min in the presence of increasing doses of cerein 8A and then viable cells were counted. Each point represents the mean of three independent experiments.

triggered in *L. monocytogenes* after cell death. At a concentration of 400 AU/ml, viable counts decreased drastically, reaching zero in 75 min (Fig. 2B).

The addition of bacteriocin (120 AU/ml final concentration) to a cell suspension of *B. cereus* caused a large decrease in the number of viable cells over a period of 10 h (Fig 2C). The OD_{600} of bacteriocin-treated *B. cereus* suspensions remained nearly constant during this period (Fig. 2C). At a higher dose of cerein 8A (400 AU/ ml), there was a rapid decrease in viable cell counts, which reached zero within 60 min, in parallel with a decrease in the OD_{600} (Fig. 2D).

Effect on gram-negative bacteria. Similarly, when a cell suspension of *Salmonella* Enteritidis was treated with 400 AU bacteriocin/ml (final concentration) the viable counts were much lower than those of the controls without bacteriocin (Fig. 3A). The addition of bacteriocin plus EDTA resulted in greater inhibition than obtained with bacteriocin alone. Similar results were obtained with *E. coli* (Fig. 3B).

Hemolysis, hemagglutination, and hydrolytic activities. The hemolytic and hemagglutination activities of cerein 8A were assayed and were negative against human and sheep

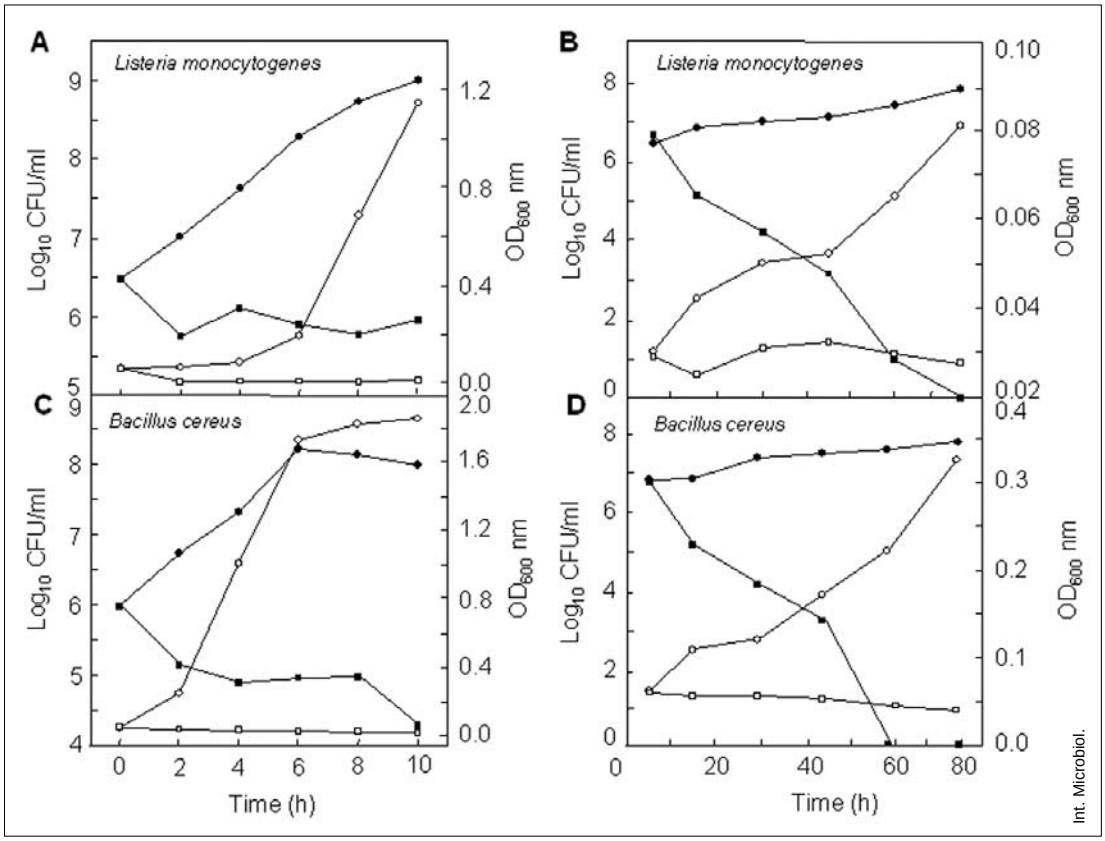

Fig. 2. Effect of cerein 8A on the growth of *Listeria monocytogenes* and *Bacillus cereus*. Optical density (open symbols) and viability (black symbols) were monitored in control (circles) and treated (squares) cells using a final concentration of 120 AU/ml (**A**, **C**) or 400 AU/ml (**B, D**). Each point represents the mean of three independent experiments.

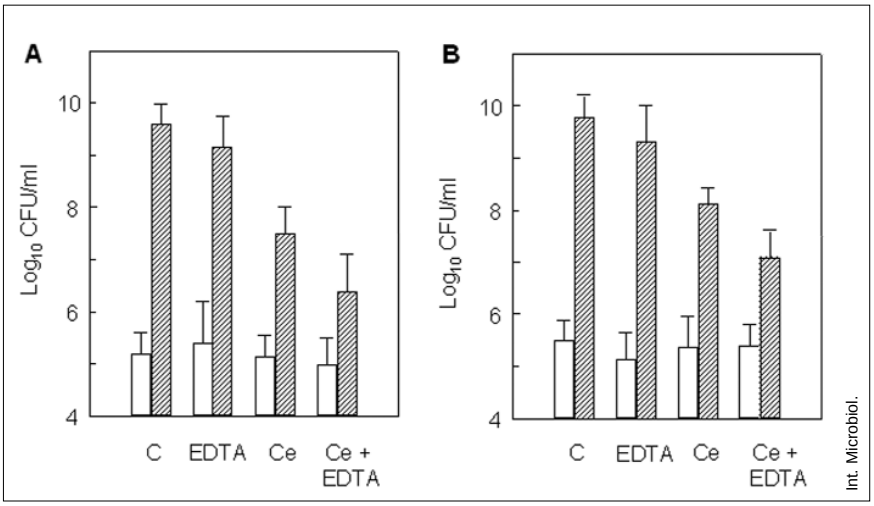

Fig. 3. Effect of cerein 8A on gram-negative bacteria. The viability of *Salmonella* Enteritidis (**A**) and *Escherichia coli* (**B**) was monitored after treatment with 400 AU cerein 8A/ml, 20 mmol EDTA/l, or cerein 8A plus EDTA. Bars are the mean ± SEM of three independent experiments. C, control; Ce, cerein.

Table 1. Fourier transform infrared (FTIR) spectroscopy absorption bands of *Listeria monocytogenes* and *Bacillus cereus*

Frequency (cm^{-1})	Possible assignment*
3280	H-bonded OH groups, NH_2 stretching
2960–2920	Aliphatic C–H stretching (fatty acids)
1715	C=O stretching (carbonic acid)
1660–1535	NH_2 bending, C=O, C=N stretching (amides I and II)
1450	C–H deformations in aliphatics
1400	C=O stretching (symmetric) of COO^-
1398–1390	C–H bending, $–CH_3$ stretching (fatty acids)
1310–1240	C–N stretching (amide III)
1250–1220	P=O stretching antisymmetric $>PO_2^-$
1200–1000	C–O, PO_2^-, C–C stretching, C–O–H, C–O–C deformation (glycopeptides, ribose, polysaccharides, phosphodiester)

*Assignments according to [12,20,33].

erythrocytes. Negative reactions for muramidase, phospholipase, and protease were also observed (data not shown).

Infrared spectroscopy. The effect of cerein 8A on macromolecular structures of *L. monocytogenes* and *B. cereus* was analyzed by FTIR spectroscopy. Table 1 shows the possible assignments of the individual bands.

Treated cells of *L. monocytogenes* showed an important frequency increase at 2920 cm^{-1} and a decrease at 1400 cm^{-1} (Fig. 4). A displacement was observed at 1230 cm^{-1} (phospholipids). Smaller differences were observed in the range 1000–1100 cm^{-1} (deformation of carbohydrates). FTIR spectra of the cell biomass of *B. cereus* treated with cerein 8A showed very similar changes.

Electron microscopy. *L. monocytogenes* and *B.cereus* harvested from an early-stationary-phase culture (10^8 cfu/ml) were incubated with 320 AU cerein 8A/ml for 60 min. After incubation, the microorganisms were separated and prepared for transmission electron microscopy. Both *L. monocytogenes* and *B. cereus* cells showed vesiculization of the protoplasm, pore formation, and complete disintegration of the cells (Fig. 5A–C).

Discussion

Cerein 8A, an antimicrobial peptide produced by *B. cereus* 8A showed inhibitory activity against major food pathogens and spoilage bacteria [4]. Our results suggest that this substance has a bactericidal effect against *L. monocytogenes* and *B. cereus*, based on the observed decline in the number of living cells of either *B. cereus* or *L. monocytogenes* within 2 h after the addition of cerein 8A. A bactericidal effect for this compound is also supported by the decrease in OD_{600}, which indicated that the cells of these indicator strains had lysed. However, the effect may depend on the specific assay conditions, such as the amount and purity of the bacteriocin, the indicator strain, and its cellular concentration [11]. Indeed, Boucabeille et al. [5] found linenscin OC2 to be bactericidal and bacteriolytic towards *L. innocua*, while at a low dose of linenscin OC2 bacteriostasis was observed. Similar results were reported for a bacteriocin produced by *Brevibacterium linens* ATCC 9175 [25].

Cerein 8A displayed similar kinetics against *B. cereus* and *L. monocytogenes*, killing the bacterial cells within 60 min. This bacteriocin, which inhibits several gram-positive bacteria, seems to be most active against *Bacillus* spp. [4]. Indeed, a higher dose was needed to kill *L. monocytogenes* than to kill *B. cereus*. This result agrees with reports that several bacteriocins are mostly active against closely related species [17,35]. Although bacteriocin cerein 8A appears not to kill *E. coli* and *Salmonella* Enteritidis cells, it greatly inhibited their growth. This inhibitory effect increased when the chelating agent EDTA was added, and was similar to the

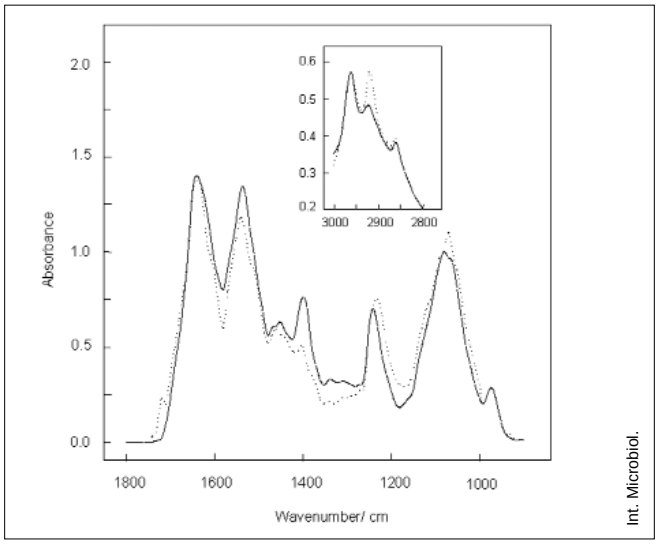

Fig. 4. Fourier transform infrared (FTIR) spectroscopy spectra of cell biomass of *Listeria monocytogenes*. Cell suspensions of *L. monocytogenes* were treated with 400 AU cerein 8A/ml for 60 min, then washed and dried onto a ZnSe optical plate. The infrared spectra of treated (dashed line) or untreated (solid line) biomass were recorded using the ATR method. Inset: detail of spectra in the range 3000–2800 cm^{-1}.

inhibitory effect reported for nisin [9]. In both cases, inhibition is most likely due to weakening of the outer plasma membrane following sequestration of magnesium ions by the chelating agents [24]. Antimicrobial activity has been associated with molecules frequently exported by bacteria, such as hemolysins or hydrolytic enzymes [19,31]. Some bacteriocins produced by bacteria isolated from food, such as linescin OC2, have hemolytic activity [5]. However, these activities are not associated with the antimicrobial activity.

FTIR spectroscopy of *B. cereus* and *L. monocytogenes* treated with cerein 8A revealed major changes in the assignments for symmetric C=O bonds (1400 cm^{-1}), C–H antisymmetric stretching of CH_2 bonds (2920 cm^{-1}) and for P=O antisymmetric bonds, in the region corresponding to membrane fatty acids [12,16,33]. By affecting the bacterial membrane, cerein 8A may cause dissipation of the proton-motive force as well as leakage of intracellular contents. FTIR has been used to identify microrganisms [20,21], but few attempts have been made to use this technique to investigate antimicrobial mechanisms. The interaction of surfactin [7] and gramicidin S [1] with membrane lipids has been demonstrated by FITR studies. Since cerein 8A does not possess phospholipase activity, it could instead be a pore-forming peptide. This mechanism is supported by electron microscopy, which showed cell lysis after treatment with cerein 8A. Cell damage caused by cerein 8A resembles that observed with a crude bacteriocin produced by *Lactobacillus salivarius* [27]. Type-A lantibiotics, to which nisin, pediocin, and epidermin, and many other amphiphilic antimicrobial peptides belong, exert their activity by disrupting the functional barrier of microbial cytoplasmic membranes [10,22]. In addition to nisin and epidermin, lipid I and II binding has been observed for subtilin and mesarcidin produced by *Bacillus* spp. [6,22]. The hydrophobic bacteriocin cerein 7, produced by *B. cereus* Bc7, has also been characterized as a membrane-active compound [29].

In conclusion, our results suggest that cerein 8A has a bactericidal effect apparently by disrupting the membrane function of target organisms.

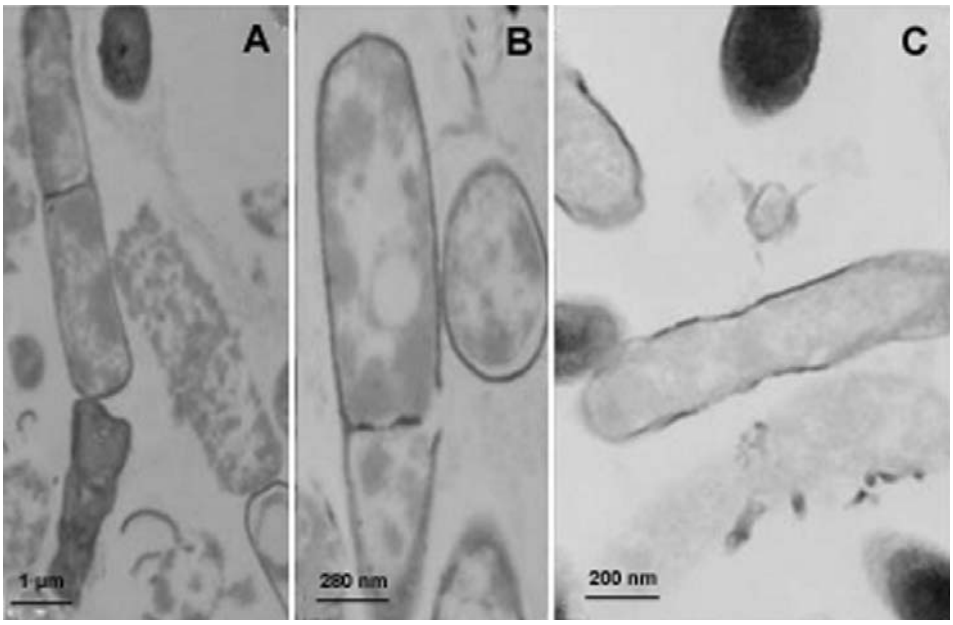

Fig. 5. Transmission electron microscopy of cells of *Bacillus cereus* (**A**) and *Listeria monocytogenes* (**B**, **C**) after treatment with cerein 8A.

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Actividad antibacteriana de la cereína 8A, un péptido de tipo bacteriocina producido por *Bacillus cereus*

Resumen. Se investigó el modo de acción de la cereína 8A, una bacteriocina producida por la bacteria del suelo *Bacillus cereus* 8A. El efecto de la cereína 8A fue probado contra *Listeria monocytogenes*, obteniendo un efecto bactericida a concentraciones de 400 unidades arbitrarias (AU)/ml. La cereína 8A

Atividade antibacteriana da cereina 8A, um peptídeo tipo bacteriocina produzido por *Bacillus cereus*

Resumo. O modo de ação da cereina 8A, uma bacteriocina produzida pela bactéria do solo *Bacillus cereus* 8A, foi investigado. O efeito da cereina 8A foi testado contra *Listeria monocytogenes*, resultando em um efeito bactericida com uma concentração de 400 unidades arbitrárias (UA)/ml. A cereina

también tuvo un efecto bactericida contra *Bacillus cereus* a una concentración de 200 AU/ml. La bacteriocina inhibió el crecimiento de *Escherichia coli* y *Salmonella* Enteritidis. Mayor inhibición contra estas bacterias gram-negativas se consiguió cuando a la bacteriocina se le añadió el agente quelante EDTA. El efecto de la cereína 8A sobre *B. cereus* y *L. monocytogenes* también fue investigado por espectroscopía de infrarrojos de transformación de Fourier (FTIR). Las células tratadas mostraron un importante crecimiento en frecuencia de 2920 cm^{-1} y un decrecimiento de 1400 cm^{-1} de banda, correspondiéndose con la asignación de los ácidos grasos. La microscopía electrónica de transmisión mostró que las células habían padecido daños en la pared celular, con pérdida de material protoplásmico. Los resultados sugieren que el modo de acción de la cereína 8A se produce mediante su intervención en las membranas celulares y en la pared celular. [**Int Microbiol** 2005; 8(2):125-131]

Palabras clave: *Bacillus cereus* · *Listeria monocytogenes* · péptidos antimicrobianos · bacteriocinas · espectroscopía de infrarrojos

8A também foi bactericida contra *Bacillus cereus* à 200 UA/ml. A bacteriocina inibiu o crescimento de *Escherichia coli* e *Salmonella* Enteritidis. Uma maior inibição contra estas bactérias gram-negativas foi alcançada quando o agente quelante EDTA foi adicionado junto com a bacteriocina. O efeito da cereina 8A sobre *B. cereus* e *L. monocytogenes* também foi investigado por espectroscopia de infravermelho com transformada de Fourier (FTIR). As células tratadas mostraram um importante aumento de frequência em 2920 cm^{-1} e uma diminuição na banda de 1400 cm^{-1}, correspondendo a designações de ácidos graxos. A microscopia eletrônica de transmissão mostrou que as células apresentaram danos na parede celular com perda de protoplasma. Estes resultados sugerem que o modo de ação da cereina 8A é interferindo nas membranas celulares e parede celular. [**Int Microbiol** 2005; 8(2):125-131]

Palavras chave: *Bacillus cereus* · *Listeria monocytogenes* · peptideo antimicrobiano · bacteriocinas · espectroscopia de infravermelho