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ORIGINAL PAPER

Antimicrobial compounds produced by *Lactobacillus sakei* subsp. *sakei* 2a, a bacteriocinogenic strain isolated from a Brazilian meat product

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Abstract Bacteriocins produced by lactic acid bacteria are gaining increased importance due to their activity against undesirable microorganisms in foods. In this study, a concentrated acid extract of a culture of *Lactobacillus sakei* subsp. *sakei* 2a, a bacteriocinogenic strain isolated from a Brazilian pork product, was purified by cation exchange and reversed-phase chromatographic methods. The amino acid sequences of the active antimicrobial compounds determined by Edman degradation were compared to known protein sequences using the BLAST-P software. Three different antimicrobial compounds were obtained, P1, P2 and P3, and mass spectrometry indicated molecular masses of 4.4, 6.8 and 9.5 kDa, respectively. P1 corresponds to classical sakacin P, P2 is identical to the 30S ribosomal protein S21 of *L. sakei* subsp. *sakei* 23 K, and P3 is identical to a histone-like DNA-binding protein HV produced by *L. sakei* subsp. *sakei* 23 K. Total genomic DNA was extracted and used as target DNA for PCR amplification of the genes *sak*, *lis* and *his* involved in the synthesis of P1, P2 and P3. The fragments were cloned in pET28b expression vector and the resulting plasmids transformed in *E. coli* KRX competent cells. The transformants were active against *Listeria monocytogenes*, indicating that the activity of the classical sakacin P produced by *L. sakei* 2a can be complemented by other antimicrobial proteins.

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Introduction

Lactic acid bacteria produce a variety of substances with antimicrobial activity, including antimicrobial peptides collectively known as bacteriocins [23], which are able to inhibit foodborne pathogens and spoilage bacteria [12, 14, 15, 20, 32]. Several classifications have already been proposed [12, 30, 31], but according to the most recent one [2, 23, 36], bacteriocins are grouped into four classes, based on their structure and function: class I: lantibiotic peptides, class II: small non-modified peptides with molecular mass <10 kDa, class III, large proteins with molecular mass >10 kDa. Bacteriocins of class II are subdivided into three subgroups: type IIa corresponds to pediocin-like bacteriocins, type IIb are multicomponent bacteriocins and type IIc are miscellaneous bacteriocins, a diverse group that includes sakacins Q, T and X.

Lactobacillus sakei subsp. *sakei* 2a is a bacteriocinogenic lactic acid bacterium isolated from a Brazilian pork sausage that is capable of inhibiting the growth and the pathology of *Listeria monocytogenes* in culture media, in food systems and in the murine gastrointestinal tract [4, 13, 33]. The bacteriocin produced by this strain forms pores in the membrane of target cells, dissipates the proton motive force in sensitive *L. monocytogenes* Scott A, reduces the intracellular ATP concentration with no detectable increase in extracellular ATP and mediates a concentration-dependent efflux of 5(6)-carboxyfluorescein from liposomes prepared from *L. monocytogenes* lipids [8, 35, 39, 42].

The purification protocol used by Rosa et al. [39] was based on salt extraction, cation exchange and reversed-phase chromatography. Many other strategies have been reported in the literature for the purification of antimicrobial peptides and proteins produced and secreted by bacteria [1, 3, 9, 21, 34, 40, 41, 44, 47]. Generally, the first step is a precipitation with ammonium sulphate [29] or an aqueous extraction of secreted compounds by medium acidification to release cationic compounds bound to the bacteria cell wall [27]. An obvious strategy for the enrichment of those cationic antimicrobial peptides and proteins is a fractionation by ion-exchange chromatography [5, 6, 11].

The fractionation of antimicrobial compounds by cation exchange and reversed-phase chromatography suggests that the *Lactobacillus sakei* subsp. *sakei* 2a strain is capable of producing more than one antimicrobial peptide. The purpose of this study was to purify and characterize these molecules, using Edman degradation and comparison to protein sequences deposited on the Basic Local Alignment Search Tool (BLAST-P) program.

Materials and methods

Bacterial cultures and media

The following strains were used (Table 1): For use, *L. sakei* 2a was grown at 25°C for 18 h in MRS broth (Difco, Detroit, MI), whereas *L. monocytogenes* and the other cultures were grown at 37°C for 24 h in BHI broth (Difco, Detroit, MI).

Assay for antibacterial activity

Antibacterial activity was assayed by the double layer diffusion test, according to Farias et al. [16]. Ten microliters of the testing material was spotted onto the surface of plates containing BHI agar (Difco). The plates were allowed to dry, over-layed with 5 ml semi-solid BHI agar containing 40 µl of a culture of the indicator

Table 1 Bacteriogenic and target strains used

Bacteriocinogenic strain
Lactobacillus sakei 2a
Target strains
Bacillus cereus ATCC 11778
Bifidobacterium bifidum (BB 12)
Enterobacter aerogenes ATCC 13048
Enterococcus canis 33
Enterococcus faecalis ATCC 19483
Enterococcus faecium 988
Enterococcus hirae 28
Escherichia coli ATCC 8739
Escherichia coli O157:H7 ATCC 35150
Lactobacillus acidophilus (LA 5)
Lactobacillus acidophilus (LAC 4)
Lactobacillus casei (BL 20)
Lactobacillus helveticus 1176
Lactobacillus sakei ATCC 15521
Lactobacillus paracasei (LBC 82)
Lactococcus lactis subsp. *lactis* 9
Listeria innocua Li7
Listeria monocytogenes ATCC 7644
Listeria monocytogenes Scott A
Listeria seeligeri
Salmonella enteriditis ATCC 13076
Salmonella typhimurium ATCC 14028
Staphylococcus aureus subsp. *aureus* ATCC 6541
Staphylococcus aureus subsp. *aureus* ATCC 29213
Staphylococcus epidermidis
Shigella sonnei
Pseudomonas aeruginosa 25723
Pseudomonas mirabilis

microorganism (10^8 CFU ml^{-1}) and incubated at 37°C for 24 h. The formation of an inhibition halo around the spotted material indicated a positive antagonistic result. The double layer diffusion test was used to determine bacteriocin activity at each step of the purification. Bacteriocin activity was determined using titers of a twofold dilution. A unit of bacteriocin activity was defined as the reciprocal of the highest dilution having a detectable zone of inhibition and expressed as AU ml^{-1}.

Extraction of the antimicrobial compounds adsorbed to the *L. sakei* 2a cells

Eight liters of MRS broth was inoculated with 1% (v/v) of an overnight *L. sakei* 2a culture in MRS broth at 25°C, and *L. sakei* 2a was grown to early stationary phase (about 5×10^8 CFU ml^{-1}). Then, pH was adjusted to 6.0 and the medium heated to 70°C for 30 min before cell harvesting by centrifugation at 10,000*g* for 15 min. Supernatant activity was assayed for bacteriocin not adsorbed onto the cells. After washing with 5 mmol l^{-1} 2-(*N*-morpholin)-ethanesulphonate (MES) buffer, pH 6.5, the cells were suspended in 400 ml of 100 mmol l^{-1} NaCl, pH 1.5 (adjusted with 5% phosphoric acid), and mixed with a magnetic stirrer for 1 h at 4°C [49]. Cell suspensions were centrifuged at 10,000*g* for 20 min, and the supernatant was concentrated ten times by ultra-filtration in an Amicon System (Millipore Ind. Com. Ltda, SP, Brazil) with a 1.000-molecular-weight-cutoff membrane and then freeze-dried. The protein concentration in the product was determined by the Bradford method [7].

Purification of the antimicrobial compounds

The acid extract from *L. sakei* 2a was fractionated by cation exchange chromatography using a MONO-S (HR5/5-Amersham) column coupled to a FPLC system (Pharmacia, GMI, Ramsey, MN). The mobile phases were the following: (1) 50 mmol l^{-1} MES buffer, pH 6.5, and (2) 50 mmol l^{-1} MES buffer, pH 6.5, plus 1.0 mmol l^{-1} of NaCl. Proteins were eluted at 1.0 ml min^{-1} by a linear NaCl gradient, from 0 to 1 mol l^{-1} in 75 min with detection at 280 nm. Fractions presenting antimicrobial activity against *L. monocytogenes* Scott A and *Enterococcus faecalis* ATCC 19483 were separately injected in a C_{18}-reversed-phase column (Shim-Pack ODS, 250 × 4.6 mm, 5 µm) coupled to a HPLC system (Shimadzu, Kyoto, Japan). Mobile phases used were as follows: (1) aqueous solution of trifluoroacetic acid (TFA) at 0.1% (by volume) and (2) aqueous solution of acetonitrile at 80% (by volume) containing 0.1% TFA (by volume). The compounds were eluted by a linear gradient at 1.0 ml min^{-1}, increasing phase B from 0 to 100% during 30 min. The eluted compounds presenting absorbance at 280 nm were assayed against *L. monocytogenes* Scott A and *E. faecalis* ATCC 19483, and those presenting activity were submitted to characterization by N-terminal protein sequencing and mass spectrometry.

Protein sequencing

The amino acid sequences of the active antimicrobial compounds were determined by Edman degradation in a automated protein sequencer (Shimadzu PPSQ-21A, Shimadzu, Kyoto, Japan) coupled to a reversed-phase separation of PTH-amino acids in a WAKOSIL-PTH (4.6 × 250 mm) column (Wako, Osaka, Japan) at 1.0 ml min^{-1}, with detection at 235 nm. The obtained sequences were compared to known protein sequences using the BLAST-P software.

Mass spectrometry

The average molecular mass of the antimicrobial compounds was determined by electrospray ionization mass spectrometry (ESI-Q-TOF Micro®, Micromass, UK) in the positive ion mode. Mass spectrometer calibrations were made using sodium iodide in the 100–2,500 m/z range. The analytes were solubilized in 100 µl of 50% (v/v) acetonitrile in aqueous 0.2% (v/v) formic acid at a final concentration ranging from 10 to 30 µmol l^{-1}, and applied to the mass spectrometer by a syringe pump system at a flow rate of 10 µl min^{-1}. The capillary and the cone voltages were 2.5 kV and 40 V, respectively. The final spectrum was the result of 20 combined scans. Original data (m/z) were treated (base line subtraction, smoothing and centring), and mass spectrum data were analyzed by the Masslynx® 4.0 software. The multiply-charge distribution spectra were converted to singly charged spectra by using the computer algorithm "Transform included in pack of analysis of Q-tof Micromass."

Cloning of genes involved in the synthesis of P1, P2 and P3 in *E. coli*

Total genomic DNA was extracted from *L. sakei* 2a using a Wizard® Genomic DNA Purification System (Promega, Madison, WI) and used as target DNA for PCR amplification of the genes (*sak*, *lis* and *his*) involved in the synthesis of three antimicrobial compounds (P1, P2 and P3) selected among those produced by the *L. sakei* 2a strain, after their purification and sequencing. For PCR amplification of *sak* (P1), *lis* (P2) and *his* (P3) genes, the following primers were used: (5′-CCATGGATGAAATATTATGGTA ACGGTGAG-3′ and 5′-GGATCCTTATTTATTCCAGCC AGCGTTTC-3′), (5′-CCATGGGCAAGACAGTCGTTCG-3′

and 5′-GGATCCTTAGAATTTCTTACGTTTTCTTGC-3′) and (5′-CCATGGCAAACAAAGCACAATTG-3′ and 5′-GG ATCCTTATTAACAGAATCCTTTAAAG-3′), respectively. The fragments of *sak*, *lis* and *his* genes were cloned in pET28b expression vector (Novagen/EMD/Merck, USA) according to the procedure recommended by the manufacturer, and the resulting plasmids (pET:sak, pET:lis and pET:his), using *Nco*I and *Bam*HI sites, transformed in *E. coli* KRX competent cells (Promega, USA) [22]. Transformants were selected on Luria-Bertani (LB) plates with neomycin 50 μg ml^{-1} at 37°C for 24 h. The selection and identification of colonies containing plasmids *sak*, *lis* and *his* with inserts in the correct orientation were performed by PCR amplification of DNA from single *E. coli* colonies using primers sak, lis and his and T7 Terminator (5′-GCTAGTTATTGCTCAGCGG-3′). Transformed *E. coli* KRX was grown on LB medium, supplemented with neomycin 50 μg ml^{-1}, and incubated at 37°C. Induction of the T7 promoter of pET28b (Novagen) was carried out as follows: an overnight culture was diluted 1:200 into fresh medium and incubated at 37°C until the optical density at 600 nm reached 0.6. The culture was then induced by adding 0.1% of rhamnose. A non-induced culture was used as control. Cultures were incubated at 25°C overnight and the cells harvested by centrifugation at 6,000*g* for 10 min. Cells were broken by the Mini Bead-Beater-8 (BioSpec Products, Inc., Bartlesville, OK) in PBS buffer, and broken cells were centrifuged at 6,000*g* for 10 min. Supernatants were evaluated for antimicrobial activity using *L. monocytogenes* Scott A as indicator strain, using the double-layer diffusion assay [16]. The molecular mass of the cloned peptides was confirmed by mass spectrometry, as described previously.

Heat treatment and effect of enzymes

To characterize the antimicrobial compounds, the producer strain was cultivated in MRS broth for 18 h at 37°C. Cells were removed by centrifugation (13,000*g*, 10 min, 4°C), and the cell-free culture supernatant fluid was considered to be crude antimicrobial compounds. First, the influence of the proteases trypsin, proteinase K and pronase on antilisterial activity was tested. All enzymes were dissolved in 3 mmol l^{-1} potassium phosphate buffer (pH 7·5) and added to the cell-free culture supernatant fluid sample. After incubation for 24 h at 37°C, the inhibitory activity was evaluated. The heat sensitivity was tested. The cell-free culture supernatant fluid was heated at 60°C for 15, 30, 45 and 60 min, at 98°C for 15, 30 and 45 min, and at 121°C for 15 min, before testing the activity. To test the influence of pH, the solution of the antimicrobial compounds was adjusted to a pH of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 or 10.0 with HCl or NaOH, mixed and allowed to stand for a few minutes before testing the inhibitory activity. The activity was checked as described above.

Measurements of proton motive force and pH gradient

The membrane potential ($\Delta\Psi$) of *L. monocytogenes* Scott A cells was qualitatively measured with the fluorescent probe 3,39-dipropylthiadicarbocyanine iodide [(DiSC3(5)] (Molecular Probes Inc, Eugene, OR). Cells were harvested in the log phase (optical density at 660 nm), washed twice with ice-cold 50 mmol l^{-1} potassium HEPES (K-HEPES) buffer, pH 7.0, resuspended in the same buffer to 1/100 of their initial volume and stored on ice. Glucose-energized *L. monocytogenes* Scott A cells (final OD660, 0.6) were added to a stirred cuvette containing 2 ml of the K-HEPES buffer and 10 μl DiSC3(5) (5 mmol l^{-1}). Next, 2 μl nigericin (1.5 nmol l^{-1}), which dissipates the pH gradient (ΔpH), and purified antimicrobial compound (10 nmol l^{-1}, 100 nmol l^{-1} e 1 μmol l^{-1}) were added.

Fluorescence measurements were performed with a Cary Eclipse spectrofluorometer (Varian, CA) with a band-pass width of 5 nm and wavelengths of 643 and 666 nm for excitation and emission, respectively. The transmembrane ΔpH was measured by loading *L. monocytogenes* Scott A cells (OD660, 0.6) with the fluorescent probe 2′-7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF AM) (Molecular Probes Inc.) by using an acid shock. Glucose-energized, BCECF-loaded cells (final OD660, 0.6) were added to a stirred cuvette containing 2 ml of 50 mmol l^{-1} KPi buffer, pH 6.0. Next, 2 μl valinomycin (1.5 nmol l^{-1}), which dissipates the $\Delta\Psi$, and purified antimicrobial compound (10 nmol l^{-1}, 100 nmol l^{-1} e 1 μmol l^{-1}) or 2 μl nigericin (1.5 nmol l^{-1}) were added. Fluorescence was measured with band-pass widths of 5.0 and 15.0 nm and wavelengths of 500 and 525 nm for excitation and emission, respectively.

Results and discussion

Bacteriocins are secreted in the culture medium, but due the cationic characteristics and high isoelectric points, large amounts remain bound to the producing bacteria cell wall. Harsh treatment of the cells, such as heating for 30 min at 70°C and then exposure to pH 1.5 for 60 min, are strategies to release the proteins bound to the cell wall without causing extensive leakage from the cells [17, 39]. In the present study, the same active compounds were detected in the supernatant and in the acid-treated material. In addition, the crude acid extract did not contain typical intracellular proteins, confirming that the cell leakage was minimal or even absent.

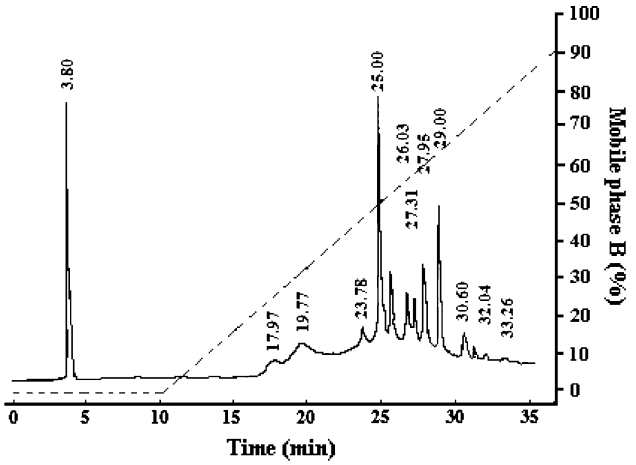

Fig. 1 Reversed-phase HPLC profile of active fractions obtained by cation-exchange fractionation of the acid extract of *L. sakei* 2a. Separation was conducted in a Shim-Pack ODS column (250 × 4.6 mm, 5 μm) at room temperature. Mobile phases A and B were 0.1% aqueous trifluoroacetic acid (TFA) and 80% aqueous acetonitrile containing 0.1% TFA, respectively; flow rate was 1.0 ml min^{-1}, and detection was conducted at 280 nm. The *dashed line* indicates the variation of percentage of mobile phase B

Application of the acid extract of *L. sakei* 2a culture onto a cation-exchange Mono-S column resulted in an active fraction in the range from 0.35 to 0.55 mol l^{-1}NaCl (Fig. 1). As shown in Table 2, the procedure resulted in a satisfactory purification factor (55.2). It is important to point out that this active fraction is still a mixture of different cationic antimicrobial peptides and proteins. The application of the pooled fractions onto a C_{18}-reversed-phase C18 column separated several substances active against *L. monocytogenes* Scott A and *E. faecalis* ATCC 19483, as well as against some other target strains (Table 3). Protein fractions exhibiting antibacterial activity were pooled and re-fractionated using C_{18}-reversed-phase chromatography, until purity levels higher than 95% were achieved. The protein identities were confirmed by mass spectrometry and Edman degradation. Three proteins (P1, P2 and P3) were consistently present in all repetitions of the purification experiments. It was not possible to calculate the purification factor for these isolated proteins and peptide due to the reduced amount of material obtained, which was employed for antimicrobial assays and protein identification. The ESI Q-TOF mass spectra and the profiles of re-fractioned proteins in C_{18}RPC-HPLC for the P1, P2 and P3 proteins are shown in Fig. 2. Table 4 shows their N-terminal amino acid sequences, molecular masses measured by ESI-MS and predicted isoelectric points.

Compound P1 presented the *N*-terminal sequence **KYYGNGVHXGKHSXTV**, containing the consensus region YGNGV, considered a signature of class IIa bacteriocins [15], and also described in bacteriocin Sakacin P [26]. This result confirms the previous report of Rosa et al. [39] for the bacteriocin produced by *L. sakei* 2a. Sakacin P produces pores on bacterial cell membranes probably by insertion of its N-terminal region into the bilayer and interaction of the C-terminal domain with specific receptors, causing leakage of some cellular components, but without lysis since the pore formation is transient. Investigations using tryptophan fluorescence spectroscopy on the interaction of Sakacin P analogues with liposomes suggest that this bacteriocin is very selective for bilayers containing anionic phospholipids that are typical of bacterial membranes [17].

The sequence **GKTVVRSNESLDDALRRFKRSVSK AGTIQEYRKR**, obtained by Edman degradation for compound P2, is identical to the 30S ribosomal protein S21 of *L. monocytogenes* 1/2a F6854, *E. faecalis* v583 and of *L. sakei* subsp. *sakei* 23 K [10]. This similarity suggests that this protein may be active against *L. monocytogenes* and *E. faecalis*, probably by interfering with ribosome assembly and function, thus hampering protein synthesis and thus leading to the cell death. Other ribosomal proteins were also identified by Edman degradation and mass spectrometry (omitted data), but their antimicrobial activity could be assayed due to the low amount. Nevertheless, this result indicates that *L. sakei* 2a secretes many ribosomal proteins.

The N-terminal sequence determined for compound P3 was NKAQLIENVASKTGLTKKDATAAVDAVFGSIQ DTLKQGDKVQLIXFGTF, identical to a DNA-binding HV histone produced by *L. sakei* subsp. *sakei* 23 K [10]. Like P2, this component may also interfere in the DNA structure and replication of *L. monocytogenes* and *E. faecalis* targets, causing cell death.

The successful cloning and expression of the genes for sakacin (P1), 30S ribosomal protein (P2) and histone (P3) in transformed *E. coli* KRX confirmed that each one of these compounds presented antilisterial activity in addition

Table 2 Partial purification of antimicrobial compounds produced by *L. sakei* subsp. *sakei* 2a in MRS broth

Sample	Volume (ml)	Activity (UA/ml)	Total activity (UA)	Protein (mg/ml)	Total protein (mg)	Specific activity (UA/mg)	Purification factor	Yield (%)
Supernatant (broth)	3,000	200	6.0×10^5	0.29	870	690	1	100
Crude extract obtained by H_3PO_4 treatment (pH 1.5)	200	100	2.0×10^4	0.050	10	2,000	2.90	3.33
Mono-S cation-exchange chromatography	24	800	1.9×10^4	0.021	0.504	38,000	55.2	3.2

Table 3 Antibacterial spectrum of P1, P2 and P3 proteins produced by *L. sakei* subsp. *sakei* 2a, assayed by the spot on the lawn test

Target strains	P1	P2	P3
Bacillus cereus ATCC 11778	−	−	−
Bifidobacterium bifidum (BB 12)	−	−	−
Enterobacter aerogenes ATCC 13048	−	−	−
Enterococcus canis 33	+	−	−
Enterococcus faecalis ATCC 19483	+	+	+
Enterococcus faecium 988	+	−	−
Enterococcus hirae 28	+	−	−
Escherichia coli ATCC 8739	−	−	−
Escherichia coli O157:H7 ATCC 35150	−	−	−
Lactobacillus acidophilus (LA 5)	−	−	−
Lactobacillus acidophilus (LAC 4)	−	−	−
Lactobacillus casei (BL 20)	−	−	−
Lactobacillus helveticus 1176	−	−	−
Lactobacillus sakei ATCC 15521	+	+	+
Lactobacillus paracasei (LBC 82)	−	−	−
Lactococcus lactis subsp. *lactis* 9	−	−	−
Listeria innocua Li7	+	+	+
Listeria monocytogenes ATCC 7644	−	−	−
Listeria monocytogenes Scott A	+	+	+
Listeria seeligeri	+	+	+
Salmonella enteriditis ATCC 13076	−	−	−
Salmonella typhimurium ATCC 14028	−	−	−
Staphylococcus aureus subsp. *aureus* ATCC 6541	−	−	−
Staphylococcus aureus subsp. *aureus* ATCC 29213	−	−	−
Staphylococcus epidermidis	+	+	+
Shigella sonnei	−	−	−
Pseudomonas aeruginosa 25723	−	−	−
Pseudomonas mirabilis	−	−	−

+, antimicrobial activity; −, absence of antimicrobial activity

to their own primary function in the cases of the ribosomal protein and the histone.

All these anti-*Listeria* peptides present isoelectric points higher than 9.0 and typically around 10.0. A high affinity for the anionic surfaces of the bacteria cell wall and phosphate group of nucleic acids is thus expected. The secretion pathway is not well understood, but it is well known that polycationic peptides, especially those rich in arginine residues, can cross lipid bilayers without causing leakage of cell components [19, 38, 45, 46]. Other researchers suggest that cationic peptides and proteins cross the cell membranes through an endocytosis process [18, 28]. A prototype for a cell-penetrating antimicrobial peptide is Buforin II, a histone fragment isolated from the gastric tissue of the Asian toad (*Bufo bufo garagrizans*). Buforin II is capable of crossing lipid bilayers with minimal perturbation, but causes cell death by intracellular effects because of its strong affinity for nucleic acids [37]. Concerning the ribosomal proteins, it can be hypothesized that if a given bacteria secretes part of their synthesized ribosomal proteins, it can interfere with ribosomal assembly of closely related bacteria. Wool [48] cited various non-lethal functions for ribosomal proteins, and the present study suggests that ribosomal peptides secreted by *L. sakei* 2a can be viewed as new examples of cell-penetrating antimicrobial proteins [48].

Preliminary characterization of the antagonistic activity confirmed the proteinaceous nature of the antimicrobial compounds secreted by *L. sakei* 2a. These compounds were sensitive to proteolytic enzymes, heat stable (maintaining the biological activity after heating at 60, 98 and 121°C for 15 min) and active in a broad range of pH (1.5 up to 10.0) (Lima et al. submitted). Their antibacterial spectra of the peptides and proteins are similar (Table 3), and probiotic cultures and gram-negative strains were resistant to all these compounds. Despite presenting antilisterial activity, preliminary results indicate that the mechanism of action of these three peptides is not the same. Tests with the fluorescence probes DiSC3(5) and BCECF indicated that the three compounds caused dissipation of membrane potential ($\Delta\Psi$) in *L. monocytogenes* Scott A (Fig. 3), but only P1 caused dissipation of ΔpH (Fig. 4). The P2 and P3 proteins do not disrupt the pH gradient, but have a significant impact on the membrane potential of this sensitive strain. This is consistent with the idea that the ribosomal protein and the histone do not cause extensive membrane disruption to affect the ATP synthase function. Other experiments indicated that the three antimicrobial compounds caused more dissipation of $\Delta\Psi$ and ΔpH than nisin (1 mmol l^{-1}) and enterocin CRL35 (1 μmol l^{-1}). Modification of $\Delta\Psi$ has been described already with other class IIa bacteriocins like enterocin P [24, 25] or piscicocin CS526 [43].

Recently, Vera Pingitore et al. [47] analyzed the generation of a ΔpH in *E. faecalis* MP97 cells by determining changes in intracellular pH with the pH-sensitive fluorescent probe cFDASE. Modifications in the fluorescence intensity of this reagent by extrusion or loss of intracellular cFDASE indicated depletion of membrane ΔpH. The fluorescence intensity of the probe was increased upon addition of Salα and Salβ (different bacteriocins), indicating depletion of membrane ΔpH of the sensitive strain. The capacity of Salα and Salβ to dissipate $\Delta\psi$ in the *E. faecalis* MP97-sensitive strain was determined using the fluorescence intensity of the cyanine dye DiSC3(5). In cells energized with glucose, rapid quenching of fluorescence was detected upon addition of the dye, showing the generation of $\Delta\psi$. After the addition of Salα and Salβ, an increase in the fluorescence intensity of DiSC3(5) was observed, indicating that the $\Delta\psi$ of *E. faecalis* MP97 was dissipated. Salα and Salβ dissipated the $\Delta\psi$ of the sensitive

Table 4 Molecular mass, N-terminal sequence and predicted isoelectric point of the antimicrobial compounds P1, P2 and P3 produced by *Lactobacillus sakei* subsp. *sakei* 2a purified by reversed-phase chromatography

Compound		Homologous proteins found in the Swiss-Prot databank			
N-terminal amino acid sequence	Experimental molecular mass	N-terminal amino acid sequence	Identification[a]	Molecular mass	Predicted isoelectric point
P1 KYYGNGVHXGKHSXTV	4,432.7	**KYYGNGVHCGKHSCT**VDWGTAI GNIGNNAAANWATGGNAGWNK	Sakacin P	4,437.8	8.8
P2 GKTVVRSNESLDDALRRF KRSVSKAGTIQE YRKR	6,789.2	**GKTVVRSNESLDDALRRFKRSVSK AGTIQEYRKR**EFYEKPSVKRKLK SEAARKRKKF	Identical to 30S ribosomal protein S21 of *Lactobacillus sakei* subsp. *sakei* 23 K.	6,789.9	10.9
			Homologous to 30S ribosomal protein S21 *Listeria monocytogenes* 1/2a F6854 and *E. faecalis* v583		
P3 ANKAQLIENVASKTGLTKKDATAAVDA VFGSIQDTLKQGD KVQLI	9,521.5	**ANKAQLIENVASKTGLTKKDATAAVDAV FGSIQDTLKQGDKVQLI**GFGTFEV RERAARKGRNPQTGAEIKIPASKVPAFKPGKALKDSVK	Histone-like DNA-binding protein HU of *Lactobacillus sakei* subsp. *sakei* 23 K	9,523.1	10.1

Calculated molecular masses are average values, as calculated from the Protein Prospector website (http://prospector.ucsf.edu) by using the MS-product search protocol

The isoelectric point was predicted by using the Protein Prospector website (http://prospector.ucsf.edu)

[a] Based on BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)

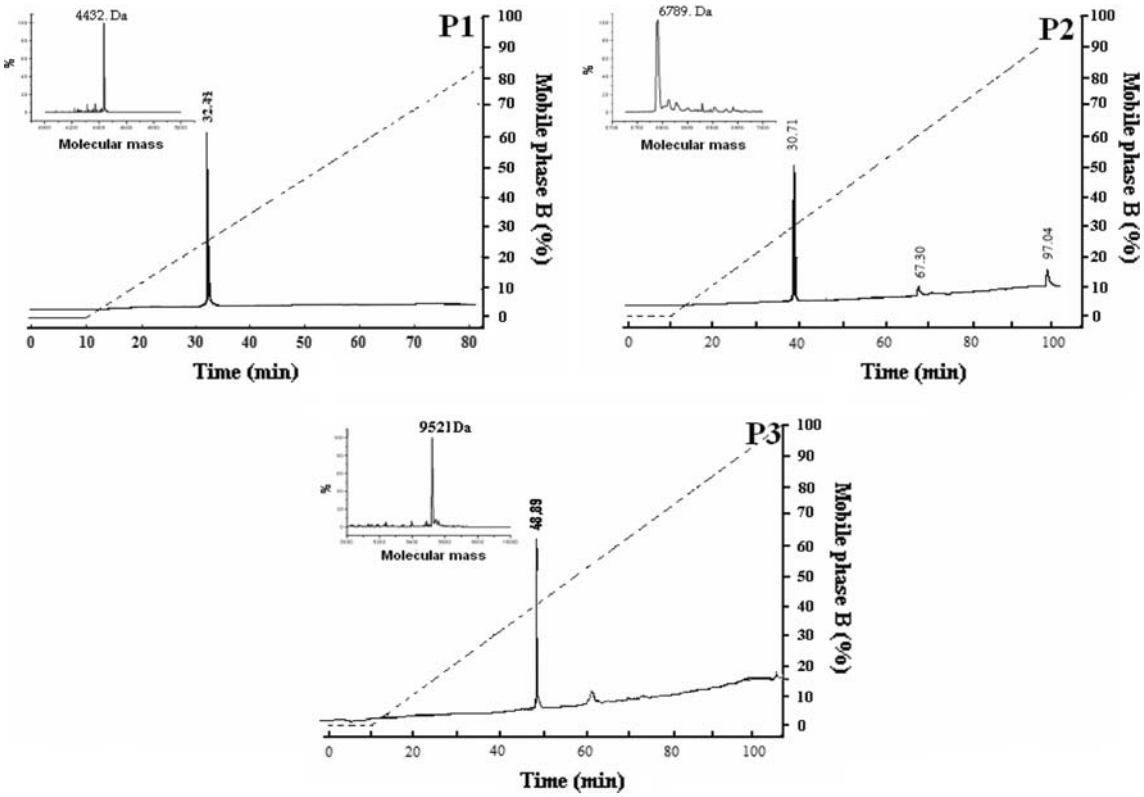

Fig. 2 Reversed-phase chromatographic profile of the purified bacteriocin (P1), ribosomal protein (P2) and histone (P3). Separation was conducted in a Shim-Pack ODS column (250 × 4.6 mm, 5 μm) at room temperature. Mobile phases A and B were 0.1% aqueous trifluoroacetic acid (TFA) and 80% aqueous acetonitrile containing 0.1% TFA, respectively; the flow rate was 1.0 ml min^{-1}, and detection was conducted at 280 nm. The *dashed line* indicates the variation of percentage of mobile phase B. The *inserts* show the deconvoluted ESI Q-TOF (Micromass, Manchester, UK) mass spectra and the calculated molecular masses

strain more gradually than that obtained with the K^+ ionophore valinomycin at 1 μM.

This is the first report showing that the activity of the classical sakacin P produced by *L. sakei* 2a can be complemented by other antimicrobial proteins with unexpected and distinct antibacterial mechanisms. This diversity of antagonistic compounds probably results in different

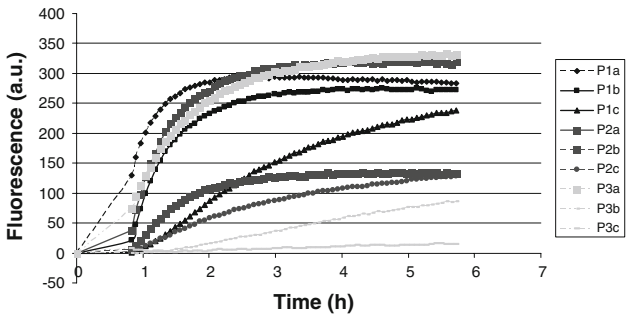

Fig. 3 Effect of the purified P1, P2 and P3 antimicrobial compounds produced by *L .sakei* 2a on the ΔΨ of *L. monocytogenes* Scott A cells. Fluorescence levels before the addition of purified P1, P2 and P3 antimicrobial compounds were arbitrarily designated zero, and the increase in fluorescence upon the addition of bacteriocins was expressed in arbitrary units (a.u.), where: *a* (1 μmol l^{-1}), *b* (100 nmol l^{-1}) and *c* (10 nmol l^{-1})

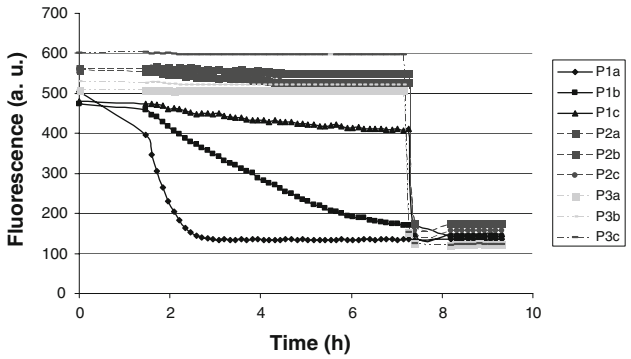

Fig. 4 Effect of the purified P1, P2 and P3 antimicrobial compounds produced by *L. sakei* 2a on the ΔpH of *L. monocytogenes* Scott A cells. Fluorescence levels before the addition of purified P1, P2 and P3 antimicrobial compounds were arbitrarily designated zero, and the increase in fluorescence upon the addition of bacteriocins was expressed in arbitrary units (a.u.). After 7 min, nigericin (1.5 nmol l^{-1}) was added, where: *a* (1 μmol l^{-1}), *b* (100 nmol l^{-1}) and *c* (10 nmol l^{-1})

mechanisms acting simultaneously, which makes the development of bacterial resistance more difficult.

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