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ORIGINAL PAPER

Characterization of multiple antilisterial peptides produced by sakacin P-producing *Lactobacillus sakei* subsp. *sakei* 2a

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Abstract

Antimicrobial compounds produced by lactic acid bacteria can be explored as natural food biopreservatives. In a previous report, the main antimicrobial compounds produced by the Brazilian meat isolate *Lactobacillus sakei subsp. sakei* 2a, i.e., bacteriocin sakacin P and two ribosomal peptides (P2 and P3) active against *Listeria monocytogenes*, were described. In this study, we report the spectrum of activity, molecular mass, structural identity and mechanism of action of additional six antilisterial peptides produced by *Lb. sakei* 2a, detected in a 24 h-culture in MRS broth submitted to acid treatment (pH 1.5) and proper fractionation and purification steps for obtention of free and cell-bound proteins. The six peptides presented similarity to different ribosomal proteins of *Lb. sakei* subsp *sakei* 23K and the molecular masses varied from 4.6 to 11.0 kDa. All peptides were capable to increase the efflux of ATP and decrease the membrane potential in *Listeria monocytogenes*. The activity of a pool of the obtained antilisterial compounds [enriched active fraction (EAF)] against *Listeria monocytogenes* in a food model (meat gravy) during refrigerated storage (4 °C) for 10 days was also tested and results indicated that the populations of *L. monocytogenes* in the food model containing the acid extract remained lower than those at time 0-day, evidencing that the acid extract of a culture of *Lb. sakei* 2a is a good technological alternative for the control of growth of *L. monocytogenes* in foods.

Keywords *Lactobacillus sakei* subsp. *sakei* 2a · *Listeria monocytogenes* · Multiple antimicrobial peptides · Meat gravy · Biopreservation

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Chemical compounds studied in this article: sodium chloride (PubChem CID: 5234), phosphoric acid (PubChem CID: 1004), trifluoroacetic acid (PubChem CID: 45039676), acetonitrile (PubChem CID: 6342), hepes potassium Salt (PubChem CID: 23702134), glucose (PubChem CID: 82400), nisin (PubChem CID: 16130280), valinomycin (PubChem CID: 5649), potassium chloride (PubChem CID: 4873).

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Introduction

Lactobacillus sakei subsp. *sakei* 2a is a lactic acid bacteria isolated from a Brazilian pork product, capable to inhibit the growth of *Listeria monocytogenes* in vitro and in meat products by the production of bacteriocin(s) (De Martinis

and Franco 1998). The mechanism of action of the bacteriocin produced by this strain was described in the early 2000s (Rosa et al. 2002) and a study conducted afterwards has shown that the main bacteriocin produced by this strain is sakacin P (peptide P1), along with two additional antilisterial peptides (P2 and P3) (Carvalho et al. 2010). Cation-exchange and reversed-phase chromatographic purification, followed by mass spectrometry and determination of amino acid sequences, indicated that P2 and P3 are identical to the 30S ribosomal protein S21 and to a histone-like DNA-binding protein HV of *Lb. sakei* subsp *sakei* 23K, respectively. Sakacin P and the peptides P2 and P3 were capable of affecting the membrane electrochemical potential of *L. monocytogenes*. Genes *sak, lis* and *his* in the DNA of *Lb. sakei* 2a, involved in the production of sakacin P and peptides P2 and P3, respectively, were cloned in pET28B expression vector and the resulting plasmids were transformed in *Escherichia coli* KRX competent cells. The resulting transformants effectively inhibited several *L. monocytogenes* strains (Carvalho et al. 2010).

Control of *L. monocytogenes* in foods and the food processing environment is of great relevance due to the occurrence of numerous food-borne listeriosis cases and outbreaks, affecting pregnant women, the sick and elderly, neonates, infants and immuno-compromised patients. Control of growth of *L. monocytogenes* in foods and the food industry environment is challenging because *L. monocytogenes* presents high physiological resistance to acidic pH, low temperature and high salt concentration, and capability to form persistent biofilms on the surfaces of equipment and utensils in food processing plants. *L. monocytogenes* can be particularly threatening in ready-to-eat foods, where there is no kill step to eliminate or reduce the pathogen before consumption (Buchanan et al. 2017).

Some studies have reported that some lactic acid bacteria strains are capable of production of multiple bacteriocins. Sawa et al. (2013) have shown that *Lactobacillus sakei* D98, isolated from rice malt, produced at least three class IIa-like or class IId bacteriocins (sakacins D98a, D98b and D98c), with different mode of action and differences in the amino acids sequences and position of the disulfite bridge compared to other class IIa bacteriocins. Also, Ustyugova et al. (2012) reported that *Lactococcus lactis* subsp *lactis* 194-K, isolated from cow milk in Buryatia, Russia, produces nisin A and another polypeptide (194-D) capable to suppress growth of Gram-positive and Gram-negative bacteria. *Lactococcus lactis* LMG2081, isolated from the European pear, produces two bacteriocins: the lantibiotic lacticin LMG (class I bacteriocin) and lactococcin G (Class IIb bacteriocin) (Mirkovic et al. 2016). Another raw milk isolate (*Enterococcus faecium* GGN7) obtained in Tunisia produced both enterocins A and B, plus a 3215.5 peptide active against Gram-negative bacteria (Gaaloul et al. 2014). *Weissella helenica* QU13, isolated from pickles, produces weissellicins Y and M, depending on the nutrition conditions (Masuda et al. 2015). The Brazilian meat isolate *Lb. sakei* 2a produces sakacin P and several antilisterial compounds, two of them (peptides P2 and P3) described in Carvalho et al. (2010). In this study, we report the properties (spectrum of activity, molecular mass, structural identity and mechanism of action) of other six antilisterial compounds produced by this strain, obtained after submitting a 24-h culture in MRS broth to proper purification steps for obtention of free and cell-bound peptides. The activity of these antilisterial compounds against *Listeria monocytogenes* in a food model (meat gravy) during refrigerated storage (4 °C) for 10 days was also tested.

Material and methods

Bacterial cultures and media

The bacteriocinogenic strain used in the study was *L. sakei* subsp. *sakei* 2a, isolated from Brazilian porcine sausage (De Martinis and Franco 1998). The target strains used in the study are listed in Table 1. *Lactobacillus* sp., *Bifidobacterium bifidum* Bb12, *Enterococcus* sp., *Lactococcus lactis* and *Lb. sakei* 2a were grown in MRS broth (Difco, Detroit, MI, USA) at 25 °C for 18 h, and the other bacteria were grown in BHI (Difco) at 37 °C for 24 h. Antimicrobial activity of *Lb. sakei* 2a was confirmed by the double-layer diffusion test, according to Farias et al. (1994), using *Listeria monocytogenes* Scott A as indicator. Results were expressed as Arbitrary Units per milliliter ($AU{\cdot}mL^{-1}$), which corresponded to the highest dilution that resulted in an inhibition halo against *L. monocytogenes*. Nisin (1000 $UI{\cdot}mL^{-1}$, Nisaplin, Danisco, Denmark) and distilled water were used as positive and negative controls, respectively.

Obtention of the free and cell-bound antimicrobial compounds produced by *Lb. sakei* 2a

Eight liters of a culture of *Lb. sakei* 2a, in MRS broth incubated at 25 °C to reach the early stationary phase (c.a. 5×10^8 $CFU{\cdot}mL^{-1}$), were submitted to pH adjustment at 6.0 and heated at 70 °C for 30 min. Cells were harvested by centrifugation at 10,000*g* for 15 min and the supernatant assayed for bacteriocin activity (free antimicrobials).

For extraction of the antimicrobial compounds bound to the *Lb. sakei* 2a cells, the resulting pellet was washed with 5 $mmol{\cdot}L^{-1}$ 2-(*N*-morpholin)-ethanesulphonate (MES) buffer, pH 6.5, and suspended in 400 mL of 100 $mmol{\cdot}L^{-1}$ NaCl, pH 1.5, adjusted with an aqueous solution of 5% phosphoric acid. The suspension was homogenized for 1 h at 4 °C by magnetic stirring and centrifuged at 10,000*g* for 20 min (Yang et al. 1992). The pH of the acid extract was adjusted to

Table 1 Antimicrobial activity ($AU \cdot mL^{-1}$) of the six antimicrobial compounds ($1000\ nmol \cdot L^{-1}$) produced by *Lactobacillus sakei* subsp. *sakei* 2a

Target strain	Antimicrobial compounds					
	P4	P5	P6	P7	P8	P9
Bacillus cereus ATCC 11778	–[a]	–	–	–	–	–
Bacteroides sp. ATCC 49056	–	–	–	–	–	–
Bifidobacterium bifidum BB 12	–	–	–	–	–	–
Enterobacter aerogenes ATCC 13048	–	–	–	–	–	–
Enterococcus canis 33	120	90	–	–	100	–
Enterococcus faecalis ATCC 19483	120	90	140	120	100	140
Enterococcus faecium 988	120	90	–	–	–	–
Enterococcus hirae 28	120	90	–	120	–	–
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	120	90	140	120	100	140
Lactobacillus paracasei LBC 82	–	–	–	–	–	–
Lactococcus lactis subsp. *lactis* 9	–	–	–	–	–	–
Listeria innocua Li7	120	90	140	120	100	140
Listeria monocytogenes ATCC 7644	120	90	140	120	100	140
Listeria monocytogenes Scott A	120	90	140	120	100	140
Listeria seeligeri ATCC 35967	120	90	140	120	100	140
Salmonella enteriditis ATCC 13076	–	–	–	–	–	–
S*almonella typhimurium* ATCC 14028	–	–	–	–	–	–
Staphylococcus aureus ATCC 6541	–	–	–	–	–	–
Staphylococcus aureus ATCC 29213	–	–	–	–	–	–
Staphylococcus epidermidis ATCC 12228	120	90	140	120	100	–
Shigella sonnei ATCC 25931	–	–	–	–	–	–
Pseudomonas aeruginosa 25723	–	–	–	–	–	–
Pseudomonas mirabilis ATCC 22906	–	–	–	–	–	–

[a]Absence of activity in the tested concentration

6.0 (neutralized acid extract), and assayed for antimicrobial activity using the double-layer diffusion test, according to Farias et al. (1994), using *Listeria monocytogenes* Scott A as indicator. The neutralized acid extract was concentrated ten times by ultra-filtration in an Amicon System (Millipore-Merck, Billerica, MA, USA) with 1000 Da molecular-weight-cut-off membrane and then freeze-dried. The protein concentration in the neutralized acid extract was determined by the Bradford method (Bradford 1976).

Characterization of the antimicrobial compounds

The procedures for purification of the antimicrobial compounds, the protein sequencing and determination of molecular mass by mass spectrometry were the same previously published (Carvalho et al. 2010). The influence of the proteases trypsin, proteinase K and pronase on antilisterial activity of each purified fraction containing the antimicrobial was tested as described in Carvalho et al. 2010.

The mechanism of action of each purified fraction containing the antimicrobial was determined by means of two measurements: (1) proton motive force depletion, according to Carvalho et al. (2010) and (2) efflux of ATP, according to Guihard et al. (1993). The detection of efflux of ATP was based in a bioluminescence assay, where *L. monocytogenes* Scott A cells were cultivated in 25 mL of BHI broth at 30 °C until absorbance reached values in the range from 0.6 to 1.0 at 600 nm (corresponding to 2×10^9 cells). Bacterial cells were centrifuged at 3000*g* for 10 min, washed twice with $50\ mmol \cdot L^{-1}$ MES buffer, pH 6.5, and suspended in 2.5 mL of a $10\ mmol \cdot L^{-1}$ KCl solution containing 1% ($w \cdot v^{-1}$) D-glucose. For total ATP measurement, 20 µL of *L. monocytogenes* Scott A suspension was added to 80 µL of dimethyl sulfoxide and the suspension was diluted with 4.9 mL of distilled water. For measurement of extracellular

ATP, 20 µL of *L. monocytogenes* Scott A suspension was added to 50 mmol·L^{-1} MES buffer, pH 6.5, and 100 µL of this suspension was mixed with 100 µL of the ATP solution (Life Technologies) and different concentrations of the purified protein or nisin (positive control) (0.1, 0.25, 0.5, and 1.0 µmol·L^{-1}). The MES buffer and ATP solution (without the antibacterial proteins or nisin) was used as negative control. Bioluminescence was measured after 15, 17, 27 and 37 min and expressed as nmol per milligram of dry weight cells (nmol·mg^{-1}), based on a standard curve built correlating bioluminescence intensity with ATP concentration. The standard curve was obtained using the standard ATP of the ATP assay kit (Life Technologies).

Test for activity against *listeria monocytogenes* in a food model (meat gravy)

A simulated meat gravy was prepared mixing proteose peptone (1.8% w·v^{-1}), meat extract (1.2% w·v^{-1}), yeast extract (0.6% w·v^{-1}) and corn starch (2.0% w·v^{-1}) (Alves et al. 2003). Peptone, meat and yeast extracts were from Oxoid Ltd—Thermo Scientific, Hampshire, Basingstoke, UK—and starch was from Corn Products Brazil, Sao Paulo, SP, Brazil. The gravy was sterilized by autoclaving at 121 °C for 20 min. Six liters of MRS were inoculated with *Lb. sakei* 2a and used to prepare the neutralized acid extract, following the procedure described in 2.2. This extract was submitted to reversed-phase chromatography and the fraction eluted at 10–17 min retention time, corresponding to the most active fraction (EAF—enriched active fraction), was used in the test. The meat gravy was divided into five 50 mL portions (A–E) and *L. monocytogenes* Scott A (10^3 CFU·mL^{-1}) and the following additives/cultures: A—none (control); B—culture of *Lb. sakei* 2a – 10^6 CFU·mL^{-1}; C—EAF: 0.1% w·v^{-1}; D—EAF: 0.2% w·v^{-1}; E nisin: 0.1% w·v^{-1} were added. The inoculated gravy portions were maintained at 4 °C for 10 days, and counts of *L. monocytogenes* Scott A and *Lb. sakei* 2a were performed at times 0, 5 and 10 days. For counting, gravy portions were submitted to serial decimal dilutions in 0.85% NaCl (w·v^{-1}) solution and 100 µL of each dilution were spread plated onto two plates of Oxford agar (Oxoid) for the enumeration of *L. monocytogenes* Scott A and two plates of MRS agar (Oxoid) agar for enumeration of *Lb. sakei* 2a. Plates were incubated at 37 °C for 48 h, colonies were counted and results expressed as log CFU·mL^{-1}. All experiments were done three times.

Statistical analysis

Variance analysis (ANOVA) was applied to results of microbial counts in the simulated meat gravy. Data were expressed as means ± standard error. The growth of *L. monocytogenes* Scott A during storage of meat gravy was evaluated using regression analyses. Statistical analysis was performed using the Fast Statistic software (Fatesoft Version 2.0 Demo, 2014). Results with $p < 0.05$ were considered significant.

Results and discussion

The treatment of the culture of *Lb. sakei* 2a in MRS with an acidified solution (pH 1.5) and extraction of the antimicrobial compounds bound to the cell walls combined with reversed-phase chromatography purification, followed by mass spectrometry and Edman degradation for identification, resulted in the detection of six new antilisterial compounds produced by the strain (P4–P9), in addition to sakacin P and peptides P2 and P3 previously described (Carvalho et al. 2010). The six antimicrobial compounds were consistently present in the cell-free supernatants in separate experiments. All compounds were sensitive to the tested proteolytic enzymes, indicating their proteinaceous nature. The peptides P4 to P9 were heat stable, maintaining the biological activity after heating at 60, 98 and 121 °C for 15 min, and were active in a broad range of pH, from 1.5 up to 10.0.

The activity of the six peptides against the target bacteria is shown in Table 1. All six compounds were active against the tested *Listeria* spp., *Enterococcus faecalis* and *Lb. sakei* strains, and five of them were active also against *Staphylococcus epidermidis*. Some activity was also observed against *Enterococcus canis, Enterococcus faecium* and *Enterococcus hirae*, in a compound-dependent manner. Food and commensal strains, as well as Gram-negative strains were resistant to all compounds. *S. epidermidis* was sensitive whereas *S. aureus* was resistant. These results are in agreement with those reported for sakacin P (Carvalho et al. 2010) and most bacteriocins produced by acid lactic bacteria (Todorov et al. 2013; Barbosa et al. 2015). This is a typical profile of antimicrobial compounds produced by microbial strains for niche occupation (O'Connor et al. 2015).

The N-terminal sequences of the six antilisterial compounds, their molecular masses, protein sequences and similarity with other proteins are presented in Table 2. Noteworthy is that these compounds were detected in all repetitions of the purification experiments. All compounds were similar to 30S or 50S ribosomal proteins of *Lb. sakei* subsp *sakei* 23K, but P6 was similar to a protein of unknown function. The observed average molecular masses were identical to the calculated average molecular masses, confirming the identification of the compounds.

The concentration of each compound, including sakacin P and peptides P1 and P1 described previously, was achieved by the fractionation of the acid extract of the culture of *Lb. sakei* 2a and the purification steps varied between 1.00 and 16.05 µg·L^{-1} (Table 3). The activity of each compound against *L. monocytogenes* Scott A is shown in Fig. 1, where

Table 2 N-terminal sequences obtained by Edman degradation, molecular masses furnished by ESI-mass spectrometry and identification of active peptides produced and secreted by *Lactobacillus sakei* subsp. *sakei* 2a

	N-terminal sequence	Observed average molecular mass (kDa)	Identification	Complete protein sequence[a]	Calculated average molecular mass (kDa)
P4	SRSLKKGPFADAHLLNKIEAQADSEK-KQVI KTWSR	10,412.7	30S ribosomal protein S19 of *Lb. sakei* subsp. *sakei* 23K Uniprot ID: Q38UR6_LACSS	**SRSLKKGPFADAHLLNKIEAQADS**EK-KQVIKTWSRRSTIFPSFIGYTIAVY-DGRKHVPVFISDDMVGHKLGEFVP-TRTFHGHGNDDKKTKAR	10,412
P5	MKAKDIIELTTAEMLEKEHQ	7,657.7	50S ribosomal protein L29 of *Lb. sakei subsp. sakei*23K Uniprot ID Q38US0_LACSS	**MKAKDIIELTTAEMLEKEHQ**Y-KEELFNLRFQQATGQLEN-TARLKQVRQNIARIKTVLRQQELNK	7,657.9
P6	SQNLHVSVSGNRRPI	4,635.3	Hypothetical small protein with unknown function of *Lb. sakei* subsp. *sakei*23K Uniprot ID Q38Z88_LACSS	**SQNLHVSVSGNRRPI**NQKNAKKR-NAETTAVLNFLKERKSAK	4,635.3
P7	AKDDVIEIEG	7,990.0	Translation initiation factor IF-1 of *Lb. sakei* subsp. *sakei*23K Uniprot ID Q38UT3_LACSS	**AKDDVIEIEG**KVTDTLPNAMFKVELEN-GAVILAHVSGKIRKNYIKILPGDRVTV-ELSPYDLTKGRITYRFK	7,990.4
P8	MFVKTGDKVKVISGKDKGKEGTI	11,035.6	50S ribosomal protein L24 of *Lb. sakei* subsp. *sakei* 23K Uniprot ID Q38US3_LACSS	**MFVKTGDKVKVISGKDKGKEGTII-KAMPKEGRVVVEGINTI**KKHVKP-NAQNPNGGIVDTEASIDASNVMLIDP-SNNEATRVGYKVVDGKKVRVSKKT-GESIDK	11,035.9
P9	PQIKSAMKRVKTIEKANNRNASQLST-MRSAIKKFKAA	8,998.1	30S ribosomal protein S20 of *Lb. sakei* subsp. *sakei* 23K Uniprot ID Q38WR3_LACSS	**PQIKSAMKRVKTIEKANNRNASQL-STMRSAIKKFKA**AQAAGN-EEAADLLKAATRAIDMASTKGLIHAN-KAGRDKSRLNKMMAK	8,998.6

[a]Complete sequences of the identified proteins are from Chaillou et al. (2005)

Table 3 Concentration ($\mu g \cdot L^{-1}$) of the antimicrobial compounds obtained by cation-exchange fractionation of the acid extract of *Lactobacillus sakei* subsp. *sakei* 2a

Antimicrobial compound	Concentration ($\mu g \cdot L^{-1}$)
P1[a]	7.65
P2[a]	16.05
P3[a]	15.62
P4	1.90
P5	2.90
P6	3.40
P7	1.00
P8	5.48
P9	5.51

Separation was performed in a C_{18}-column (250×4.6 mm, 5 µm, 100 Å) 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 $mL \cdot min^{-1}$ and detection was conducted at 280 nm

[a]Described in Carvalho et al. 2010

a dose-dependent activity can be seen for all, except P5. The activity of P6 and P9 was similar to nisin, for the same concentration.

The compounds P4–P9 presented similar mode of action: they decreased the membrane potential ($\Delta\Psi$) and increased the efflux of ATP of *L. monocytogenes* (Figs. 2, 3, 4), though with varied intensity. However, they did not cause disruption of the pH gradient (ΔpH), as occurred with sakacin P (data not shown). A decrease in $\Delta\Psi$ of about 90–140 a.u. was observed when the concentration was 1000 $nmol \cdot L^{-1}$, which is smaller decrease than that reported for sakacin P (Carvalho et al. 2010).

The decrease of membrane potential ($\Delta\Psi$) accompanied by increase of ATP efflux observed in the present study is due to the well-known mechanism by which class II bacteriocins inhibit target cells (Snyder and Worobo 2014). Modification of $\Delta\Psi$ is commonly observed for typical class IIa bacteriocins, as demonstrated for enterocin P (Herranz et al. 2001a, b), piscicocin CS526 (Suzuki et al. 2005) and sakacin P (Carvalho et al. 2010). The decrease of membrane potential ($\Delta\Psi$) was the same for all antilisterial compounds together or when the EAF was used (unpublished data). The same occurred with the purified acid extract (1600 $AU \cdot mL^{-1}$).

Results of this study suggest that some antimicrobial compounds produced by *Lb. sakei* 2a are moonlight proteins, i.e., besides their predominant function, such as ribosomal or DNA-binding, they can also have an antibacterial activity. Since these proteins are highly cationic, they can bind to nucleic acids and anionic constituents of the surface of Gram-positive bacteria and can be transported through their cell wall and membranes (Drider et al. 2006). Although unable to promote extensive membrane disruption, these proteins can affect some membrane processes such as ATP transport and maintenance of the electrochemical gradient (Chen and Montville 1995). It is important to point out that ribosomal- and nucleic acid-binding proteins may cooperate with sakacin P by presenting the same mechanism of action such as disruption of membrane potential and increase of ATP.

Production of multiple antimicrobial compounds by different species of lactic acid bacteria seems to be quite common (Ustyugova et al. 2012; Sawa et al. 2013; Gaaloul et al. 2014; Masuda et al. 2015; Mirkovic et al. 2016). In the study of Sawa et al. (2013), the authors observed that *Lb.*

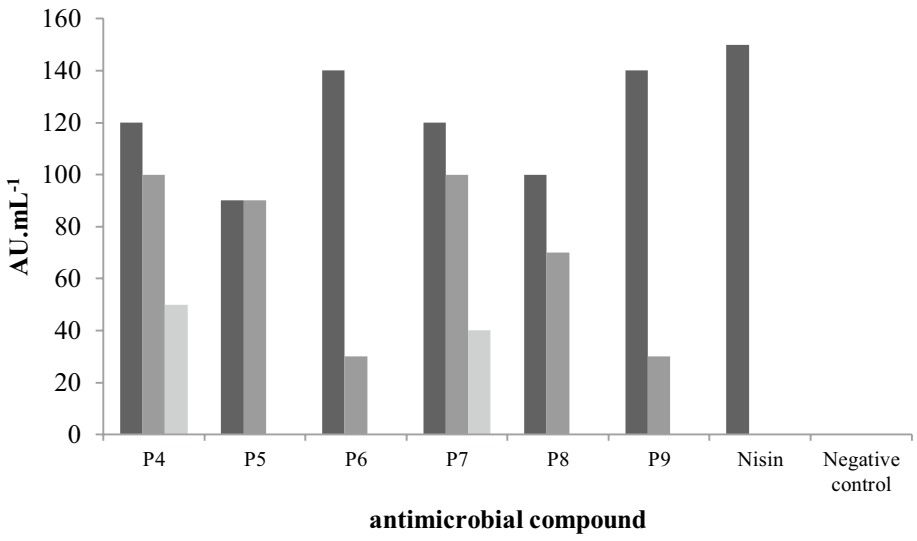

Fig. 1 In vitro antilisterial activity ($AU \cdot mL^{-1}$) of the antimicrobial compounds produced by *Lactobacillus sakei* subsp. *sakei* 2a and nisin. (Dark square) 1000 $nmol \cdot L^{-1}$, (dark gray square) 100 $nmol \cdot L^{-1}$, (light gray square) 10 $nmol \cdot L^{-1}$

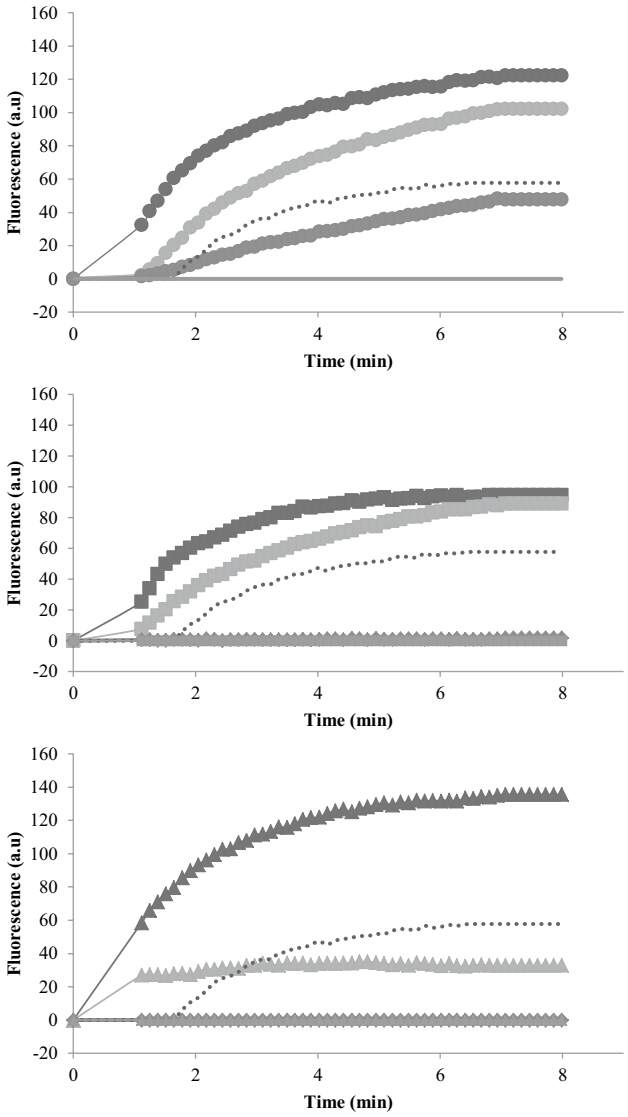

Fig. 2 Effect of compounds P4, P5 and P6 produced by *Lactobacillus sakei* subsp. *sakei* 2a on the membrane potential ($\Delta\Psi$) of *Listeria monocytogenes* Scott A cells. Fluorescence levels before the addition of the compounds were arbitrarily set to zero, and the increase in fluorescence upon the addition of compounds was expressed in arbitrary units (a.u). Antimicrobial compounds and concentrations were: (black dot dash dot line) P4, 1000 nmol·L^{-1}, (light gray dot dash dot line) P4, 100 nmol·L^{-1},(dark gray dot dash dot line) P4, 10 nmol·L^{-1},(black dash square dash line) P5, 1000 nmol·L^{-1}, (light gray dash square dash line) P5, 100 nmol·L^{-1}, (dark gray dash square dash line) P5, 10 nmol·L^{-1}, (black dash triangle dash line) P6, 1000 nmol·L^{-1}, (light gray dash triangle dash line) P6, 100 nmol·L^{-1},(dark gray dash triangle dash line) P6, 10 nmol·L^{-1}, (thick dash) negative control, (dark gray square dot square) nisin, 1000 nmol·L^{-1}

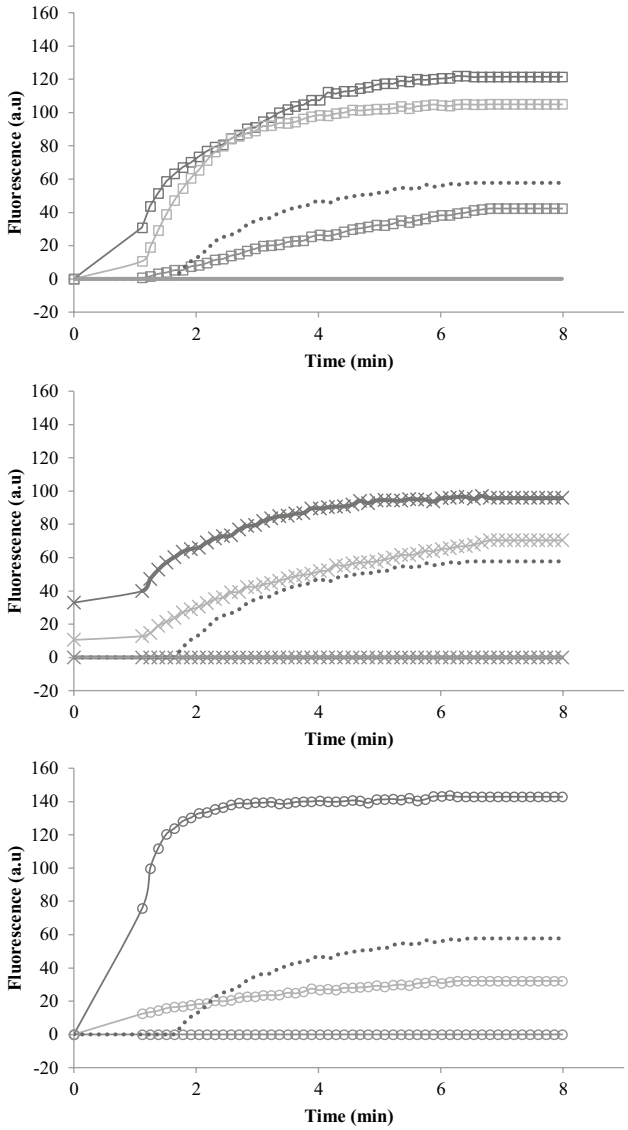

Fig. 3 Effect of the compounds P7, P8 and P9 produced by *Lactobacillus sakei* subsp. *sakei* 2a on the membrane potential ($\Delta\Psi$) of *Listeria monocytogenes* Scott A cells. Fluorescence levels before the addition of the compounds were arbitrarily designated zero, and the increase in fluorescence upon the addition of proteins was expressed in arbitrary units (a.u..). Antimicrobial compounds and concentrations were: (black dash square dash line) P7, 1000 nmol·L^{-1}, (light gray dash square dash line) P7, 100 nmol·L^{-1}, (dark gray dash square dash line) P7, 10 nmol·L^{-1}, (black dash times dash line) P8, 1000 nmol·L^{-1}, (light gray dash times dash line) P8, 100 nmol·L^{-1}, (dark gray dash times dash line) P8, 10 nmol·L^{-1}, (black dash circle dash line) P9, 1000 nmol·L^{-1}, (light gray dash circle dash line) P9, 100 nmol·L^{-1}, (dark gray dash circle dash line) P9, 10 nmol·L^{-1}, (dark dash) negative control, (dark gray square dot square), nisin, 1000 nmol·L^{-1}

sakei D98 isolated from rice malt (Shubo) produced sakacin with a pediocin box-like sequence, and also two class IId bacteriocins (sakacins D98a and D98c). These bacteriocins presented different antimicrobial mechanisms, amino acid sequences and disulfite bridges in their molecules, which are characteristics also observed for the mat isolate *Lb sakei* 2a. *Lb. sakei* D98 is part of the starter culture used in the production of Shubo, so Sawa et al. (2013) considered that

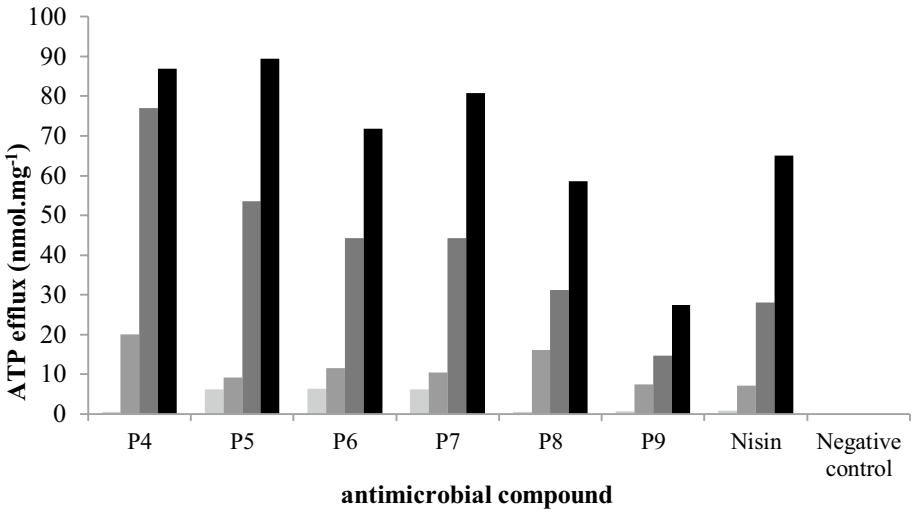

Fig. 4 Effect of the antimicrobial compounds produced by *Lactobacillus sakei* subsp. *sakei* 2a on the ATP efflux from *Listeria monocytogenes* Scott A cells. Bioluminescence levels before the addition of the compounds were arbitrarily designated zero, and the increase in bioluminescence upon the addition of the compounds was expressed in ATP concentration (nmol·mg^{-1}). The compounds and nisin concentrations were: (black square) 1.0 µmol·L^{-1}, (dark gray square) 0.5 µmol·L^{-1}, (light gray square) 0.25 µmol·L^{-1}, (lighter gray square) 0.1 µmol·L^{-1}

it may play an important role in controlling growth of other microorganisms by the production of bacteriocins in addition to organic acids. However, the multiple bacteriocins produced by *Lb. sakei* D98 did not present a synergistic effect, as also observed for the strain tested in the present study (*Lb. sakei* 2a).

Other authors have also studied peptides with antimicrobial activity which are not recognized as classical bacteriocins. Miao et al. (2016) evaluated a novel peptide (F1) produced by *Lactobacillus paracasei* subsp. *tolerans* FX-6, isolated from Tibetan kefir. F1 showed a wide antimicrobial spectrum against bacteria and fungi, and stability to protease, pH and heat treatments. This peptide contained 18 amino acids and its antimicrobial mechanism against *Escherichia coli* was related to the membrane permeability and the leakage of the cytoplasmic β-galactosidase and potassium ions. The results showed that the antimicrobial peptide F1 could penetrate and accumulate into cell causing disruption of cell membrane functions, in addition to a DNA-binding ability. Hence, F1 depicted multiple cell targets in the killing of *E. coli*.

Xiao and Niu (2015) verified the production of the antilisterial peptides released by enzymatic hydrolysis from grass carp proteins and then evaluated their antilisterial activity against *L. monocytogenes* inoculated in surimi noodle for storage at 4 and 25 °C up to 20 days. The results indicated that antilisterial peptides produced by enzymatic hydrolysis from grass carp proteins can inhibit the growth of *L. monocytogenes* in surimi noodle, which was useful as natural food preservatives of meat products.

When tested in the meat gravy model simulating a processed food, both the *Lb. sakei* 2a culture and the enriched active fraction (EAF) of the culture inhibited the growth of *L. monocytogenes* (Fig. 5), regardless the final concentration of the acid extract in the gravy (0.1 or 0.2% w·v^{-1}). In the control experiment, the count of *L. monocytogenes* Scott A in the gravy stored at 4 °C was 5.0 log CFU·mL^{-1} after 5 days and remained the same until the 10th day. In the gravy containing the bacteriocinogenic strain or the acid extract, the growth of *L. monocytogenes* was inhibited to an extent that the population of the pathogen in the product stored at 4 °C for 5 days was 0.6 to 1 log lower than the initial population (10^3 CFU·mL^{-1}). These differences were statistically significant ($p < 0.05$), supporting the potential application of *Lb. sakei* 2a or the EAF in foods for biopreservation.

The EAF from *Lb. sakei* 2a presented similar and even better inhibitory activity against *L. monocytogenes* than the culture in a simulated meat product during storage under refrigeration up to 10 days. This enhanced activity of the extract when compared to the culture may be due to the lag time needed for the bacteriocinogenic strain to adapt to the meat gravy model before starting production of the antimicrobial compounds. Furthermore, the in situ production of these compounds may be hampered by components in the food matrix and unfavorable environmental conditions for growth, such as pH and salt or sugar content (Urso et al. 2006).

Castellano et al. (2016) evaluated the antilisterial peptides from Spanish dry-cured hams produced during the ripening from the hydrolysis of the muscle proteins by muscle peptidases. Ten peptides showed antilisterial activity and these results proved novel natural strategies and alternative to chemical compounds for potential antimicrobial action against *L. monocytogenes*.

The combination of one or more antilisterial compounds either produced in situ or added to the product is effective to reduce the survival or regrowth of *Listeria* populations in model systems. Normally, the resistance of the strains is observed in foods because changes in the fatty acid composition of the membrane and alterations in the cell envelope (Abee 1995). Therefore, a useful alternative would be the combined use of one or more antilisterial compounds to

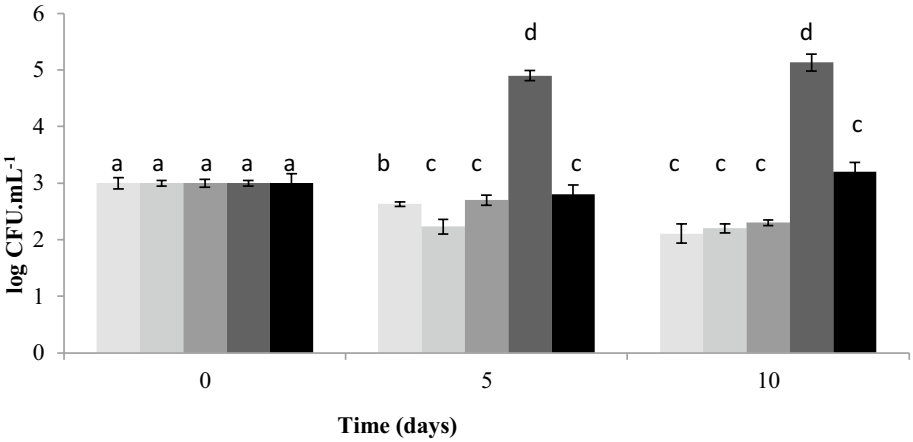

Fig. 5 Counts of *Listeria monocytogenes* (log CFU·mL^{-1}) in the meat gravy model containing (lighter gray square) *L. monocytogenes* + enriched active fraction (EAF) 0.1% (w·v^{-1}); (light gray square) *L. monocytogenes* + enriched active fraction (EAF) 0.2% (w·v^{-1}); (dark gray square) *L. monocytogenes* + *Lb. sakei* subsp *sakei* 2a (darker gray square) *L. monocytogenes*, (black square) *L. monocytogenes* + nisin 0.1% (w·v^{-1}). [a,b,c,d]Different letters indicate statistically significant differences ($p < 0.05$)

ensure the food safety and avoid the emergence of resistant bacteria strains.

In conclusion, results obtained from this study indicate that *Lb. sakei* 2a is capable to produce several antimicrobial compounds and these compounds or a culture of this strain have a potential for application in processed food by the inhibition of growth of *L. monocytogenes* and other closely related bacteria thereby increasing the shelf life of the products. Further scale-up studies, where enough amount of each antilisterial peptide will be obtained and tested in food models, will clarify the role of each peptide in the inhibition of *L. monocytogenes*, and a possible synergistic effect could be evaluated and explored as strategy for effective biopreservation of foods.

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Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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