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ORIGINAL ARTICLE

Characterization of bacteriocins produced by strains of *Pediococcus pentosaceus* isolated from Minas cheese

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Abstract

Interest in obtaining bacteriocin-producing strains of lactic acid bacteria (LAB) from different sources has been increasing in recent years due to their multiple applications in health and food industries. This study focused on the isolation and characterization of metabolically active populations of bacteriocinogenic LAB and the evaluation of their antimicrobial substances as well as of some nutritional requirements of them. One hundred and fifty colonies of LAB from artisanal cheeses produced in Minas Gerais state (Brazil) were isolated and screened for their antimicrobial activity. According to their activity against *Listeria monocytogenes*, ten strains were selected and subsequently identified using biochemical and molecular techniques including 16s rRNA amplification and sequencing as *Enterococcus faecalis*, *Lactobacillus* spp., and *Pediococcus pentosaceus*. Antimicrobial substances produced by four of the selected strains, *P. pentosaceus* 63, *P. pentosaceus* 145, *P. pentosaceus* 146, and *P. pentosaceus* 147, were biochemically characterized, and presented sensitivity to proteolytic enzymes (suggesting their proteinaceous nature) and to extreme pH. Antimicrobial activity showed stability after treatment with lipase, catalase, α-amylase, and chemicals. Growth kinetics of the *P. pentosaceus* selected showed maximal bacteriocin production at 37 °C during the end of the exponential growth phase (25,600 AU/mL) and stable production during 24 h of incubation. Dextrose, maltose, and a mixture of peptone, meat extract, and yeast extract increased bacteriocin production. This study demonstrated that dairy products provide a good alternative for obtaining LAB, with the ability to produce antimicrobial substances such as bacteriocins that have potential use as biopreservatives in food.

Keywords Antimicrobial activity · Bacteriocin · Lactic acid bacteria · *Pediococcus*

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Introduction

Milk and dairy products represent important ecological niches that are sources of bacteriocinogenic strains of lactic acid bacteria (LAB) (Furtado et al. 2014). Minas cheese, produced in Brazil (Minas Gerais state), is an artisanal product which is a ripened cheese made mostly from raw cow's milk. Producers require approximately 60 days to complete maturation of the product and, during this period, a reduction of the most common pathogen population (Martins et al. 2015; Perin et al. 2015) such as *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus* occurs (Freitas et al. 2013). After this time, cheeses reach quality standards according to Brazilian food production regulations of *L. monocytogenes* and *Salmonella* spp. absence and 10^3 CFU/g as maximal count of coagulase-positive staphylococci (CPS) (Moraes et al. 2009). The LAB isolated from dairy products belong to genera *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Lactococcus* (Luiz et al. 2017) and have as important

characteristics the production of organic acids, carbon dioxide, hydrogen peroxide, diacetyl, and bacteriocins (Ammor et al. 2006; Khan et al. 2010). Pediocins are class II bacteriocins produced by *Pediococcus* strains as a primary metabolite with antimicrobial activity against *Listeria monocytogenes*. Pediocins are generally small peptides (with 36–48 residues) and non-modified after translation, with some exceptions such as pediocin AcH/PA-1 (Papagianni and Anastasiadou 2009).

Different studies of *Pediococcus* strains have been focused on their antimicrobial activity. Numerous strains of *Pediococcus* spp. have been reported to be producers of various bacteriocins, including pediocin PA-1/AcH (*P. acidilactici* PAC 1.0, *P. acidilactici* H, E, F, and M), JD (*P. acidilactici* SJ-1), pediocin 5 (*P. acidilactici* UL5), pediocin A (*P. pentosaceus* FBB-61), pediocin N5p (*P. pentosaceus*), pediocin ST18 (*P. pentosaceus*), and pediocin PD-1 (*P. damnosus*) (Anastasiadou et al. 2008). A recent study reported a bacteriocinogenic strain *Pediococcus pentosaceus* ST65ACC from Minas cheese with activity against two strains of *Listeria monocytogenes* (Cavicchioli et al. 2017). Application of bacteriocins, such as pediocins from *Pediococcus* spp. strains, is an alternative means of controlling food-borne pathogenic bacteria and may lead to reduced use of chemical preservatives and the production of healthier food products (Udhayashree et al. 2012).

Knowledge of the optimal production conditions for bacteriocins is important for obtaining maximum activity. Information on inoculation conditions, environmental factors (pH and temperature), and nutritional requirements are key to obtain amounts of bacteriocins that of use in industrial applications (Malheiros et al. 2015). Studies investigating these requirements are necessary because some nutrients can stimulate or limit expression of bacteriocins (Todorov et al. 2012; Abbasiliasi et al. 2017).

In the present study, we report on the isolation, identification, and characterization of LAB with bacteriocinogenic potential from Minas cheese. Based on a preliminary screening, four strains of *Pediococcus pentosaceus* were selected and a biochemical and molecular characterization of their bacteriocins was conducted. Finally, the effect of modifications to the growth medium on bacteriocin production was studied.

Materials and methods

Isolation of bacteriocin-producing strains

Two different samples of Minas cheese were obtained from a dairy store selling artisanal products in Viçosa (Minas Gerais state, Brazil). Screening for LAB bacteriocin-producing strains was performed as previously described by Todorov et al. (2010). Eleven grams of Minas cheese were homogenized in 99 mL of physiological solution (0.85% NaCl, *w*/*v*). Serial dilutions of the homogenized cheese were prepared, plated onto man, rogosa, sharpe (MRS) agar (Difco, BD), and covered with a thin layer of bacteriological agar. Plates were incubated at 37 °C for 24–48 h and total microbial populations were counted. Plates with less than 50 separated colonies were covered with BHI medium containing 1.0% (*w*/*v*) agar (Oxoid) and inoculated with a culture of *Listeria monocytogenes* 104, *L. monocytogenes* 712, or *L. monocytogenes* ATCC 7644 (final concentration of 10^6 CFU/mL). After incubation for an additional 24 h at 37 °C, 150 colonies that presented inhibition zones were selected and cultured in MRS broth (Difco, BD) for 24 h. Bioactivity of the selected strains against *L. monocytogenes* 104, *L. monocytogenes* 712, and *L. monocytogenes* ATCC 7644 was verified using the agar spot-test according to Murua et al. (2013). Briefly, cell-free supernatants (CFS) of isolated LAB were obtained by centrifugation (8000×*g* at 4 °C for 10 min). The pH of the supernatants was adjusted to 6.0 with sterile 1 N NaOH to eliminate the effect of lactic acid produced by the strains. Potential generation of proteolytic enzymes and H_2O_2 was prevented by heat treatment of CFS (10 min at 80 °C).

Antimicrobial activity was measured using the spot-on-the-law method. Twofold dilutions of the CFS were made in phosphate buffer (100 mM, pH 6.5). Aliquots (10 μL) of each dilution were spotted onto soft BHI agar (1% agar) inoculated with 10^6 CFU/mL of *L. monocytogenes* 104. Tests were conducted in three independent repetitions. Antimicrobial activity was expressed as arbitrary units per milliliter (AU/mL) and defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition and calculated according to the equation:

$$\frac{\mathrm{AU}}{\mathrm{mL}} = a^b \times 100$$

where $a = 2$ (factor dilution) and b = value of the highest dilution showing at least 2-mm inhibition zone (Murua et al. 2013).

Morphology of the studied cultures was examined using Gram staining. Pure cultures were stored at − 20 °C in MRS broth supplemented with 20% (*w*/*v*) glycerol.

Differentiation and identification

Basing on preliminary screening for bacteriocin production, conducted with *L. monocytogenes* 104, *L. monocytogenes* 712, and *L. monocytogenes* ATCC 7644, 150 colonies with potential for bacteriocin production were isolated. However, 18 isolates were confirmed to be bacteriocin producers according to the applied agar spot-on-lawn test reported by Murua et al. (2013). Random amplification of polymorphic DNA (RAPD-PCR) analysis was performed in order to obtain differentiation of the selected 18 isolates with primers OPL-

04, OPL-05, and OPL-20 (www.operon.com/products/downloads/OperonsRAPD10merSequences.xls). Total DNA from the 18 LAB isolates was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA). The DNA concentration was estimated on a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA). Amplification reactions were performed according to Dos Santos et al. (2015). The 25 μL reaction volume contained the following: 2 μL total DNA, 5 μL of 10 mM primer, 2.5 μL of buffer (BioLab), 10 μL of 5 mM $MgCl_2$ (Fermentas), 1 μL Milli-Q water, 4 μL dNTP (Fermentas), and 0.5 μL Taq DNA polymerase (BioLab). Amplifications were performed on a DNA MasterCycler® (Eppendorf Scientific, Hamburg, Germany) as follows: 45 cycles of 1 min at 94 °C and 1 min at 28 °C, followed by an increase to 72 °C for 2 min. Extension of the amplified product was at 72 °C for 5 min. Amplified products were separated by electrophoresis on 1.2% (*w*/*v*) agarose gels in TAE buffer at 120 V for 1 h. Gels were stained with GelRed (Biotium Inc., Hayward, CA, USA). A 100-bp DNA ladder (Fermentas) was used as a molecular weight marker.

Bacteriocin-producing LAB strains were identified according to physiological and biochemical characteristics as previously described by Todorov et al. (2013). Carbohydrate fermentation profiles were recorded using APICHL50 (Biomérieux, Marcy-l′Etoile, France). In addition, molecular identification was confirmed by 16s rRNA sequencing. Total DNA was isolated and quantified as described previously. PCR was performed with primers 8F: 5′-AGTTTGATCCTGGCTCAG-3′ and 1512R: 5′-ACGGCTACCTTGTTACGACTT-3′, according to the method described by Felske et al. (1997). PCR amplification was performed using a DNA MasterCycler® with a 20-μL reaction volume containing 0.1 μL of each primer 10 mM, 2 μL buffer (BioLab), 8 μL of 5 mM MgCl2 (Fermentas), 1.95 μL dNTP (Fermentas), and 0.05 μL Taq DNA polymerase (BioLab). Amplification conditions were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 5 min at 94 °C, and 10 s at 61 °C, followed by an increase to 72 °C for 2 min. Final extension of the amplified product was at 72 °C for 75 min. The obtained amplicons were purified with a QIAquick PCR Purification Kit (Qiagen), following the manufacturer's instructions, and submitted to sequencing at the Center for Human Genome Studies, Institute of Biomedical Sciences, University of Sao Paulo, Brazil. The sequences were compared to those deposited in GenBank, using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). After identification, four of the isolates were used for analysis.

Screening for the presence of bacteriocin genes

Total DNA was isolated as previously described and amplified by PCR using primers, targeting different bacteriocin genes (nisin, pediocin PA-1, enterocin A, enterocin B, enterocin L50B, enterocin P, plantaricin W, plantaricin S, and plantaricin NC8). PCRs were performed using a DNA MasterCycler® with conditions based on previous studies (Stephens et al. 1998; du Toit et al. 2000; Holo et al. 2001; Maldonado et al. 2003; Kruger et al. 2013; Todorov et al. 2016) and based on the specification of the primers, which are summarized in Table 1. The amplified products were visualized on agarose gel and stained with GelRed.

Table 1 Primers

Target gene	Primers	Annealing, T°	Fragment size (bp)	Ref.
Nisin	ATGAGTACAAAAGATTTCAACTT TTATTTGCTTACGTGAACGC	48 °C	203	Kruger et al. (2013)
Pediocin PA-1	CAAGATCGTTAACCAGTTT CCGTTGTTCCCATAGTCTAA	44 °C	1238	Todorov et al. (2016)
Enterocin A	AAATATTATGGAAATGGAGTGTAT GCACTTCCCTGGAATTGCTC	34 °C	452	du Toit et al. (2000)
Enterocin B	GAAAATGATCACAGAATGCCTA GTTGCATTTAGAGTATACATTTG	41 °C	159	du Toit et al. (2000)
Enterocin L50B	STGGGAGCAATCGCAAAATTAG ATTGCCCATCCTTCTCCAAT	44 °C	135	du Toit et al. (2000)
Enterocin P	TATGGTAATGGTGTTTATTGTAAT ATGTCCCATACCTGCCAAAC	41 °C	216	du Toit et al. (2000)
Plantaricin NC8	GGTCTGCGTATAAGCATCGC AAATTGAACATATGGGTGCTTTAAATTCC	35 °C	207	Maldonado et al. (2003)
Plantaricin S	GCCTTACCAGAGTAATGCCC CTGGTGATGCAATCGTTAGTTT	45 °C	450	Stephens et al. (1998)
Plantaricin W	TCACACGAAATATTCCA GGCAAGCGTAAGAAATAAATGAG	41 °C	165	Holo et al. (2001)

Effect of enzymes, temperature, pH, and surfactants on bacteriocin activity

Strains were grown in MRS broth for 18 h at 37 °C. Cells were separated by centrifugation (8000 *g*, 10 min, 4 °C), and the CFS was adjusted to pH 6.0 with 1 M NaOH. One milliliter of CFS was incubated for 1 h at 37 °C in the presence of 1 mg/mL (1%) proteinase K, papain, pepsin, and lipase and 0.1 mg/mL α-amylase and catalase (all from Sigma-Aldrich). In a separate experiment, 1% (*w*/*v*) sodium dodecyl sulfate (SDS), Tween 80, Triton X-100, and NaCl (all from Sigma-Aldrich) were added to the CFS; these were also were incubated for 1 h at 37 °C. Untreated CFS and chemicals at their respective concentrations in water were used as controls. Effect of different pH on the activity of bacteriocins was tested by correcting pH of the CFS, prepared as described before, to pH 2.0, 4.0, 6.0, 8.0, and 10 adjusted with sterile 1 M NaOH or 1 M HCl. Samples were incubated for 1 h at 25 °C, and after incubation, they were re-adjusted to pH 6.5 with sterile 1 M NaOH or 1 M HCl. Effect of temperature on the bacteriocin activity was tested by incubating CFS at 4, 25, 30, 37, 45, 60, 80, and 100 °C for 1 h and at 121 °C for 20 min. After each treatment, antimicrobial activity was tested by using the agar spot test method, as previously described and *L. monocytogenes* 104 was used as the target strain. Results were expressed as percentages of reduction of activity by comparing the diameters of the inhibition zones of treated CFS with untreated CFS (control). Tests were conducted in three independent repetitions.

Adsorption of bacteriocin on producer cells

Determination of the adsorbed bacteriocin onto the surface of the producer cells was performed as previously proposed by Yang et al. (1992). Briefly, after incubation in MRS broth for 18 h at 37 °C, the cultures were adjusted to pH 6.0 with 1 M NaOH and the cells then harvested (10,000 *g*, 15 min, 4 °C) and washed with 10 mL of sterile phosphate buffer (0.1 M, pH 6.5). The CFS samples were stored for use as controls. The cells were re-suspended in 10 mL 100 mM NaCl (pH 2.0), stirred for 1 h at 4 °C, and then harvested (12,000×*g*, 15 min, 4 °C). The CFS supernatant obtained was neutralized to pH 7.0 with sterile 1 N NaOH and tested for activity using the agar spot-test (Murua et al. 2013). Tests were conducted in three independent repetitions.

Growth dynamics and bacteriocin production

Growth dynamics and bacteriocin production were evaluated using the turbidity and spot-on-the-law methods, respectively. MRS broth (100 mL) was inoculated with 2% overnight culture and incubated at 37 °C for 24 h. Changes in optical density at 600 nm (OD600) and pH were monitored hourly for 24 h. Antimicrobial activity was measured every 3 h using the spot-on-the-law method (Murua et al. 2013). Twofold dilutions of the CFS were made in phosphate buffer (100 mM, pH 6.5). Aliquots (10 μL) of each dilution were spotted onto soft BHI agar (1% agar) inoculated with 10^6 UFC/mL of *L. monocytogenes* 104.

Growth of *Listeria monocytogenes* 104 in the presence of CFS

One hundred milliliters of BHI was inoculated with 2 mL overnight culture of *L. monocytogenes* 104 and incubated at 37 °C. After 3 h of incubation, 20 mL aliquots of CFS (pH 6.5) of *P. pentosaceus* 63, *P. pentosaceus* 145, *P. pentosaceus* 146, or *P. pentosaceus* 147 were filter-sterilized (0.20 mm, Millipore) and added. The incubation was continued. Control without addition of CFS served as a comparison of *L. monocytogenes* 104 growth. Optical density measurements (600 nm) were recorded at 1-h intervals during the subsequent 12 h according to Todorov et al. (2010). Tests were conducted in three independent repetitions.

Adsorption onto target cell

The adsorption of the bacteriocins produced by *P. pentosaceus* 63, *P. pentosaceus* 145, *P. pentosaceus* 146, and *P. pentosaceus* 147 onto *L. monocytogenes*104, *Lb. sakei* ATCC 15521, and *Enterococcus faecalis* ATCC 19443 was measured according to Biscola et al. (2013). The target microorganisms were grown overnight in 10 mL of BHI (for *L. monocytogenes* 104) and MRS broth (for *Lb. sakei* and *E. faecalis*) at 37 °C. Biomass was recovered by centrifugation (8000×*g*, 15 min, 4 °C). Cells were washed twice with sterile 5 mM phosphate buffer (pH 6.5) and re-suspended in the same buffer to reach an equal to 1.0 of OD600. One milliliter of each cell suspension was mixed with 1 mL of CFS prepared as described before and incubated at 37 °C for 1 h. The antimicrobial activity, using the spot-on-the-law method against *L. monocytogenes* 104 as previously described, of unbound bacteriocin in the CFS was measured after removal of cells (8000×*g*, 15 min, 4 °C). Reduction of bacteriocin activity results in adsorption of bacteriocin onto cell surface of target cells and being unavailable for detection in cell-free supernatant. In addition, the effect of pH (4.0, 6.0, 8.0, and 10.0), temperature (4, 25, 30, and 37 °C), and the presence of 1% (*w*/*v*) of NaCl, Tween 80, glycerol, and SDS on the adsorption of the bacteriocin was determined (Biscola et al. 2013). The adsorbed bacteriocins were determined as follows:

$$\%\text{Adsorption} = \left(100 - \frac{\text{AU/mL}_1}{\text{AU/mL}_0}\right) \times 100$$

where AU/mL_0 is the bacteriocin activity before treatment, and AU/mL_1 is the bacteriocin activity after treatment. Tests were conducted in three independent repetitions.

Effect of medium composition on the production of bacteriocins

To investigate the effect of nitrogen and carbon sources and also the requirements of micronutrients on the growth and antimicrobial activity of the studied strains, different modified MRS broths were developed. Strains were grown in 10 mL MRS broth at 37 °C for 18 h. Aliquots (100 μL) of the cultures were used to inoculate 10 mL of the following media: (a) MRS broth without organic nutrients, supplemented with peptone (25 g/L), meat extract (25 g/L), and yeast extract (25 g/L) or supplemented with combinations of peptone (12.5 g/L) plus meat extract (12.5 g/L), peptone (15 g/L) plus yeast extract (7.5 g/L), meat extract (15 g/L) plus yeast extract (7.5 g/L), or peptone (10 g/L), meat extract (10 g/L), and yeast extract (5 g/L); (b) MRS broth, replacing the carbon source with fructose, sucrose, lactose, mannose, raffinose, mannitol, or maltose (20 g/L); (c) MRS broth modified to contain 0, 2, 5, or 10 g/L K_2HPO_4; (d) MRS broth modified to contain 0, 0.1, or 0.5 g/L of $MgSO_4$ and 0, 0.05, or 0.20 g/L of $MnSO_4$; (e) MRS broth supplemented with 0, 0.5, 1, 2, 5, or 10 g/L glycerol; (f) MRS broth modified to contain 0, 2, or 5 g/L of ammonium citrate; (g) MRS broth modified to contain 0, 1, 2, or 5 g/L of Tween 80; and (h) MRS broth with pH adjusted to 2, 4, 6, 8, 10, or 12. Incubation in all tests was at 37 °C for 24 h. Activity levels of bacteriocins were determined as described before in the "Isolation of bacteriocin-producing strains" section. Tests were conducted in three independent repetitions.

Partial bacteriocin purification and determination of approximate molecular mass by SDS-PAGE

Partial bacteriocin purification was performed according to Martinez et al. (2013), with some modifications. Strains were cultured in 1 L of MRS for 18 h at 37 °C and CFS then obtained by centrifugation for 15 min at 12000×*g* at 4 °C. Proteins from the CFS were precipitated by 80% saturation with ammonium sulfate at 4 °C (overnight), and the precipitate was then centrifuged for 60 min at 12,000 *g* at 4 °C. The pellets were resuspendend in 10 mL of 25 mM phosphate buffer (pH 6.5), and antimicrobial activity against *L. monocytogenes* 104 was determined as described before. In the next step, the resulting material was loaded on an activated SepPakC18 column (Waters, Millipore, MA, USA) and different fractions were eluted using 20, 40, 60, and 80% isopropanol in 25 mM phosphate buffer (pH 6.5). Antimicrobial activity of the obtained fractions was determined as described previously, using *L. monocytogenes* 104.

SDS-PAGE electrophoresis was performed according to Laemmli (1970), and sample preparation was performed according to Schagger (2006). All examined fractions were loaded in duplicate, and SDS-PAGE electrophoresis was performed at 200 V and 60 mA for first 10 min and then at 200 V and 35 mA. One part of the gel was stained with Coomassie Blue, as described by Schagger (2006), and the second part was used for an overlay assay, according to Cytryńska et al. (2001). Overlay gel was irradiated with UV for 30 min to prevent potential antimicrobial contamination and covered with a soft BHI agar (1%) inoculated with *Listeria monocytogenes* 104 (approx. 10^5 CFU/mL) in order to localize the protein bands with antibacterial activity.

Results and discussion

Isolation, differentiation, and identification

One hundred and fifty LAB grown on MRS agar, and that had formed clear inhibition zones against *Listeria* spp. incorporated in the third agar layer, were isolated from two samples of artisanal cheese produced in Viçosa municipality (Minas Gerais, Brazil). According to the results of additional antimicrobial tests (Murua et al. 2013) using the CFS (pH 6.5) of the 150 isolated colonies, on spot agar test 18 of them (isolates 54, 56, 59, 63, 64, 65, 66, 67, 68, 70, 87, 91, 127, 145, 146, 147, 148, and 149) produced more than 10 mm of inhibition zones using the *Listeria* spp. strains (the other strains did not produce inhibition after pH correction) and were selected for further analysis. Based on RAPD-PCR performed with 18 selected isolates, 10 presented unique profile and were selected for further studies. From them, seven presented cocci and three rods morphology. Analysis of the 16s rRNA amplified fragments showed that isolates 54, 87, and 91 presented homology with *Enterococcus faecalis* (*Enterococcus faecalis* 54, *Enterococcus faecalis* 87, and *Enterococcus faecalis* 91), isolates 56 and 127 with *Lactobacillus plantarum* (*Lb. plantarum* 56 and *Lb. plantarum* 127), and isolate 70 with *Lactobacillus rhamnosus* (*Lb. rhamnosus* 70). Isolates 63, 145, 146, and 147 presented homology with *Pediococcus pentosaceus* (*P. pentosaceus* 63, *P. pentosaceus* 145, *P. pentosaceus* 146, and *P. pentosaceus* 147). Biochemical characterization of the 10 selected strains was performed using carbohydrate fermentation reactions and was recorded according to the API50CHL® test.

Artisanal cheeses produced in Minas Gerais state (Brazil) are considered to be a cultural heritage and are traditional products made with raw milk and serum collected from cheeses prepared the previous day (Lima et al. 2009). Lima et al. (2009) reported on *Lactococcus lactis*, *Enterococcus* spp., *Enterococcus faecalis*, and *Streptococcus agalactiae*, isolated from Minas cheese.

Another type of Brazilian cheese made with raw milk is the coalho cheese, a traditional product of the North-West region of Brazil. Different species of *Lactobacillus* spp. such as *Lb. acidophilus*, *Lb. casei, Lb. fermentum*, and *Lb. rhamnosus* and *Lactococcus* spp. such as *Lc. lactis* and *Lc. raffinolactis* were reported to be isolated from this type of cheese (Neto et al. 2005). LAB belonging to the genera *Pediococcus* have rarely been isolated from dairy products, generally being isolated from meat products. Nevertheless, there are some reports on the occurrence of *Pediococcus* spp. strains in Minas cheese in Brazil (Cavicchioli et al. 2017; Luiz et al. 2017). Strains of *P. acidilactici* and *P. pentosaceus*, isolated from South African farm-style cheese (pasteurized Gouda, young and matured; un-pasteurized aged Bouquet, aged and matured Gouda), were also reported (Gurira and Buys 2005). In another study, strains of *P. acidilactici* were isolated from traditional Colombian double-cream cheese (non-matured acid cheese), prepared from a mixture of fresh and acidified cow milk. The process of milk acidification of the Colombian cheese as well as maturation of Minas cheese occurs naturally as a result of native microbiota containing LAB, which promotes the organoleptic, physico-chemical, and microbiological characteristics of the finished product (Londoño-Zapata et al. 2017).

Table 2 shows the bioactivity of the 10 identified strains against *Listeria monocytogenes* 104 using the agar spot-test. The most active were the *Pediococcus* strains; *Lactobacillus* presented the lowest activity, while the *Enterococcus* presented intermediate activity. Similar results have been reported for these genera. Two strains of *Pediococcus acidilactici* HA-6111-2 and HA-5692-3 were isolated from alheira and showed 1600 AU/mL of antimicrobial activity against *Listeria innocua* N27 (Albano et al. 2007). Another study reported higher activity (6400 AU/mL) for the previously mentioned *P. acidilactici* HA-6111-2 under high pressure (Castro et al. 2015). Cavicchioli et al. (2017) isolated *Enterococcus hirae* ST57ACC and *P. pentosaceus* ST65ACC from Minas cheese; these two bacteriocinogenic strains showed antimicrobial activity against 101 different strains of *Listeria* spp., 8 *Enterococcus* spp., 9 *Lactobacillus* spp., 1 *Leuconostoc* spp., 2 *Pediococcus* spp., and 2 *Streptococcus* spp. In another study, *P. pentosaceus* FBBGl (ATCC 43200) presented antimicrobial activity of 3200 AU/mL (Piva and Headon 1994). *Pediococcus* strains isolated in this study presented activity of 51,200 AU/mL, recorded against *L. monocytogenes* 104. *E. faecalis* 54, *E. faecalis* 87, and *E. faecalis* 91 showed activity of 3200 AU/mL, recorded against *L. monocytogenes* 104. Activity of *E. faecium* SD1, SD2, SD3, and SD4 strains, isolated from goat's milk, was reported as 51,200 AU/mL for strains SD1 and SD2, 3200 AU/mL for SD3, and 800 AU/mL for SD4 (Schirru et al. 2012). Casaburi et al. (2016) described activity of 6400 AU/mL for *Lactobacillus curvatus* 54 M16, isolated from traditional fermented sausages of Campania region (Italy). In the present study, activities of 200, 800, and 3200 AU/mL were reported for *Lb. plantarum* 56, *Lb. rhamnosus* 70, and *Lb. plantarum* 127, respectively. Similar results of 800 AU/mL were reported for *Lb. rhamnosus* EM253 (dos Santos et al. 2015) and less than 800 AU/mL for *Lb. plantarum* HKN01 isolated from dairy products (Sharafi et al. 2013). However, these levels of activity may be unreliable, since bacteriocin activity depends on the specificity of the expressed antibacterial protein and on the specific characteristics of the microorganisms investigated. The optimal scenario would be if the same test microorganisms were used in all studies, which would facilitate comparison of the investigated bacteriocins.

Table 2 Antimicrobial activity (AU/mL) of isolates recorded against *L. monocytogenes* 104

Isolates	AU/mL[a]
Enterococcus faecalis 54	3200
Lactobacillus plantarum 56	200
Pediococcus pentosaceus 63	51,200
Lactobacillus rhamnosus 70	800
Enterococcus faecalis 87	3200
Enterococcus faecalis 91	3200
Lactobacillus plantarum 127	3200
Pediococcus pentosaceus 145	51,200
Pediococcus pentosaceus 146	51,200
Pediococcus pentosaceus 147	51,200

[a] All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%, and single data points are presented in the table without standard deviation

Screening for the presence of bacteriocin genes in total DNA

When total DNA was screened for presence of genes related to bacteriocin production, positive results were only generated for the presence of pediocin PA-1 gene in DNA obtained from *P. pentosaceus* 63, 145, 146, and 147 strains. There was no evidence of the presence of genes related to nisin, enterocin A, enterocin B, enterocin L50B, enterocin P, plantaricin NC8, plantaricin S, or plantaricin W. Figure 1 shows the bands obtained with the *Pediococcus* strains using the primer to amplify the gene of PA-1 (1044 bp).

P. pentosaceus 63, *P. pentosaceus* 145, *P. pentosaceus* 146, and *P. pentosaceus* 147 harbor a 1044 bp fragment corresponding to that reported for pediocin PA-1 (Fig. 1). The size of the obtained amplicon was consistent with that reported for

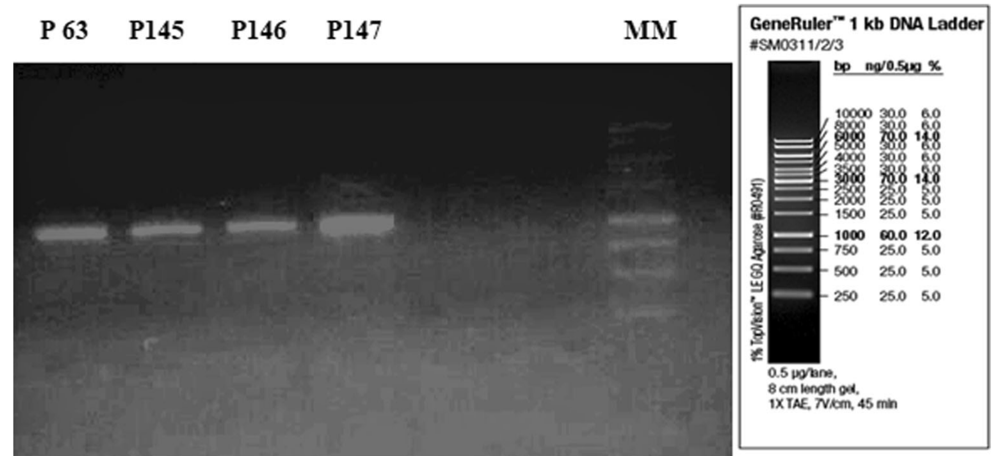

Fig. 1 Amplification of total DNA from *Pediococcus* strains using a primer of PA-1 gen. P63 *P. pentosaceus* 63, P145 *P. pentosaceus* 145, P146 *P. pentosaceus* 146, P147 *P. pentosaceus* 147

pediocin PA-1 by Marugg et al. (1992). Pediocin PA-1 biosynthesis involves a DNA fragment of approximately 3.5 kb with the presence of four genes pedA, pedB, pedC, and pedD (Marugg et al. 1992). However, amplicon sequencing can confirm the fact that *P. pentosaceus* 63, *P. pentosaceus* 145, *P. pentosaceus* 146, and *P. pentosaceus* 147 studied are producers of pediocin PA-1.

Effect of enzymes, temperature, pH, and surfactants on bacteriocin activity

All tests were performed with CFS from each strain in MRS broth incubated at 37 °C for 24 h and pH was corrected (pH 6.5) each time. Table 3 shows percentage reduction of activity for each isolate. CFS from *P. pentosaceus* 63 lost at

Table 3 Percentages of reduction of activity after different treatments

		P. pentosaceus 63	*P. pentosaceus* 145	*P. pentosaceus* 146	*P. pentosaceus* 147
Enzymes	Proteinase K	53	95	95	94
	Papain	95	95	50	50
	Pepsin	53	63	95	94
	Lipase	74	47	45	39
	Catalase	37	42	50	39
	α-Amylase	32	42	45	33
Chemicals	NaCl	16	11	15	17
	SDS	0	0	0	0
	Tween 80	32	26	25	0
	Triton X-100	11	11	0	0
	Skim milk	21	21	25	22
pH	2	21	16	20	11
	4	21	0	15	0
	6	11	11	15	0
	8	11	21	25	6
	10	16	16	20	6
Temperatures	25	37	32	35	17
	30	37	32	35	28
	37	32	32	30	22
	60	32	32	35	22
	80	21	32	30	28
	100	21	26	30	22
	20 min at 121 °C	32	32	35	28

All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%, and single data points are presented in the table without standard deviation

least 50% of its activity by treatment with proteinase K, and pepsin. Papain produced a reduction almost of the 100% and lipase 74%. An antimicrobial activity reduction of 95% of CFS from *P. pentosaceus* 145 was produced by proteinase K and papain, less reduction was obtained with pepsin (63%). Lipase, catalase and α-amylase produced a reduction almost of the 50%. *P. pentosaceus* 146 and *P. pentosaceus* 147 lost almost 100% of their activity with proteinase K and pepsin and the rest of the enzymes caused 50% or least reduction. The effect of α-amylase was very low for all isolates. The CFS of each strain presented a small partial loss of activity at 25, 30, and 37 °C, remaining active after 1 h at 60, 80, and 100 °C, also with the treatment at 121 °C for 20 min. This heat tolerance, characteristic of the bacteriocins, obeys to their small size and makes them a good option as biopreservatives in foods ((Karumathil et al. 2016; Parada et al. 2007). Similar results have been reported by different authors (Todorov and Dicks 2005a; Albano et al. 2007; Murua et al. 2013; Seo et al. 2014). Low pH, such as 2.0 and 4.0, had little effect on antimicrobial activity, as did pH 8.0. Ghanbari et al. (2013) reported this tolerance to low pH with bacteriocins produced by *Lb.* casei AP8 and *Lb. plantarum* H5, isolated from the intestinal bacterial flora of beluga (*Huso huso*) and Persian sturgeon (*Acipenser persicus*); inactivation at pH 10.0 was reported to be due to proteolytic degradation, protein aggregation, and instability of proteins.

Treatment with Triton X-100, Tween 80, SDS, NaCl, or skimmed milk had no significant effect on the antimicrobial activity. Sharafi et al. (2013) reported the lack of effect of these treatments in bacteriocins from *Lb. plantarum* HKN01 isolated from Iranian traditional dairy products. Todorov and Dicks (2005a, b) reported that pediocin ST18 produced by *Pediococcus pentosaceus* ST18, isolated from boza (a cereal-fermented non-alcoholic beverage from Bulgaria), was not sensitive to SDS, Tween 20, Tween80, urea, *N*-lauroylsarcosine, or Triton X-100. The effect of different chemicals, pH, and temperature is dependent on the specific structure and amino acid sequence of the bacteriocins studied. Moreover, these results may have a practical application in subsequent experiments, including in their planning, and investigations of bacteriocin use in food biopreservation.

Adsorption on the cell surface of producer cells

Secretion of the bacteriocins normally is performed via ABC transporter system or sec-dependent (Cintas et al. 2000; Kumar et al. 2011). Yang et al. (1992) showed that some bacteriocins can be secreted and then be adsorbed onto the cell surface of the producer cells. This adsorption could be a result of some affinity or because of charge-specific interaction. High levels of adsorbed bacteriocins on the cell surface of producer cells could be considered an opportunity to facilitate the purification process of produced bacteriocins, and this was applied by Yang et al. (1992). However, in the case of the bacteriocins studied here, only low levels were found to be adsorbed on the surface of *P. pentosaceus* 63, *P. pentosaceus* 147, *P. pentosaceus* 146, and *P. pentosaceus* 147 (Fig. 2). This was found to be the case for most of the investigated bacteriocins. For instance, similar results were reported for bacteriocins produced by *Lactococcus lactis* subsp. *lactis* B14 isolated from boza (Ivanova et al. 2000) and for bacteriocin bacST8KF produced by *L. plantarum* ST8KF isolated from

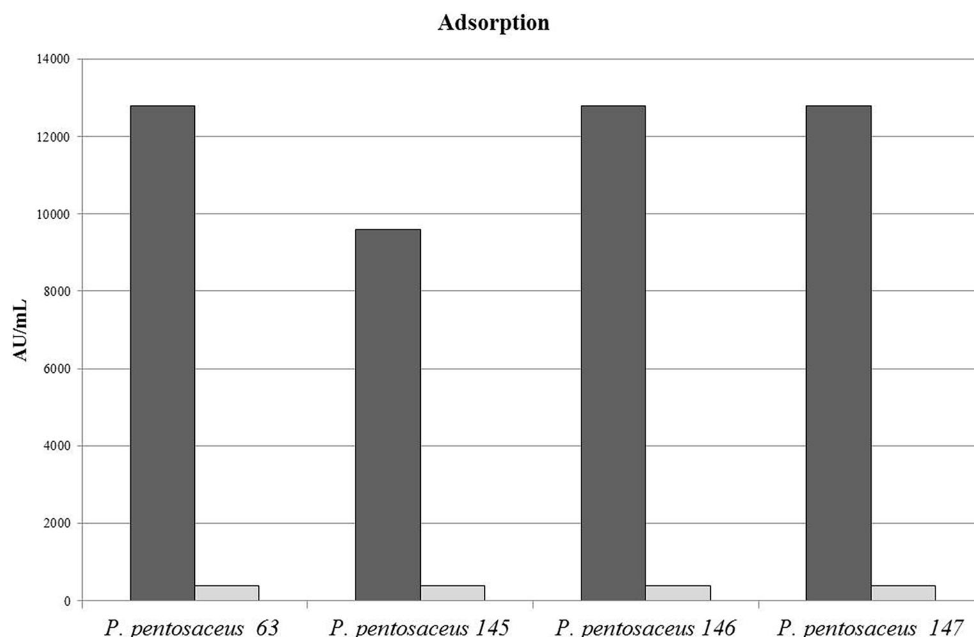

Fig. 2 Adsorption of the bacteriocins produced to the own surfaces of the studied strains of *Pediococcus*. Antimicrobial activity (AU/mL) of the isolates. Light gray column: recovered from cell surface (after desadsorption) and black column: in cell-free supernatant. Titles presented reduction along the development of the study. All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%, and single data points are presented in the figures without standard deviation bars

kefir (Powell et al. 2007). Two bacteriocins from *Lb. curvatus* and *Lb.* sakei, isolated from salpicao, a traditional fermented pork sausage produced in Portugal, also presented low levels of bacteriocin adsorption onto the cell surface of producer cells (Todorov et al. 2013).

Growth dynamics and bacteriocin production

Figure 3 shows the relationship between bacterial growth of the selected strains and produced bacteriocin with activity against *L. monocytogenes* 104 during a 24-h period of culture in MRS broth at 37 °C. *P. pentosaceus* 63 reached its stationary phase at 15 h with OD600 of 4.82. Antimicrobial activity was reported early in the exponential growth phase (3 h), with bacteriocin levels of 1600 AU/mL and at 6 h increased to 3200 AU/mL. Maximum activity was recorded after 9 h of incubation (12,800 AU/mL) and remained stable until 24 h, with an OD600 of 4.692 (Fig. 3a). Antimicrobial activity of bacteriocin produced by *P. pentosaceus* 145 started during the exponential phase, with 1600 AU/mL and OD600 of 0.298 at 3 h; maximum activity (25,600 AU/mL) was after the beginning of the stationary phase after 12 h of incubation, with OD600 of 3.368; at 24 h, OD600 was 3.49 (Fig. 3b). Similar dynamics were observed with *P. pentosaceus* 146, presenting 25,600 AU/mL at 24 h and OD600 of 3.588 (Fig. 3c). *P. pentosaceus* 147 reached maximum activity in the middle of the exponential phase with OD600 of 1.13 and continued until 24 h with OD600 of 3.58 (Fig. 3d). The results are according to other studies that report optimal production on stationary phase, for example, of bacteriocins EM485 and EM925 (produced by *E. faecium* EM485 and *E. faecium* EM925 isolated from Brazilian cheese) (dos Santos et al. 2014) and bacteriocins produced by *E. faecium* ET05, ET12, and ET88 isolated from smoked salmon that were produced during stationary growth (Tomé et al. 2009). Maximal production occurs during the stationary phase, which suggests that bacteriocins are secondary metabolites, according to other studies (Albano et al. 2007). Another study reported pediocin PD-1 production by *P. damnosus* NCFB 1832 during logarithmic phase (1600 AU/mL) and an increment during the stationary phase (Nel et al. 2001). *P. acidilactici* P9, isolated from pickles, started production at 8 h and, during the stationary phase (after 16 h of incubation), reached maximum production, and remained constant until 24 h of incubation (Wang et al. 2014). For strains of *Lactobacillus* spp., production was reported during the logarithmic phase of growth, as in the case of *Lb. plantarum* ST71KS, with maximum production (6400 AU/mL) during

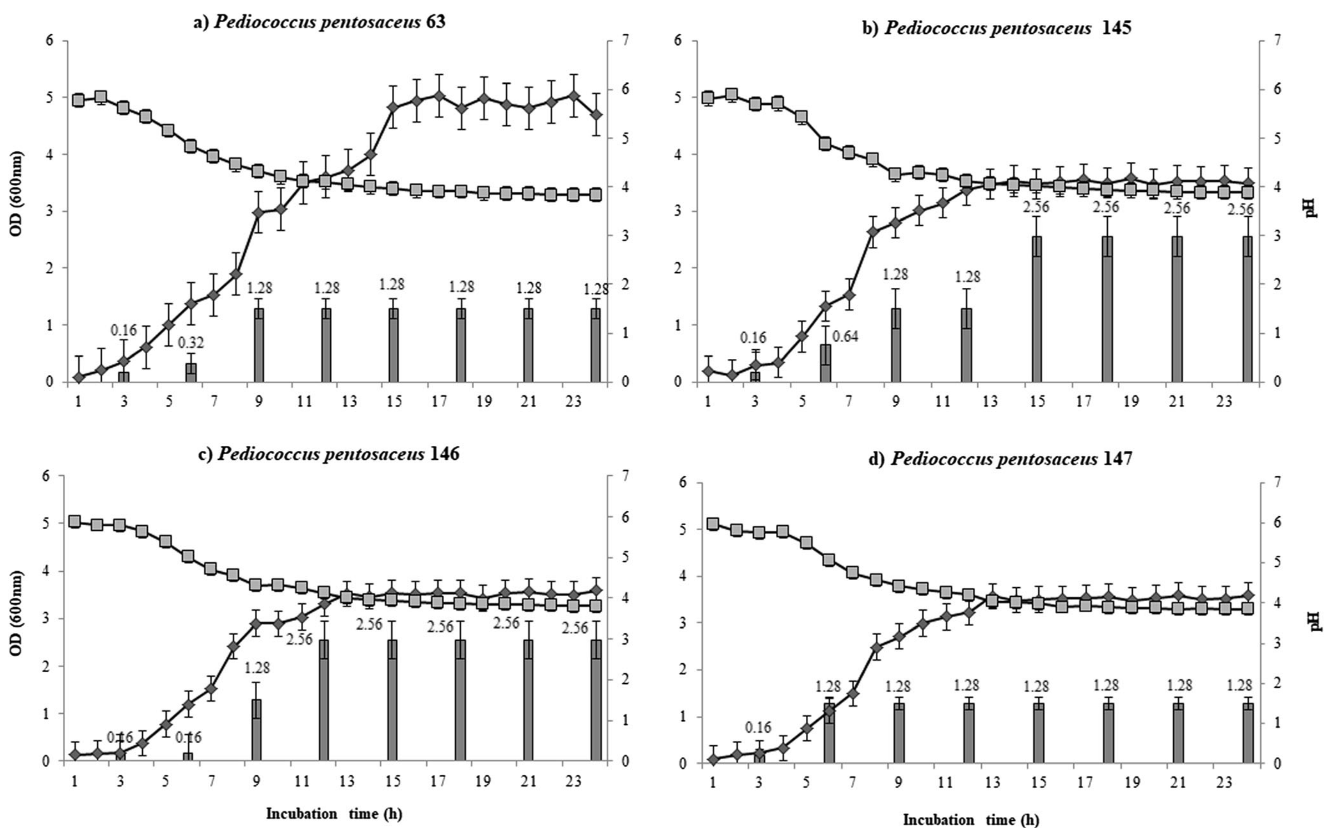

Fig. 3 Growth dynamics. **a** *P. pentosaceus* 63. **b** *P. pentosaceus* 145. **c** *P. pentosaceus* 146. **d** *P. pentosaceus* 147. Optical density (OD600) (filled diamond); pH (filled square). Bars represent antimicrobial activity (kAU/mL) (1 kAU/mL = 1000 AU/mL)

the stationary phase (Martinez et al. 2013), similar results were reported for *Lb. curvatus* 54 M16 (Casaburi et al. 2016).

Growth of *Listeria monocytogenes* 104 in the presence of CFS

Visualization of the effect of bacteriocin containing CFS on actively growing *L. monocytogenes* 104 is presented in Fig. 4. After 3 h of incubation of *L. monocytogenes* 104, values of OD600 reached an average of 0.64. The addition of bacteriocin containing CFS of *P. pentosaceus* 63, *P. pentosaceus* 145, *P. pentosaceus* 146, and *P. pentosaceus* 147 to the *L. monocytogenes* 104 actively growing in culture resulted in growth inhibition after 1 h (hour 4, Fig. 4) with *P. pentosaceus* 63 seeing almost no change of OD600 from 0.511 (hour 3) to 0.585 (hour 4), the same happened with *P. pentosaceus* 146 (OD600 from 0.574 to 0.594) and *P. pentosaceus* 147 (OD600 from 0.749 to 0.620). *P. pentosaceus* 145 allowed initial growth of *L. monocytogenes* 104, and the values of OD600 were very close to the control (without CFS). However, 2 h after the addition of CFS, growth was limited and similar OD600 values were observed in all cases and remained at this level. The control reached a maximum OD600 after 8 h of incubation (6.128); this decreased to 4.296 at the end of the test. The results suggest that CFS of isolates can inhibit growing cultures of *L. monocytogenes* 104. Similar results have been reported for *P. pentosaceus* ST65ACC against *L. monocytogenes* 211 and *L. monocytogenes* 422 (Cavicchioli et al. 2017) and for *L. casei* AP8 isolated from sturgeon fish against *L. monocytogenes* ATCC 19115 (Ghanbari et al. 2013).

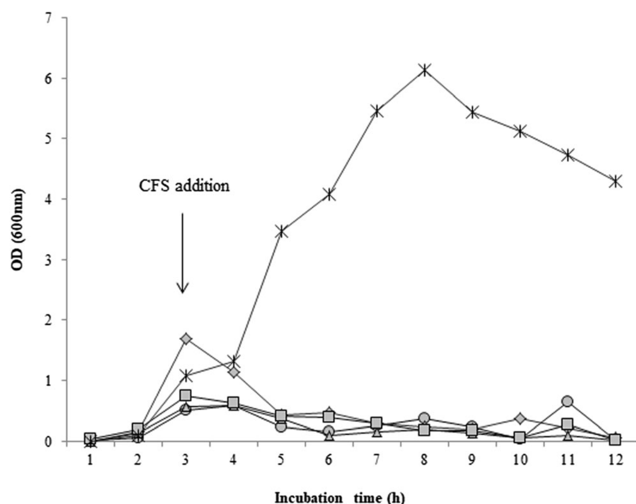

Fig. 4 Growth kinetics of *L. monocytogenes* 104 on BHI with added CFS of the studied *P. pentosaceus* strains. Optical density (at 600 nm) measurements of the medium with the following: circle: *P. pentosaceus* 63, diamond: *P. pentosaceus* 145, triangle: *P. pentosaceus* 146, square: *P. pentosaceus* 147, and asterisk: control without CFS. All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%, and single data points are presented in the figures without standard deviation bars

Adsorption onto target cell

The aim of this test is to find how much bacteriocin was able to bind to the target cell surface comparing antimicrobial activity before and after contact of CFS with target cells. Bacteriocin adsorption is considered as first step for bacteriocin mode of action. This information about potential efficacy of the bacteriocin and its ability to bind on the surface is important for the technological applications of bacteriocins exploration. CFS from *Pediococcus* strains were incubated with *Lb. sakei* and *E. faecalis* during 1 h on 5 mM phosphate buffer, a short time and poor nutritional conditions that did not allow the target strains to produce bacteriocins to interfere with the test. Figure 5 shows the effect of different conditions on the adsorption of bacteriocins onto *L. monocytogenes* 104, *E. faecalis* ATCC 19443, and *Lb. sakei* ATCC 15521. Under natural conditions (pH 6.5 and 25 °C), the highest adsorption for *P. pentosaceus* 63, *P. pentosaceus* 145, *P. pentosaceus* 146, and *P. pentosaceus* 147 was with *L. monocytogenes* 104 (98.4, 96.9, 96.9, and 98.4%, respectively). Adsorption onto *E. faecalis* ATCC 19443 surface was 93.8% for all isolates, except *P. pentosaceus* 145 which presented a lower value (87.5%). *P. pentosaceus* 63 showed 96.9% of adsorption to *Lb. sakei* ATCC 15521, and *P. pentosaceus* 145 and *P. pentosaceus* 146 presented 93.8%. The lowest adsorption value was for *P. pentosaceus* 147 with 70%.

Very low influence of temperature over adsorption of bacteriocins was observed in tests with *L. monocytogenes* 104. An increase in adsorption of *P. pentosaceus* 63 and *P. pentosaceus* 146 at 37 °C onto *E. faecalis* ATCC 19443 and a reduction at 4 °C were observed. For *Lb. sakei* ATCC 15521, *P. pentosaceus* 63, *P. pentosaceus* 145, and *P. pentosaceus* 146, the lowest adsorption was at 37 °C and 4 °C, and for *P. pentosaceus* 147, the lowest adsorption was at 25 °C. Low pH affected the adsorption of all isolates onto *L. monocytogenes* 104. The same effect occurred with *E. faecalis* ATCC 19443 and, at pH 10.0, adsorption also decreased. Adsorption onto *Lb. sakei* ATCC 15521 increased at low pH with *P. pentosaceus* 63 and *P. pentosaceus* 146 and decreased with *P. pentosaceus* 147. *P. pentosaceus* 146 had decreased adsorption onto *Lb. sakei* ATCC 15521 at pH 8.0. Percentage of adsorption of all isolates decreased in the presence of chemicals. SDS was the chemical that most affected adsorption onto target cells, especially onto *L. monocytogenes* 104. Glycerol only affected adsorption onto *L. monocytogenes* 104. Adsorption onto *E. faecium* ATCC 19443 was affected by all chemicals, except glycerol with *P. pentosaceus* 63 and *P. pentosaceus* 145.

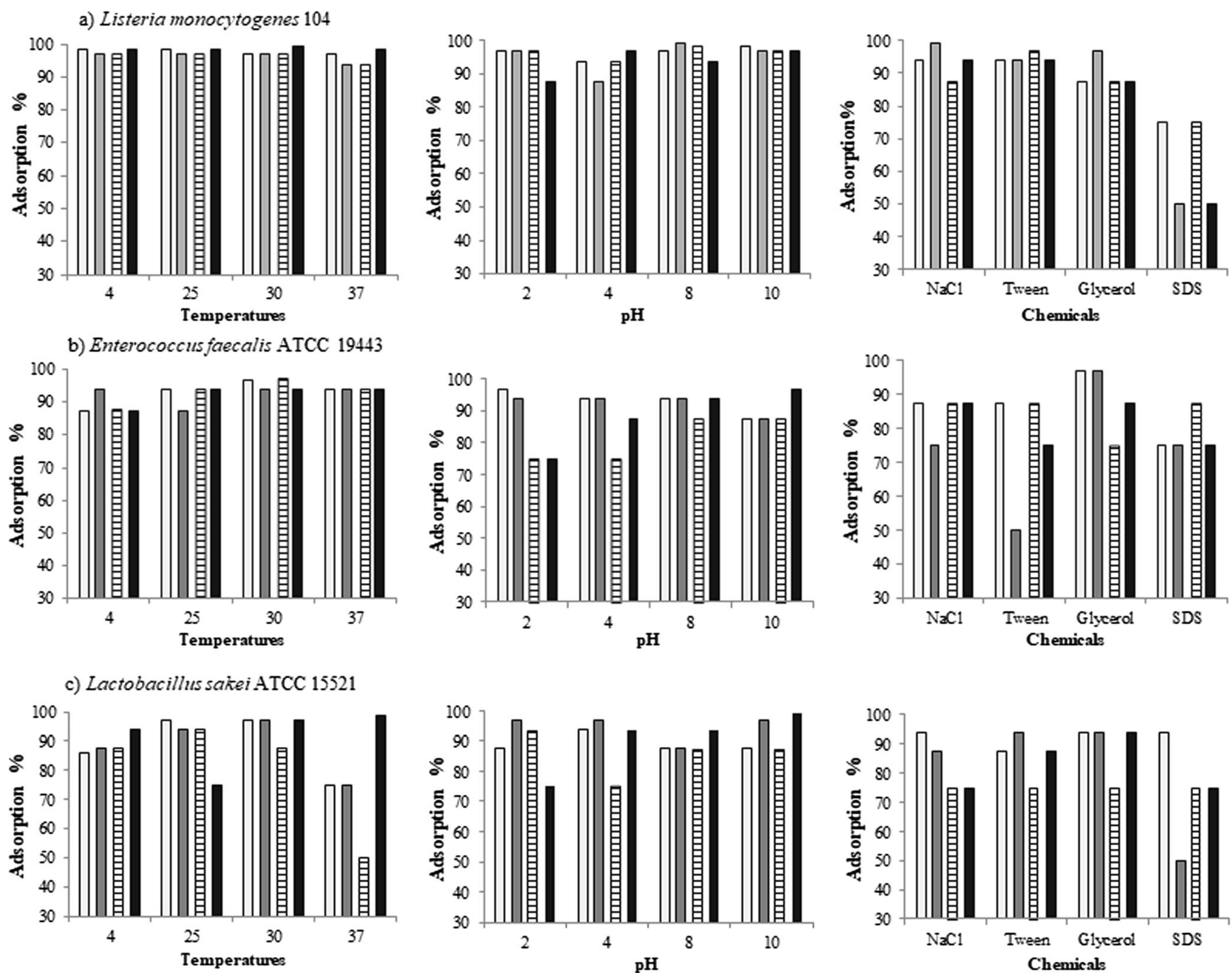

Fig. 5 Percentage of adsorption of bacteriocins onto target cells under different treatments of the CFS. **a** *L. monocytogenes* 104. **b** *E. faecalis* ATCC 19443. **c** *Lb. sakei* ATCC 15521. □: *P. pentosaceus* 63, ■: *P. pentosaceus* 145; ■: *P. pentosaceus* 146, ≡: *P. pentosaceus* 147

It is important to note that different conditions common in the food industry can affect the ability of bacteriocins to bind to the microorganism surface; nevertheless, the results reported demonstrate the high affinity of bacteriocins for target cells and indicate that bacteriocins have a potential use in industry for controlling growth of microorganisms because they showed that bacteriocins continue active. Other studies have also investigated the effect of pH, temperature, and chemical agents and concluded that effect of temperature is minimal, similar to pH with values close to neutrality, and that chemicals may affect adsorption the most (Biscola et al. 2013; Furtado et al. 2014).

Effect of medium composition on the production of bacteriocins

Table 4 shows the results of bacteriocin production, expressed in arbitrary units per milliliter, with each modified MRS broth. *P. pentosaceus* 63 produced the maximum antimicrobial activity using dextrose and maltose as carbon sources (12,800 and 25,600 AU/mL, respectively); with raffinose and mannitol, production was minimal. Antimicrobial activity of 6400 AU/mL was obtained using peptone, meat extract, and yeast extract; in combination, these generated 128,000 AU/mL. Without K_2HPO_4 or with K_2HPO_4 at more than 2 g/L, production decreased to 6400 AU/mL. The absence of $MnSO_4$ or $MnSO_4$ at more than 0.05 g/L also caused decreased bacteriocin activity by *P. pentosaceus* 63. The same was observed with different concentrations of sodium acetate. The absence of $MgSO_4$ and glycerol had no effect, and bacteriocin production was 12,800 AU/mL. High amounts of Tween 80 had no effect on production, but its absence caused a decrease. The absence of ammonium citrate had no effect on bacteriocin production, but a high amount increased production. Extreme pH decreased the production of antimicrobial compound by *P. pentosaceus* 63.

Table 4 Antimicrobial activity (AU/mL) on different modified MRS

Media		UA/mL			
	g/L	*P. pentosaceus* 63	*P. pentosaceus* 145	*P. pentosaceus* 146	*P. pentosaceus* 147
Lactose	20.0	1600	1600	800	3200
Sucrose	20.0	1600	800	400	0
Mannitol	20.0	800	200	400	0
Dextrose	20.0	12,800	12,800	12,800	12,800
Fructose	20.0	6400	6400	12,800	6400
Maltose	20.0	25,600	12,800	12,800	12,800
Raffinose	20.0	400	400	200	0
Peptone	25.0	6400	6400	6400	3200
Meat extract	25.0	6400	6400	12,800	6400
Yeast extract	25.0	6400	6400	12,800	6400
Peptone	12.5	6400	6400	12,800	6400
Meat extract	12.5				
Peptone	15.0	6400	6400	12,800	6400
Yeast extract	7.5				
Meat extract	15.0	6400	12,800	6400	6400
Yeast extract	7.5				
Peptone	10.0	12,800	12,800	12,800	12,800
Meat extract	10.0				
Yeast extract	5.0				
K_2HPO_4	0	6400	6400	6400	6400
	2.0	12,800	12,800	12,800	12,800
	5.0	6400	6400	6400	3200
	10.0	6400	6400	3200	3200
$MgSO_4$	0	12,800	6400	6400	6400
	0.1	6400	12,800	12,800	12,800
	0.5	6400	6400	6400	6400
$MnSO_4$	0	6400	400	12,800	6400
	0.05	12,800	12,800	12,800	12,800
	0.2	6400	400	6400	6400
Sodium acetate	0	6400	400	6400	6400
	5.0	6400	6400	3200	6400
	10.0	6400	400	12,800	6400
Ammonium citrate	0	6400	400	6400	6400
	2.0	6400	6400	12,800	12,800
	5.0	12,800	400	6400	6400
Tween 80	0	3200	400	6400	1600
	1.0	12,800	12,800	12,800	12,800
	2.0	12,800	400	12,800	6400
	5.0	12,800	400	12,800	6400
pH	2	400	400	800	200
	4	6400	400	6400	6400
	6	12,800	12,800	12,800	12,800
	8	6400	400	6400	6400
	10	3200	400	3200	3200
	12	0	0	0	0
Glycerol	0	12,800	25,600	6400	12,800
	0.5	6400	12,800	6400	12,800
	1.0	6400	12,800	6400	6400
	2.0	6400	6400	6400	6400
	5.0	6400	6400	6400	6400
	10.0	6400	6400	6400	3200

All data represent an average of three repeats. The values recorded in each experiment did not vary by more than 5%, and single data points are presented in the table without standard deviation

P. pentosaceus 145 also produced the maximum of antimicrobial activity (12,800 AU/mL) using dextrose and maltose as carbon source. Maximum production (12,800 AU/mL) was obtained with a mixture of yeast (15 g/L) and meat extract (7.5 g/L) or a mixture of peptone (10 g/L), meat extract (10 g/L), and yeast extract (5 g/L) as nitrogen sources. The amount of K_2HPO_4 and $MnSO_4$ had no effect on production and changes in sodium acetate, ammonium citrate, and Tween 80 decreased production. Only pH 6.0 of the range of pH values tested registered antimicrobial activity (6400 AU/mL). The absence of glycerol increased production. *P. pentosaceus* 146, in addition to a preference for dextrose and

maltose, exhibited antimicrobial activity of 12,800 AU/mL with fructose as the carbon source. The use of peptone alone or meat and yeast mixture caused decrease in production until (6400 AU/mL). Increasing amounts of K_2HPO_4 or absence of K_2HPO_4 caused decrease in antimicrobial activity; similar effects occurred with 0.05 g/mL of $MgSO_4$ or its absence. $MnSO_4$ in 0.05 g/L favored bacteriocin production and the opposite occurred with sodium acetate. The absence of Tween 80 decreased antimicrobial activity and the same occurred with extreme pH (2.0 or 12). *P. pentosaceus* 147 had no activity when sucrose, mannitol, or raffinose was used as the carbon source, and a mixture of peptone (10 g/L), meat extract (10 g/L), and yeast extract (5 g/L) generated maximum activity. The absence of K_2HPO_4 or ammonium citrate or more than 2 g/L of each of these chemicals caused decreased antimicrobial activity; the same occurred without $MgSO_4$ or with $MgSO_4$ at more than 1 g/L and without $MnSO_4$ or with $MnSO_4$ at more than 0.05 g/L. Different amounts of sodium acetate generated the same activity (6400 AU/mL). Less or more than 1 g/mL of Tween 80 added to the broth caused a decrease in activity, as well as extreme pH values.

In all cases, extremely, pH limited bacterial growth generating very low or no antimicrobial activity. Similar results have been reported in a study of optimization of bacteriocin ST22Ch production by *Lb. sakei* isolated from salpicao in which glucose, as the carbon source, was found to promote production of the antimicrobial substance. The same study reported that a combination of different sources of nitrogen (meat and yeast extract or tryptone and meat extract) stimulated production. The same happened with high concentrations of $MgSO_4$ and Tween 80. The absence of $MgSO_4$ decreased production, and the presence of glycerol had no effect (Todorov et al. 2012). Another study reported that optimal production of *P. acidilactici* LAB5 isolated from a fermented meat product was obtained with a mixture of tryptone, yeast extract as a nitrogen source, glucose as a carbon source, and a buffer composed of sodium citrate, sodium acetate, and K_2HPO_4 (0.2 g/L of each) (Mandal et al. 2008). Suganthi and Mohanasrinivasan (2015) used a process of optimization to obtain maximal production (25,600 AU/mL) of the bacteriocin from *P. pentosaceus* KC692718, isolated from mixed vegetable pickles (India), using sucrose (24 g/L) as a carbon source and soyatone (10.3 g/L) as a nitrogen source. Kaur et al. (2013) enhanced pediocin BA28 production by *P. acidilactici* using peptone (10 g/L), beef extract (10 g/L), meat extract (10 g/L), tryptone (10 g/L), KH2PO4 (2 g/L), potassium sodium tartrate (2 g/L), dextrose (50 g/L), and Tween 80 0.1 g/L.

Partial bacteriocin purification and determination of approximate molecular mass by SDS-PAGE

Precipitation with 80% ammonium sulfate saturation was successful in obtaining all antimicrobial proteins produced by the investigated strains. However, when proteins were separated using SepPack chromatography, almost all fractions presented activity against *L. monocytogenes* 104. Nevertheless, the most active of the isopropanol-eluted fractions was with 60% isopropanol presenting activities of 25,600, 12,800, 5600, and 25,600 AU/mL, respectively, for *P. pentosaceus* 63, *P. pentosaceus* 145, *P. pentosaceus* 146, and *P. pentosaceus* 147. Miteva et al. (1998) reported a difference with this study with activity of 50% fractions obtained with a strain of *Lactobacillus* spp. 1043 against Gram-positive and Gram-negative indicator strains.

The results presented in Fig. 6, representing the Tricine-SDS-PAGE gel, indicate that the approximate molecular weight of the bacteriocins studied was between 3.5 and 6.5 kDa. The antimicrobial activity was confirmed by inhibition zones against both *L. monocytogenes* 104 in the same place as the proteins bands. Similar weights of peptides were reported for bacteriocin PA-1 produced by *P. pentosaceus* NCDC 273 (Vijay Simha et al. 2012); for pediocin ST71KS produced by *Lb. plantarum* ST71KS, isolated from

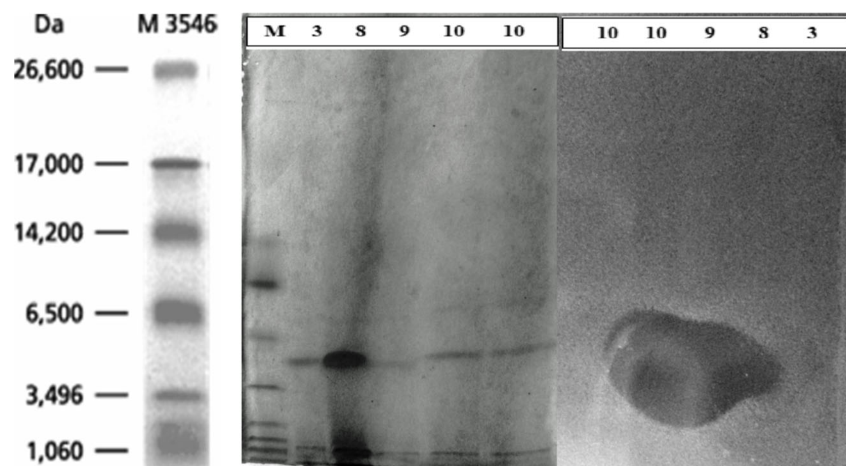

Fig. 6 Separation of the proteins obtained after precipitation by ammonium sulfate and separation by SepPack and subjected to SDS-PAGE electrophoresis. Stained electrophoresis gel (left) and inhibition zone observed using *L. monocytogenes* 104 as indicator strain with the non-stained electrophoresis gel (right)

homemade goat feta cheese (Martinez et al. 2013); for pediocin ST44AM produced by *P. pentosaceus* ST44AM (Todorov and Dicks 2009); and for bacteriocins BacHA-6111-2 and bacHA-5692-3 produced by strains of *P. acidilactici* (Albano et al. 2007).

Conclusions

LAB isolated from dairy products are a good alternative for obtaining antimicrobial substances such as bacteriocins. LAB that occur naturally in dairy products generally belong to species with well-proven GRAS status. However, additional research is required to confirm safety aspects of isolated LAB in order to recommend their application or their expressed bacteriocins as non-hazardous agents in food production. Although bacteriocins are recognized to be non-toxic proteinaceous molecules, their safety needs to be carefully examined prior to their use as food additives or therapeutic agents. Biochemical characteristics of bacteriocins allow better design for their possible application in the food industry. Pediocins have been reported to be a good option for food biopreservation, instead of conventional treatments used to preserve food products (Papagianni and Anastasiadou 2009). In our study, strains isolated from Minas cheese presented remarkable antimicrobial activity against three *L. monocytogenes* strains from different serological groups. Based on the specific characteristics of the bacteriocins studied, produced by four *P. pentosaceus* strains, it is necessary to be conducted a future research in order to explore the possibilities of the application of the strains as protector cultures or the expressed bacteriocins in the control of food spoilage in fermented food products.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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