Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal

Helena Albano [a,1], Svetoslav D. Todorov [b,2], Carol A. van Reenen [b,2], Tim Hogg [a,1], Leon M.T. Dicks [b,*], Paula Teixeira [a,1]

[a] *Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[b] *Department of Microbiology, Stellenbosch University, Stellenbosch, Private Bag X1, Matieland 7602, South Africa*

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Abstract

Lactic acid bacteria were isolated from "Alheira" sausages that have been sampled from different regions in Portugal. The sausages were produced according to different recipes and with traditional starter cultures. Two isolates (HA-6111-2 and HA-5692-3) from different sausages were identified as strains of *Pediococcus acidilactici*. Each strain produces a bacteriocin, designated as bacHA-6111-2 and bacHA-5692-3. Both bacteriocins are produced at low levels after 18 h of growth in MRS broth (3200 AU/ml against *Enterococcus faecium* HKLHS and 1600 AU/ml against *Listeria innocua* N27). BacHA-6111-2 and bacHA-5692-3 are between 3.5 kDa and 6.5 kDa in size, as determined by tricine-SDS-PAGE. Complete inactivation or significant reduction in antimicrobial activity was observed after treatment of cell-free supernatants with proteinase K, pronase and trypsin. No change in activity was recorded when treated with catalase. Both bacteriocins are sensitive to treatment with Triton X-114 and Triton X-100, but resistant to Tween 20, Tween 80, SDS, Oxbile, NaCl, urea and EDTA. The bacteriocins remained stable after 2 h at pH 6.0. A decrease in antibacterial activity was recorded after 60 min at 100 °C. After 60 min at 80 °C, 60 °C and 25 °C the antibacterial activity against *L. innocua* N27 decreased by 25%. Addition of bacHA-6111-2 and bacHA-5692-3 (1600 AU/ml) to a mid-log (5-h-old) culture of *L. innocua* N27 inhibited growth for 7 h. Addition of the bacteriocins (3200 AU/ml) to a mid-log (5-h-old) culture of *E. faecium* HKLHS repressed cell growth. The bacteriocins did not adhere to the surface of the producer cells. Both strains contain a 1044 bp DNA fragment corresponding in size to that recorded for pediocin PA-1. Sequencing of the fragments from both bacteriocins revealed homology to large sections of *pedA* (188 bp), *pedB* (338 bp) and *pedC* (524 bp) of pediocin PA-1 and the bacteriocins are considered similar to pediocin PA-1.

Introduction

Fermented meat products are part of the daily diet in rural areas of Portugal and have become very popular in urban centres. "Alheira" is a traditional fermented meat product typical of the Northern regions (Trás-os-Montes) in Portugal. The specific characteristics of the product depend on the raw materials used, the agro-ecosystem of the production area, and the traditional production methods used. "Alheira" is produced from a combination of pork meat, pork lard, poultry, wheat bread and olive oil mixed with salt, garlic and spices. The paste is stuffed in casings made from animal products and is smoked for a maximum of 8 days. "Alheira" contains mainly lactic acid bacteria and *Micrococcaceae*. *S*ome pathogens, such as *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus* are occasionally present (Ferreira et al., 2006).

Selected strains of lactic acid bacteria, used as starter cultures, may inhibit spoilage microorganisms by production

* Corresponding author. Tel.: +27 21 808 5849; fax: +27 21 808 5846.
E-mail addresses: lmtd@sun.ac.za (L.M.T. Dicks), paula@esb.ucp.pt (P. Teixeira).
[1] Tel.: +351 225 580 001; fax: +351 225 580 111.
[2] Tel.: +27 21 808 5849; fax: +27 21 808 5846.

of organic acids, hydrogen peroxide, diacetyl and bacteriocins. Strains of *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* spp. are the most commonly used starter cultures (Liepe, 1983; Stiles and Hastings, 1991). Although many strains of these species produce bacteriocins, only nisin produced by *Lactococcus lactis* subsp. *lactis* has GRAS (generally recognized as safe) status and has been approved by the USA FDA (Food and Drug Administration) as food preservative.

Pediococcus acidilactici, *Pediococcus pentosaceus* and *Pediococcus parvulus* isolated from meat produce various bacteriocins. Pediocin AcH (PA-1), produced by *P. acidilactici*, was the first thoroughly characterized class IIa bacteriocin (Bhunia et al., 1988; Pucci et al., 1988; Nieto-Lozano et al., 1992; Cintas et al., 1995). The same bacteriocin is also produced by strains of *P. parvulus* isolated from vegetables (Bennik et al., 1997) and a strain of *Lactobacillus plantarum* isolated from cheese (Ennahar et al., 1996; Loesner et al., 2003). Pediocin-like bacteriocins share 40 to 60% DNA homology (Eijsink et al., 1998) and are all active against *L. monocytogenes* (Aymerich et al., 1996; Cintas et al., 1998; Bennik et al., 1999; Guyonnet et al., 2000).

In this study, a class IIa pediocin-like bacteriocin produced by *P. acidilactici* HA-6111-2 and *P. acidilactici* HA-5692-3 isolated from "Alheira" is described.

Materialas and methods

Screening for bacteriocin-producing lactic acid bacteria

Lactic acid bacteria, isolated from "Alheira" sausages produced in different regions of Portugal and according to traditional recipes, were cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) and screened for bacteriocin production according to the method described by Van Reenen et al. (1998). The target strains and their growth media are listed in Table 1. Antimicrobial activity was expressed as arbitrary units (AU) per ml. One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (Van Reenen et al., 1998). Cell-free supernatants with antimicrobial activity were treated with proteinase K (1 mg/ml; Roche, Indianopolis, USA) to determine if activity is caused by the presence of a bacteriocin. All strains were stored at − 80 °C in the presence of 15% (v/v) glycerol.

Table 1
Growth medium and incubation temperature of indicator strains and spectrum of antimicrobial activity recorded for bacteriocins HA-6111-2 and HA-5692-3

Indicator strain	Origin	Growth media and temperature	HA-6111-2	HA-5692-3
Lactobacillus brevis 25, 54 [a]	"Alheira"	30 °C, MRS	−	−
Lactobacillus curvatus DF38 [b]	Salami	30 °C, MRS	−	−
Lactobacillus pentosus NCFB 363 [T c]	Unknown	30 °C, MRS	−	−
L. pentosus ST151BR, ST112BR [b]	Barley beer	30 °C, MRS	−	−
Lactobacillus paraplantarum ATCC 700211 [T d]	French beer	30 °C, MRS	−	−
Lactobacillus paracasei subsp. *paracasei* ST11BR [b]	Barley beer	30 °C, MRS	−	−
Lactobacillus plantarum AMA-K [b]	Amasi	30 °C, MRS	−	−
L. plantarum 423 [b]	Sorghum beer	30 °C, MRS	−	−
L. plantarum ST8KF [b]	Kefir	30 °C, MRS	−	−
L. plantarum ST13BR [b]	Barley beer	30 °C, MRS	−	−
Lactobacillus rhamnosus 50, 10, 78, 56 [a]	"Alheira"	30 °C, MRS	−	−
Lactobacillus sakei DSM 20017 [e]	Meat	30 °C, MRS	−	−
L. sakei 36 [a]	"Alheira"	30 °C, MRS	−	−
Lactococcus lactis subsp *lactis* HV219 [b]	Human vagina	30 °C, MRS	+	+
Leuconostoc mesenteroides 23 [a]	"Alheira"	30 °C, MRS	−	−
Listeria innocua LMG 13568 [f]	Bovine	37 °C, BHI	−	−
L. innocua N27 [g]	Cheese	37 °C, BHI	+	+
Listeria monocytogenes 4855 [a]	Unknown	37 °C, BHI	+	+
L. innocua 2030c, PHLS [a]	Unknown	37 °C, BHI	−	−
L. monocytogenes 7973 [a]	Unknown	37 °C, BHI	−	−
L. monocytogenes ScottA [a]	Smoked salmon	37 °C, BHI	+	+
L. monocytogenes 54 [a]	Unknown	37 °C, BHI	−	−
Listeria ivanovii subsp. *ivanovii* NCTC 11846 [h]	Unknown	37 °C, BHI	+	+
Enterococcus faecalis ATCC 29212 [d]	Urine	37 °C, BHI	−	−
E. faecalis FAIR E90 [b]	Piglets	37 °C, BHI	−	−
E. faecalis FAIR E80 [b]	Piglets	37 °C, BHI	−	+
E. faecalis FAIR E77, FA2 [b]	Piglets	37 °C, BHI	+	+
E. faecalis FAIR E88, FAIR E92 [b]	Piglets	37 °C, BHI	+/−	+/−
Enterococcus mundtii PTA-7278 [d]	Soy beans	37 °C, BHI	−	+
Enteroccocus faecium HKLHS [b]	Piglets	37 °C, BHI	+	+
Enterococcus sp. 9, 57 [a]	"Alheira"	37 °C, BHI	+	+
E. faecalis BFE1071 [b]	Piglets	37 °C, BHI	−	−
Escherichia coli NCTC 9001 [h]	Urine	37 °C, BHI	−	−
E. coli 0:157: H7 [a]	Unknown	37 °C, BHI	−	−
Staphylococcus aureus ATCC 29213 [d]	Wound	37 °C, BHI	−	−
Salmonella typhimurium [a]	Unknown	37 °C, BHI	−	−
Salmonella enteritidis NCTC 5188 [h]	Unknown	37 °C, BHI	−	−
Streptococcus caprinus ATCC 700065 [d]	Goat	37 °C, BHI	+	+
Streptococcus sp. TL1, TL2R, TL2W [b]	Goat	37 °C, BHI	+	+
Streptococcus macedonicus ST91KM [b]	Goat yogurt	37 °C, BHI	−	−

Notes to Table 1
− = no activity, + = zone larger than 2 mm, +/− = zone smaller than 2 mm.
[a] Escola Superior de Biotecnologia, UCP, Porto, Portugal.
[b] Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa.
[c] National Collection of Food Bacteria, Reading, UK.
[d] American Type Culture Collection, Manassas, VA, USA.
[e] Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.
[f] Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.
[g] Department of Microbiology, University of Western Cape, Cape Town, South Africa.
[h] National Collection of Type Cultures, Central Public Laboratory Service, London, UK.

Identification of bacteriocin-producing strains

Bacteriocin-producing strains were identified according to physiological and biochemical characteristics, as described by Schillinger and Lücke (1987). Further identification was by PCR with species-specific primers (PacF: CGA ACT TCC GTT AAT TGA TTA T and PuR: ACC TTG CGG TCG TAC TCC) according to the method described by Mora et al. (1997). Lambda DNA, digested with *Eco*R1 and *Hin*dIII (Roche, Indianopolis, USA) was used as marker.

Differentiation of the strains was by random amplification of polymorphic DNA (RAPD) PCR. Total DNA was isolated according to the method of Dellaglio et al. (1973). Primers M13 (5′-GAG GGT GGC GGT TCT-3′) and D8635 (5′-GAG CGG CCA AAG GGA GCA GAC-3′) were used (Huey and Hall, 1989). Amplification reactions were performed according to Andrighetto et al. (2001). The 25 µl reaction volume contained 0.99 mM primer M13, 1× PCR Buffer (MBI Fermentas, Mundolsheim, France), 2.5 mM $MgCl_2$ (MBI Fermentas), 0.15 mM dNTP (Abgene, Surrey, UK) and 1 U *Taq* DNA polymerase (MBI Fermentas). The second amplification contained 0.88 mM primer D8635, 1× PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP and 1 U *Taq* DNA polymerase. Amplification was in a DNA thermal cycler (My Cycler™ Thermal Cycler Firware, BioRad) as follows: initial denaturation at 94 °C for 2 min, 35 cycles of 1 min per cycle at 94 °C, and 1 min at 46.9 °C, followed by an increase to 72 °C over 90 s. Extension of the amplified product was at 72 °C for 10 min. The amplified products were separated by electrophoresis in 1.2% (w/v) agarose gels in 1× TAE buffer (4.84 g Tris-base, 1.09 g glacial acetic, 0.29 g ethylenediaminetetraacetic acid, 1 l distilled water) at 80 V for 2 h. Gels were stained in TAE buffer containing 0.5 µg/ml ethidium bromide (Sigma Diagnostics, St. Louis, Mo., USA). A 100-bp DNA ladder (BioRad Laboratories, Richmond, CA) was used as a molecular weight marker. Banding patterns were analysed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium).

Bacteriocin production during growth

MRS broth (100 ml; Biolab) was inoculated with 1% (v/v) of an overnight culture and incubated at 37 °C. Changes in pH and optical density (600 nm) were recorded every hour for 24 h. Bacteriocin activity (AU/ml) in the cell-free supernatant was recorded every 3 h for 24 h, as described by Van Reenen et al. (1998). *Listeria innocua* N27 and *Enterococcus faecium* HKLHS were used as target strains.

Molecular size of bacteriocins

Strains were grown in MRS broth for 18 h at 37 °C. The cells were harvested (8000 ×*g*, 10 min, 4 °C) and bacteriocins precipitated from the cell-free supernatants with 40% saturated ammonium sulphate (Sambrook et al., 1989). The precipitate was re-suspended in one tenth-volume 25 mM ammonium acetate buffer (pH 6.5) and separated by tricine-SDS-PAGE, as described by Schägger and Von Jagow (1987). A low molecular weight marker with sizes ranging from 2.5 to 45 kDa (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) was used. The gels were fixed and one half stained with Coomassie Brilliant Blue R250 (Saarchem, Krugersdorp, South Africa). The positions of the active bacteriocins were determined by overlaying the other half of the gel (not stained and extensively pre-washed with the sterile distilled water) with cells of *L. innocua* N27 (10^6 CFU/ml), embedded in Brain Heart Infusion (BHI) agar (0.7% agar w/v).

Effect of enzymes, temperature, pH, surfactants and proteaseinhibitorsonbacteriocinactivity

Strains were grown in MRS broth for 18 h at 37 °C. The cells were harvested (8000 ×*g*, 10 min, 4 °C) and the cell-free supernatant adjusted to pH 6.0 with 1 M NaOH. One millilitre cell-free supernatant was incubated for 2 h in the presence of 1 mg/ml and 0.1 mg/ml of each of proteinase K, pronase, papain, pepsin and trypsin (Boehringer Mannheim GmbH, Germany), α-amylase (Sigma) and catalase (Boehringer Mannheim), respectively. Antimicrobial activity was monitored by using the agar-spot test method (Van Reenen et al., 1998). In a separate experiment, 1% (w/v) sodium dodecyl sulphate (SDS), Tween 20, Tween 80, urea, Triton X-114, Triton X-100, Oxbile and NaCl were added to bacteriocin-containing cell-free supernatants. EDTA was added to cell-free supernatants to yield final concentrations of 0.1, 2.0 and 5.0 mM. Untreated cell-free supernatants and detergents at these respective concentrations in water were used as controls. All samples were incubated at 37 °C for 5 h and then tested for antimicrobial activity by using the agar-spot test method as described before.

The effect of pH on the activity of bacteriocins was tested by adjusting cell-free supernatants from pH 2.0 to 12.0 (at increments of two pH units) with sterile 1 M NaOH or 1 M HCl. After 1 h of incubation at room temperature (25 °C), the samples were re-adjusted to pH 6.5 with sterile 1 M NaOH or 1 M HCl and tested for antimicrobial activity by using the agar-spot test method. *L. innocua* N27 and *E. faecium* HKLHS were used as target strains. The effect of temperature on bacteriocin activity was tested by incubating cell-free supernatants, adjusted to pH 7.0 at 4, 25, 30, 37, 45, 60, 80, and 100 °C, respectively, for 60 and 120 min. Bacteriocin activity was also tested after 20 min at 121 °C. The agar-spot test method was used in all tests. *L. innocua* N27 and *E. faecium* HKLHS served as target strains.

Cell lysis

Twenty millilitres of a bacteriocin-containing cell-free supernatant (1600 AU/ml, pH 6.0) was filter-sterilized and added to 100 ml early exponential phase (5-h-old) cultures of *L. innocua* N27 and *E. faecium* HKLHS, respectively. Optical density readings at 600 nm were taken every hour for 13 h.

In a separate experiment, 18-h-old cultures of *E. faecium* HKLHS and *L. innocua* N27, respectively, were harvested

(5000 ×*g*, 5 min, 4 °C), washed twice with sterile saline water and re-suspended in 10 ml. Equal volumes of the cell suspensions and filter-sterilized (0.20 μm, Minisart®, Sartorius) bacHA-6111-2 containing cell-free supernatant were mixed. Viable cell numbers were determined before and after incubation for 1 h at 37 °C by plating onto MRS agar (Biolab). The experiment was repeated with bacHA-5692-3. Cell suspensions of *E. faecium* HKLHS and *L. innocua* N27 without added bacteriocins served as controls.

Adsorption studies

Adsorption of bacteriocins to producer cells was studied according to the method described by Yang et al. (1992). Bacteriocin-producing cells were cultured for 18 h at 37 °C. The pH of the culture was adjusted to 6.0 with 1 M NaOH to allow maximal absorption of the bacteriocin to the producer cells. The cells were then harvested (12,000 ×*g*, 15 min, 4 °C) and washed with sterile 0.1 M phosphate buffer (pH 6.5). The pellet was re-suspended in 10 ml 100 mM NaCl (pH 2.0) and agitated for 1 h at 4 °C to allow delaminating bacteriocin from the cells. The cells were then harvested (12,000 ×*g*, 15 min, 4 °C), the cell-free supernatant neutralized to pH 7.0 with sterile 1 M NaOH and tested for bacteriocin activity as described before.

Partial purification

Bacteriocin-producing strains were cultured in 400 ml MRS broth at 37 °C until early stationary-phase (18-h-old cultures). Cells were harvested (12,000 ×*g*, 20 min, 4 °C) and ammonium sulphate gradually added to the supernatant to 40% saturation. After 4 h of slow stirring at 4 °C, the proteins were harvested (12,000 ×*g*, 1 h, 4 °C). Precipitated proteins in the pellet and floating on the surface were collected and solubilized in 25 mM ammonium acetate buffer (pH 6.5). All samples were stored at − 20 °C.

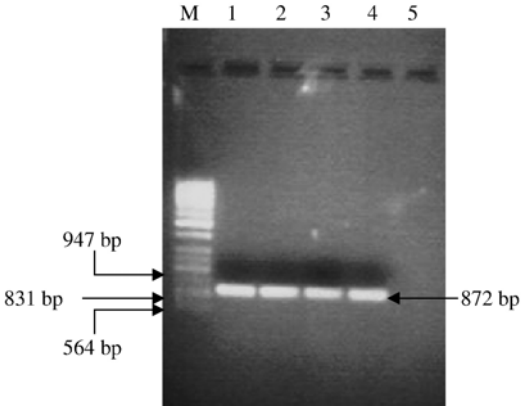

Fig. 1. Amplification with species-specific primers PacF and PuR yielded a 872 bp fragment characteristic for *P. acidilactici.* Lane 1: strain HA-6111-2; lane 2: strain HA-5692-3; lanes 3 and 4: *P. acidilactici* ATCC 12697; lane 5: *P. pentosaceus* ATCC 13561; lane M: λ, digested with *Eco*RI and *Hin*dIII.

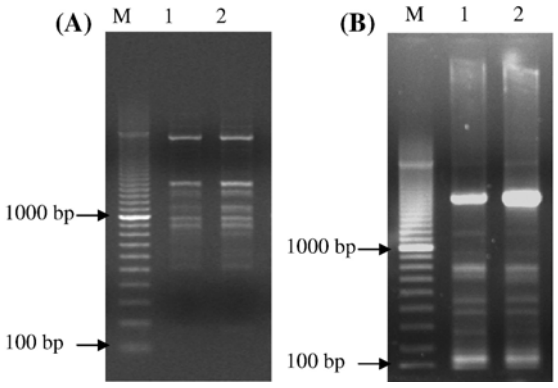

Fig. 2. Differentiation between strains HA-6111-2 and HA-5692-3 by RAPD-PCR with primers M13 (A) and D8635 (B). Lane 1: strain HA-6111-2; lane 2: strain HA-5692-3; lane M: DNA molecular size markers (100 bp ladder).

Identification of genes encoding bacteriocin production

DNA was isolated according to the method of Dellaglio et al. (1973). Primers Pedpro (5′-CAA GAT CGT TAA CCA GTT T-3′) and Ped 1041 (5′-CCG TTG TTC CCA TAG TCT AA-3′) were designed from the operon encoding pediocin PA-1 (accession number M83924) and synthesised by Genosys Biotechnologies (Europe) Ltd. (Cambridgeshire, United Kingdom). PCR reactions were performed using a GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, USA). The following conditions were used: an initial denaturation step of 94 °C for 1 min, followed by 35 cycles of 1 min at 94 °C, 30 s at 50 °C and 1 min at 72 °C, and final extension at 72 °C for 5 min. The amplified product was visualized in a 0.8% (w/v) agarose gel stained with ethidium bromide. For each strain, a band corresponding to the correct size was purified from the gel using the QIAquick PCR purification kit according to the instructions of the manufacturer (QIAGEN GmbH). Products were ligated into pGEM-T® Easy Vector (Promega, Madison, USA) and transformed into *E. coli* DH5α according to the instructions of the manufacturer. Plasmids were isolated using a QIAGEN Plasmid Mini Kit and fragments sequenced on an automatic sequencer (ABI Genetic Analyzer 3130XI, Applied Biosystems) using bigdye terminator chemistry (Biosystem, Wanington, England). Sequences were analysed using DNAMAN *for Windows*® (Lynnon Biosoft, Quebec, Canada).

Results and discussion

Strains HA-6111-2 and HA-5692-3, isolated from “Alheira” produced by different companies, were identified as *P. acidilactici* by physiological and biochemical characteristics. The majority of lactic acid bacteria isolated from “Alheira” were identified as enterococci, present at levels of approximately 10^7 cfu/ml (Ferreira et al., 2006). Little is known about the presence of pediococci in the product. The cells formed tetrads and displayed carbohydrate fermentation reactions typical to that recorded for *P. acidilactici* (not shown). Amplification with species-specific primers (PacF and PuR) yielded

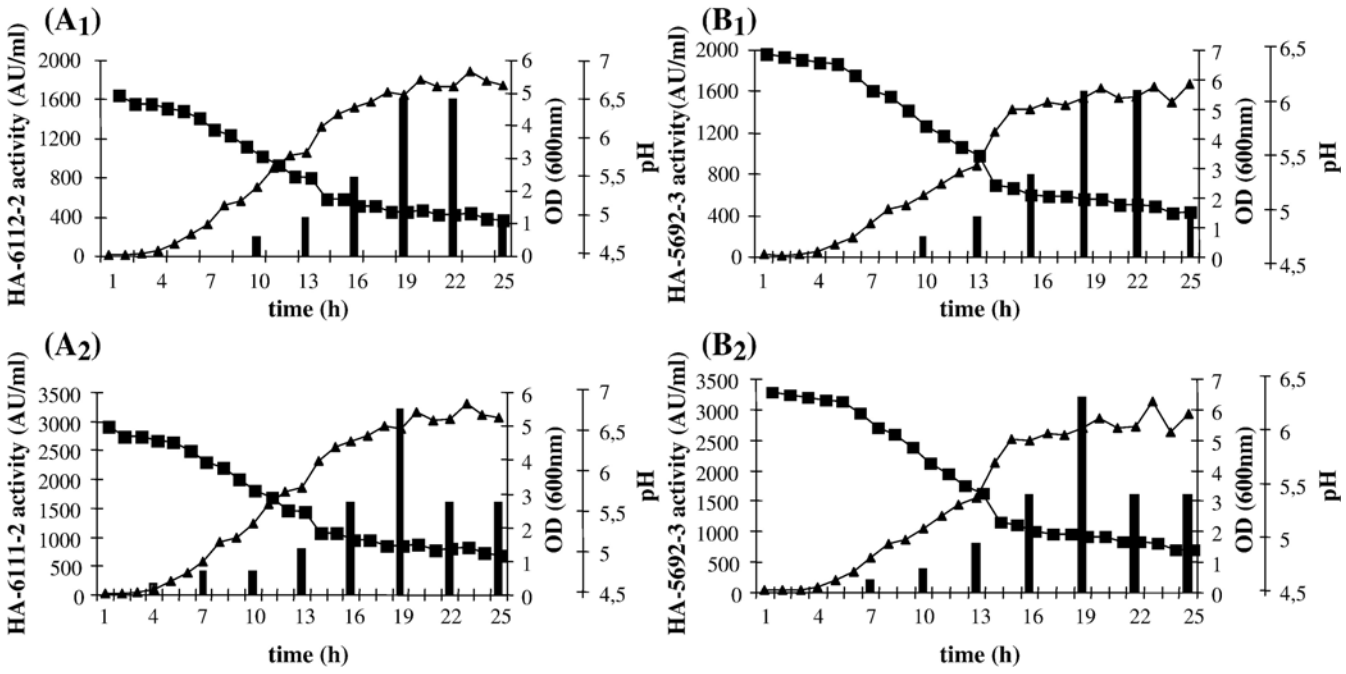

Fig. 3. Production of bacteriocins HA-6111-2 (graph A) and HA-5692-3 (graph B) in MRS broth (pH 6.4) at 37 °C. Antimicrobial activity of cell-free supernatants is presented as AU/ml (bars). 1: Sensitive strain *L. inoccua* N27; 2: sensitive strain *E. faecium* HKLHS. Changes in optical density (▲) and pH (■) are indicated.

a 872 bp fragment (Fig. 1), characteristic for the species. The RAPD-PCR banding patterns of the two strains were identical (Fig. 2).

The cell-free supernatant of strains HA-6111-2 and HA-5692-3 inhibited the growth of *L. lactis* subsp. *lactis*; *L. monocytogenes* ScottA, 4855 and 54; *L. innocua* N27; *L. ivanovii* subsp. *ivanovii* NCTC 11846; *E. faecalis* FAIR E77. FAIR E88. FAIR E92 and FA2; *Enterococcus* sp. 9 and 57 (isolated from "Alheira"); *Streptococcus* sp. TL1, TL2R and TL2W; and *Streptococcus caprinus* ATCC 700065 (Table 1) *E. faecium* HKLHS; *E. faecalis* FAIR E80 and *E. mundtii* PTA-7278 were inhibited by strain HA-5692-3 and not by strain HA-6111-2. No antimicrobial activity was recorded against Gram-negative bacteria. The broad spectrum of antimicrobial activity recorded against Gram-positive bacteria is characteristic of many class IIa bacteriocins. The activity of pediocin PA-1 against *L. monocytogenes* is particularly relevant (Rodríguez et al., 2002). Activity against Gram-negative bacteria is an unusual phenomenon and has so far only been reported for thermophylin 81, produced by *Streptococcus thermophylus*, bacteriocins produced by *L. paracasei* subsp. *paracasei* L126 and L134, a bacteriocin produced by *L. lactis* KCA2386, and plantaricin 35d produced by *L. plantarum* (Ivanova et al., 1998; Ko and Ahn, 2000; Caridi, 2002; Messi et al., 2001). The structure and

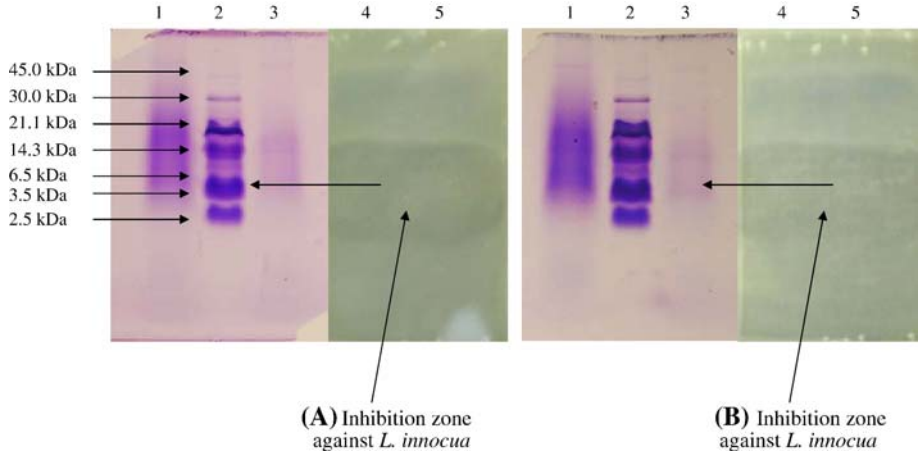

Fig. 4. Tricine-SDS-PAGE of bacteriocins HA-6111-2 (A) and HA-5692-3 (B). Lane 1: peptide bands stained with Coomassie Blue R250 (40% ammonium sulphate saturated); lane 2: molecular mass marker; lane 3: peptide bands stained with Coomassie Blue R250 (60% ammonium sulphate saturated); lane 4: zone of growth inhibition, corresponding to the position of the peptide bands in lane 1; lane 5: zone of growth inhibition, corresponding to the position of the peptide bands in lane 3. The gel in lanes 4 and 5 was covered with viable cells of *L. innocua* N27 (approx. 10^6 CFU/ml), imbedded in BHI agar. Incubation was at 37 °C for 24 h.

composition of the outer membrane of Gram-negative bacteria does not allow access of pediocin to the cytoplasmic membrane. However, many Gram-negative organisms, (e.g. *Salmonella typhimurium*, *Escherichia coli*, *Serratia liquefaciens* and *Pseudomonas fluorescens*) are sensitive to pediocin PA-1 after sublethal injuries such as freezing, gentle heating, exposure to lactic acid or EDTA, and hydrostatic-pressure pasteurization to the outer membrane (Kalchayanand et al., 1992). Only a few *P. pentosaceus* strains are inhibitory to Gram-negative organisms (Spelhaug and Harlander, 1989). *P. damnosus* and *P. pentosaceus* isolated from beer exhibit antibacterial activity against a range of Gram-negative organisms (Skytta et al., 1993). Pediocin PA-1 from *P. acidilactici* PAC 1.0 adsorbed to both sensitive and resistant indicator strains (Gonzalez and Kunka, 1987; Pucci et al., 1988). Pediocin AcH from *P. acidilactici* binds to non-specific receptors, probably lipoteichoic acid. When present in high concentrations, the molecules bind to specific receptor(s) and change the integrity of the membrane (Bhunia et al., 1991).

BacHA-6111-2 and bacHA-5692-3 were produced at maximum levels (3200 AU/ml against *E. faecium* HKLHS and 1600 AU/ml against *L. innocua* N27) after 18 h of growth in MRS broth (Fig. 3A and B). During the first 24 h of growth, the medium pH of strain HA-6111-2 decreased from 6.51 to 4.95 and the cell density increased from 0.05 to 5.45 (dilution factor taken into account) (Fig. 3A). Low levels of bacHA-6111-2 activity (approximately 200 AU/ml against *L. innocua* N27) were recorded after 9 h of growth in MRS broth. Identical results were obtained when cells were grown at 30 °C and 37 °C. This is in agreement with the results recorded for pediocin PA-1 (Ray, 1992). Optimal levels of pediocin PA-1 were recorded in growth media that supported high biomass production, e.g. MRS and TGE (tryptone glucose extract) (Biswas et al., 1991; Ray, 1992; Yang and Ray, 1994). All further experiments were conducted at 37 °C.

Maximal activity of bacHA-6111-2 (1600 AU/ml) was recorded after 18 h in MRS broth at pH 5.1. Bacteriocin activity against *E. faecium* HKLHS was detected after 3 h at pH 6.34 and maximal activity (3200 AU/ml) recorded after 18 h (Fig. 3B). Similar results were recorded for bacHA-5692-3. The culture pH decreased from 6.38 to 4.92 and the cell density increased from $OD_{600\ nm}$ 0.074 to 5.85 (dilution factor taken into account). Maximal activity was recorded at pH 5.05. The constant bacteriocin activity levels recorded at pH values below 4.5 suggest that production is blocked (Todorov and Dicks, 2005b). Genetic studies on the expression of the genes encoding bacteriocin production will have to be done to confirm the latter hypothesis.

BacHA-6111-2 and bacHA-5692-3 are between 3.5 and 6.5 kDa in size, as determined by SDS-PAGE (Fig. 4). The small peptides shown in Fig. 4 are within the range of most bacteriocins reported for the genus *Pediococcus.* The molecular weight of pediocin PA-1, calculated from the amino acid sequence, is 4629 Da (Henderson et al., 1992; Fimland et al., 1996).

Complete inactivation or significant reduction in antimicrobial activity was observed after treatment of the cell-free supernatant with proteinase K, pronase and trypsin (Table 2). Treatment with papain at 0.1 mg/ml decreased the activity of bacHA-6111-2 by approximately 50%. Treatment with pepsin (1 mg/ml) reduced the activity levels of the bacteriocins by more than 75%, but only 25% with 0.1 mg/ml, showing that the concentration is an important factor. No change in activity was recorded when treated with catalase (Table 2), indicating that H_2O_2 was not responsible for inhibition. Treatment with α-amylase did not change the antimicrobial activity (Table 2), suggesting that both bacteriocins were not glycosylated and thus similar to most other bacteriocins (De Vuyst and Vandamme, 1994). Leuconocin S, produced by *Leuconostoc paramesenteroides* (Lewus et al., 1992) and carnocin 54, produced by *Leuconostoc carnosum* (Keppler et al., 1994) are typical examples of amylase-sensitive bacteriocins. Pediocin PA-1 activity is unaffected by treatment with phospholipase C, catalase, lysozyme, DNAses, RNAses or lipases, but is lost after incubation with proteolytic enzymes such as trypsin, papain, chymotrypsin, protease IV, protease XIV, protease XXIV, and

Table 2
Reduction of antimicrobial activity of bacteriocins HA-6111-2 and HA-5692-3 (expressed in percentage values) after incubation at different conditions

		HA-6111-2		HA-5692-3	
		L. innocua N27	*E. faecium* HKLHS	*L. innocua* N27	*E. faecium* HKLHS
pH	2	50%	50%	50%	25%
	4	25%	25%	25%	25%
	6	0%	0%	0%	0%
	8	50%	25%	50%	50%
	10	75%	50%	75%	50%
	12	100%	100%	100%	75%
Temperature	4 °C	0%	25%	0%	25%
	25 °C	25%	25%	0%	25%
	30 °C and 37 °C	0%	0%	0%	0%
	45 °C	25%	0%	0%	25%
	60 °C	25%	0%	25%	25%
	80 °C	25%	0%	25%	50%
	100 °C	50%	50%	50%	75%
	121 °C	100%	100%	100%	100%
Enzymes	Proteinase $K_{1.0 \text{ and } 0.1 \text{ mg/ml}}$	100%	100%	100%	100%
	$Pronase_{1.0 \text{ and } 0.1 \text{ mg/ml}}$	100%	100%	100%	100%
	$Papain_{1.0 \text{ mg/ml}}$	100%	100%	100%	100%
	$Papain_{0.1 \text{ mg/ml}}$	50%	50%	100%	100%
	$Pepsin_{1.0 \text{ mg/ml}}$	100%	100%	75%	75%
	$Pepsin_{0.1 \text{ mg/ml}}$	25%	25%	0%	0%
	$Trypsin_{1.0 \text{ and } 0.1 \text{ mg/ml}}$	100%	100%	100%	100%
	$\alpha\text{-amylase}_{1.0 \text{ mg/ml}}$	0%	25%	25%	25%
	$\alpha\text{-amylase}_{0.1 \text{ mg/ml}}$	0%	0%	0%	0%
	$Catalase_{1.0 \text{ and } 0.1 \text{ mg/ml}}$	0%	25%	0%	25%
	Tween 20 and Tween $80_{0.01 \text{ g/ml}}$	0%	0%	0%	0%
	Triton X-114 and Triton $X\text{-}100_{0.01 \text{ g/ml}}$	100%	50%	100%	50%
	$SDS_{0.01 \text{ g/ml}}$	0%	0%	0%	0%
	$EDTA_{0.1 \text{ mM}}$	0%	0%	0%	0%
	$EDTA_{5.0 \text{ mM}}$	0%	0%	0%	0%
	$Oxbile_{0.01 \text{ g/ml}}$	0%	0%	0%	0%
	Urea and $NaCl_{0.01 \text{ g/ml}}$	0%	0%	0%	0%

proteinase K (Gonzalez and Kunka, 1987; Bhunia et al., 1988; Ray et al., 1989).

Both bacteriocins are sensitive to treatment with 1% (m/v, final concentration), Triton X-114 and Triton X-100 (Table 2). However, treatment with Tween 20, Tween 80, SDS, Oxbile, NaCl and urea at 1% (m/v) or EDTA (0.1 mM, 2.0 mM or 5.0 mM) had no affect on antimicrobial activity (Table 2). Similar results were recorded for plantaricin 423 (Verellen et al., 1998), pediocin AcH (Biswas et al., 1991), lactacin B (Barefoot and Klaenhammer, 1984) and lactocin 705 (Vignolo et al., 1995). NaCl concentrations of 0.1 M were used to study the release of pediocin AcH (identical to PA-1) from the surface of the producer cells (Ray, 1994). Even in the presence of 1.0 M NaCl the activity was not affected (Ray, 1994). Bacteriocins HA-6111-2 and HA-5692-3 remained stable after incubation for 2 h at pH 6.0 (Table 2). Antimicrobial activity was reduced at pH values below 5.0 and above 7.0 (Table 2), suggesting that the peptides are sensitive to acidic and alkaline conditions. This corresponded to the pH stability reported for pediocin PA-1 (Gonzalez and Kunka, 1987; Bhunia et al., 1988). A decrease in antibacterial activity was recorded after 60 min at 100 °C, similar to that recorded for pediocin PA-1 produced by *P. acidilactici* (Rodríguez et al., 2002).

Addition of bacHA-6111-2 and bacHA-5692-3 (1600 AU/ml) to a mid-log (5-h-old) culture of *L. innocua* N27 ($OD_{600\ nm} \approx 0.2$) inhibited the growth for 7 h (Fig. 5A). Addition of bacHA-6111-2 and bacHA-5692-3 (3200 AU/ml) to a mid-log (5-h-old) culture of *E. faecium* HKLHS ($OD_{600\ nm} \approx 0.3$) repressed cell growth in a similar way (Fig. 5B). However both bacteriocins acted bactericidal to stationary-phase (18-h-old) cells of *E. faecium* HKLHS and bacteriostatic to stationary-phase (18-h-old) cells of *L. innocua* N27 (Table 3). No changes in cell numbers of *E. faecium* HKLHS and *L. innocua* N27 were recorded in the untreated (control) samples. Similar results were recorded in the treatment of the lower concentration of *L. innocua* N27 and *E. faecium* HKLHS by bacHA-6111-2 and bacHA-5692-3 (data not shown).

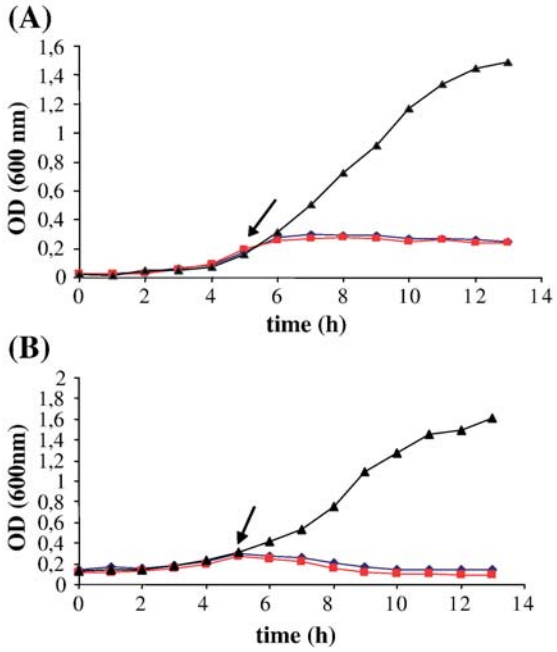

Fig. 5. Effect of bacteriocins HA-6111-2 (■) and HA-5692-3 (♦) on (A) *L. innocua* N27 and (B) *E. faecium* HKLHS cultured at 37 °C. The symbol (▲) represents the growth of *L. innocua* N27 (A) and *E. faecium* HKLHS (B) without added bacteriocins (controls). The arrow indicates the point at which the bacteriocins were added (5 h).

Table 3
Effect of bacteriocins HA-6111-2 and HA-5692-3 on cell growth of *L. innocua* N27 and *E. faecium* HKLHS

Treatment:	*E. faecium* HKLHS (CFU/ml)		*L. innocua* N27 (CFU/ml)	
	Before treatment with bacteriocin	After treatment with bacteriocin	Before treatment with bacteriocin	After treatment with bacteriocin
BacHA-6111-2	1.5×10^{10}	<10	1.0×10^{11}	2.8×10^{7}
BacHA-5692-3	2.3×10^{10}	<10	1.9×10^{11}	2.9×10^{7}
None (control)	2.7×10^{10}	3.1×10^{10}	1.3×10^{11}	1.9×10^{11}

No bacteriocin activity was detected after treatment of strains HA-6111-2 and HA-5692-3 with 100 mM NaCl pH 2.0 (data not shown), suggesting that the bacteriocins did not adhere to the surface of the producer cells. Similar results were reported for plantaricin ST31 (Todorov et al., 1999), pediocin ST18 (Todorov and Dicks, 2005a) and bozacin B14 (Ivanova et al., 2000).

Pediocin PA-1 biosynthesis involves a DNA fragment of approximately 3.5 kb, comprising the four genes *pedA*, *pedB*, *pedC*, and *pedD* (Marugg et al., 1992). Strains HA-6111-2 and HA-5692-3 have a 1044 bp DNA fragment identical to that recorded for pediocin PA-1 (Fig. 6). Sequencing of the 1044 bp fragments revealed homology to large sections of *pedA* (bp 1076–1264), *pedB* (1302–1640) and *pedC* (bp 1664–2188) of pediocin PA-1 (Genbank accession number M83924; Marugg et al., 1992). Bacteriocins bacHA-6111-2 and bacHA-5692-3 are thus considered similar to pediocin PA-1.

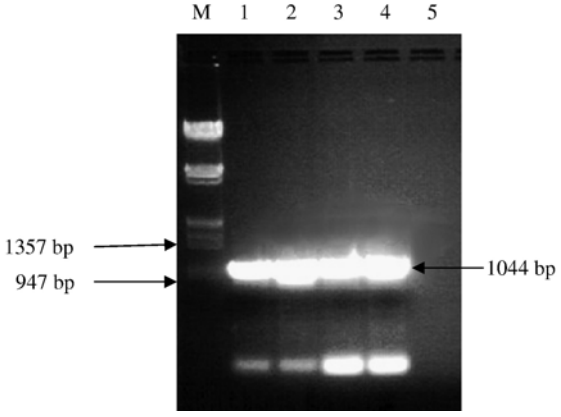

Fig. 6. Amplification of DNA of strains HA-6111-2 and HA-5692-3 with primers specific for pediocin PA-1 yielded a 1044 kb DNA fragments. Lane 1: strain HA-6111-2; lane 2: strain HA-5692-3; lanes 3 and 4: *P. acidilactici* ATCC 12697; lane 5: *P. pentosaceus* ATCC 13561; lane M: λ, digested with *Eco*RI and *Hin*dIII.

Most bacteriocins (pediocin and pediocin-like) produced by *Pediococcus* spp. have antilisterial activity, are thermostable and fall within the size range from 2867 to 4685 Da (Henderson et al., 1992; Daba et al., 1994; Fimland et al., 2002; Bauer et al., 2005; Diep et al., 2006). Pediocin AcH, produced by *P. acidilactici* H, is identical to pediocin PA-1 produced by *P. acidilactici* PAC-1 (Gonzalez and Kunka, 1987; Bhunia et al., 1988; Henderson et al., 1992; Motlagh et al., 1992). Pediocin PA-1 is one of the best studied bacteriocins (Henderson et al. 1992; Marugg et al. 1992; Nieto-Lozano et al. 1992; Bukhtiyarova et al., 1994; Motlagh et al., 1994; Venema et al. 1995) and is considered a good biopreservative (Bhunia et al. 1988, 1991; Pucci et al. 1988; Yousef et al., 1991; Foegeding et al., 1992). BacHA-6111-2 and bacHA-5692-3 described in this study are similar to pediocin PA-1. Further research on the technological properties of the two strains (*P. acidilactici* HA-6111-2 and HA-5692-3) has to be done to determine if they can be used as commercial starter cultures for production of "Alheiras".

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