Characterization of anti-*Listeria* bacteriocins isolated from shellfish: Potential antimicrobials to control non-fermented seafood

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A B S T R A C T

This work had as main objectives to characterize two bacteriocins produced by lactic acid bacteria (LAB) previously isolated from non-fermented seafood, in order to evaluate their potential as new food protective agents. The two bacteriocinogenic isolates were identified by Polymerase Chain Reaction (PCR) using genus- and species-specific primers, and confirmed by 16S rDNA sequencing, as *Enterococcus faecium* and *Pediococcus pentosaceus*. The antimicrobial spectrum of each strain included several indicator microorganisms, some of them also isolated from seafood. Growth of *Listeria innocua*, *L. monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and other LAB species were inhibited, although no inhibition of Gram-negative microorganisms was observed. Proteolytic, but not lipolytic or glycolytic enzymes, completely inactivated the antimicrobial effect of both cell-free supernatants confirming the proteinaceous nature of the inhibitors. The antimicrobial activity was maintained after treatment with NaCl, SDS, Triton X-100, Tween 20, Tween 80 and EDTA after 2 h or 5 h of exposure and both bacteriocins were stable over a wide range of pH and temperatures. Production of bacteriocin by *E. faecium* (bacALP7) was detected initially at exponential phase and reached a maximum activity of 25,600 AU/ml in the early stationary phase, whereas bacteriocin production by *P. pentosaceus* ALP57 (bacALP57) reached the maximum at exponential phase with 12,800 AU/ml. The bacteriocins did not kill *L. monocytogenes* ESB54 nor *L. innocua* 2030c however, cellular growth was reduced. The partially purified bacteriocins, bacALP7 and bacALP57, were below 6.5 kDa in size as determined by Tricine-SDS gel electrophoresis. *E. faecium* and *P. pentosaceus* contained DNA fragments corresponding in size to those recorded for enterocin B and pediocin PA-1, respectively. Sequencing of the fragments from both bacteriocins confirmed the homology. To our knowledge, for the first time two LAB producing bacteriocins similar to pediocin PA-1 and enterocin B, were isolated from non-fermented shellfish. The adaptation of the cultures to seafood matrices may be advantageous in terms of application as a biopreservation strategy for reduction of *L. monocytogenes* levels in seafood products.

Introduction

Thousands of new fish and seafood products have been prepared and launched worldwide, motivated by the new lifestyles and consumers' demand for new products, more convenient, natural and safe. The new trends constitute considerable challenges to the food and medical industries (Devlieghere et al., 2004). From a natural food preservation point of view, the search for and study of new antimicrobials such as the LAB bacteriocins, generally regarded as safe (GRAS status) (Deegan et al., 2006), are desirable in order to stimulate interest and evaluate industrial potential. Several studies have reported improvement of the quality and shelf-life of fish products (Katla et al., 2001; Nilsson et al., 2004; Brillet et al., 2005; Calo-Mata et al., 2007). Combination methods have been tested for efficacy of pathogen inhibition in fish and shellfish systems. Examples include synergistic combinations of bacteriocins and other technologies (Zuckerman and Bem Avraham, 2002; Al-Holy et al., 2004; Elotmani and Assobhei, 2004), for instance, active packaging with adsorbed bacteriocins (Chen and Hoover, 2003).

The spectrum of activity of most LAB bacteriocins is rather narrow and may not inhibit the variety of microorganisms found in foods. This is particularly important regarding Gram-negative bacteria. Bacteriocin resistance is also one of the major current limitations for application of bacteriocins in foods (Chen and Hoover, 2003). Moreover, food matrices affect the activity of bacteriocins and growth of bacteria in several ways (Katla et al., 2001). In fact, improvement in knowledge

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and understanding of seafood matrices, their microbial ecology, with particular attention to LAB, would be advantageous. The search for new bacteriocins from fermented and non-fermented food products could open new application horizons in terms of biopreservation.

The occurrence of the food borne pathogen *Listeria monocytogenes*, the causative agent of listeriosis (Katla et al., 2001), has motivated several studies regarding its control in foods, particularly in those that pose higher degrees of risk. Biopreservation technology has gained special attention for application to minimally processed seafood such as cold-smoked salmon and has been already been promoted as a strategy for *L. monocytogenes* control in seafood.

L. monocytogenes is able to grow at low temperatures and under conditions of high salt content (Ben Embarek, 1994; Handa et al., 2005; Pinto et al., 2006). In fact, listeriosis outbreaks caused by the consumption of fresh seafood has been reported (Brett et al., 1998). In the same food niche, the presence of LAB has been reported for several fish species (Ringo and Gatesoupe, 1998), seafood products such as surimi (Yamazaki et al., 2003); fresh fish (Bucio et al., 2006), aquacultured fish (Michel et al., 2007), and intestinal contents of marine coastal fish (Itoi et al., 2008). Some strains of *Carnobacterium* (Pilet et al., 1995; Nilsson et al., 2004; Tahiri et al., 2004), *Lactococcus lactis*, *Enterococcus* spp., and *Pediococcus* spp. (Campos et al., 2006; Tomé et al., 2008) have received attention as bacteriocin-producing cultures. In comparison with other food products such as dairy and meat (Cleveland et al., 2001), few bacteriocinogenic strains and their bacteriocins recovered from aquatic environments, fish products and even less from non-fermented seafood, have been identified and characterized. The acquisition of strains already acclimatized to a seafood habitat would be advantageous in terms of biopreservation (Tahiri et al., 2004), not only to be used as live bacterial (protective) cultures to be applied to foods but also, incorporated into feedstuffs, with application in aquaculture (for a review see Balcázar et al., 2006). Taking this into account, our research group has recently isolated LAB from marine non-fermented shellfish and tested the isolates for antimicrobial activity by bacteriocin production against *L. monocytogenes*. The present study focuses on the characterization of two bacteriocins, produced by *E. faecium* ALP7 and *P. pentosaceus* ALP57, obtained from the initial screening.

Materials and methods

Bacterial strains and cultures conditions

All LAB strains used in this study were maintained as stock cultures at −80 °C in All Purpose Tween (APT) broth (Difco Laboratories, Detroit, USA) and the remaining strains in Brain Heart Infusion (BHI; LAB M, Bury, UK) broth with 30% (v/v) glycerol. The strains were propagated twice in the respective media at 30 °C or 37 °C before use. Reference strains used for the identification tests were *E. faecium* ESB82, *E. faecalis* ATCC29212, *P. acidilactici* PAC-1.0, and *P. pentosaceus* ESB663.

Identification of bacteriocin-producing strains

The two strains (given code ALP7 and ALP57) used in this study were selected from a group of 78 bacteriocinogenic LAB previously isolated from non-fermented shellfish, including oysters, mussels, and clams. The selection was based on phenotypical studies such as growth at different temperatures, pH, salt content and atmosphere conditions, maintenance of stability and functionality after freeze- and spray-drying; antimicrobial activity and inhibition spectrum, and also safety requirements involving antibiotic resistance, biogenic amines production and haemolytic activity (Pinto et al. unpublished results). Before total DNA extraction, the strains were cultivated for 24 h in APT broth at 30 °C according to the method described by Dellaglio et al. (1973). Further identification was done

Table 1
Antimicrobial activity spectrum of *E. faecium* ALP7 and *P. pentosaceus* ALP57; growth medium and incubation temperature of indicator strains

Indicator organism	Strain	Source	Growth media and temperature	*E. faecium*	*P. pentosaceus*
				ALP7	ALP57
Gram-negative					
Salmonella typhimurium	IPIMAR-SD28	Seafood (shellfish)	37 °C, BHI	−	−
Salmonella enteritidis	NCTC 3046	Unknown	37 °C, BHI	−	−
Salmonella enteritidis	NCTC 5188	Unknown	37 °C, BHI	−	−
Salmonella spp.	IPIMAR–SD1	Seafood (shellfish)	37 °C, BHI	−	−
Escherichia coli	NCTC 9001	Urine	37 °C, BHI	−	−
Escherichia coli	O157:H7	Unknown	37 °C, BHI	−	−
Escherichia coli	ATCC 8739	Faeces	37 °C, BHI	−	−
Pseudomonas aeruginosa	ESB 03	Unknown	37 °C, BHI	−	−
Shewanella putrefaciens	IPIMAR-SP4	Seafood (shellfish)	37 °C, BHI	−	−
Gram-positive					
Listeria monocytogenes	ALP211	Seafood (bivalve molluscs)	37 °C, BHI	+++ (++)	+++ (++)
Listeria monocytogenes	ALP17	Seafood (bivalve molluscs)	37 °C, BHI	+++ (++)	+++ (++)
Listeria monocytogenes	ALP18	Seafood (bivalve molluscs)	37 °C, BHI	+++ (+)	+++ (++)
Listeria monocytogenes	NCTC 10357	Rabbit	37 °C, BHI	+++ (+)	++ (++)
Listeria monocytogenes	ESB54	Seafood (smoked salmon)	37 °C, BHI	+++ (++)	+++ (++)
Listeria monocytogenes	NCTC 11994	Rabbit	37 °C, BHI	+++ (+)	+++ (+)
Listeria monocytogenes	ESB 7946	"Alheira" *	37 °C, BHI	+++ (+)	+++ (+)
Listeria monocytogenes	ESB 121	Seafood (smoked salmon)	37 °C, BHI	+++ (++)	+++ (++)
Listeria innocua	NCTC 11288	Cow brain	37 °C, BHI	+++	++
Listeria innocua	NCTC 10528	Unknown	37 °C, BHI	++	++
Listeria innocua	ESB 1325	Unknown	37 °C, BHI	+++	+++
Staphylococcus aureus	ATCC 6538	Human lesion	37 °C, BHI	−	−
Staphylococcus aureus	ATCC 29213	Wound	37 °C, BHI	+	−
Bacillus subtilis	NCTC 3610	Unknown	37 °C, BHI	+	+
Bacillus cereus	ATCC 11778	Unknown	37 °C, BHI	+	−
Bacillus cereus	NCTC 2599	Unknown	37 °C, BHI	−	−
Pediococcus acidilactici	ESB 663	Meat	30 °C, APT	−	−
Pediococcus acidilactici	PAC-1.0	Unknown	30 °C, APT	−	−
Carnobacterium maltaromaticum	DSM 20730	Seafood (rainbow trout)	30 °C, APT	−	−
Carnobacterium divergens	MAAE 3.3	Seafood (shrimps)	30 °C, APT	−	−
Enterococcus faecalis	ESB 05	Seafood (smoked salmon)	30 °C, APT	+	+
Enterococcus faecalis	ATCC 29212	Urine	30 °C, APT	+	+
Enterococcus faecium	ESB 82	Cheese	30 °C, APT	−	−
Lactobacillus brevis gravensis	LMG 7934	Wine	30 °C, APT	+	+
Lactobacillus curvatus	ESB 114	Cheese	30 °C, APT	+	+
Lactobacillus curvatus	MF 411	Pork loin	30 °C, APT	−	−

(continued on next page)

Table 1 (*continued*)

Indicator organism	Strain	Source	Growth media and temperature	*E. faecium* ALP7	*P. pentosaceus* ALP57
Lactobacillus curvatus	MF 368	Pork loin	30 °C, APT	+	+
Lactobacillus curvatus	MF 379	Pork loin	30 °C, APT	−	−
Lactobacillus sakei	MF 473	"Servelat" **	30 °C, APT	+	+
Lactococcus lactis subsp lactis	ATCC 11454	Unknown	30 °C, APT	−	−
Lactococcus lactis	ESB 533	Meat	30 °C, APT	+	+
Lactobacillus plantarum	ESB 1752	Meat	30 °C, APT	−	−
Lactobacillus plantarum	CETC 305	Unknown	30 °C, APT	+	+
Lactobacillus rhamnosus	ESB78	"Alheira"	30 °C, APT	−	−
Lactobacillus rhamnosus	ESB50	"Alheira"	30 °C, APT	−	−
Lactobacillus sakei	MF 1186	Seafood (smoked salmon)	30 °C, APT	+	+
Lactobacillus sakei	Lb 706	Fresh beef meat	30 °C, APT	+	+
Lactobacillus sakei	CETC 494	Unknown	30 °C, APT	−	−
Lactobacillus sakei	ESB 256	"Alheira"	30 °C, APT	−	−
Lactobacillus sakei	MF 468	"Servelat"	30 °C, APT	+	+
Leuconostoc mesenteroides	ESB 882	Meat	30 °C, APT	++	+

−, no zone of inhibition; +, 1 mm<zone<5 mm; ++, 5 mm<zone<10 mm; +++, zone>10 mm. In brackets, inhibition results after 6 months at 4 °C. * Portuguese traditional fermented sausage generally made from pork meat, poultry, wheat bread, olive oil and spices. ** Norwegian Bologna type sausage, made from beef and pork meat. MF — Matforsk, Norwegian Food Research Institute, Ås, Norway; ESB — Universidade Católica Portuguesa, Escola Superior de Biotecnologia, Porto, Portugal; NCTC — National Collection of Type Cultures, Central Public Health Laboratory Service, London, UK; ATCC — American Type Culture Collection, Manassas, VA, USA; LMG — Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; CETC — Spanish Type Culture Collection, Valencia, Spain.

by PCR with genus- and species-specific primers. The following primers were used: *Enterococcus* spp, EntF: 5′-TAC TGA CAA ACC ATT CAT GAT G-3′ and EntR: 5′-AAC TTC GTC ACC AAC GCG AAC-3′ (Ke et al., 1999); *Pediococcus pentosaceus*, PpeF: 5′-CGA ACT TCC GTT AAT TGA TCA G-3′ and PuR: 5′-ACC TTG CGG TCG TAC TCC-3′ (Mora et al., 1997), and *Pediococcus acidilactici*, PacF: 5′-CGA ACT TCC GTT AAT TGA TTA T-3′ and PuR: 5′-ACC TTG CGG TCG TAC TCC-3′ (Mora et al., 1997); each primer was acquired from Amersham, Bioscience, UK Limited (Buckinghamshire, UK). A 100 bp DNA ladder (Bio-Rad Laboratories, Richmond, CA) was used as molecular weight marker. Reference strains of pediococci and enterococci were used as positive controls (described in Section 2.1) and samples without genomic DNA were used as negative controls. 16S rDNA sequencing was also used to confirm identification since one of the strains was identified only to the genus level. Amplification of the 16S rDNA was carried out with the primers 27F (AGA GTT TGA TCC TGG CTC AGG) and 1492R (GGT TAC CTT GTT ACG ACT T) using the following temperature profile: primary DNA denaturation step at 95 °C for 5 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C, with an extension of the amplified product at 72 °C for 10 min. Amplification reactions were performed in a MyCycler Thermal Cycler System (Bio-Rad). Following amplification, 5 μl of product was separated at 90 V for 50 min in a 1% (w/v) agarose gel in 1× TAE buffer (4.84 g Tris-base, 1.09 g glacial acetic acid, 0.29 g ethylenediaminetetraacetic acid, 1 l distilled water), and then stained with 0.5 μg/ml of ethidium bromide. A 1-kb ladder molecular weight marker (Bio-Rad) was used. PCR products, used as templates, were previously purified with the GFX PCR DNA and Band Purification kit (GE HealthCare, Amersham Biosciences, Amersham, UK) and sent to STABVIDA (Lisbon, Portugal) for sequencing. On-line similarity searches were performed with the BLAST program in GenBank (http://www.ncbi.nlm.nih.gov).

Screening for bacteriocinogenic activiry

The detection of antimicrobial activity of strains ALP7 and ALP57 was performed according to the method described by Van Reenen et al. (1998) against several indicator microorganisms, some of them also isolated from seafood (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).

In a separate experiment, the cell-free supernatants (CFS) were preserved at 4 °C over a period of 6 months and evaluated regarding the bacteriocinogenic activity against *L. monocytogenes*.

Growth and bacteriocin production

For each strain, an overnight culture was inoculated (1% v/v) into 100 ml of APT broth and incubated at 30 °C. Samples were taken every hour during 24 h, and the changes in pH and optical density (600 nm) were recorded. Bacteriocin activity (AU/ml) in the CFS was recorded every 3 h, as described by Van Reenen et al. (1998), testing against two strains, namely, *L. monocytogenes* ESB54 and *L. innocua* 2030c (Central Public Health Laboratory, Colindale, London).

Mode of action

Twenty ml of each bacteriocin-containing CFS previously filter-sterilized, were added to 150 ml of early exponential phase cultures (≈10^6 cfu/ml) of *L. monocytogenes* ESB54 (4 h) and *L. innocua* 2030c (5 h). Optical density readings at 600 nm of target strains were determined every hour during 12 h. *L. monocytogenes* ESB54 and *L. innocua* 2030c cultures without added bacteriocins were used as controls.

Effects of enzymes, chemicals, pH and temperature on activity of bacteriocins

Cultures were grown in APT broth for 18 h at 30 °C followed by centrifugation (10,000 ×*g*, 10 min, 4 °C) and pH adjustment of the CFS to pH 6.0 with 1 M NaOH. To test the sensitivity to the enzymes, 1 ml of the CFS was incubated for 2 h at 30 °C in the presence of proteinase K, pronase E, papain, pepsin (Boehringer Mannheim GmbH, Germany), lipase and α-amylase (Sigma) at enzyme concentrations of 1 mg/ml and 0.1 mg/ml. Also, 1% (w/v) of sodium dodecyl sulphate (SDS), Tween 20, Tween 80, urea, Triton X-110, ox-bile and NaCl were added to the supernatants. EDTA was added to CFS to yield final concentrations of 0.1, 2.0 and 5.0 mM. Untreated CFS and detergents at these respective concentrations in water were used as controls. All samples were incubated at 30 °C for 5 h.

The effect of pH on the activity of bacteriocins was tested by adjusting CFS 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 as appropriate, and tested for antimicrobial activity. The effect of temperature on the bacteriocins activity was tested by incubating the CFS, adjusted to pH 6.5 at 4, 10, 25, 30, 37, 60, 80 and 100 °C for 120 min, except for 121 °C (20 min). The antimicrobial activity of the CFS after these treatments was tested against the target strains as described by Van Reenen et al. (1998).

Adsorption of bacteriocins to the producer cells

Bacteriocin-producing cells were cultured at 30 °C for 18 h. The pH of the cultures was adjusted to 6.0 with 1 M NaOH to allow maximal adsorption of the bacteriocin to the producer cells, according to the method described by Yang et al. (1992). 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 of 100 mM NaCl

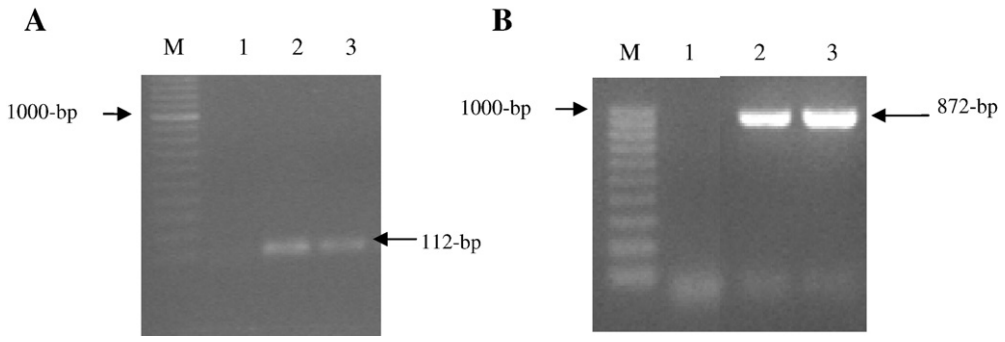

Fig. 1. Agarose gel electrophoresis of PCR products banding patterns obtained after PCR amplification with genus- and species-specific primers. A) Primers EntF and EntR yielding a 112 bp fragment characteristic for *Enterococcus* spp. Lane M: 100 bp molecular marker; Lane 1: negative control; Lane 2: strain ALP7 and Lane 3; positive control. B) Primers PepF and PuR yielding a 872 bp fragment characteristic for *Pediococcus pentosaceus*. Lane M: 100 bp molecular marker; Lane 1: negative control; Lane 2: strain ALP57 and Lane 3; positive control.

(pH 2.0) and stirred slowly for 1 h at 4 °C. The suspension was then centrifuged (12,000 ×*g*, 15 min, 4 °C), the CFS adjusted to pH 7.0 with sterile 1 M NaOH and the bacteriocin activity tested as described above.

Partial purification and molecular size of bacteriocins

Strains ALP7 and ALP57 were inoculated (1% v/v) into 400 ml of APT broth, and incubated without agitation at 30 °C until early stationary phase (18 h) corresponding to maximum bacteriocin production. The cells were harvested (12,000 ×g, 20 min, 4 °C) and the peptides precipitated from the CFS with 40%, 60% and 80% saturated ammonium sulphate, gradually added by slow stirring during 4 h at 4 °C, in independent experiments (Sambrook et al., 1989). Precipitated peptides in the pellet and floating on the surface were collected and re-suspended in one-tenth volume 25 mM ammonium acetate buffer (pH 6.5). The samples were stored at −20 °C for one week. Activity tests were performed according to the method previously described of Van Reenen et al. (1998).

For determination of bacteriocins molecular size, precipitated peptides re-suspended in 25 mM ammonium acetate buffer (pH 6.5) were 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. After fixing the gels, one half was stained with Coomassie Brilliant Blue R250 (Bio-Rad). In order to determine the positions of the active peptide bands, the other half of the gel (not stained and extensively pre-washed with sterile distilled water) was overlaid with 2 ml of *L. monocytogenes* ESB54 (10^6 cfu/ml) embedded in 0.7% (w/v) BHI agar.

PCR detection and sequencing of genes encoding bacteriocins

DNA extraction was performed according to the method described by Dellaglio et al. (1973). The following primer sequences were used for pediocin PA-1, Pedpro: 5′-CAA GAT CGT TAA CCA GTT T-3′ and Ped1041: 5′-CCG TTG TTC CCA TAG TCT AA-3′ (Albano et al., 2007); and for enterocin B, 5′-GAA AAT GAT CAC AGA ATG CCT A-3′ AND 5′-GTT GCA TTT AGA GTA TAC ATT TG-3′ (Casaus et al., 1997) synthesised by Eurofins MWG Operon GmbH (Ebersberg, Germany). PCR reactions were performed using a MyCycler Thermal Cycler System (Bio-Rad). The PCR reactions were the following for pediocin PA-1: an initial

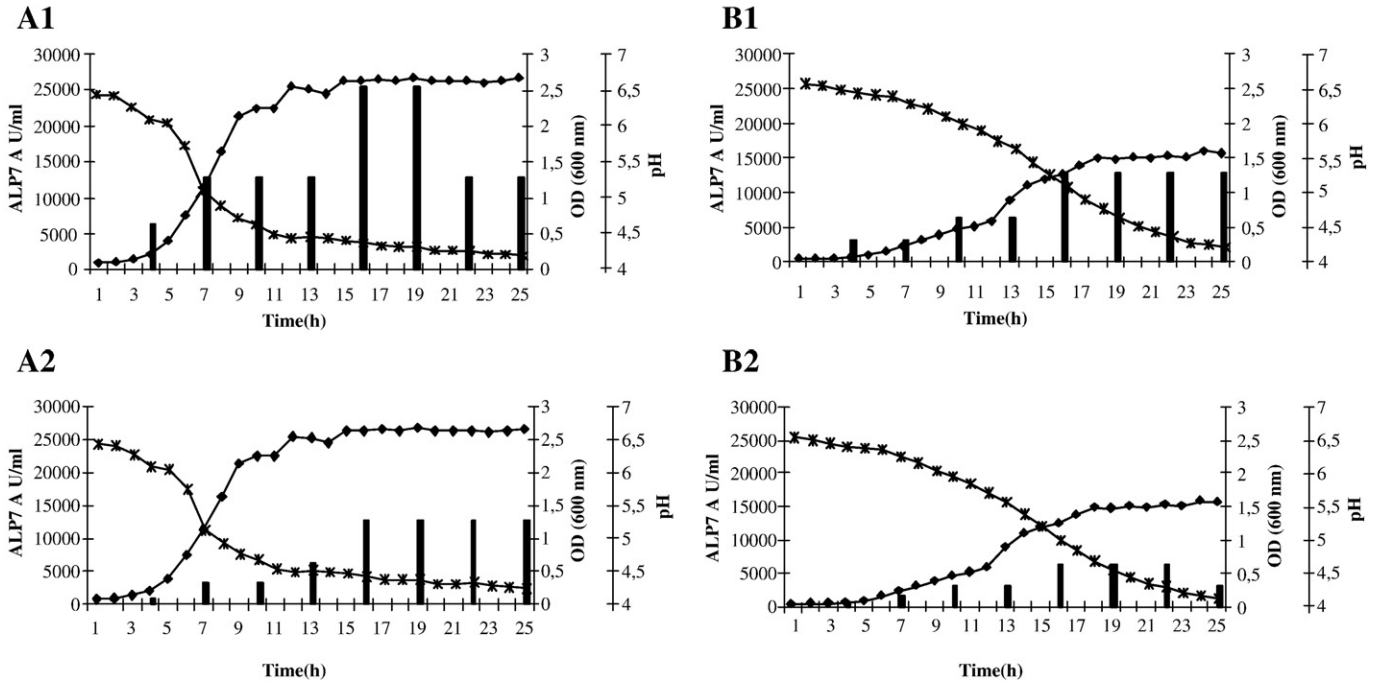

Fig. 2. Growth of strains *E. faecium* ALP7 (A) and *P. pentosaceus* ALP57 (B) in APT broth (pH 6.5) at 30 °C. Production of bacteriocin ALP7 and bacteriocin ALP57 was expressed in AU/ml (bars) against *L. monocytogenes* ESB54 (A1 and B1) and *L. innocua* 2030c (A2 and B2). Optical density (♦) and pH (*) are also indicated.

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, with a final extension at 72 °C for 5 min; and for enterocin B: 5 min denaturation at 95 °C; followed by 30 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C; this followed by 5 min at 72 °C and a cool down to 4 °C (Strompfová et al., 2008). The amplified products were visualized in a 1% (w/v) agarose gel stained with 0.5 μg/ml of ethidium bromide (Sigma) and visualized by UV light. A 1-kb ladder molecular weight marker (Bio-Rad) was used. Strains *P. acidilactici* HA-6111-2 and *E. faecium* ESB 48 were used as the positive control strains. For each strain, the band corresponding to the correct size was purified from the gel using the GFX PCR DNA and Band Purification kit (GE HealthCare, Amersham Biosciences, Amersham, UK) and sent to STABVIDA (Lisbon, Portugal) for sequencing. On-line similarity searches were performed with the BLAST program in GenBank (http://www.ncbi.nlm.nih.gov).

Results

Identification of bacteriocin-producing strains

The strain ALP7 was identified as *Enterococcus* spp. based on positive Gram-reaction, absence of catalase, morphology (cocci in pairs) and genus-specific PCR yielding a 112 bp fragment (Fig. 1A). Further identification to species level, *E. faecium*, was based on 16S rDNA sequencing. Strain ALP57 was identified as *Pediococcus pentosaceus* by physiological and biochemical characteristics namely, Gram-reaction (positive), absence of catalase and cell morphology (tetrads) and confirmed by PCR with species-specific primers yielding a 872 bp fragment characteristic for the species (Fig. 1B). The sequence data of rDNA for ALP7 and ALP57 exhibited 99% and 100% similarities to the 16S rDNA sequences of *E. faecium* (accession no. DQ672262) and *P. pentosaceus* (accession no. CP000422). Both strains showed carbohydrate fermentation reactions characteristic for the species (data not shown).

Antimicrobial activity screening

With the exception of Gram-negative strains, *E. faecium* ALP7 and *P. pentosaceus* ALP57 CFS demonstrated antagonistic activity against the majority of the indicator organisms investigated. Greater inhibition was observed against *L. monocytogenes* and *L. innocua* strains in comparison with the remaining indicators (Table 1).

Growth and bacteriocin production

During the 24 h of growth at 30 °C, the pH of both cultures decreased from 6.5 to 4.3 (*E. faecium* ALP7) and from 6.4 to 4.1 (*P. pentosaceus* ALP57) (Fig. 2). Detectable levels of the bacteriocins

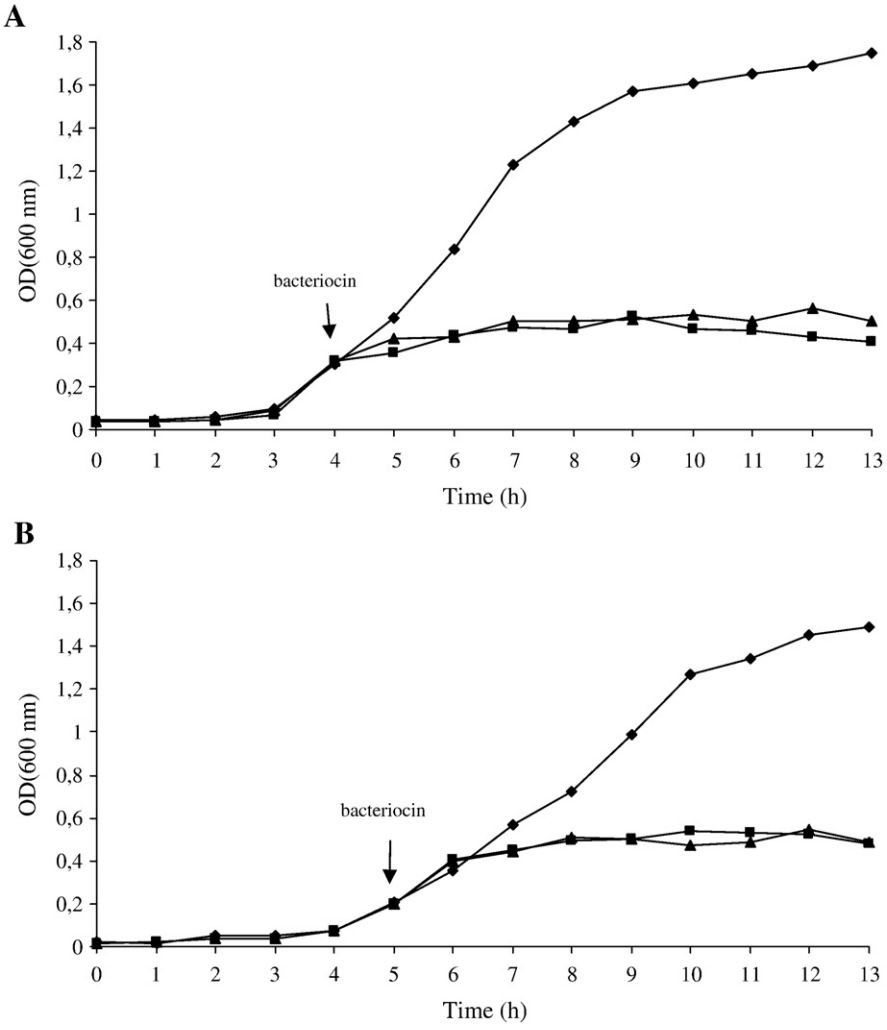

Fig. 3. Activity effect of bacteriocins bacALP7 (▲) and bacALP57 (■) on (A) *L. monocytogenes* ESB54 and (B) *L. innocua* 2030c, incubated at 30 °C. The growth of the indicator strains (A) *L. monocytogenes* ESB54 and (B) *L. innocua* 2030c without added bacteriocins (control) are represented by the symbol (◆). The arrow indicates the point at which the bacteriocins were added.

were recorded after 4 h of growth (approximately 6400 AU/ml and 3200 AU/ml against *L. monocytogenes* ESB54). Maximum production of bacALP7 (25,600 AU/ml against *L. monocytogenes* ESB54) was reached after 16 h at pH 4.4, in the beginning of the stationary phase (Fig. 2A1), followed by a decrease to 12,800 AU/ml after 21 h of incubation. Maximum activity yield against *L. innocua* 2030c was also observed at 16 h of growth, pH 4.5 (12,800 AU/ml) remaining stable as observed in Fig. 2A2. *P. pentosaceus* ALP57 exhibited maximum activity (12,800 AU/ml) against *L. monocytogenes* ESB54 (Fig. 2B1) and 6400 AU/ml against *L. innocua* 2030c (pH 4.8) both at the end of exponential phase. In this case, a decrease of bacteriocin activity to 3200 AU/ml was recorded at the end of stationary phase (Fig. 2B2). Activity of both bacteriocinogenic CFSs was maintained against *L. monocytogenes* ESB54 during storage at refrigeration temperature (4 °C) up to 6 months (data not shown).

Mode of action

Addition of bacALP7 (25,600 AU/ml) and bacALP57 (12,800 AU/ml) to an early-log culture of *L. monocytogenes* ESB54 (4 h-old, $OD_{600\ nm} \approx 0.3$) repressed pathogen growth for 9 h (Fig. 3A). Addition of bacALP7 (12,800 AU/ml) and bacALP57 (6400 AU/ml) to an early-log culture of *L. innocua* 2030c (5 h-old, $OD_{600\ nm} \approx 0.2$) repressed cell growth in a similar way (Fig. 3B). Both bacteriocins demonstrated a bacteriostatic mode of action towards the *Listeria* strains. In the untreated (control) samples, no repression or inhibition of growth was observed.

Effects of chemicals, enzymes, temperature and pH on activity of bacteriocins

Both bacteriocins were completely inactivated after treatment with the proteolytic enzymes trypsin, proteinase K, pronase E and papain (Table 2). BacALP7 was completely inactivated by pepsin at 1 mg/ml but only partially at a concentration of 0.1 mg/ml. Activity of bacALP57 was reduced at both concentrations. The exposure of bacteriocins to different pH values showed that both remained fully active in the pH range of 2.0–8.0, and also at pH 10.0 in the case of *P. pentosaceus* ALP57. Reduced activity of both bacteriocins was found after treatment at pH 12.0 (Table 2). At higher temperatures, both bacteriocins revealed some degree of resistance, maintaining 50% of the activity when subjected to 121 °C for 20 min. *P. pentosaceus* ALP57 maintained stability at low temperatures, including at 4 °C, in contrast to *E. faecium* ALP7 which showed a partial loss of activity. Both bacteriocins remained stable when treated with 1% (w/v) of NaCl, SDS, Triton X-100, Tween 20, Tween 80 and EDTA (0.1 mM, 2 mM and 5 mM). However, the antimicrobial activity was affected after treatment with Ox-bile (1% w/v). The addition of urea to both bacteriocins did not reduce the antimicrobial activity against *L. monocytogenes* ESB54 in contrast to the effect produced on *L. innocua* 2030c. Overall, *L. innocua* 2030c appear to be less sensitive to both bacteriocins after treatments (Table 2).

Adsorption of bacteriocins to the producer cells

The loss of bacteriocin activity after the treatment of strains ALP7 and ALP57 with 100 mM NaCl at pH 2.0 suggested that the bacteriocins did not adhere to the producer cells (data not shown).

Partial purification and molecular size of bacteriocins

The bacteriocins bacALP7 and bacALP57 were isolated from 18-h-old cultures in APT medium. The bacteriocins were concentrated by precipitation with 60% (w/v) ammonium sulphate. After precipitation, the inhibitory activity of both bacteriocins against *L. monocytogenes* ESB54 showed a slight increase (≈10%).

The analysis by Tricine-SDS-PAGE gel electrophoresis showed peptide bands for bacALP7 and bacALP57 below 6.5 kDa in size (Fig. 4).

Table 2
Effect of different treatments on the activity of bacteriocins *E. faecium* ALP7 and *P. pentosaceus* ALP57 against the target organisms *L. monocytogenes* ESB54 and *L. innocua* 2030c, expressed in arbitrary units (AU/ml) and corresponding percentage values (%)

Treatment	Bacteriocin activity of *E. faecium* ALP7		Bacteriocin activity of *P. pentosaceus* ALP57	
	AU/ml (%)		AU/ml (%)	
	L. monocytogenes ESB54	*L. innocua* 2030c	*L. monocytogenes* ESB54	*L. innocua* 2030c
Without treatment	25,600 (100%)	12,800 (100%)	12,800 (100%)	6400 (100%)
Treatment with 1%				
NaCl, SDS, Triton X-100, Tween 20, Tween 80	25,600 (100%)	12,800 (100%)	12,800 (100%)	6400 (100%)
Urea	25,600 (100%)	800 (6.25%)	12,800 (100%)	3200 (50%)
Ox-bile	3200 (12.5%)	400 (3.125%)	400 (3.125%)	200 (3.25%)
EDTA				
0.1 mM, 2 mM, 5 mM	25,600 (100%)	12,800 (100%)	12,800 (100%)	6400 (100%)
Effect of enzymes (0.1 mg/ml/1 mg/ml)				
Proteinase K, Pronase E, Papain	0	0	0	0
Lipase	25,600 (100%)	12,800 (100%)	12,800 (100%)	6400 (100%)
α-Amylase	25,600 (100%)	12,800 (100%)	12,800 (100%)	6400 (100%)
Pepsin	3200 (12.5%)/0	0	3200 (25%)/400 (3.25%)	1600 (25%))/0
Effect of temperature (°C) after 2 h				
4	6400 (25%)	1600 (12.5%)	12,800 (100%)	3200 (50%)
10	12,800 (50%)	1600 (12.5%)	12,800 (100%)	6400 (100%)
25	25,600 (100%)	3200 (25%)	12,800 (100%)	3200 (50%)
30, 37	25,600 (100%)	3200 (25%)	12,800 (100%)	1600(25%)
60	6400 (25%)	3200 (25%)	3200 (25%)	800 (12.5%)
80	12,800 (50%)	3200 (25%)	9600 (75%)	1600 (25%)
100, 121 (20 min)	12,800 (50%)	1600 (12.5%)	6400 (50%)	1600 (25%)
Effect of pH after 1 h				
2	25,600 (100%)	1600 (12.5%)	12,800 (100%)	1600 (25%)
4, 6	25,600 (100%)	12,800 (100%)	12,800 (100%)	3200 (50%)
8	25,600 (100%)	1600 (12.5%)	12,800 (100%)	1600 (25%)
10	12,800 (50%)	800 (6.25%)	12,800 (100%)	1600 (50%)
12	6400 (25%)	400 (3.125%)	1600 (12.5%)	400 (6.25%)

PCR detection and sequencing of genes encoding bacteriocins

The amplification of DNA of strains ALP7 and ALP57 with specific primers for enterocin B and pediocin PA-1 yielded 360 kb and 1044 kb fragments, respectively (Fig. 5). The fragments revealed homology to enterocin B (GenBank Accession number U87997.1; Casaus et al., 1997) and pediocin PA-1 (GenBank Accession number AY316525; Miller et al., 2005). Bacteriocins bacALP7 and bacALP57 are thus considered similar to enterocin B and pediocin PA-1, respectively.

Discussion

The two bacteriocin-producing strains isolated from marine non-fermented shellfish were identified pheno- and geno-typically as *E. faecium* ALP7 and *P. pentosaceus* ALP57. Other studies, previously mentioned, also showed the occurrence of LAB in seafood products, although *Carnobacterium* spp. was the dominant genus in most studies. The inhibitory substances produced by both strains were inactivated by proteolytic enzymes (Table 2), confirming their proteinaceous nature and indicating the presence of bacteriocins (Todorov and Dicks, 2005). Moreover, their activities were not affected by lipolytic or glycolytic

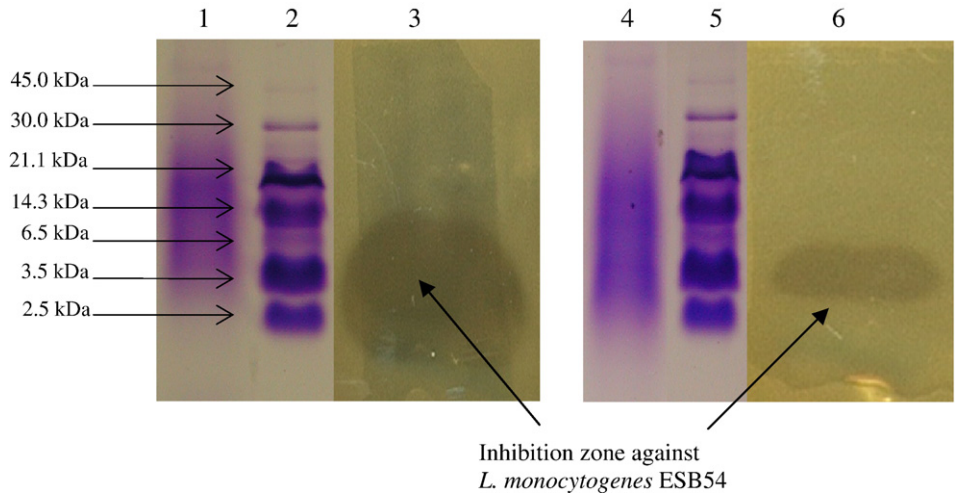

Fig. 4. Tricine-SDS-PAGE gel of bacteriocins *E. faecium* ALP7 and *P. pentosaceus* ALP57. Lane 1: peptide (precipitated by 60% saturated ammonium sulphate) band of strain ALP7 stained with Coomassie Blue R250; Lane 2: molecular mass marker; Lane 3: zone of growth inhibition by bacteriocin ALP7; Lane 4: peptide (precipitated by 60% saturated ammonium sulphate) band of strain ALP57 stained with Coomassie Blue R250; Lane 5: molecular mass marker; Lane 6: zone of growth inhibition of bacteriocin ALP57. The gel in lanes 3 and 6 was overlaid with *L. monocytogenes* ESB54 (approx. 10^6 CFU/ml), embedded in BHI agar, after incubation at 30 °C for 24 h.

enzymes suggesting that the active moiety was not a lipid or a glucan, respectively (Tomé et al., 2006). The inhibitory effect was also enhanced at refrigeration temperatures particularly for *P. pentosaceus* ALP57. Like most of the described bacteriocins (e.g. Messi et al., 2001; Todorov and Dicks, 2005), the bacteriocins described in this study were also heat tolerant, despite the reduction in activity. In contrast, bacteriocin ST15, produced by *E. mundtii*, was inactivated after 10 min at 90–121 °C (De Kwaadsteniet et al., 2005). Treatments with 1% NaCl, Triton X-100, Tween 20, Tween 80, and EDTA did not affect the bacteriocins (Table 2) as reported similarly for enterocin EJ97, produced by *E. faecalis* (Gálvez et al., 1998), pediocin HA-6111-2 produced by *P. acidilactici* (Albano et al., 2007) and bacteriocin ST15 produced by *E. mundtii* (De Kwaasdsteniet et al., 2005). Other treatments, such as addition of ox-bile and urea (in the case of *L. innocua* 2030c) to the CFS, reduced the activity of the bacteriocins. In general, bacteriocins demonstrated higher activity against *L. monocytogenes* ESB54 after all treatments, in comparison with *L. innocua* 2030c. It would be interesting to further investigate the structural and functional changes of the bacteriocins induced by some treatments. This would help to understand the different behaviour observed for *L. monocytogenes* and *L. innocua* strains.

The antimicrobial spectra observed for *E. faecium* ALP7 and *P. pentosaceus* ALP57 included several genera indicating a broad spectrum of activity against Gram-positive organisms. A high level of inhibitory activity against *L. monocytogenes* was evident, including strains isolated from seafood. Strong antilisterial bacteriocins have been allocated to Class IIa (Nes et al., 2001). No activity was observed against the Gram-negative bacteria tested. Inhibition of Gram-negative bacteria is unusual, and has thus far only been reported for a few LAB bacteriocins (e.g. Messi et al., 2001; Todorov and Dicks, 2004; De Kwaadsteniet et al., 2005). The bacteriocinogenic activity of both bacteriocins against all *L. monocytogenes* strains, remained, although reduced, after 6 months at 4 °C. Testing the strains' ability to survive and maintain the functionality at −20 °C for several months would be useful for an evaluation of their application as biopreservatives.

Like most bacteriocins (Pilet et al., 1995; Tahiri et al., 2004; Tomé et al., 2006), those investigated in the current study were secreted into the culture medium in the early exponential phase of growth. The detectable levels of the bacteriocins recorded after 4 h of growth could indicate that the peptide is a primary metabolite as reported for other bacteriocins produced by *L. lactis* subsp. *lactis* A164 (Cheigh et al., 2002), *P. acidilactici* (Nieto-Lozano et al., 2002), and *E. mundtii* ST15 (De Kwaadsteniet et al., 2005). An increase in the production during the log phase was observed for *E. faecium* ALP7, reaching their maximum concentration at the beginning of stationary phase. Then, a decrease in activity against *L. monocytogenes* ESB54 was observed from 25,600 to 12,800 AU/ml below pH 4.3. The biosynthesis of *P. pentosaceus* ALP57 bacteriocin was

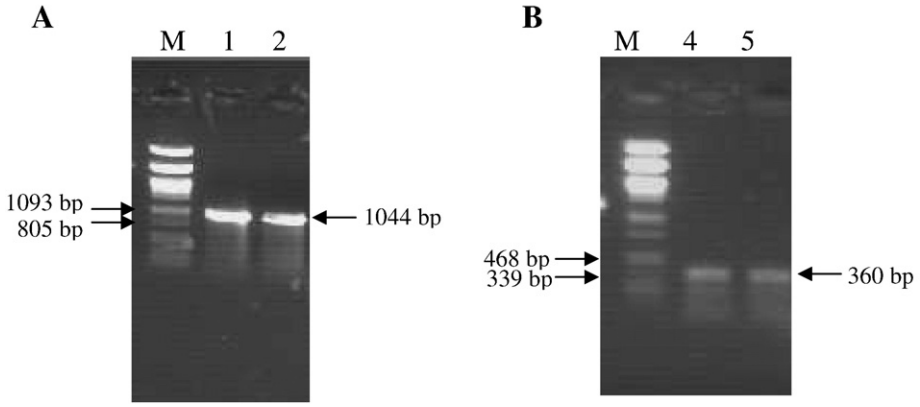

Fig. 5. Amplification of DNA of strains *E. faecium* ALP7 and *P. pentosaceus* ALP57 with specific primers for (A) enterocin B and (B) pediocin PA-1, respectively. (A): Lane M: lambda DNA/*PstI* Marker; Lane 1: Control strain *P. pentosaceus* HA-6111-2; Lane 2: Strain *P. pentosaceus* ALP57; (B) Lane M: lambda DNA/*PstI* Marker; Lane 3 Control Strain *E. faecium* ESB 48; Lane 4: Strain *E. faecium* ALP7.

maximal at the end of the exponential phase, in agreement with other studies (Yamazaki et al., 2003, Campos et al., 2006).

The bacteriocins revealed a bacteriostatic mode of action towards the target indicators. However, particularly in the case of *L. innocua* 2030c, a slow recovery of growth was observed for 2 h followed by stability in optical density units. As reported by Tomé et al. (2006), the time when a bacteriocin is added to a food system might influence significantly their mode of action on the target strain. The same authors reported that the addition of *P. acidilactici* ET34 at 5 h of growth of *L. innocua* 2030c had a bacteriostatic effect but, when added at the beginning or after just 3 h of incubation, the result would be a bactericidal activity on the target indicator.

More accurate techniques could be used to determine the molecular mass of molecules, yet the SDS-PAGE technique provides valuable information about the presence of the peptides (Moreno et al., 2002). The molecular weights of the bacteriocins were close to that reported for other small bacteriocins (<10 kDa) produced by enterococci and pediococci, e.g., bacteriocin ST15 from *E. mundtii* (De Kwaadsteniet et al., 2005), bacteriocin ET05 from *E. faecium* and bacteriocin ET34 from *P. acidilactici* (Tomé et al., 2008). Partial purification of the produced bacteriocins was attempted as described by Yang et al. (1992). No adsorption of bacteriocins to their producer cells was observed, in concordance with other authors [e.g. Ivanova et al., 2000 (bozacin B14), Todorov and Dicks, 2005 (pediocin ST18); Albano et al., 2007 (pediocins HA-6111-2 and HA-5692-3)] thus, the semi-purification was performed by peptides precipitation with 60% ammonium sulphate (Sambrook et al., 1989) in subsequent experiments.

The bacteriocin produced by *P. pentosaceus* ALP57 showed similarity to pediocin PA-1/AcH (Miller et al., 2005), a so-called pediocin-like bacteriocin (class IIa; Nes et al., 2001). By other hand, the bacteriocin produced by *E. faecium* ALP7 demonstrated similarity with enterocin B (Casaus et al., 1997) a linear nonpediocin-like enterocin which belongs to the Class IIc (Klaenhammer, 1993) or Class II3 under the recent proposal by Frank et al. (2007). The pediocin PA-1/AcH was the first class IIa bacteriocin characterized and is extensively studied (Nieto-Lozano et al., 1992; Chen and Hoover, 2003). Synthesis initially was detected in *P. acidilactici* strains (Bhunia et al., 1988; Gonzalez and Kunka, 1987) but *P. pentosaceus* has also ability to produce pediocin AcH as recently described by Bagenda et al. (2008) in a marine fermented seafood product. Other pediocins have been associated to *P. pentosaceus*, including pediocin A (Fleming et al., 1975), pediocin N5p (Strasser de Saad and Manca de Nadra, 1993), pediocin P (Osmanağaoğlu et al., 2001), pediocin ACCEL (Wu et al., 2004), pediocin SM-1 (Anastasiadou et al., in press). In recent years, there have been numerous reports on bacteriocin-producing enterococci, primarily among *E. faecium* associated with food systems (Giraffa, 2003). Enterococcal bacteriocins (A, B, I and P) proved to be strong inhibitors of pathogens as *L. monocytogenes* (Ennahar et al., 2001). Enterocin B was firstly described by Casaus et al. (1997). Both pediocin PA-1/AcH and enterocin B have been tested as natural food preservatives in many food systems, particularly dairy and meat products (Drider et al., 2006) but neither of the bacteriocins have been used in seafoods. Other class IIa bacteriocins have been applied, for instance, divercin V41 (Duffes et al., 1999) and sakacin P (Katla et al., 2001) both to cold-smoked salmon, bavaricin A to brined shrimp (Einarsson and Lauzon, 1995), and piscicosin CS526 to surimi (Yamazaki et al., 2003).

To our knowledge this is the first report of a pediocin PA-1/AcH as well as enterocin B isolated from marine non-fermented shellfish.

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