Characterization of *Pediococcus acidilactici* strains isolated from rainbow trout (*Oncorhynchus mykiss*, Walbaum) feed and larvae: safety, DNA fingerprinting and bacteriocinogenicity

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ABSTRACT

The use of Lactic Acid Bacteria (LAB) as probiotics constitutes an alternative or complementary strategy to chemotherapy and vaccination for disease control in aquaculture. The objectives of this work were (i) the *in vitro* safety assessment of 8 *Pediococcus acidilactici* strains isolated from rainbow trout feed and larvae; (ii) the evaluation of their genetic relatedness; (iii) the study of their antimicrobial/bacteriocin activity against fish pathogens, and (iv) the biochemical and genetic characterization of the bacteriocin produced by the strain displaying the greatest antimicrobial activity. Concerning the safety assessment, none of the pediococci showed antibiotic resistance nor produced hemolysin or gelatinase, degraded gastric mucin or deconjugated bile salts. Four strains (50%) produced tyramine or putrescine, but the corresponding genes were not successfully amplified by PCR. ERIC-PCR fingerprinting allowed clustering of the pediococci into 2 well-defined groups (68% similarity). From the 8 pediococci displaying direct antimicrobial activity against, at least, 3 out of 9 fish pathogens, 6 strains (75%) were identified as bacteriocin producers. The bacteriocin produced by *P. acidilactici* L-14 was purified, and mass spectrometry and DNA sequencing revealed its identity to be pediocin PA-1 (PedPA-1). Altogether, our results allowed the identification of 4 (50%) putatively safe pediococci, including 2 bacteriocinogenic strains. ERIC-PCR fingerprinting was a valuable tool for genetic profiling of *P. acidilactici* strains. This work reports for the first time the characterization of a PedPA-1-producing *P. acidilactici* strain isolated from an aquatic environment (rainbow trout larvae), which shows interesting properties related to its potential use as probiotic in aquaculture.

Keywords: Aquaculture, rainbow trout (*Oncorhynchus mykiss*, Walbaum), Lactic Acid Bacteria, *Pediococcus acidilactici*, anti-fish pathogen activity, fish probiotics

INTRODUCTION

One of the major challenges for modern aquaculture is fish mortality, mainly at the larval stage, attributed to opportunistic and pathogenic bacteria and resulting in important economic losses (Villamil et al. 2010, Pérez-Sánchez et al. 2014). Several approaches have been used to prevent and/or treat bacterial diseases, including (i) effective management of stock, soil, water, nutrition and environment; (ii) sanitary prophylaxis; (iii) water disinfection, (iv) vaccination, and (v) chemotherapy (mainly the use of antibiotics) (Ringø et al. 2010). However, the use of antibiotics is expensive and may induce resistance in the bacterial pathogens through mutations of indigenous genes and/or acquisition of antibiotic resistance genes by mobile genetic elements (Panigrahi & Azad 2007, Ringø et al. 2010). Despite the fact that vaccination seems to constitute the ideal control method, effective commercial vaccines against some fish pathogens are not available yet. Vaccine efficacy can differ due to variable pathogenicity mechanisms displayed by a single pathogen species, and vaccines cannot prevent disease outbreaks in immunologically immature individuals (Subasinghe et al. 2009, Toranzo et al. 2009). In addition, vaccination is laborious, costly, and highly stressful to the animals, retarding their growth (EFSA 2008, Gillor et al. 2008).

A suitable alternative for disease control is the use of probiotics, which do not induce the adverse effects of antibiotics and other chemotherapeutic agents (Nayak 2010). In aquaculture, probiotics are considered as live beneficial microbial adjuncts that modify the host-associated or ambient microbial community, improve feed use or its nutritional value, enhance the host response to diseases, and/or improve the physico-chemical and microbiological quality of the surrounding environment (Verschuere et al. 2000, Pérez-Sánchez et al. 2014, Hai 2015). Moreover, inactivated forms of bacteria (*i.e.*, dead cells), cell components, or extra-cellular compounds have also shown probiotic effects

(Nayak 2010). Lactic Acid Bacteria (LAB) are the bacterial group most commonly proposed as probiotics in aquaculture (Gatesoupe 2008, Pérez-Sánchez et al. 2014, Ringø et al. 2014); nevertheless, to date, only *Pediococcus acidilactici* CNCM MA18/5 M (Bactocell®) has been legally authorized for this purpose in the European Union [Commission Regulation (EC) No. 911/2009 and Commission Implementing Regulation (EU) No. 95/2013]. Most LAB are considered as non-pathogenic and non-opportunistic microorganisms and awarded the Qualified Presumption of Safety (QPS) status granted by the European Food Safety Authority (EFSA) in Europe (Liu et al. 2009, Gaggìa et al. 2010). Recently, LAB isolated from rainbow trout have shown the ability to inhibit the growth of fish pathogens and these strains have been proposed as probiotics to prevent bacterial fish diseases (Pérez-Sánchez et al. 2014, Ringø et al. 2014). The genus *Pediococcus* is one of the best characterized LAB groups, and pediococci have been isolated from ripened cheese, processed meat, plant materials and fish (Cai et al. 1999, Todorov & Dicks 2009, Araújo et al. 2015a) and are used as starter cultures for the production of fermented foods (Lee et al. 2014). Furthermore, some pediococci produce ribosomally-synthesized antimicrobial peptides referred to as bacteriocins (pediocins), and PA-1/AcH (PedPA-1) is the most thoroughly characterized pediocin (Papagianni & Anastasiadou 2009). The operon of this bacteriocin includes four genes: the structural gene (*pedA*), the immunity gene encoding an immunity protein that protects the bacteriocin producer from its own bacteriocin (*pedB*), the gene encoding the ABC transporter for secretion (*pedC*), and the gene encoding a complementary protein of unknown function (*pedD*) (Papagianni & Anastasiadou 2009). PedPA-1 was firstly identified in *P. acidilactici* PAC1.0 (Marugg et al. 1992), and is the subclass IIa bacteriocin most effective against spoilage and food-borne pathogens (Devi & Halami 2011). A previous study demonstrated that repeated doses

(250 µg/day for three consecutive days) of purified pediocin PA-1 given to *Listeria monocytogenes*-infected mice resulted in a significant reduction of fecal listerial counts and slowed pathogen translocation into the liver and spleen, leading to the disappearance of *L. monocytogenes* infection in both organs within 6 days (Dabour et al. 2009).

The objectives of this work were (i) the *in vitro* safety assessment of 8 *P. acidilactici* strains isolated from rainbow trout (*Oncorhynchus mykiss*, Walbaum) feed and larvae using a previously described subtractive screening method (Muñoz-Atienza et al. 2013); (ii) the evaluation of their genetic relatedness; (iii) the study of their antimicrobial/bacteriocin activity against fish pathogens, and (iv) the biochemical and genetic characterization of the bacteriocin produced by the strain (first determined to be safe) which showed the most interesting antimicrobial properties to be used as probiotic in aquaculture.

MATERIALS AND METHODS

Sampling procedure and LAB isolation

Samples of rainbow trout feed and larvae were obtained from a rainbow trout farm located in the south of Spain. Due to commercial confidentiality, the feed brand used in the farm is not given. However, the feed did not contain any bacterial additive and it was confirmed that no additional supplements were added to feed or fish in the farm. The feed was received in a sterilized condition from the manufacturer, and the hypothesis was that feed was colonized by bacterial species from the farm environment.

LAB were isolated from commercial rainbow trout feed (a pool of 3 different samples, 1 g each), and independent composite samples from whole larvae (10 specimens; 5 days post-hatching) disinfected with benzalkonium chloride (Sigma-Aldrich Co., Ltd., St. Louis, Missouri, USA) (0.1% v/v, 30 s) and rinsed twice with 10 mM phosphatebuffered saline (PBS; pH 7.2). The samples were 10-fold diluted in sterile peptone water (Oxoid, Ltd., Basingstoke, United Kingdom), and homogenized in a Stomacher. Then, the sample was pour-plated (three plates per dilution) in de Man, Rogosa and Sharpe (MRS, Oxoid, Ltd., Basingstoke, United Kingdom) agar (1.5% w/v) and incubated at 15 °C in microaerobiosis (plate overlying with an agar medium layer) for 3-7 days.

Bacterial strains and growth conditions

In this study, we used the strain *P. acidilactici* L-14, previously isolated from aquacultured rainbow trout larvae by Araújo et al. (2015a) (not labelled as L-14 in that study), and the strains isolated here from aquacultured rainbow trout feed. *P. acidilactici* L-14, the strains isolated from feed, the Gram-positive fish pathogens (*Lactococcus garvieae* JIP29-99, *Lc. garvieae* CECT5807, *Lc. garvieae* CF01144, *Lc. garvieae* CF00021 and *Carnobacterium maltaromaticum* LMG14716), and the indicator microorganism *Pediococcus damnosus* CECT4797 were aerobically grown in MRS at 30 °C. *Streptococcus iniae* LMG14521 was aerobically grown in Brain Heart Infusion (BHI) broth (Oxoid) at 37 °C. The Gram-negative fish pathogens *Yersinia ruckeri* LMG3279 and *Aeromonas salmonicida* LMG3776 were aerobically grown in Tryptone Soya Broth (TSB; Oxoid) at 28 °C, while *Vibrio campbellii* LMG21363 was aerobically grown in TSB supplemented with NaCl (1% w/v; Panreac Química S.A.U, Barcelona, Spain) at 28 °C. *L. monocytogenes* CECT4032 and *L. innocua* CECT910 were cultured in Brain Heart Infusion (BHI, Oxoid) at 30 °C.

Direct antimicrobial activity assays

A total of 26 LAB isolates recovered from rainbow trout feed and the strain *P. acidilactici* L-14 were assayed for antimicrobial activity against the 9 fish pathogens cited above by a stab-on-agar test (SOAT) as previously described by Cintas et al. (1995). Briefly, each candidate strain was stabbed onto MRS agar and incubated at 30 °C for 5 h, and then 40 ml of the corresponding soft agar (0.8% w/v) medium containing about 1 × 10^5 CFU ml^{-1} of the pathogen was poured onto the plates. After incubation at 28–37 °C for 16–24 h, depending on the optimum growth conditions for each tested pathogen, the plates were checked for inhibition zones (absence of visible microbial growth around the stabbed cultures), and only inhibition halos with diameters above 3 mm were considered positive.

Taxonomic identification

The LAB isolates from rainbow trout feed showing antimicrobial activity against, at least, 3 of the tested fish pathogens were taxonomically identified by DNA sequencing of the PCR-amplified gene encoding a fragment of the 16S rRNA subunit (*16S rDNA*) (Kullen et al. 2000). PCR-amplifications were performed from total bacterial DNA purified using the InstaGene Matrix resin (Bio-Rad Laboratories Inc., Hercules, CA, USA), in 50 µl reaction mixtures with 5 to 50 ng of purified DNA, 0.7 µmol l^{-1} of each primer and 25 µl of MyTaq PCR mix (Bioline, London, UK) in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). The oligonucleotide primers used for PCR amplification of *16S rDNA* were obtained from Sigma-Genosys Ltd. (Cambridge, United Kingdom) (Table 1). PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose (Pronadisa, Madrid, Spain) gels stained with GelRed (Biotium, California, USA), and visualized with the Gel Doc 1000 documentation system (Bio-Rad, Madrid, Spain). HyperLadder II (Bioline GmbH, Germany) was used as molecular size marker. The amplicons were purified by using the NucleoSpin Extract II kit (Macherey & Nagel, Düren, Germany) and both DNA strands were sequenced at the Unidad de Genómica (Parque Científico de Madrid, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Spain). Analysis of *16S rDNA* sequences was performed with the BLAST program available at the National Center for Biotechnology Information (NCBI; blast.ncbi.nlm.nih.gov). Only sequence similarities above 97% were considered significant for bacterial identification at the species level.

Antibiotic susceptibility determination

The minimum inhibitory concentrations (MICs) of 8 antibiotics against the 8 *P. acidilactici* strains, identified following *16S rDNA* sequencing, were determined by a broth microdilution test (Klare et al. 2005). The tested antibiotics were ampicillin (0.5 to 32 μg ml^{-1}), gentamicin (2 to 128 μg ml^{-1}), kanamycin (4 to 256 μg ml^{-1}), streptomycin (4 to 256 μg ml^{-1}), erythromycin (0.12 to 8 μg ml^{-1}), clindamycin (0.12 to 8 μg ml^{-1}), tetracycline (1 to 64 μg ml^{-1}), and chloramphenicol (0.5 to 32 μg ml^{-1}). Individual colonies were suspended in 5 ml of saline solution (0.85% NaCl) to a turbidity of 1 in the McFarland scale (*ca*., 3×10^8 CFU ml^{-1}) and subsequently 1000-fold diluted in LSM broth, consisting of Iso-sensitest (IST) (Oxoid) and MRS broth (IST:MRS, 9:1; pH 6.7). A volume of 50 μl of the diluted suspensions was added to microplate wells containing 50 μl of LSM broth with the different antibiotic concentrations. After incubation at 37 °C for 18 h, MICs were interpreted according to the breakpoints established by the EFSA (2012). Strains showing MICs higher than the respective breakpoint were considered as resistant. *Enterococcus faecalis* CECT795 and *Staphylococcus aureus* CECT794 were used for quality control.

Hemolysin and gelatinase production

The production of hemolysin and gelatinase by the 8 *P. acidilactici* strains was determined as previously described (Eaton & Gasson 2001, Muñoz-Atienza et al. 2013). Briefly, cultures grown in MRS broth were streaked onto COH agar plates (Columbia agar + 5% [v/v] horse blood; BioMérieux, Marcy l'Étoile, France). After plate incubation at 37 °C for 1-2 days, β-hemolysin production was revealed by the presence of clear zones of hydrolysis around the colonies. Moreover, in order to evaluate the production of gelatinase, cultures grown in MRS broth were streaked onto Todd-Hewitt (Oxoid) agar plates (1.5%, w/v) containing 30 g of gelatine per liter. After overnight incubation at 37 °C, the plates were placed at 4 °C for 5 h before examination for the presence of zones of turbidity (protein hydrolysis) around the colonies. *E. faecalis* P4 (Eaton & Gasson 2001) was used as positive control in both assays.

Mucin degradation

The ability of 8 the *P. acidilactici* strains to degrade gastric mucin was determined as described by Zhou et al. (2001). Mucin from porcine stomach type III (Sigma-Aldrich) and agar were incorporated into medium B without glucose at concentrations of 0.5 and 1.5% (w/v), respectively. Briefly, 10 μl of cultures grown in MRS broth were spotted onto the surface of medium B with mucin. The plates were anaerobically (Anaerogen, Oxoid) incubated at 37 °C for 72 h. After incubation, the plates were stained with a mixture of 0.1% (w/v) amido black (Merck KGaA, Darmstadt, Germany) in 3.5 mol l^{-1} acetic acid for 30 min, and then washed with 1.2 mol l^{-1} acetic acid (Merck KGaA). The presence of a discoloured zone around the colony was considered as a positive result. A fresh fecal slurry from a healthy adult cow was used as positive control of mucinolytic activity.

Biogenic amine production

The detection of production of biogenic amines (histamine, tyramine, putrescine and cadaverine) by the 8 *P. acidilactici* strains was first carried out according to the plate assay described by Bover-Cid and Holzapfel (1999). Briefly, cultures grown in MRS broth were streaked on the improved decarboxylase differential growth medium with and without (negative control) the corresponding amino acid precursor (1% w/v, histidine, tyrosine, ornithine and lysine). After plate incubation at 37 °C for 4 days under anaerobic conditions (Anaerogen), the plates were examined for the presence of histidine decarboxylase (HDC), tyrosine decarboxylase (TDC), ornithine decarboxylase (ODC) and lysine decarboxylase (LDC) activities. Biogenic amine production was detected by a yellow to violet color change of the decarboxylase medium due to the alkalinization produced by the decarboxylation of the corresponding amino acid precursor. *Lactobacillus brevis* CECT4121 and *Lactobacillus* sp. 30a (García-Moruno et al. 2005) were used as positive controls for tyramine, and histamine and putrescine production, respectively. Subsequently, the presence of the genes encoding HDC (*hdc*), TDC (*tdc*), ODC (*odc*) and LDC (*ldc*) in the 8 *P. acidilactici* strains was analyzed by PCR. *Lactobacillus brevis* CECT4121 and *Lactobacillus* sp. 30a were used as positive controls. PCR-amplifications were performed as previously described (Le Jeune et al. 1995, Coton et al. 2004, Marcobal et al. 2005, de las Rivas et al. 2006) (Table 1) and PCR-product visualization and sequence analysis were performed as described above.

Bile salt deconjugation

The ability of the 8 *P. acidilactici* to deconjugate primary and secondary bile salts was determined according to Noriega et al. (2006). Bile salt plates were prepared by adding 0.5% (w/v) sodium salts of taurocholate or taurodeoxycholate (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) to MRS agar (1.5%, w/v) supplemented with 0.05% (w/v) L-cysteine (Merck KGaA). Briefly, 10 µl of cultures grown in MRS broth were spotted onto agar plates and incubated at 37 °C for 72 h under anaerobic conditions (Anaerogen, Oxoid). The presence of precipitated bile acid around the spotted-culture (opaque halo) was considered as a positive result. A fresh fecal slurry of a healthy adult cow was used as positive control.

Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR)

Genetic profiling of the 8 *P. acidilactici* strains by ERIC-PCR was performed using the primers ERIC-1R and ERIC-2 (Table 1) as previously described (Versalovic et al. 1991). PCR-amplifications were performed from total bacterial DNA, obtained by using the InstaGene Matrix resin (Bio-Rad Laboratories Inc.), in 50 µL reaction mixtures with 2 µL of purified DNA, 0.7 µmol l^{-1} of each primer, 3 mmol l^{-1} of $MgCl_2$ and 25 µl of MyTaq PCR mix in a Eppendorf Mastercycler thermal cycler. PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gels stained with GelRed at 90V for 90 min. PCR-product visualization and analysis were performed as described above. The molecular size marker used was the 1 Kb Plus DNA ladder (Invitrogen, Madrid, Spain). Cluster analyses were performed using the unweighted-pair-group method with arithmetic averages (UPGMA) using the PhoretixTM 1D Advanced (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) software. *P. acidilactici* 347 (Cintas et al. 1998) and *P. acidilactici* MA18/5M (Bactocell®) were used as controls.

Extracellular antimicrobial (bacteriocin) activity assay

The antimicrobial activity of cell-free supernatants from the 8 *P. acidilactici* cultures grown in MRS broth at 30 °C for 16 h against the indicator microorganisms *P. damnosus* CECT4797, *L. monocytogenes* CECT4032, *L. innocua* CECT910, *Lc. garvieae* CF01144, *Lc. garvieae* CECT5807, *Lc. garvieae* CF00021 and *A. salmonicida* LMG3776 was determined by an agar well-diffusion test (ADT) as previously described by Cintas et al. (1995). Briefly, supernatants were obtained by centrifugation of cultures at 10,000 × g at 4 °C for 10 min, adjusted to pH 6.2 with 1 mol l^{-1} NaOH, filter-sterilized through 0.22 μm-pore-size filters (Millipore Corp., Bedford, Massachussets, USA) and stored at −20°C until use. Fifty-μl aliquots of supernatants were placed into wells (6-mm diameter) cut in cooled MRS (0.8%, w/v) plates previously seeded (1×10^5 CFU ml^{-1}) with the indicator microorganisms. After 2 h at 4 °C, the plates were incubated under the conditions mentioned in the bacterial strains and growth conditions section and then analyzed for the presence of inhibition zones around the wells. To determine the nature and thermostability of the antimicrobial compounds, the supernatants showing antimicrobial activity were (i) treated with proteinase K (10 mg ml^{-1}) (AppliChem GmbH, Germany) at 37 °C for 2 h and then heated at 100 °C for 10 min to inactivate the enzyme, and (ii) heated at 100 °C for 10 min. After treatments, samples were assayed for residual antimicrobial activity by an ADT as described above, using *P. damnosus* CECT4797 and *L. monocytogenes* CECT4032 as indicator microorganisms. Non-treated supernatants were used as positive controls.

Bacteriocin purification and mass spectrometry analysis

The bacteriocin produced by *P. acidilactici* L-14 was purified using a modification of the multi-chromatographic procedure described by Cintas et al. (1995). Briefly, the bacteriocin was purified from a 1-liter culture grown in MRS at 30 °C until the early stationary phase (approximately, 16 h). The cell-free culture supernatant was subjected to protein precipitation with ammonium sulphate (50% [w/v]; Merck). The pellet and floating materials were mixed and solubilized in 100 ml of 20 mM sodium phosphate buffer (NaP) (pH 6.0), and subsequently desalted by gel filtration (PD-10 columns; GE Healthcare Life Sciences, Barcelona, Spain). The resulting fractions were further subjected to cationic-exchange (SP Sepharose Fast Flow, GE Healthcare) and hydrophobic-interaction (Octyl Sepharose CL-4B, GE Healthcare) chromatographies, followed by reversed-phase chromatography (PepRPC HR 5/5) in a Fast Protein Liquid Chromatography system (ÄKTA-FPLC) (GE Healthcare). The antimicrobial activity of the fractions obtained during the purification procedure was determined by a microtiter plate assay (Cintas et al. 1998) using *L. monocytogenes* CECT4032 as indicator microorganism. Briefly, two-fold serial dilutions of the purified bacteriocin in MRS broth were prepared in microtiter plates. The wells were then filled up to 200 µl by the addition of 150 µl of a diluted (in BHI broth) fresh overnight culture of the indicator microorganism (*ca*., 1×10^5 CFU ml^{-1}). After incubation at 30 °C for 16 h, growth inhibition of *L. monocytogenes* CECT4032 was measured spectrophotometrically at 620 nm with a microtiter plate reader (Labsystems iEMS Reader MF, Labsystems, Helsinki, Finland). One bacteriocin unit (BU) was defined as the reciprocal of the highest dilution of supernatant or purified bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin). Fractions displaying a high and specific bacteriocin activity were pulled together and rechromatographed on the same reversed-phase column until chromatographically pure bacteriocin peptides were obtained. The purified peptide was subjected to mass spectrometry analysis in a Matrix-Assisted Laser Desorption Ionization-Time Of Flight Voyager-DE STR mass spectrometer (MALDI-TOF MS) (PerSeptive Biosystems, Foster City, California, USA), at the Mass Spectrum Service of the Centro de Genómica y Proteómica (Parque Científico-UCM, Madrid, Spain).

Nucleotide sequencing of the PA-1 structural and immunity genes

The nucleotide sequence encoding the structural (*pedA*) and immunity (*pedB*) genes of PedPA-1 was determined using specific primer-pairs based on the published DNA sequence of the PedPA-1 operon in *P. acidilactici* PAC1.0 (GenBank accession number M83924) (Marugg et al. 1992). Overlapping PCR products of approximately 400-bp in length were obtained and subsequently electrophoresed, visualized and purified, and both DNA strands sequenced and analyzed, as described above.

RESULTS

Isolation of LAB from rainbow trout feed and direct antimicrobial activity

A total of 26 LAB isolates were recovered from the rainbow trout feeds, of which 7 displayed direct antimicrobial activity against, at least, 3 out of the 9 tested fish pathogens (Table 2). In addition, *P. acidilactici* L-14, isolated previously in Araújo et al. (2015) from rainbow trout larvae, was the most active strain, inhibiting a total of 7 fish pathogens. According to the sizes of the inhibition halos, the most susceptible indicators were *Lc. garvieae* JIP29-99, *Lc. garvieae* CF00021 and *Lc. garvieae* CF01144, followed by *Lc. garvieae* CECT5807, *A. salmonicida* LMG3776 and *C. maltaromaticum* LMG14716. On the contrary, *S. iniae* LMG14521, *Y. ruckeri* LMG3279 and *V. campbellii* LMG21363 were the most resistant indicator microorganisms. The strains *P. acidilactici* L-14 (isolated from larvae) and *P.*

acidilactici NF1-1, NF1-5, NF1-7 and NF1-25 (isolated from feed) displayed a broad antimicrobial spectrum and inhibited most of the tested *Lc. garvieae* strains.

Taxonomic identification

The 7 isolates recovered from rainbow trout feed showing antimicrobial activity against, at least, 3 of the tested fish pathogens were identified by nucleotide sequencing of *16S rDNA* as *P. acidilactici*, showing 100% similarity to the sequences available at the NCBI database.

Safety assessment

The 7 selected *P. acidilactici* strains isolated from rainbow trout feed and *P. acidilactici* L-14 were submitted to a comprehensive *in vitro* safety assessment procedure including several microbiological, biochemical and genetic assays. MIC distribution of the tested antibiotics is summarized in Table 3. The microbiological breakpoints established by EFSA (2012) were used to categorize the 8 pediococci as susceptible or resistant strains. According to our results, all the strains were susceptible to the antibiotics tested in this work (Table 3). Moreover, none of the 8 pediococci produced hemolysin or gelatinase, degraded gastric mucin or deconjugated primary or secondary bile salts (results not shown). On the other hand, a total of 4 (50%) strains produced one of the tested biogenic amines. With regard to this, 2 strains (25%) (*P. acidilactici* NF1-5 and NF1-12) produced tyramine and 2 strains (25%) (*P. acidilactici* NF1-1 and NF-10) produced putrescine; however, the respective genes, *tdc* and *odc*, were not detected by PCR in these strains. None of the tested strains produced histamine or cadaverine (results not shown). Altogether, our results showed that 4 strains (*P. acidilactici* L-14, NF1-7, NF1-18 and NF1-25) are putatively safe pediococci.

Genetic profiling

ERIC-PCR fingerprinting of the 8 *P. acidilactici* strains revealed 2 well-defined groups (G1 and G2; 68% similarity). Furthermore, the 8 pediococci were clustered in 3 well-defined sub-groups (SG1.1, SG1.2 and SG2.1) with similarity coefficients above 75% (Fig. 1). The strains NF1-12 and NF1-25 were the most closely related, presenting 100% of similarity (SG1.2).

Extracellular antimicrobial activity

Six out of the 8 *P. acidilactici* strains (NF1-1, NF1-5, NF1-7, NF1-10, NF1-12 and *P. acidilactici* L-14) (75%) displayed extracellular antimicrobial activity in their supernatants against *P. damnosus* CECT4797, but only 2 (25%) of them (*P. acidilactici* L-14 and NF1-1) were active against the 2 tested *Listeria* spp, with *P. acidilactici* L-14 displaying the strongest antilisterial activity. None of the supernatants from tested strains inhibited the growth of the fish pathogens *Lc. garvieae* CF01144, *Lc. garvieae* CECT5807, *Lc. garvieae* CF00021 and *A. salmonicida* LMG3776 (Table 4). Interestingly, the antimicrobial activity of the supernatants disappeared completely after proteinase K treatment, but was not sensitive to heating, revealing the proteinaceous nature and heat stability of the secreted antimicrobial compounds (*i.e.*, heat-stable bacteriocins). Interestingly, from the 6 bacteriocinogenic strains, only *P. acidilactici* NF1-7 and *P. acidilactici* L-14 were considered as putatively safe.

Purification and genetic characterization of the bacteriocin produced by *P. acidilactici* L-14

The results of the purification of the bacteriocin produced by *P. acidilactici* L-14 are summarized in Table 5. The final RP-FPLC resulted in a major well-separated absorbance peak with antimicrobial activity against *L. monocytogenes* CECT4032, which eluted at 19% (v/v) 2-propanol in aqueous 0.1% (v/v) trifluoroacetic acid (Fig. 2). This fraction showed an increment of 8.1×10^{8}% of the bacteriocin activity originally present in the culture supernatant, and an outstanding 6.3×10^{11}-fold increase in the specific antimicrobial activity (Table 5). The purity and molecular mass of the bacteriocin was confirmed by MALDI-TOF MS, which revealed a major peptide with a molecular mass of 4,621.8 Da, as well as a second peptide peak with a molecular mass of 4,637.6 Da (Fig. 3).

Blast analysis of the sequence of 770 contiguous nucleotides (including the genes *pedA* and *pedB*) of the PedPA-1 gene cluster of *P. acidilactici* L-14 revealed 100% similarity to the sequence of the bacteriocin operon from *P. acidilactici* PAC1.0 (Marugg et al. 1992).

DISCUSSION

In this work, 26 LAB isolates were recovered from rainbow trout feed samples. From these isolates, a total of 7 were selected for further characterization due to their direct antimicrobial activity against, at least, 3 of the tested fish pathogens, and were taxonomically identified as *P. acidilactici*. In addition, *P. acidilactici* L-14, a strain previously isolated from a rainbow trout larvae (Araújo et al. 2015a), showed direct antimicrobial activity against 7 of the tested fish pathogens. The extracellular antimicrobial (bacteriocin) activity, safety assessment and genetic relatedness of these 8 *P. acidilactici* strains were determined in order to select the most suitable strain to be used as a probiotic for aquaculture.

Our group has recently demonstrated the *in vivo* effectiveness of nisin Z, a peptide produced by *Lactococcus lactis,* in protecting rainbow trout against lactococcosis caused by *Lc. garvieae* (Araújo et al. 2015b). In this study, the 8 *P. acidilactici* strains displayed a broad antimicrobial spectrum against fish pathogens, which could be due to different antimicrobial metabolites such as organic acids (mainly lactic acid), hydrogen peroxide, diacetyl, acetaldehyde and/or bacteriocins, with the organic acids and bacteriocins being the key weapons in inhibiting pathogens of relevance for aquaculture (Gillor et al. 2008, Desriac et al. 2010). In this respect, 6 out of 8 *P. acidilactici* strains were found to be bacteriocin producers, based on the proteinaceous nature and heat stability of the antimicrobial agent, which is in agreement with previous studies, where a remarkable high number of LAB displaying antimicrobial activity against fish pathogens were potential bacteriocin producers (Muñoz-Atienza et al. 2013, Gómez-Sala et al. 2015). However, the results indicated that the antimicrobial activity against *Lc. garvieae* exerted by the pediococci was not due to the production of bacteriocins as the antimicrobial effect was only observed in the presence of pediococcal cells. Similarly, Muñoz-Atienza et al. (2013) described several lactic acid bacteria strains with direct but not extracellular antimicrobial activity against *Lc. garvieae*, which can be explained by the fact that the direct antimicrobial activity may due to the competition for nutrients, which is considered as a desirable probiotic property.

The species *P. acidilactici* is awarded with the QPS status since these bacteria are considered neither pathogenic nor opportunistic microorganisms (Liu et al. 2009, Gaggìa et al. 2010). With regard to this, according to EFSA, the demonstration of their safety only requires confirmation of the absence of genes encoding resistance to antibiotics of clinical significance in human and veterinary medicine. Nevertheless, in this work we followed a comprehensive previously described *in vitro* subtractive screening (Muñoz-Atienza et al. 2013) with criteria more exhaustive than that established by EFSA to assess the safety of *P. acidilactici* strains, which included not only the evaluation of antibiotic resistance but also hemolysin and gelatinase production, mucin degradation and biogenic amine production.

The irresponsible use of antibiotics in aquaculture has contributed to the emergence and spread of antibiotic resistance (Cabello 2006). The genetic determinants encoding antibiotic resistance may be acquired by LAB, such as pediococci, and subsequently horizontally transferred to other bacteria, including animal and human pathogens (Liu et al. 2009). Danielsen et al. (2007) reported the presence of the gene *erm*(B) in a *P. acidilactici* strain, which confers acquired resistance to erythromycin, and Tenorio et al. (2001) described the presence of the gene *aac(6')Ie-aph(2'')Ia* in a strain of *P. acidilactici*, which confers high-level resistance to gentamicin. Furthermore, the presence of the genes *mef*(A/E) and *lnu*(A), conferring resistance to erythromycin and lincosamides, respectively, was reported in pediococcal strains (Muñoz-Atienza et al. 2013). Interestingly, none of the *P. acidilactici* strains evaluated in our study showed resistance to any of the tested antibiotics. Moreover, none of them produced the virulence factors hemolysin or gelatinase, which is in agreement with previous studies (Albano et al. 2009, Muñoz-Atienza et al. 2013, Mabrouk et al. 2014). In addition, the 8 tested strains did not display mucinolytic activity indicating their low invasive and toxigenic potential at the mucosal barrier, which supports that LAB do not degrade mucin *in vitro* and is in agreement with previously published works (Zhou et al. 2001, Delgado et al. 2007, Muñoz-Atienza et al. 2013). The production of biogenic amines (low molecular weight organic bases with biological activity), by the decarboxylation of precursor amino acids through substrate-specific enzymes, by many LAB strains has been associated with undesirable toxic effects for humans (Buňková et al. 2009, Talon

& Leroy 2011). In this sense, tyramine poisoning is characterized by hypertension and headache, while histamine or scombroid poisoning is associated with facial flush, vomit, diarrhea, edema, and heart palpitations amongst other symptoms. In the European Union, the presence of histamine is already regulated in some fish products (European Commission, 2005). Other biogenic amines, such as putrescine and cadaverine interfere with the enzymes that metabolize tyramine and histamine, enhancing the adverse effects even if not being toxic by themselves (Landete et al. 2007, Buňková et al. 2009). In this work, we found that 4 *P. acidilactici* strains (50%) produced tyramine or putrescine. In a previous study, it was reported that strains of the genus *Pediococcus* were the main producers of tyramine in beer (Izquierdo-Pulido et al. 2000). Moreover, *P. acidilactici* strains were identified as low concentration tyramine producers in Robiola di Roccaverano cheese (Bonetta et al. 2008), but other strains were shown as producing high tyramine concentrations in a sardine infusion decarboxilase broth (Kuley et al. 2013). In contrast, several studies showed that pediococcal strains isolated from fermented sausages (Fadda et al. 2001, Albano et al. 2009), traditionally processed fish products (Thapa et al. 2006) and fish and fish products (Muñoz-Atienza et al. 2011) were unable to produce tyramine, cadaverine, histamine and/or putrescine. Interestingly, the respective genetic determinants in the 4 biogenic-amine producing strains identified in our study were not detected by PCR analysis, which may be due to the existence of still unknown genes, to point mutations of the respective genes that could hamper their PCR-amplification or to false-positives. With regard to this, and accordingly to the precautionary principle, we did not consider as safe these 4 *P. acidilactici* strains. On the other hand, the ability of probiotic strains to deconjugate bile salts could disrupt the formation of micelles, lipid digestion and absorption of fatty acids and monoglycerides in the intestine, due to the ineffectiveness in the emulsification of dietary lipids, and seriously affect the animal production (Begley et al. 2005). None of the 8 tested *P. acidilactici* deconjugated bile salts. It should be mentioned that, according to EFSA, the 8 *P. acidilactici* strains tested in this work could be considered as safe microorganisms to be used in food and feed; however, the *in vitro* subtractive screening procedure used in this work to assess the safety of these *P. acidilactici* strains revealed that, based on our more stringent criteria, as discussed above, only 4 of them were considered as safe.

PCR fingerprinting methods, such as ERIC-PCR, are valuable tools for genetic typing of microorganisms, and more useful than pulsed-field gel electrophoresis due to a fast and easy execution and the possibility of evaluate many samples simultaneously (Ventura & Zink 2002). A previous work reported the suitability of ERIC-PCR to determine the genetic variability of *P. acidilactici*, *Pediococcus pentosaceus* and *Pediococcus lolii* (Doi et al. 2009). In our work, ERIC-PCR fingerprinting allowed the clustering of the 8 *P. acidilactici* strains isolated from rainbow trout feed and larvae in 2 well-defined groups. The 2 groups displayed equal percentages of safe pediococcal strains (50%). Furthermore, the 6 pediococcal strains identified as bacteriocinogenic were grouped in the 3 different subgroups (2 strains by each subgroup [NF1-1 and NF1-12 in SG1.1; L-14 and NF1-10 in SG1.2, and NF1-5 and NF1-7 in SG2.1]). Only 2 out of the 6 bacteriocinogenic strains, included in 2 different subgroups (*P. acidilactici* L-14 and *P. acidilactici* NF1-7), were considered as safe. The bacteriocinogenic strains *P. acidilactici* NF1-5 and NF1-7 were the most closely related to the commercial probiotic *P. acidilactici* MA18/5M (Bactocell), with a similarity of 82%. Moreover, the PedPA-1 producer strains *P. acidilactici* L-14 and *P. acidilactici* 347 (Cintas et al. 1998) presented 83% of similarity, suggesting genetic differences that may be due to the different source of isolation (*i.e.* rainbow trout larvae and dry-fermented sausage).

Based on its strong antimicrobial activity and safety, we selected the bacteriocinogenic strain *P. acidilactici* L-14 for the characterization of its bacteriocin, using biochemical and genetic methods. The purified bacteriocin produced by *P. acidilactici* L-14 was analyzed by MALDI-TOF MS (Fig. 3) revealing a major peptide with a molecular mass (4,621.8 Da) closely similar to that of the PedPA-1 (4,628.1 Da) firstly identified in *P. acidilactici* PAC1.0 (Henderson et al. 1992). This difference may be explained by the formation in the purified PedPA-1 of 2 disulphide bonds between the cysteine residues Cys9 and Cys14, and Cys24 and Cys44, and by the deamidation of the 3 asparagine residues (Asn27, Asn28 and Asn41). Moreover, a second peptide with a molecular mass of 4,637.6 Da was identified, which may have resulted from a spontaneous oxidation of the methionine residue (Met31) to a methionine sulfoxide (MetSO31), thus increasing the molecular mass by 16 additional Da. In this respect, it has been reported that during bacteriocin purification, Met usually becomes spontaneously oxidized, leading to loss or reduction of the bacteriocins antimicrobial activity (Basanta et al. 2010). Taking into account that the PedPA-1 activity quantified in the last RP-FPLC fraction was higher than that of the culture supernatant, the oxidation events cited above could have taken place during the subsequent treatment of the purified bacteriocin for MALDI-TOF MS analysis, similar to suggested reasons for reduction in enterocin L50 (L50A and L50B) produced by *E. faecium* L50 (Basanta et al., 2010). The genetic analysis of *pedA* and *pedB* in *P. acidilactici* L14 supported the results obtained by mass spectra, since both genes were identical to the respective genes from *P. acidilactici* PAC1.0 (Marugg et al. 1992). The gene *pedA* gene encodes a 62 amino acid peptide (prepediocin PA-1) containing a 18 N-terminal amino acid leader sequence which is cleaved off during secretion yielding a 44 amino acid peptide corresponding to the mature pediocin PA-1 (Marugg et al. 1992). The gene *pedB* encodes a 112 amino acid protein responsible for the immunity of the cells (Marugg et al. 1992). The operon of this bacteriocin is completed with two more genes involved in secretion, *pedC* and *pedD,* encoding a 174 amino acid membrane fusion protein and a 724 amino acid ATP-binding cassette (ABC) transporter, respectively (Marugg et al. 1992, Venema et al. 1995). Pediocin-producing pediococci strains are highly widespread in nature and have been isolated from several sources, including a large variety of plants and fruits (Bennik et al. 1997, Carr et al. 2002, Todorov & Dicks 2009), fermented meat and meat products (Bauer et al. 2005, Albano et al. 2007), boza (cereal-based fermented beverage) (Todorov & Dicks 2005), the gastrointestinal tract of poultry (Juven et al. 1991), ducks (Kurzak et al. 1998) and sheep (Hudson et al. 2000), and human feces (Millette et al. 2007).

CONCLUSION

The results of this work reveal that rainbow trout feed and larvae constitute an interesting source for the isolation of bacteriocinogenic pediococci with potential application as probiotics in aquaculture. To our knowledge, this is the first study reporting the characterization of a PedPA-1-producing *P. acidilactici* strain isolated from an aquatic environment (rainbow trout larvae). Further *in vitro* and *in vivo* studies should be carried out in order to evaluate the probiotic effects and to confirm the lack of toxicity of this strain before its proposal as probiotic to prevent fish diseases in aquaculture.

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LITERATURE CITED

Albano H, Pinho C, Leite D, Barbosa J, Silva J, Carneiro L, Magalhães R, Hogg T, Teixeira P (2009) Evaluation of a bacteriocin-producing strain of *Pediococcus acidilactici* as a biopreservative for "Alheira", a fermented meat sausage. Food Control 20:764–770

Albano H, Todorov SD, van Reenen CA, Hogg T, Dicks LMT, Teixeira P (2007) Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. Int J Food Microbiol 116:239–247

Araújo C, Muñoz-Atienza E, Nahuelquín Y, Poeta P, Igrejas G, Hernández PE, Herranz C, Cintas LM (2015a) Inhibition of fish pathogens by the microbiota from rainbow trout (*Oncorhynchus mykiss*, Walbaum) and rearing environment. Anaerobe 32:7–14

Araújo C, Muñoz-Atienza E, Pérez-Sánchez T, Poeta P, Igrejas G, Hernández PE, Herranz C, Ruiz-Zarzuela I, Cintas LM (2015b) Nisin Z production by *Lactococcus lactis* subsp. *cremoris* WA2-67 of aquatic origin as a defense mechanism to protect rainbow trout (*Oncorhynchus mykiss*, Walbaum) against *Lactococcus garvieae*. Submitted for publication

Basanta A, Gómez-Sala B, Sánchez J, Diep DB, Herranz C, Hernández PE, Cintas LM (2010) Use of the yeast *Pichia pastoris* as an expression host for secretion of enterocin L50, a leaderless two-peptide (L50A and L50B) bacteriocin from *Enterococcus faecium* L50. Appl Environ Microbiol 76:3314–3324

Bauer R, Chikindas ML, Dicks LMT (2005) Purification, partial amino acid sequence and mode of action of pediocin PD-1, a bacteriocin produced by *Pediococcus damnosus* NCFB 1832. Int J Food Microbiol 101:17–27

Begley M, Gahan CG, Hill C (2005) The interaction between bacteria and bile. FEMS Microbiol Rev 29:625–651

Bennik M, Smid EJ, Gorris L (1997) Vegetable-associated *Pediococcus parvulus* produces pediocin PA-1. Appl Environ Microbiol 63:2074-2076

Bonetta S, Bonetta S, Carraro E, Coïsson JD, Travaglia F, Arlorio M (2008) Detection of biogenic amine producer bacteria in a typical Italian goat cheese. J Food Protect 71:205-209

Bover-Cid S, Holzapfel WH (1999) Improved screening procedure for biogenic amine production by lactic acid bacteria. Int J Food Microbiol 53:33–41

Buňková L, Buňka F, Hlobilová M, Vakátková Z, Nováková D, Dráb V (2009) Tyramine production of technological important strains of *Lactobacillus*, *Lactococcus* and *Streptococcus*. Eur Food Res Technol 229:533–538

Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol 8:1137–1144

Cai Y, Suyanandana P, Saman P, Benno Y (1999) Classification and characterization of lactic acid bacteria isolated from the intestines of common carp and freshwater prawns. J Gen Appl Microbiol 45:177–184

Carr FJ, Chill D, Maida N (2002) The lactic acid bacteria: a literature survey. Crit Rev Microbiol 28:281–370

Cintas LM, Casaus P, Fernández MF, Hernández PE (1998) Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin A and lactocin S against spoilage and foodborne pathogenic bacteria. Food Microbiol 15:289–298

Cintas LM, Rodríguez JM, Fernández MF, Sletten K, Nes IF, Hernández PE, Holo H (1995) Isolation and characterization of pediocin L50, a new bacteriocin from

Pediococcus acidilactici with a broad inhibitory spectrum. Appl Environ Microbiol 61:2643–2648

Coton M, Coton E, Lucas P, Lonvaud A (2004) Identification of the gene encoding a putative tyrosine decarboxylase of *Carnobacterium divergens* 508. Development of molecular tools for the detection of tyramine-producing bacteria. Food Microbiol 21:125–130

Dabour N, Zihler A, Kheadr E, Lacroix C, Fliss I (2009) *In vivo* study on the effectiveness of pediocin PA-1 and *Pediococcus acidilactici* UL5 at inhibiting *Listeria monocytogenes*. Int J Food Microbiol 133:225–233

Danielsen M, Simpson PJ, O'Connor EB, Ross RP, Stanton C (2007) Susceptibility of *Pediococcus* spp. to antimicrobial agents. J Appl Microbiol 102:384–389

de las Rivas B, Marcobal Á, Carrascosa AV, Muñoz R (2006) PCR detection of foodborne bacteria producing the biogenic amines histamine, tyramine, putrescine, and cadaverine. J Food Protect 69:2509–2514

Delgado S, O'Sullivan E, Fitzgerald G, Mayo B (2007) Subtractive screening for probiotic properties of *Lactobacillus* species from the human gastrointestinal tract in the search for new probiotics. J Food Sci 72:M310–315

Desriac F, Defer D, Bourgougnon N, Brillet B, Le Chevalier P, Fleury Y (2010) Bacteriocin as weapons in the marine animal-associated bacteria warfare: inventory and potential applications as an aquaculture probiotic. Mar Drugs 8:1153–1177

Devi S, Halami P (2011) Detection and characterization of pediocin PA-1/AcH like bacteriocin producing lactic acid bacteria. Curr Microbiol 63:181–185

Doi K, Nishizaki Y, Fujino Y, Ohshima T, Ohmomo S, Ogata S (2009) *Pediococcus lolii* sp. nov., isolated from ryegrass silage. Int J Syst Evol Microbiol 59:1007–1010

Eaton TJ, Gasson MJ (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microbiol 67:1628–1635

EFSA (2008) Scientific opinion of the Panel on Animal Health and Animal Welfare on a request from the European Commission on the animal welfare aspects of husbandry systems for farmed trout. EFSA J. 796:1−97

EFSA (2012) Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J 10:2740–2749

European Commission, 2005. Commission Regulation (EU) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. O. J. L 338/1

Fadda S, Vignolo G, Oliver G (2001) Tyramine degradation and tyramine/histamine production by lactic acid bacteria and *Kocuria* strains. Biotechnol Lett 23:2015–2019

Gaggìa F, Mattarelli P, Biavati B (2010) Probiotics and prebiotics in animal feeding for safe food production. Int J Food Microbiol 141:S15–S28

García-Moruno E, Carrascosa AV, Muñoz R (2005) A rapid and inexpensive method for the determination of biogenic amines from bacterial cultures by thin-ayer chromatography. J Food Protect 68:625–629

Gatesoupe FJ (2008) Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. J Mol Microbiol Biotechnol 14:107–114

Gillor O, Etzion A, Riley MA (2008) The dual role of bacteriocins as anti- and probiotics. Appl Microbiol Biotechnol 81:591–606

Gómez-Sala B, Muñoz-Atienza E, Sánchez J, Basanta A, Herranz C, Hernández PE, Cintas LM (2015) Bacteriocin production by Lactic Acid Bacteria isolated from fish, seafood and fish products. Eur Food Res Technol 241:341–356

Hai NV (2015) The use of probiotics in aquaculture. J Appl Microbiol 119:917–935

Henderson JT, Chopko AL, van Wassenaar PD (1992) Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0. Arch Biochem Biophys 295:5–12

Hudson JA, Cai Y, Corner RJ, Morvan B, Joblin KN (2000) Identification and enumeration of oleic acid and linoleic acid hydrating bacteria in the rumen of sheep and cows. J Appl Microbiol 88:286–292

Izquierdo-Pulido M, Mariné-Font A, Vidal-Carou MC (2000) Effect of tyrosine on tyramine formation during beer fermentation. Food Chem 70:329–332

Juven BJ, Meinersmann RJ, Stern NJ (1991) Antagonistic effects of lactobacilli and pediococci to control intestinal colonization by human enteropathogens in live poultry. J Appl Bacteriol 70:95–103

Klare I, Konstabel C, Müller-Bertling S, Reissbrodt R, Huys G, Vancanneyt M, Swings J, Goossens H, Witte W (2005) Evaluation of new broth media for microdilution antibiotic susceptibility testing of Lactobacilli, Pediococci, Lactococci, and Bifidobacteria. Appl Environ Microbiol 71:8982–8986

Kuley E, Ozogul F, Balikci E, Durmus M, Ayas D (2013) The influences of fish infusion broth on the biogenic amines formation by lactic acid bacteria. Braz J Microbiol 44:407–415

Kullen MJ, Sanozky-Dawes RB, Crowell DC, Klaenhammer TR (2000) Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. J Appl Microbiol 89:511–516

Kurzak P, Ehrmann MA, Vogel RF (1998) Diversity of lactic acid bacteria associated with ducks. Syst Appl Microbiol 21:588–592

Landete JM, Ferrer S, Pardo I (2007) Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. Food Control 18:1569–1574

Le Jeune C, Lonvaud-Funel A, Ten Brink B, Hofstra H, Van der Vossen JM (1995) Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. J Appl Bacteriol 78:316–326

Lee KW, Park JY, Sa HD, Jeong JH, Jin DE, Heo HJ, Kim JH (2014) Probiotic properties of *Pediococcus* strains isolated from jeotgals, salted and fermented Korean sea-food. Anaerobe 28:199–206

Liu C, Zhang Z-Y, Dong K, Yuan J-P, Guo X-K (2009) Antibiotic resistance of probiotic strains of Lactic Acid Bacteria isolated from marketed foods and drugs. Biomed Environ Sci 22:401–412

Mabrouk AM, Effat BA, Sadek ZI, Tawfiek NF, Hassan ZM, Magdoub MN (2014) Antibacterial activity of some Lactic acid Bacteria isolated from Egyptian Dairy Products. Int J ChemTech Res 6:1139–1150

Marcobal A, De Las Rivas B, Moreno-Arribas MV, Muñoz R (2005) Multiplex PCR method for the simultaneous detection of histamine-, tyramine-, and putrescine-producing lactic acid bacteria in foods. J Food Protect 68:874–878

Marugg JD, Gonzalez CF, Kunka BS, Ledeboer AM, Pucci MJ, Toonen MY, Walker SA, Zoetmulder LC, Vandenbergh PA (1992) Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl Environ Microbiol 58:2360–2367

Millette M, Dupont C, Archambault D, Lacroix M (2007) Partial characterization of bacteriocins produced by human *Lactococcus lactis* and *Pediococccus acidilactici* isolates. J Appl Microbiol 102:274–282

Muñoz-Atienza E, Gómez-Sala B, Araújo C, Campanero C, del Campo R, Hernández PE, Herranz C, Cintas LM (2013) Antimicrobial activity, antibiotic susceptibility and virulence factors of lactic acid bacteria of aquatic origin intended for use as probiotics in aquaculture. BMC Microbiol 13:15

Muñoz-Atienza E, Landeta G, de las Rivas B, Gómez-Sala B, Muñoz R, Hernández PE, Cintas LM, Herranz C (2011) Phenotypic and genetic evaluations of biogenic amine production by lactic acid bacteria isolated from fish and fish products. Int J Food Microbiol 146:212–216

Nayak SK (2010) Probiotics and immunity: A fish perspective. Fish Shellfish Immunol 29:2–14

Noriega L, Cuevas I, Margolles A, de los Reyes-Gavilán CG (2006) Deconjugation and bile salts hydrolase activity by *Bifidobacterium* strains with acquired resistance to bile. Int Dairy J 16:850–855

Panigrahi A, Azad IS (2007) Microbial intervention for better fish health in aquaculture: the Indian scenario. Fish Physiol Biochem 33:429-440

Papagianni M, Anastasiadou S (2009) Pediocins: the bacteriocins of pediococci. Sources, production, properties and applications. Microb Cell Fact 8:3

Pérez-Sánchez T, Ruiz-Zarzuela I, de Blas I, Balcázar JL (2014) Probiotics in aquaculture: a current assessment. Rev Aquaculture 6:133–146

Ringø E, Løvmo L, Kristiansen M, Bakken Y, Salinas I, Myklebust R, Olsen RE, Mayhew TM (2010) Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. Aquac Res 41:451–467

Ringø E, Olsen R, Jensen I, Romero J, Lauzon H (2014) Application of vaccines and dietary supplements in aquaculture: possibilities and challenges. Reviews in Fish Biology and Fisheries 24:1005–1032

Subasinghe R, Basurco B, Rogers C (2009) Disease control in aquaculture and the responsible use of veterinary drugs and vaccines: the issues, prospects and challenges. In: The use of veterinary drugs and vaccines in Mediterranean aquaculture, Vol 86. CIHEAM, Zaragoza, p 5–11

Talon R, Leroy S (2011) Diversity and safety hazards of bacteria involved in meat fermentations. Meat Sci 89:303–309

Tenorio C, Zarazaga M, Martinez C, Torres C (2001) Bifunctional enzyme 6'-*N*-aminoglycoside acetyltransferase-2''-*O*-aminoglycoside phosphotransferase in *Lactobacillus* and *Pediococcus* isolates of animal origin. J Clin Microbiol 39:824–825

Thapa N, Pal J, Tamang JP (2006) Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas. Int J Food Microbiol 107:33–38

Todorov SD, Dicks LMT (2005) Pediocin ST18, an anti-listerial bacteriocin produced by *Pediococcus pentosaceus* ST18 isolated from boza, a traditional cereal beverage from Bulgaria. Process Biochem 40:365–370

Todorov SD, Dicks LMT (2009) Bacteriocin production by *Pediococcus pentosaceus* isolated from marula (*Scerocarya birrea*). Int J Food Microbiol 132:117–126

Toranzo AE, Romalde JL, Magariños B, Barja JL, Basurco B, Rogers C (2009) Present and future of aquaculture vaccines against fish bacterial diseases. In: The use of veterinary drugs and vaccines in Mediterranean aquaculture, Vol 86. CIHEAM, Zaragoza, p 155–176

Venema K, Kok J, Marugg JD, Toonen MY, Ledeboer AM, Venema G, Chikindas ML (1995) Functional analysis of the pediocin operon of *Pediococcus acidilactici* PAC1.0: PedB is the immunity protein and PedD is the precursor processing enzyme. Mol Microbiol 17:515–522

Ventura M, Zink R (2002) Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. FEMS Microbiol Lett 217:141–154

Versalovic J, Koeuth T, Lupski R (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerpriting of bacterial genomes. Nucleic Acids Res 19:6823–6831

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671

Villamil L, Figueras A, Planas M, Novoa B (2010) *Pediococcus acidilactici* in the culture of turbot (*Psetta maxima*) larvae: administration pathways. Aquaculture 307:83–88

Zhou JS, Gopal PK, Gill HS (2001) Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin in vitro. Int J Food Microbiol 63:81–90

Figure legends

Fig. 1. Cluster analysis of ERIC-PCR fingerprinting of the 8 *P. acidilactici* strains isolated from rainbow trout feed and larvae. The dendrogram was constructed using the Unweighted Pair Group Method (UPGMA) using arithmetic averages with correlation levels expressed as percentage values of the Dice correlation coefficient.

Fig. 2. RP-FPLC of the bacteriocin produced by *P. acidilactici* L-14. The black arrow indicates the peak showing bacteriocin activity against *L. monocytogenes* CECT4032. Bacteriocin activity was expressed in bacteriocin units (BU, *i.e.* the reciprocal of the highest dilution of the bacteriocin causing 50% growth inhibition).

Fig. 3. Mass spectrometry analysis of purified PedPA-1 from *P. acidilactici* L-14.

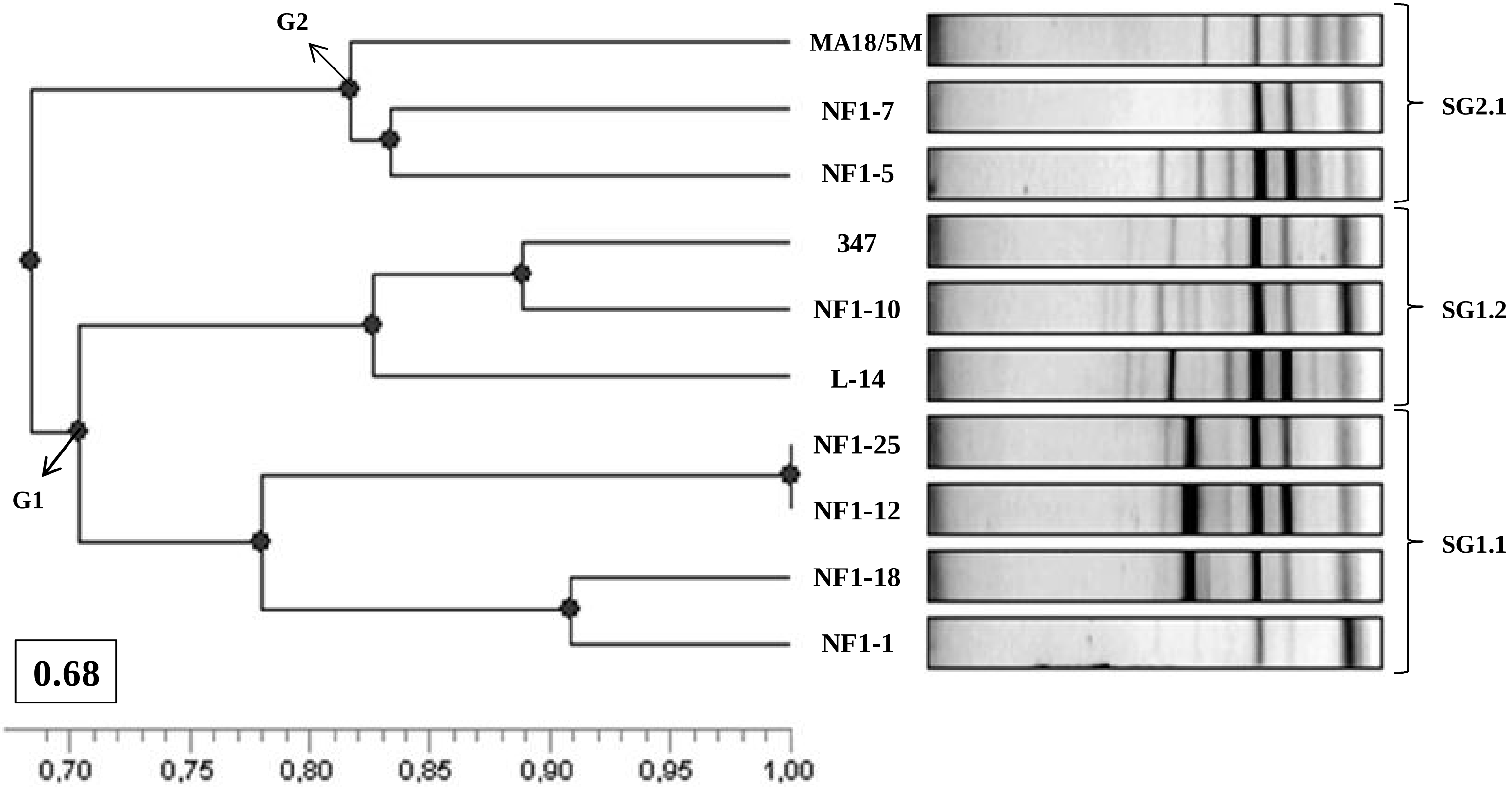

G2
MA18/5M
NF1-7
NF1-5
347
NF1-10
L-14
NF1-25
NF1-12
NF1-18
NF1-1
G1
0.68
SG2.1
SG1.2
SG1.1
0,70
0,75
0,80
0,85
0,90
0,95
1,00

Fig. 1

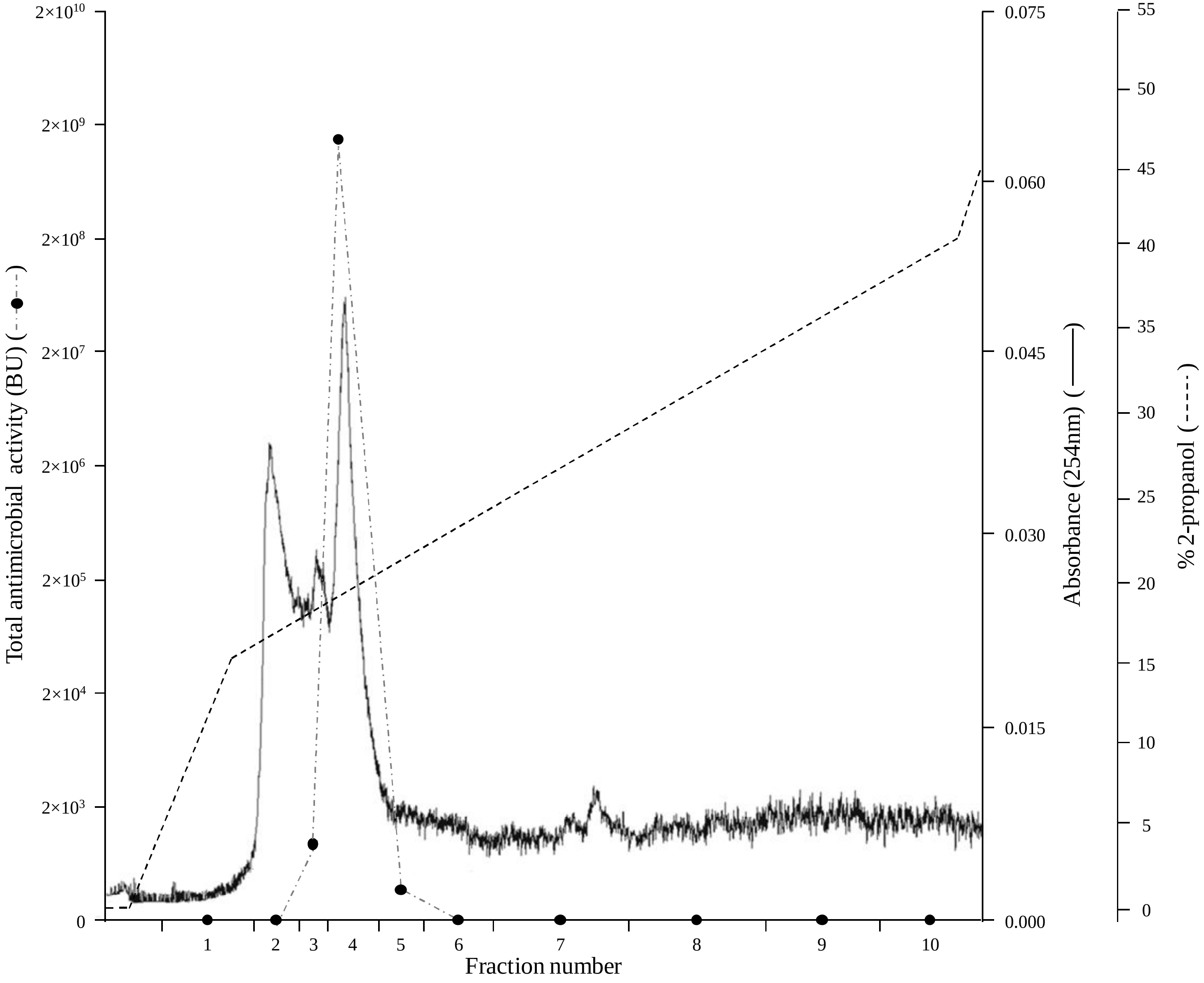
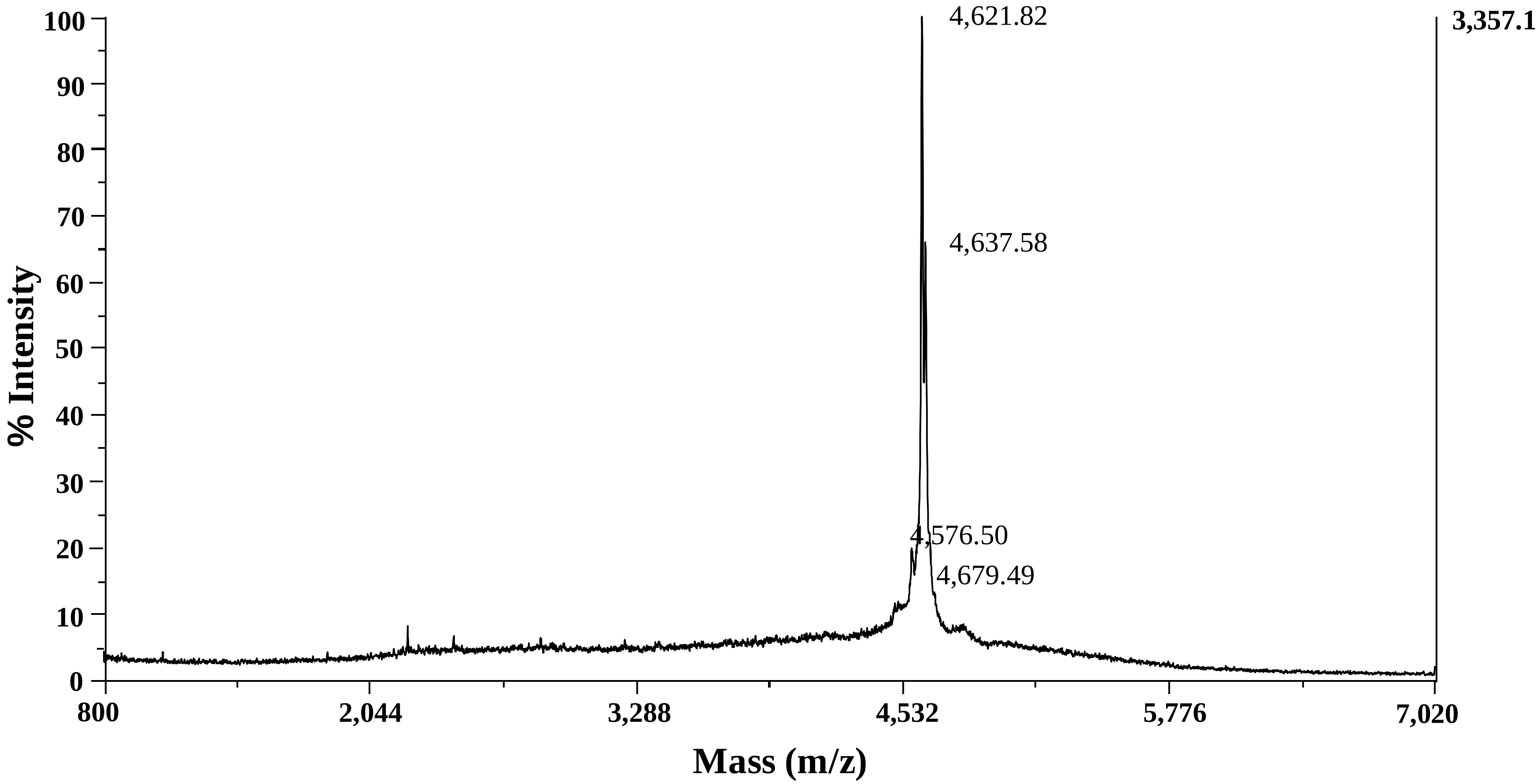

Total antimicrobial activity (BU) (-·●·-)
2×10^10
2×10^9
2×10^8
2×10^7
2×10^6
2×10^5
2×10^4
2×10^3
0
Absorbance (254nm) (——)
0.075
0.060
0.045
0.030
0.015
0.000
% 2-propanol (-----)
55
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Fraction number

Fig.2



4,621.82
3,357.1
4,637.58
4,576.50
4,679.49
% Intensity
100
90
80
70
60
50
40
30
20
10
0
800
2,044
3,288
4,532
5,776
7,020
Mass (m/z)

Fig. 3

Table 1. Oligonucleotide primers used in this study

Target gene	Primer	5’–3’ sequence[a]	PCR fragment size (bp)	Annealing temperature (°C)	Reference
16S rDNA	plb16	AGAGTTTGATCCTGGCTCAG	Variable	48	Kullen et al. (2000)
	mlb16	GGCTGCTGGCACGTAGTTAG			
hdc	CL1	CCWGGWAAWATWGGWAATGGWTA	500	48	Le Jeune et al. (1995)
	JV17HC	AGACCATACACCATAACCTT			
ldc	CAD2-F	CAYRTNCCNGGNCAYAA	1,185	53	de las Rivas et al. (2006)
	CAS2-R	GGDATNCCNGGNGGRTA			
odc	3	GTNTTYAAYGCNGAYAARACNTAYTTYGT	1,446	52	Marcobal et al. (2005)
	16	TACRCARAATACTCCNGGNGGRTANGG			
tdc	TD5	CAAATGGAAGAAGAAGTAGG	1,100	48	Coton et al. (2004)
	TD2	ACATAGTCAACCATRTTGAA			
ERIC-PCR	ERIC-1R	ATGTAAGCTCCTGGGGATTCAC	Variable	46	Versalovic et al. (1991)
	ERIC-2	AAGTAAGTGACTGGGGTGAGCG			

[a] R=A or G; W=A or T; Y=C or T; D=A, G, or T; N=A, G, C, or T.

Table 2. Direct antimicrobial activity against fish pathogens of the 8 *P. acidilactici* strains isolated from rainbow trout feed and larvae[a]

Strain[b]	Indicator microorganisms								
	L. garvieae CF00021	*L. garvieae* CF01144	*L. garvieae* JIP29-99	*L. garvieae* CECT5807	*S. iniae* LMG14521	*C. maltaromaticum* LMG14716	*Y. ruckeri* LMG3279	*A. salmonicida* LMG3776	*V. campbellii* LMG21363
L-14	++	++	++	+++	-	-	+	++	+++
NF1-1	+	+++	+++	+++	-	+	-	-	-
NF1-5	-	+++	++	+++	-	-	-	-	-
NF1-7	+	+++	++	+++	-	-	-	+	-
NF1-10	+	+++	-	-	-	-	-	+	-
NF1-12	-	-	+	+	-	+	-	-	-
NF1-18	+	-	+	-	+	+	-	+	-
NF1-25	+	+++	++	-	-	-	-	-	-

[a]Direct antimicrobial activity was determined by a stab-on-agar test and the scores reflect different degrees of growth inhibition (diameter in mm): -, no inhibition; +, 3–5 mm inhibition zone; ++, 6–9 mm inhibition zone; +++, ≥10 mm inhibition zone. [b]Strains inhibiting less than 3 fish pathogens are not shown.

Table 3. MICs distribution of 8 antibiotics for the 8 *P. acidilactici* strains isolated from rainbow trout feed and larvae

Antibiotics	Number of strains with the indicated MIC (mg/L)[a]														EFSA breakpoints (mg/L)[b]
	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	
Ampicillin						2	6								4
Gentamicin					2		3	3							16
Kanamycin								1			7				64
Streptomycin						1			4	3					64
Erythromycin	6			1	1										1
Clindamycin	6				2										1
Tetracycline						1		7							8
Chloramphenicol						7	1								4

[a] Shaded areas show the range of dilutions tested for each antibiotic. MICs which exceeded the upper or lower limit of the tested range are listed in the next dilution series.

[b] Strains with MICs higher than the EFSA breakpoints are considered as resistant.

Table 4. Extracellular antimicrobial (bacteriocin) activity of the 8 *P. acidilactici* strains isolated from rainbow trout feed and larvae[a]

Strain	Origin	Indicator microorganisms														
		P. damnosus CECT4797			*L. monocytogenes* CECT4032			*L. innocua* CECT910			*Lc. garvieae* CF01144, CECT5807 and CF00021			*A. salmonicida* LMG3776		
		SN	SN-H	SN-PK	SN	SN-H	SN-PK	SN	SN-H	SN-PK	SN	SN-H	SN-PK	SN	SN-H	SN-PK
L-14	Larva	18.7	18.7	-	17.8	17.8	-	17.4	ND	ND	-	ND	ND	-	ND	ND
NF1-1	Feed	7.9	7.8	-	7.2	7.1	-	7.2	ND	ND	-	ND	ND	-	ND	ND
NF1-5	Feed	7.2	7	-	-	-	-	-	ND	ND	-	ND	ND	-	ND	ND
NF1-7	Feed	7.1	7.1	-	-	-	-	-	ND	ND	-	ND	ND	-	ND	ND
NF1-10	Feed	7.3	7.3	-	-	-	-	-	ND	ND	-	ND	ND	-	ND	ND
NF1-12	Feed	7.1	7	-	-	-	-	-	ND	ND	-	ND	ND	-	ND	ND
NF1-18	Feed	-	-	-	-	-	-	-	ND	ND	-	ND	ND	-	ND	ND
NF1-25	Feed	-	-	-	-	-	-	-	ND	ND	-	ND	ND	-	ND	ND

[a] Antimicrobial activity (mm) of supernatants (SN), supernatants subjected to heat treatment (SN-H) and supernatants subjected to proteinase K treatment (SN-PK) as determined by an agar well-diffusion test.

ND, not determined.

Table 5. Purification of PedPA-1 produced by *P. acidilactici* L-14[a]

Supernatant and purification stage	Volume (ml)	Total A_{254}[b]	Total activity (10^3 BU)[c]	Specific activity[d]	Increase in specific activity[e] (fold)	Yield (%)
Culture supernatant	1,000	22,600	240	11	1	100
Ammonium sulfate precipitation	100	1,300	49,200	36,800	3,500	20,480
Gel filtration chromatography	156	780	9,600	12,200	1,100	3,994
Cation-exchange chromatography	50	12	1.6×10^6	1.3×10^8	1.2×10^7	6.6×10^5
Hydrophobic-interaction chromatography	15	0.3	900	3.2×10^6	3.0×10^5	384
Reversed-phase chromatography (FPLC)	1.5	0.3	1.9×10^9	6.7×10^{12}	6.3×10^{11}	8.1×10^8

[a] During the purification process, the antimicrobial activity, but not the protein concentration of the bacteriocin samples, was quantified.

[b] Absorbance at 254 nm (A_{254}) multiplied by the volume in milliliters.

[c] Antimicrobial activity in bacteriocin units per milliliter (BU/mL), as determined by an microtiter plate assay, multiplied by the total volume in milliliters.

[d] Specific antimicrobial activity expressed as the total antimicrobial activity (BU) divided by the total A_{254}.

[e] Specific antimicrobial activity of a fraction (BU/A_{254}) divided by the specific antimicrobial activity of the culture supernatant (BU/A_{254}).