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Preliminary characterization of bacteriocins from *Lactococcus lactis*, *Enterococcus faecium* and *Enterococcus mundtii* strains isolated from turbot (*Psetta maxima*) ☆

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Abstract

The aim of this work was the characterization of new strains of lactic acid bacteria (LAB) from farmed fish and with potential application as biopreservatives against both *Listeria monocytogenes* and *Staphylococcus aureus*. Twenty-five strains of LAB isolated from the muscle of farmed turbot were investigated. Genetic identification of the bacteriocin-producing LAB strains was performed by means of a PCR method using novel BAL1/BAL2 16S ribosomal-RNA-targeted primers. Maximum bacteriocin production by *Lactococcus lactis* ssp. *lactis* USC-39, *Enterococcus faecium* USC-46 and *Enterococcus mundtii* USC-51 was detected in the stationary phase of growth. Both acidification and the production of hydrogen peroxide by LAB were ruled out as the source of the inhibition. In contrast, the antimicrobial activity of all three LAB strains was inactivated by the addition of proteinase K, thus confirming the proteinaceous nature of the inhibition. The activity against *L. monocytogenes* was maintained in the 3.5–5.5 or 3.5–6.5 pH range, depending on the LAB strain. Likewise, inhibition of *S. aureus* strains was observed in the 3.5–4.5 and in the 3.5–5.5 pH ranges, depending on the LAB strain and on the *S. aureus* strain tested. Bacteriocin activity was stable in all three strains after heating the cell-free extract for 60 min at 100 °C, or even for 15 min at 121 °C, in all the three LAB strains. The acidic and heat-resistant bacteriocins produced by the three LAB strains isolated from turbot, able to inhibit the growth of both *L. monocytogenes* and *S. aureus* may find application as biopreservatives in fermented and/or heated food products.

Keywords: Bacteriocin; LAB; Fish; Biopreservation; *Enterococcus*; *Lactococcus*; *Listeria monocytogenes*; *Staphylococcus aureus*

1. Introduction

Lactic acid bacteria (LAB) have commonly been used in dairy, meat, bakery and vegetable fermentation since ancient times (Marrug, 1991). Although fish products are not the ideal habitat for LAB because of their low carbohydrate content, these microorganisms are found on several fresh fish species, fish products, or in the intestinal contents of fish (Lyhs, Björkroth, & Korkeala, 1999; Mauguin & Novel, 1994; Ringø & Gatesoupe, 1998). Basby, Jeppesen, and Huss (1998) isolated 70 LAB strains from lightly salted

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lumpfish roe. Pilet et al. (1995) and Leroy, Joffraud, and Chevalier (2000) also reported the isolation of LAB from smoked fish.

LAB may compete for nutrients or space with spoilage microorganisms, such as certain Gram-negative bacteria. Moreover, the production of organic acids, hydrogen peroxide, low molecular weight metabolites – such as diacetyl and bacteriocins – may extend the shelf-life of food products due to their inhibiting effect on the growth of spoilage and pathogenic bacteria (Huss, Jeppesen, Johansen, & Gram, 1995). For these reasons, harmless LAB exhibiting antagonistic effects against pathogenic bacteria may be of applied interest as biopreservatives – with the subsequent reduction in the use of antibiotics – in future aquaculture activities (Ringø & Gatesoupe, 1998).

Bacteriocins have been defined as proteinaceous substances exhibiting bactericidal activity against closely related species (Tagg, Adnan, & Wamna-Maker, 1976). Currently they are receiving increased attention because of their inhibitory activity against food spoilage and food-borne pathogenic bacteria such as *Listeria monocytogenes* (Yamazaki, Suzuki, Kawai, Inque, & Montville, 2003). Commercial nisin preparations have been evaluated in food systems and are now widely used as biopreservatives in the food industry due to their antibacterial properties, this allowing a more strict microbial control of a variety of commercial food products (Yang, Johnson, & Ray, 1992). Several studies addressing the bacteriocins produced by LAB isolated from dairy and meat products have been reported in the literature, but only a few bacteriocins have been isolated from fish products (Duffes, Leroi, Boyaval, & Dousset, 1999; Pilet et al., 1995; Stoffels, Nes, & Guomundsdottir, 1992; Yamazaki et al., 2003).

In a previous study we reported the extended shelf-life of refrigerated farmed turbot, this result being in agreement with the predominance of LAB strains over Gram-negative microorganisms involved in the production of histamine, trimethylamine (TMA-N) and H_2S (Rodriguez, Velázquez, Ojea, Piñeiro, & Aubourg, 2003). The purpose of the present study was to investigate those LAB strains as regards their ability to produce bacteriocins against *L. monocytogenes* and *Staphylococcus aureus*. The genetic identification of the bacteriocin-producing LAB strains was undertaken. In addition, the characterization of the heat-resistance and pH stability of the bacteriocins found was also undertaken.

2. Materials and methods

2.1. Bacterial strains and media

Two-year old farmed turbot (*Psetta maxima*) specimens were obtained from Stolt Sea Farm, S.A. (Carnota, La Coruña, Spain). Fish specimens were sacrificed in a water–ice mixture and then kept in ice for 10 h until they arrived at our laboratory. The fish were neither headed nor gutted and were stored in ice in an isothermal room at 2 °C. Twenty-five LAB strains were isolated from turbot muscle, as previously described (Rodriguez et al., 2003). The microorganisms were maintained as frozen stocks at −80 °C. Before experimental use, all LAB strains were recovered in MRS broth (Oxoid, Ltd., London, UK) and were incubated without shaking at 30 °C. Then, a 48-h culture of each strain was centrifuged at 7000 rpm for 15 min and the cell-free extract was sterilized by filtration through 0.22 μm (Millex GS, Millipore, France) and kept at 4 °C for assaying bacteriocin production. The nisin-producer *Lactococcus lactis* ATCC 11454 strain was used as a positive control to check bacteriocin production.

The following pathogenic bacterial strains were considered in the susceptibility assays: *L. monocytogenes* NCTC 11994, *L. monocytogenes* LHICA 1112, *L. monocytogenes* LHICA 835, *S. aureus* ATCC 9144, *S. aureus* ATCC 35845, and *S. aureus* LHICA 1010. All strains were maintained as frozen stocks at −80 °C and were recovered in Mueller Hinton broth (Oxoid) without shaking at 37 °C for 48 h prior to being used as bacterial lawns in susceptibility plate bioassays.

2.2. Phenotypic characterization of bacteriocin-producing LAB strains

All strains were investigated to determine their colony morphology, cell morphology, motility, Gram stain and the production of cytochrome oxidase and catalase. The phenotypic identification of LAB strains was carried out by means of miniaturized API 50 CH biochemical tests (BioMérieux, Marcy L'Etoile, France). The results of the identification tests were interpreted using the APILAB PLUS software (BioMérieux). All strains were further characterized for the ability to produce enzymes, using the API ZYM system (BioMérieux). The proteolytic phenotype of LAB was investigated in casein-agar medium (Phaff, Starmer, Lachance, & Ganter, 1994), as previously described (Ben-Gigirey, Vieites-Baptista de Sousa, Villa, & Barros-Velázquez, 2000).

2.3. Genetic identification of bacteriocin-producing LAB strains using novel BAL1/BAL2 primers and DNA sequencing

DNA from the LAB was isolated from the pellets of 1.5 ml of overnight cultures after spinning at 7500 rpm/10 min. Each pellet was resuspended in 180 μl of lysis buffer. Each 10 ml of lysis buffer was prepared by mixing 4 ml of lysozyme (10 mg/ml, in bi-distilled water), 4 ml of 50 mM Tris–HCl, 200 μl of 100 mM EDTA, 120 μl of Triton X-100 and 1.68 ml of Milli-Q water. All reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). After an incubation step at 37 °C/30 min, 25 μl of proteinase K (10 mg/ml) (Sigma) was added, this being followed by incubation at 70 °C/30 min. Then, bacterial DNA was purified from each extract by means of the DNeasy tissue minikit (QIAGEN Inc., Valencia, CA, USA), based on the use of microcolumns. The concentration of purified

DNA extract was determined by measuring the fluorescence developed after mixing with Hoechst 33258 reagent (Sigma) on a LS 50 fluorimeter (Perkin Elmer, Wellesley, MA, USA).

Genetic characterization of LAB was performed by a PCR method based on the detection of 16 S rRNA sequences using the novel set of primers BAL1 (forward: 5′-GAGTAACGCGTGGGGAATCT-3′) and BAL2 (reverse: 5′-CCGTCCCTTTCTGGGTAGTT-3′), that yielded a 402 bp PCR product with a 362 bp variable region of the 16S rRNA gene of LAB. Amplification conditions were as follows: a previous denaturing step at 94 °C for 1 min 30 s was coupled to 35 cycles of denaturation (94 °C for 10 s), annealing (57 °C for 30 s), and extension (72 °C for 40 s), and to a final extension step at 72 °C for 15 min. All amplification assays comprised 100 ng of template DNA, 25 μl of a master mix (BioMix, Bioline Ltd., London, UK) – this including reaction buffer, dNTPs, magnesium chloride and *Taq* DNA polymerase – PCR water (Genaxis, Montigny le Bretonneaux, France), and 25 pmol of each oligonucleotide primer to achieve a final volume of 50 μl. All PCR assays were carried out on a MyCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). PCR products were processed in 2.5% horizontal agarose (MS-8, Pronadisa, Madrid, Spain) gels. Prior to sequencing, the PCR products were purified by means of the ExoSAP-IT kit (GE Healthcare, Amersham Biosciences, Uppsala, Sweden). Direct sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The same primers used for PCR were considered for the sequencing of both strands of the PCR products, respectively. Sequencing reactions were analyzed in an automatic sequencing system (ABI 3730XL DNA Analyser, Applied Biosystems). The DNA sequences were carefully reviewed by eye, using the Chromas software (Griffith University, Queensland, Australia). Alignment of sequences was carried out using the CLUSTALW software.

2.4. Susceptibility plate and broth bioassays

Detection of bacteriocin activity in LAB strains was initially screened by means of a standardized agar disk diffusion method. Briefly, Mueller Hinton (Oxoid) agar plates were seeded with a bacterial lawn of *L. monocytogenes* or *S. aureus* indicator strains at a 10^5 CFU/ml concentration. Then, 20 μl portions of each extracellular extract from each LAB strain – grown at 30 °C/48 h in MRS broth without shaking – were placed on 6 mm sterile disks (Oxoid), which had previously been placed on the agar plates. The plates were incubated overnight at 37 °C, antimicrobial activity being detected as translucent halos in the bacterial lawn surrounding the disks. A positive control for antimicrobial activity – this was a nisin-producing *L. lactis* strain – was included. With a view to ruling out the possibility that the inhibition might have been caused by acidification of the media induced by LAB metabolism, an aliquot of MRS broth (Oxoid) was readjusted as regards its pH value – with a view to mimicking the pH of the LAB cultures – and sterile 20 μl portions were placed on sterile disks, as described above, and considered as negative controls. The pH value was measured by means of an automated 6 mm-diameter electrode (Crison, Barcelona, Spain).

LAB strains exhibiting a positive result in the plate bioassays were further characterized in their bacteriocin-producing capabilities by means of a critical dilution microassay (Himelbloom, Nilsson, & Gram, 2001). Briefly, extracellular extracts from bacteriocin-producing LAB strains were serially diluted in Mueller Hinton broth (Oxoid), and portions of 50 μl of the diluted extracts were pipetted into the wells of microtitre plates, together with 50 μl of a 10^5 CFU/ml culture of the *L. monocytogenes* or *S. aureus* indicator strains. Microtitre plates were incubated at 37 °C for 24 h and the detection of turbidity in the wells, as compared with the negative and positive controls, was considered as the absence of inhibition. Portions of 50 μl of positive and negative controls described above were tested in parallel. The bacteriocin titre was defined as the reciprocal of the highest dilution showing inhibition of the indicator strain and was expressed as arbitrary units/ml (AU/ml), as described elsewhere (Coventry et al., 1997).

2.5. Determination of LAB growth for maximum bacteriocin production

To elucidate the time of incubation at which the LAB strains exhibited the maximum bacteriocin production, sterile flasks containing 250 ml of MRS broth (Oxoid) were inoculated at a 1% ratio with each bacteriocin-producing LAB strain and incubated without shaking at 30 °C. Then, 1 ml portions were retrieved after 9, 21, 33, 46, 55 and 72 h, the bacterial concentrations being determined by the McFarland method, as previously described (Velázquez, Jiménez, Chomón, & Villa, 1995). These portions were then spun to obtain the extracellular extracts and were tested against *L. monocytogenes* and *S. aureus* by means of the critical dilution microassay method with a view to monitoring the production of bacteriocin along the bacterial growth.

2.6. Sensitivity of the antimicrobial activities of strains USC-39, USC-46 and USC-51 to proteinase K and catalase

To study the sensitivity of the antimicrobial activity associated with the three LAB strains with respect to proteolytic enzymes, 2.5 mg of proteinase K (EC 3.4.21.64) (Sigma) was added to 1 ml portions of extracellular extracts from each bacteriocin-producing LAB strain, and the mixture was incubated at 37 °C for 3 h. Following this, these extracts were investigated for their ability to inhibit the growth of the indicator microorganisms, using the agar disk diffusion plate bioassay.

In order to elucidate whether the antimicrobial activity might derive from the production of hydrogen peroxide

by LAB, 300 IU/ml of bovine liver catalase (EC 1.11.16) (Merck, Darmstadt, Germany) was added to 1 ml portions of the extracellular extracts of the LAB exhibiting antimicrobial activity, and the mixture was incubated at 37 °C for 24 h. Then, the treated extracts were subjected to the agar disk diffusion plate bioassay, as described above.

In both cases, a positive control sample, consisting of a portion of the extracellular extract with no enzyme added, was tested in parallel.

2.7. Effect of pH range and thermal treatment on bacteriocin activity

The pH and temperature ranges tested were chosen based on their usual levels in foods and in their processing operations. To check the pH stability of the bacteriocins, 1 ml portions of the extracellular extracts from the three LAB strains grown in MRS broth (Oxoid) at 30 °C/48 h were adjusted to pH values of 3.5, 4.5, 5.5 and 6.5 by adding the appropriate volumes of 4 N HCl or 4 N NaOH. Then, the samples were sterilized by filtration through 0.22 μm (Millex GS, Millipore, Bedford, MA, USA) and antimicrobial activity was determined by means of the critical dilution microassay described above. Negative controls, aimed at elucidating the possible role of acid pH values in the inhibition of *L. monocytogenes* and *S. aureus*, were prepared by testing portions of non-inoculated MRS broth whose pH values were adjusted to 3.5, 4.5, 5.5 and 6.5. Extracellular extracts from the nisin-producer *Lactococcus lactis* ATCC 11454 strain were used as positive controls.

The effect of temperature on bacteriocin stability was determined in extracellular extracts from LAB strains grown at 30 °C/48 h subjected to treatment at 100 °C/30 min, 100 °C/60 min or 121°C/15 min in an autoclave (Raypa, model AE 75 TIC, Sterilmatic, Barcelona, Spain). The effect of extended storage at low temperature −4 °C – on bacteriocin stability was also evaluated by placing extracellular extracts – obtained as described above – in an isothermal room at 4 °C for 7, 14 and 21 days. In all cases, the remaining bacteriocin activity was determined by the critical dilution microassay method. A positive control, consisting of freshly prepared extracellular extract, was tested in parallel.

2.8. Statistical analyses

All experiments were carried out in triplicate. The SPSS 11.5 for Windows software (SPSS Inc., Chicago, IL) was used to explore the statistical significance of the results obtained, this including multivariate contrasts and multiple comparisons by the Tukey test. A confidence interval at the 95% level ($p < 0.05$) was considered in all cases.

3. Results

3.1. Identification of bacteriocin-producing LAB strains

Five strains – USC-39, USC-46, USC-48, USC-50 and USC-51 – of the 25 LAB strains isolated from turbot muscle and initially considered in this study exhibited inhibitory activity against *L monocytogenes* and/or *S aureus* (Table 1). The diameters of the inhibition halos were in all cases within the 7.7–9.5 mm range. Strains USC-48 and USC-50, which had previously been identified as *Lactobacillus delbrueckii* ssp. *delbrueckii* by phenotypic analysis, were found to display a very low antimicrobial activity, and only exerted their inhibitory effect on *L. monocytogenes* LHICA 1112 or on *L. monocytogenes* NCTC 11994, respectively (Table 1).

The identification of strains USC-39, USC-46 and USC-51 was also carried out using a novel 16S rRNA-targeted PCR method, as previously described. The fragment of the rRNA gene amplified from strain USC-39 exhibited 100% homology with respect to *Lactococcus lactis* ssp. *lactis* strains isolated from spoiled cooked meat products (GenBank accession number AB122036), rice silage (accession number AF515226) and from a traditional Korean fermented food (accession number AY675242). Strain USC-46 exhibited 100% homology with respect to *Enterococcus faecium* strains isolated from a traditional Korean fermented food (accession number AY692451), common carp and freshwater prawns (accession number AB018210) and rabbit (accession number AJ490520). Strain USC-51 exhibited 100% homology with respect to *Enterococcus mundtii* strains such as an enterocin-producing strain (accession number AY439006), and a mundticin-producing strain exhibiting anti-listerial activity (accession number AB066266).

Table 1
Antimicrobial activity of extracellular extracts obtained from LAB against several food-borne pathogenic bacteria

LAB strain	Indicator microorganism					
	L. monocytogenes	*L. monocytogenes*	*L. monocytogenes*	*S. aureus*	*S. aureus*	*S. aureus*
	NCTC 11994	LHICA 1112	LHICA 835	LHICA 1010	ATCC 35845	ATCC 9144
L. lactis USC-39	8.4 ± 0.5	8.9 ± 0.7	–	8.5 ± 0.5	8.5 ± 0.5	–
E. faecium USC-46	9.5 ± 0.5	9.1 ± 0.5	–	9.5 ± 0.5	8.3 ± 1.5	–
L. delbrueckii USC-48	–	8.0 ± 0.3	–	–	–	–
L. delbrueckii USC-50	9.0 ± 0.4	–	–	–	–	–
E. mundtii USC-51	8.0 ± 1.0	7.7 ± 0.5	–	8.5 ± 0.5	8.0 ± 0.1	–
L. lactis ATCC 11454	9.7 ± 0.7	9.5 ± 0.6	9.0 ± 0.3	9.0 ± 0.1	8.0 ± 0.1	8.3 ± 0.5

Results are expressed as diameters of the inhibition zone and standard deviations in mm (the filter disks had a diameter of 6 mm).

L. lactis ssp. *lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 exhibited antimicrobial activity against two of the three *L. monocytogenes* and *S. aureus* strains tested (Table 1). These results prompted us to select the latter three LAB strains for further characterization. In contrast, none of the LAB strains from the turbot specimens studied exhibited antimicrobial activity against *L. monocytogenes* LHICA 835 – belonging to our laboratory collection – or with respect to *S. aureus* ATCC 9144. These two strains were only efficiently inhibited by the nisin-producing *L. lactis* ATCC 11454 strain, included in the bioassays as a positive control (Table 1).

It should also be noted that the pH of the extracellular extracts of the LAB strains exhibiting antimicrobial activity was within the range of 4.5–4.7. Negative control assays – consisting of aliquots of non-inoculated MRS broths whose pH had been adjusted to 4.5 – were tested in parallel using the agar disk diffusion bioassay. None of these aliquots exhibited any antimicrobial activity against any of the *L. monocytogenes* or *S. aureus* strains tested.

3.2. Sensitivity of bacteriocins to enzymes

The antimicrobial activities exhibited by *L. lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 were found to be sensitive to the action of protease. Thus, incubation of the extracellular extracts obtained from these strains together with proteinase K elicited a total loss of antimicrobial activity, as determined by the agar disk diffusion method. Antimicrobial activity was observed in the positive controls, consisting of untreated extracellular extracts from the respective LAB strains. The antimicrobial activities of strains USC-39, USC-46 and USC-51 were not affected by catalase, since the treated extracellular extracts remained active against *L. monocytogenes* and *S. aureus.* Again, a positive control – consisting of untreated extracellular extracts – and a negative control – consisting of a non-inoculated MRS broth portion treated with catalase – were tested in parallel.

3.3. Determination of bacterial growth for maximum bacteriocin production

Figs. 1–3 show the growth curves and the profiles of bacteriocin production of the *L. lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 strains, respectively. In all three cases, bacteriocin production was initially detected in the mid-exponential phase of growth, and the maximum levels of antimicrobial activity were found at different stages of the stationary phase of growth, depending on both the producer strain and the indicator microorganism.

L. lactis ssp. *lactis* USC-39 exhibited the maximum antimicrobial activity against *L. monocytogenes* LHICA 1112 at 33 h of incubation, the level of inhibition remaining constant up to 72 h (Fig. 1, panel A). However, the maximum antimicrobial activity of *L. lactis* ssp. *lactis* USC-39 against *L. monocytogenes* NCTC 11994 was detected after 46 h of incubation, a slight decrease in the bacteriocin activity being determined after that time (Fig. 1, panel B). Finally, *L. lactis* ssp. *lactis* USC-39 exhibited maximum inhibitory activity on *S. aureus* LHICA 1010 after 21 h of incubation, this coinciding with the end of the exponential phase of growth, and activity remaining constant during the stationary phase (Fig. 1, panel C). From these results, it was concluded that the maximum efficiency of the bacteriocin produced by *L. lactis* ssp. *lactis* USC-39 corresponded to 46-h cultures in the stationary phase of growth.

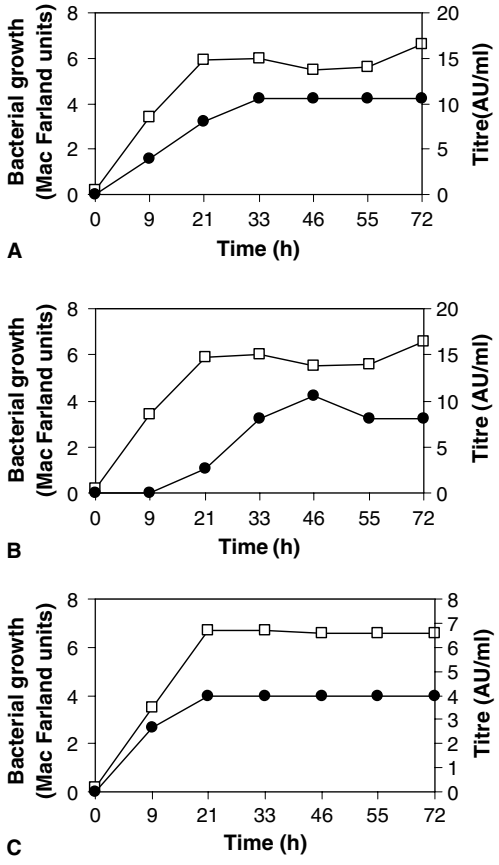

Fig. 1. Bacteriocin production and growth kinetics of *L. lactis* ssp. *lactis* USC-39 determined at 30 °C. Symbols: (□) bacterial growth expressed as McFarland units; (●) bacteriocin titre in AU/ml. Indicator microorganisms: *L. monocytogenes* LHICA 1112 (panel A), *L. monocytogenes* NCTC 11994 (panel B), and *S. aureus* LHICA 1010 (panel C).

The highest bacteriocin production by *E. faecium* USC-46 as determined against *L. monocytogenes* LHICA 1112 and *S. aureus* ATCC 35845 was observed after 21 h. This was at the beginning of the stationary phase of growth, the level of inhibition thereafter remaining constant (Fig. 2, panels A and B). Similar results were also found for *E. faecium* USC-46 as regards the inhibition of *L. monocytogenes* NCTC 11994 and *S. aureus* LHICA 1010 (data not shown).

Bacteriocin production by *E. mundtii* USC-51 exhibited its maximum inhibitory activity against *L. monocytogenes* strains after 46–55 h of incubation, this peak corresponding to an advanced phase of the stationary phase of growth (Fig. 3, panels A and B). Likewise, maximum inhibitory

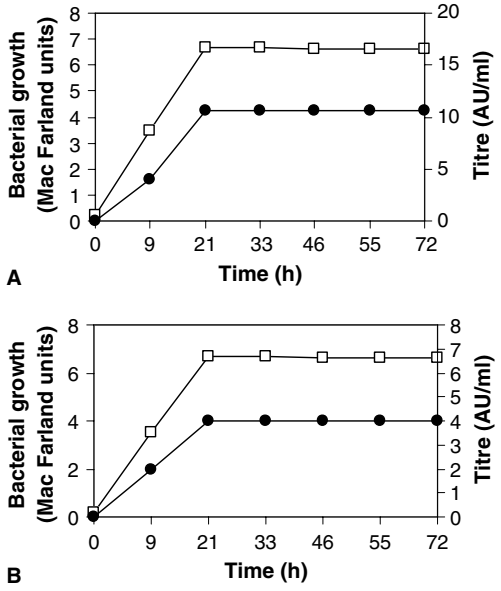

Fig. 2. Bacteriocin production and growth kinetics of *E. faecium* USC-46 determined at 30 °C. Symbols: (□) bacterial growth expressed as McFarland units; (●) bacteriocin titre in AU/ml. Indicator microorganisms: *L. monocytogenes* NCTC 1112 (panel A), and *S. aureus* ATCC 35845 (panel B).

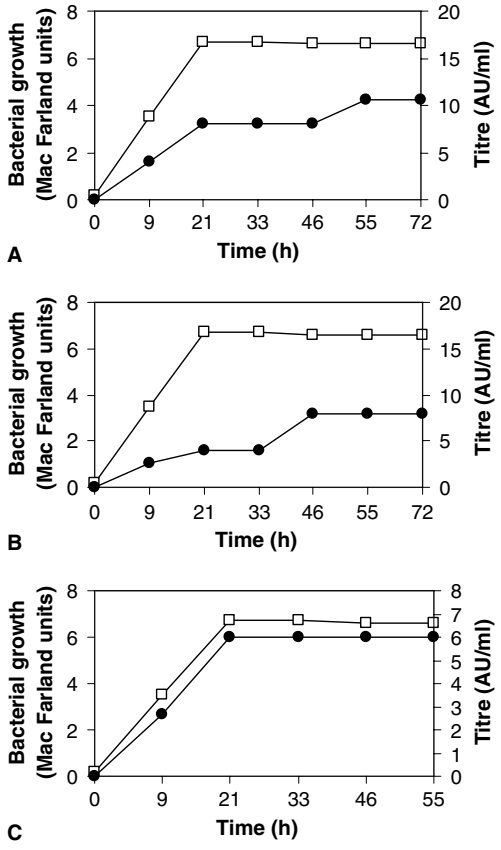

Fig. 3. Bacteriocin production and growth kinetics of *E. mundtii* USC-51 determined at 30 °C. Symbols: (□) bacterial growth expressed as McFarland units; (●) bacteriocin titre in AU/ml. Indicator microorganisms: *L. monocytogenes* LHICA 1112 (panel A), *L. monocytogenes* NCTC 11994 (panel B), and *S. aureus* LHICA 1010 (panel C).

activity of strain USC-51 against *S. aureus* LHICA 1010 was detected after 21 h of incubation – this coinciding with the beginning of the stationary phase of growth – (Fig. 3, panel C); this is in agreement with the results found for the other two bacteriocin-producing LAB strains (Fig. 1, panel C and Fig. 2, panel B).

3.4. Effect of pH range and thermal treatment on bacteriocin activity

These assays were performed with extracellular extracts corresponding to cultures of *L. lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 grown at 30 °C/48 h, since this led to maximum bacteriocin production in all three strains, as stated above. As may be observed in Table 2, the effect of pH was dependent upon the indicator microorganism tested. Thus, the three bacteriocin-producing strains tested exhibited a broader pH range of activity against *L. monocytogenes* than against *S. aureus*.

The bacteriocin produced by *E. faecium* USC-46 inhibited *L monocytogenes* strains NCTC 11994 and LHICA 1112 in the 3.5–6.5 pH range, while the bacteriocins from strains *L. lactis* ssp. *lactis* USC-39 and *E. mundtii* USC-51 were only effective against both *L. monocytogenes* strains within the 3.5–5.5 pH range (Table 2). Interestingly, the bacteriocin produced by *L. lactis* ssp. *lactis* USC-39 was also effective against *L. monocytogenes* LHICA 1112, even at a pH value of 6.5. The bacteriocins produced by *L. lactis* ssp. *lactis* USC-39 and *E. faecium* USC-46 exerted an inhibitory effect on *S. aureus* strains only at pH 3.5–5.5, while *E. mundtii* USC-51 was only effective against this food-borne pathogen in the 3.5–4.5 pH range (Table 2). It is also remarkable that antimicrobial activities increased as pH decreased from 6.5 to 3.5 in all three strains tested (Table 2), and reached maximum levels at a pH value of 3.5.

The thermal resistance of the bacteriocins produced by strains USC-39, USC-46 and USC-51 was also investigated. Thus, treatment of the extracellular extracts of all three bacteriocin-producing LAB strains at either 100 °C/60 min or at 121 °C/15 min did not elicit any loss of antimicrobial activity with respect to either *L. monocytogenes* or *S. aureus*. Interestingly, this result was observed in the 3.5–6.5 pH range (data not shown).

4. Discussion

The main goal of this study was the isolation and characterization of bacteriocin-producing LAB from turbot. Five strains out of a total of 25 LAB strains isolated from the muscle of refrigerated turbot were found to exhibit antimicrobial activity against *L. monocytogenes* and *S. aureus* strains. Such a high incidence of marine LAB strains able to produce bioactive compounds may be related to the extended shelf life of refrigerated turbot (Rodriguez et al., 2003), a flat-fish species of remarkably high commercial value in Europe. Three of such LAB strains able to

Table 2
Effect of pH on the titres (AU/ml) of the bacteriocins produced by *L. lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51

LAB strain	pH	Indicator microorganism			
		L. monocytogenes	*L. monocytogenes*	*S. aureus*	*S. aureus*
		NCTC 11994	LHICA 1112	LHICA 1010	ATCC 35845
L. lactis USC-39	3.5	32	42.7	10.7	10.7
	4.5	9.3	16	4	4
	5.5	4	2.7	2	2
	6.5	–	2	–	–
E. faecium USC-46	3.5	64	64	10.7	10.7
	4.5	10.6	16	4	4
	5.5	8	8	2	2.7
	6.5	2	2	–	–
E. mundtii USC-51	3.5	16	32	10.7	10.7
	4.5	8	10.6	6	6
	5.5	2	2	–	–
	6.5	–	–	–	–
MRS broth (negative control)	3.5	10.7	10.6	4	4
	4.5	4	4	2.7	2.7
	5.5	–	–	–	–
	6.5	–	–	–	–

The bacteriocin titre is defined as the reciprocal of the highest dilution showing inhibition of the indicator strains by means of the critical dilution microassay and expressed as Arbitrary Units/ml (AU/ml). Negative controls represent a non-inoculated broth whose pH was adjusted in the 3.5–6.5 range. Standard deviation values were in all cases within the range of 0–0.4.

inhibit the growth of the above food-borne pathogens were investigated in detail in this study, namely: *L. lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51. Although other universal primers targeted to the amplification of variable regions of the 16S rRNA of eubacteria have been widely employed for species identification purposes, such primers might not be helpful to distinguish between certain closely related LAB (Aymerich, Martín, Garriga, & Hugas, 2003). For this reason, a novel set of primers – BAL1/BAL2 – based only on sequences of the 16S rRNA gene from LAB, was considered in this study. Such set of primers successfully allowed the amplification of a variable 362 bp DNA fragment from the 16S rRNA gene of enterococci and lactococci, allowing us to distinguish between closely related enterococci. Moreover, these primers also allowed the amplification of rRNA from lactobacilli (data not shown), this underlining the potential usefulness of this PCR method as a genotyping tool for LAB strains.

The scientific literature offers examples of a number of LAB strains able to produce bacteriocins. In this sense, certain strains of *L. lactis* ssp. *lactis* produce nisin, the bacteriocin that has attracted the greatest attention in the scientific community. However, other lactococci have also been reported to be significant bacteriocin-producers. For example, the activities of lacticin 481 – produced by *L. lactis* ssp. *lactis* CNRZ 481 – lactococcin B – produced by *L. lactis* ssp. *cremoris* 9B4 – lactococcin G – produced by *L. lactis* LMG 2081 – and diplococcin – produced by *L. lactis* ssp. *cremoris* – among others, have been reported previously (Lee et al., 1999). With respect to *Enterococcus* spp., strains belonging to species such as *E. faecium*, *E. mundtii* or *E. durans* have been reported to produce bacteriocins such as enterocin A, enterocin B, enterocin I, enterocin L or enterocin P, some of them belonging to the IIa bacteriocin class. Nevertheless, most of these strains were isolated from sources other than fish, such as fresh meat, fermented sausages or fermented olives (for a review, see Ennahar, Deschamps, & Richard, 2000).

In our study, the antimicrobial activities exhibited by *L. lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 extracellular extracts were found to be sensitive to proteinase K treatment. Additionally, when subjected to thermal treatment above 100 °C, the extracellular extracts of all three strains exhibited stable antimicrobial activity, even after 21 days of storage at 4 °C. This result, which was confirmed in all the three strains – all of them exhibiting a proteolytic phenotype – seems to be related to the inactivation of bacterial proteases, thereby providing further evidence of the proteinaceous nature of the substance responsible for the antimicrobial activity in all three LAB strains. Moreover, the data reported in this study also support the idea that the antimicrobial activities against *L. monocytogenes* and *S. aureus* were not caused by the bacterial acidification of the medium – although a modest contribution of pH values below 4.5 was observed – and nor were they related to the production of hydrogen peroxide by any of the three LAB strains tested. Such observations strongly suggest that the antimicrobial activities against *L. monocytogenes* and *S. aureus* exhibited by *L. lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 would be based on the biosynthesis and secretion of bacteriocins.

The maximum levels of antimicrobial activity were determined during the stationary phase of growth, depending on both the producer strain and the indicator

microorganism. A similar trend – the maximum accumulation during the stationary phase of growth – has previously been reported for a bacteriocinogenic *Carnobacterium piscicola* strain isolated from fish (Pilet et al., 1995; Stoffels et al., 1992).

The inhibitory action of the bacteriocins studied here increased as pH decreased. These results, obtained for our LAB strains isolated from turbot, confirm previous reports referring to LAB strains of other origins that have described a stronger effect of bacteriocins at acidic pH values (Blom, Katia, Nissen, & Holo, 2001; Guerra & Pastrana, 2002; Muriana, 1996; Schillinger, Chung, Keppler, & Holzapfel, 1998). However, in our study, the effect of pH on bacteriocin activity was strain-specific and also depended on the microorganism used as indicator. This latter issue has previously been mentioned by other authors, who described a remarkable variation in the susceptibility of different strains of *Listeria* to bacteriocins belonging to the IIa class (Castellano, Farias, Holzapfel, & Vignolo, 2001; Ennahar et al., 2000). It should also be stressed that the bacteriocins obtained from LAB isolated from fish are generally effective for inhibiting *L. monocytogenes* but they apparently fail to inhibit *S. aureus* (Duffes et al., 1999; Himelbloom et al., 2001; Pilet et al., 1995; Yamazaki et al., 2003). Moreover, the bacteriocins of LAB isolated from meat, milk and dairy foods exhibit a higher activity against *Listeria* spp. than against *S. aureus* (Coventry et al., 1997), a result that has been interpreted in terms of the ability of *S. aureus* to overcome stress factors. In our study, strains USC-39 and USC-46 inhibited the growth of *L. monocytogenes* and *S. aureus* strains in the 3.5–5.5 pH range. This result suggests that the bacteriocins described in this study might have applied interest in both low and medium-acid fermented food products whose final pH values fall in such range, this including a number of fermented and ripened dairy and meat products. The acid-resistant nature of the bacteriocinogenic LAB strains isolated from farmed turbot also invites to explore the possible application of these strains as animal feed supplements, especially in the case of feeds destined to turbot or other farmed fish species.

From the methodological point of view, our study also supports the use of the critical dilution microassay as a quantitative method to evaluate the differences between the inhibitory activities exerted by LAB bacteriocins under different technical conditions. The latter trend has been reported previously by other authors, reporting that certain nisin-like bacteriocins exhibit greater activity in broth than in agar when *L. monocytogenes* and *L. seeligeri* are used as indicators (Coventry et al., 1997).

5. Conclusions

In summary, the present results clearly suggest the potential usefulness of the bacteriocins produced by *L. lactis* USC-39, *E. faecium* USC-46 and *E. mundtii* USC-51 in fermented foods at pH values below 5.5. These bacteriocin-producing strains were successfully identified using the novel 16S rRNA-targeted primers proposed by us. The fact that the bacteriocins produced by all three LAB strains were heat-stable, even after sterilization at 121 °C/15 min, is also remarkable, and may broaden the potential application of such compounds as biopreservatives for other food products subjected to pasteurization, cook-chilling, sterilization and other heat-processing treatments.

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