Single nucleotide polymorphism analysis of the enterocin P structural gene of *Enterococcus faecium* strains isolated from nonfermented animal foods

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The bacteriocins produced by two lactic acid bacteria isolated from nonfermented fresh meat and fish, respectively, and exhibiting a remarkable antilisterial activity, were characterized. Bacteriocinogenic strains were identified as *Enterococcus faecium* and the maximum bacteriocin production by both strains was detected in the stationary phase of growth. The activity against *Listeria monocytogenes* was maintained in pH range of 3 – 7 and was stable in both strains after heating at 100 or 121°C. The genes coding for enterocin P were detected, isolated, and sequenced in both *E. faecium* strains. They exhibited DNA/DNA homology in the 87.1 – 97.2% range with respect to the other four enterocin P genes reported so far. Three single nucleotide polymorphism events, silent at the amino acid level, were detected at nucleotide positions 45 (G/A), 75 (A/G), and 90 (T/C) in *E. faecium* LHICA 28 – 4 and may explain the differences reported for those *loci* in other enterocin P-producing *E. faecium* strains. This work provides the first description of enterocin P-producing *E. faecium* strains in nonfermented foodstuffs and, in the case of *E. faecium* LHICA 51, the first report of an enterocin P-producing strain isolated from fish so far.

Keywords: Bacteriocins / Enterocins / Lactic Acid Bacteria / *Listeria monocytogenes* / Single nucleotide polymorphisms

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1 Introduction

The presence of spoilage and pathogenic microorganisms in foods is a major concern for the food industry, the administration and consumers, and has moved food technologists to develop a wide variety of preservation processes aimed at destroying microbial cells or at delaying their growth [1]. However, the increasing demand of consumers for safe but minimally processed fresh products, together with the increasing concerns about the use of certain chemical preservatives, has raised a significant interest in the development and use of novel natural biopreservation methods [2, 3].

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Abbreviations: LAB, lactic acid bacteria; **SNP,** single nucleotide polymorphism; **VSP,** vacuum skin packaging

Lactic acid bacteria (LAB) have been empirically used for the production of fermented products for centuries [4]. Currently, it is well known that LAB may compete for nutrients or space with other microorganisms, also producing organic acids, hydrogen peroxide, and low M_r metabolites, such as diacetyl and bacteriocins, that may protect food products and extend their shelf-life due to their inhibiting effect on the growth of spoilage and pathogenic microorganisms [5]. Among LAB metabolites, bacteriocins are ribosomally synthetized, small, cationic, amphiphilic antibacterial peptides exhibiting a remarkable potential as alternatives to other traditional preservation methods with a view to achieve food safety [6]. Certain bacteriocins are receiving increased attention because of their inhibitory activity against food spoilage and food-borne pathogenic bacteria such as *Listeria monocytogenes* [7]. Among them, commercial preparations of the lantibiotic nisin, a class I-bacteriocin produced by certain *Lactococcus lactis* strains, have been successfully used for the control of microbial spoilage in certain types of foods [8].

Enterocins are bacteriocins produced by species of the genus *Enterococcus* [9]. Enterococci not only contribute to the organoleptic properties of fermented food products, such as dairy, meat products, or vegetables [10, 11], but their possible protective role as probiotics in nonfermented food products, due to their health-promoting abilities, has also been highlighted [9, 12]. Enterocins are found within the class I – lantibiotics, class IIa – pediocin-like antilisterial bacteriocins possessing the consensus sequence YGNGVXCXXXXVXV in their N-terminus, class IIc – bacteriocins not belonging to classes IIa and IIb, and class III – large, hydrophilic, and heat-labile proteins [9, 13]. Enterocins are mainly targeted to the cytoplasmic membrane, where they form pores, with subsequent depletion of the transmembrane potential and/or the pH gradient, these events resulting in microbial death by leakage of the intracellular content [9]. The mechanism of action of enterococcal bacteriocins might be complementary to nisin and may have applied interest in combined food biopreservation strategies [13].

In previous studies, we have reported the extended shelf-life of fresh meat and fish due to the use of advanced preservation techniques such as vacuum skin packaging (VSP) [14, 15] and storage in ice slurries [16, 17], respectively. Such studies evidenced a direct relationship between shelf-life extension in such food products and the predominance of LAB strains over other competing microorganisms [14–17]. The phenotypic and genotypic characterization of bacteriocinogenic LAB strains isolated from fish as well as the characterization of their heat-resistant antilisterial activity had previously been undertaken at our laboratory [18]. In the present study, we describe the genetic identification of the heat-resistant bacteriocins produced by two selected enterococci isolated from nonfermented fresh meat and farmed fish, respectively, and exhibiting a remarkable antilisterial activity, with special attention being focused on the elucidation of the presence of single nucleotide polymorphic events (SNPs) in the structural bacteriocin genes.

2 Materials and methods

2.1 Isolation of bacteriocinogenic LAB from fresh meat and farmed turbot

Strain LHICA 28-4 was isolated from a 200 g vacuum-packed beef sample stored for 10 days in an isothermal room at +4°C. Meat packaging was performed on a Multivac R570 CD VSP machine (Multivac, Wolfertschwenden, Germany) as previously described [14, 15]. Immediately before descending over the meat surface, the upper film was heated over 210 and 115°C in its upper and lower side, respectively. Strain LHICA 51 was isolated from the muscle of a 2-year old farmed turbot (*Psetta maxima*) specimen obtained from Stolt Sea Farm, S. A. (Carnota, La Coruña, Spain) and stored in ice for 28 days in an isothermal room at +2°C [16]. The fish specimen was neither headed nor gutted.

Both LAB strains were maintained as frozen stocks at –80°C. Before experimental use, LAB strains were recovered in MRS broth (Oxoid, London, UK) and plated on MRS agar (Oxoid). When required, 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 against *L. monocytogenes* NCTC 11994, *L. monocytogenes* LHICA 1112, *Staphylococcus aureus* ATCC 35845, *S. aureus* LHICA 1010, and *Bacillus cereus* ATCC 14893. 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 tests performed by agar disk diffusion and by critical dilution micro-assays [19], as previously described [18]. The bacteriocin titre was defined as the reciprocal of the highest dilution showing inhibition of the indicator strain and was expressed as Arbitrary U/mL (AU/mL), as described elsewhere [18, 20]. The nisin-producer *L. lactis* ATCC 11454 strain was used as a positive control to check bacteriocin production.

2.2 Phenotypic identification and characterization of bacteriocinogenic LAB

Both LAB strains were investigated to determine their colony morphology, cell morphology, motility, Gram stain, and the production of catalase. The phenotypic identification of LAB strains was carried out by means of miniaturized API 50 CH and API 20 STREP biochemical tests (BioMérieux, Marcy L'Etoile, France). The results of the identification tests were interpreted using the APILAB PLUS software (BioMérieux). The proteolytic phenotype of LAB was investigated in casein-agar medium [21], as previously described [22]. The capability of bacterial strains to grow in Chromocult® enterococci-selective agar (Merck, Darmstadt, Germany) was also evaluated as described elsewhere [23]. The production of extracellular lipases was investigated in tributyrine-agar [22]. Histamine production was screened in Niven medium [24].

2.3 Preliminary characterization of bacteriocins produced by *Enterococcus faecium* LHICA 28-4 and *E. faecium* LHICA 51

The proteinaceous nature of the inhibitory activity was confirmed by treatment with proteinase K (EC 3.4.21.64) at 37°C *per* 3 h, as previously described [18]. The potential inhibitory effect of hydrogen peroxide produced by LAB

Table 1. Primers targeting to enterocin genes tested in this study for the PCR amplification of the bacteriocins produced by *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51

Primer sets	Sequence (5′-3′)	Target	Reference
EntA	AAATATTATGGAGTGTAT GCACTTCCCTGGAATTGCTC	Enterocin A	[25]
EntB	GAAAATGATCACAGACCTA GTTGCARRRAGAGTATACATTG	Enterocin B	[25]
EntL50	STGGGAGAATCGCAAAATTAG ATTGCCCATCCTTCTCCAAT	Enterocins L50A and L50B	[25]
EntL50(2)	GATTGGAGGAGTTATATTATGGG CAAATTATAAAGAAATAATTACCTATCATTAAC	Enterocins L50A and L50B	[26]
EntP	TATGGTAATGGTGTTTATTGTAAT ATGTCCCATACCTGCCAAAC	Enterocin P	[25]
EntP2	GCTACGCGTTCATATGGTAATGGTG ATGTCCCATACCTGCCAAACCAGAAGC	Enterocin P	[26]
EntQ	GGAATAAGAGTAGTAGTGGAATACTGATATGAGAC AAAGACTGCTCTTCCGAGCAGCC	Enterocin Q	[26]
ORF	TTTTGCGGCCGCTAAGACTATAGGATAGACAA AAATGAATTCTTCATCAGAATGAATGGGAG	Mundticin KS	[27]
Mun1	GCAAACCGATAAGAATGTGGGAT TATACATTGTCCCCACAACC	Enterocin CRL35	[28]

The technical conditions of the PCR amplification with each primer set were as described elsewhere [25–28].

was ruled out with bovine liver catalase (EC 1.11.16) as described elsewhere [18]. The bacteriocin stability was evaluated in the 3.0–7.0 pH range and in the 80–121°C temperature range, as previously described [18].

Kinetics of LAB growth for optimum bacteriocin production was investigated. Briefly, extracellular extracts from bacteriocin-producing LAB strains were obtained during the different stages of the exponential and stationary phases of growth and assayed for bacteriocin activity against *L. monocytogenes* strains by the dilution microassay.

2.4 Genetic characterization of bacteriocinogenic *E. faecium* strains

Total DNA from both *E. faecium* strains was isolated from the pellets of 1.5 mL of overnight cultures after spinning at 7500 rpm/10 min, as previously described [18]. Total DNA was purified from each extract by means of the DNeasy tissue minikit (Qiagen, Valencia, CA, USA), based on the use of microcolumns. The concentration of purified DNA extracts was determined by measuring the fluorescence developed after mixing with Hoechst 33258 reagent (Sigma, St. Louis, MO) on an LS50 fluorimeter (Perkin Elmer, Wellesley, MA, USA). A novel set of 16S rRNA-targeted primers BAL5 (forward: 5′-CGTTGTCCGGATTTATTGGG-3′) and BAL6 (reverse: 5′-GTCGTCAGCTCGTGTCGTGA-3′), which allowed the PCR-based amplification of a 533 bp PCR product with a 493 bp variable region, were used. Such primers were constructed after a careful analysis of more than 200 nucleotide sequences of the 16S rRNA genes of LAB, namely: *Lactobacillus* spp., *Lactococcus* spp., and *Enterococcus* spp. retrieved from the GenBank (http://www.ncbi.nlm.nih.gov). The design of primers was performed using Primer3 and Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) softwares.

All amplification assays comprised 100 ng of template DNA, 25 µL of a master mix (BioMix, Bioline, London, UK) – this including the reaction buffer, dNTPs, magnesium chloride, and *Taq* DNA polymerase, double-distilled water (Genaxis, Montigny le Bretonneaux, France), and 25 pmol of each oligonucleotide primer to achieve a final volume of 50 µL. 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 20 s), annealing (55°C for 30 s), extension (72°C for 40 s), and to a final extension step at 72°C for 15 min. All PCR assays were carried out in duplicate 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.

2.5 Genetic identification of the structural bacteriocin genes in *E. faecium* strain LHICA 28-4 and *E. faecium* LHICA 51

This was performed by PCR using EntA [25], EntB [25], EntL50 [25], EntP [25], EntP2 [26], EntQ [26], EntL50(2) [26], ORF [27], and Mun1 [28] primers, targeting to class II enterocins produced by enterococci, namely: enterocin A, enterocin B, enterocin L50, enterocin P, enterocin Q, enterocin L50, mundticin KS, and enterocin CRL35. The nucleotide sequences of the primers used in this study are compiled in Table 1. Technical conditions were as described

elsewhere [25–28]. DNA from a mundticin KS-producer *E. mundtii* strain, kindly given by Dr. J. Shima (National Food Research Institute, Ibaraki, Japan [27]), was included as a positive control for amplification with ORF primers. DNA from an enterocin CRL35-producer *E. mundtii* strain, kindly given by Sesma [28] (CERELA-CONICET, Tucumán, Argentina) was included as a positive control for amplification with Mun1 primers.

2.6 DNA sequencing and and SNPs analysis

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 BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). 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 Analyzer, Applied Biosystems). SNP events in DNA sequences were carefully reviewed by eye, using the Chromas software (Griffith University, Queensland, Australia). Alignment of sequences was carried out using the CLUSTAL W software. Prediction of putative protein sequences was carried out using the Visual Sequence Editor software (VISED, Parksville, Canada). Homologies of the sequences were searched with the BLAST tool (National Center for Biotechnology Information). Phylogenetic and molecular evolutionary analyses were conducted by means of the MEGA software [29] using the neighbor-joining method [30] to construct distance-based trees.

3 Results

3.1 Phenotypic and genetic identification of bacteriocin-producing LAB strains

The two LAB strains were catalase-negative, nonmotile Gram-positive cocci. Both the strains survived to heating at 60°C *per* 30 min and grew as red colonies in Chromocult enterococci-selective agar. Both the strains were able to grow in broth supplemented with 6.5% NaCl and did not produce carbon dioxide as a fermentation product from glucose. Both strains were phenotypically identified as *E. faecium* according to the API miniaturized tests. Both strains produced extracellular proteases but not extracellular lipases. Bioassays in Niven medium revealed that none of the strains produced histamine.

Both strains fermented ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, *N*-acetyl-glucosamine, arbutine, esculine, salicine, cellobiose, maltose, lactose, saccharose, trehalose, and β-gentibiose. None of the two strains fermented glycerol, erythritol, D-arabinose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, L-sorbose, rhamnose, dulcitol, inositol, α-methyl-D-mannoside, α-methyl-D-glucoside, melezitose, D-raffinose, starch, glycogene, xylitol, D-lyxose, D-turanose, D-fucose, L-fucose, D-tagatose, D-arabitol, L-arabitol, 2-keto-gluconate, or 5-keto-gluconate.

The genetic identification of strains LHICA 28-4 and LHICA 51 based on 16S rRNA analysis was also undertaken. For this aim, a novel set of primers BAL5/BAL6, based on more than 200 LAB sequences, were designed, as described above. The nucleotide sequences of the 533 bp PCR products amplified from strains LHICA 28-4 and LHICA 51 were identical and shared 100% homology with other *E. faecium* strains from the GenBank (Fig. 1). The similarity of LHICA 28-4 and LHICA 51 strains with respect to the enterocin P-producing *E. faecium* strain JCM 5804T and *E. faecium* strain NCFB 942T was above 99%. On contrast, the similarity with respect to other type strains such as *E. faecium* UK1225 was significantly lower (Fig. 1).

3.2 Characterization of the antilisterial activity of *E. faecium* strains LHICA 28-4 and LHICA 51

The antilisterial activity of *E. faecium* strains LHICA 28-4 and LHICA 51 is shown in Table 2. Thus, the inhibition of the growth of *L. monocytogenes* strains was significant, and at least, as intense as that observed for an extracellular extract of the nisin-producing strain *L. lactis* ATCC 11454 used as positive control (Table 2). The extracellular extracts of both bacteriocinogenic enterococci also exhibited antimicrobial activity against other pathogenic Gram-positive such as *S.* ATCC 35845, *S. aureus* LHICA 1010, and *B. cereus* ATCC 14893 (Table 2).

The antimicrobial activities exhibited by *E. faecium* strains LHICA 28-4 and LHICA 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 caused a total loss of antimicrobial activity. Antimicrobial activity was observed in the positive controls, consisting of untreated extracellular extracts from the respective enterococcal strains. The antimicrobial activities of both strains were not affected by catalase, since the treated extracellular extracts remained active against *L. monocytogenes.* 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. This result allowed ruling out that the inhibitory activity was caused by the production of hydrogen peroxide by any of the enterococci investigated. The activity against *L. monocytogenes* was maintained in the

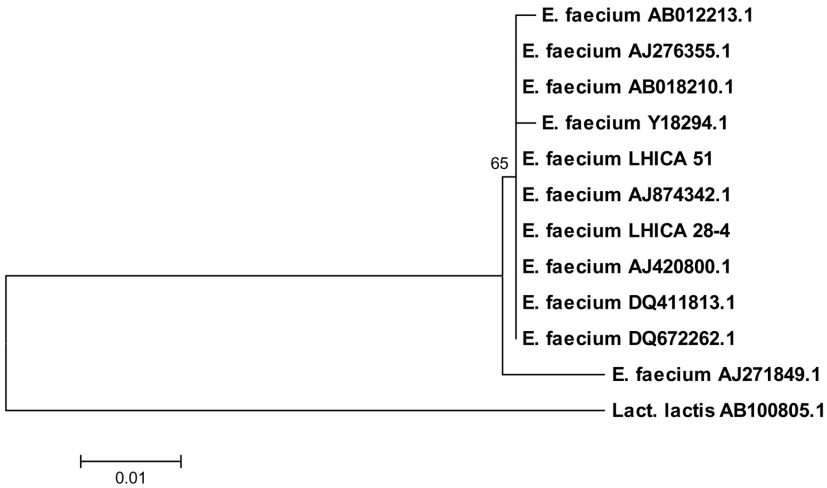

Figure 1. Phylogenetic relationships, according to the nucleotide sequences of the 533 bp 16S rRNA fragment, between the two bacteriocinogenic *E. faecium* LHICA 28-4 and 51 strains described in this work, as compared with a selection of the type of strains of *E. faecium.* The microbial strains are identified with their GenBank accession numbers: strain JCM 5804T (AB012213.1); strain E26 (AB018210.1); strain RJ16 (AJ874342.1); strain NCFB 942T (Y18294.1); strain DSM20477 (AJ276355.1); strain CICC6078 (DQ672262.1); strain CECT410T (AJ420800.1); strain ATCC 19434 (DQ411813.1); strain UK1225 (AJ271849.1). The *Lactococcus lactis* subsp. *lactis* strain ATCC 13675 (AB100805.1) has also been considered as an external reference.

Table 2. Antimicrobial activity of extracellular extracts obtained from *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51 against *L. monocytogenes*, *S. aureus*, and *B. cereus*

	Indicator microorganism				
	L. monocytogenes ATCC 11994	*L. monocytogenes* LHICA 1112	*S. aureus* ATCC 35845	*S. aureus* LHICA 1010	*B. cereus* ATCC 14893
E. faecium LHICA 28-4	11.0 ± 1.0	11.1 ± 0.9	8.0 ± 0.1	8.5 ± 0.5	12.0 ± 0.5
E. faecium LHICA 51	8.0 ± 1.0	7.7 ± 0.5	8.0 ± 0.1	8.5 ± 0.5	11.0 ± 0.1
L. lactis ATCC 11454	9.7 ± 0.7	9.5 ± 0.6	8.0 ± 0.1	9.0 ± 0.1	ND

Results are expressed as diameters of the inhibition zone and SDs in millimeter (the filter disks had a diameter of 6 mm).
The nisin-producing strain *L. lactis* ATCC 11454 strain was included as a positive control.
ND: not determined.

3–7 pH range. Negative controls consisting of noninoculated MRS broth whose pH values were adjusted in the 3–7 pH range, allowed to discard that acidification of the medium might be the cause of the inhibitory activity.

Bacteriocin activity was stable in both strains after heating the cell-free extract for 60 min at 100°C, or for 15 min at 121°C. In both the cases, bacteriocin production was initially detected in the midexponential phase of growth, and the maximum levels of antimicrobial activity were found at different stages of the stationary phase of growth, depending on the producer strain. Bacteriocin production by *E. faecium* LHICA 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. Likewise, maximum inhibitory activity of strain LHICA 28-4 was detected after 36 h of incubation – this coinciding with the beginning of the stationary phase of growth.

3.3 Nucleotide and amino acid sequences of the enterocin P gene of strains *E. faecium* LHICA 28-4 and LHICA 51

No positive amplification was achieved with the total DNA extracts of any of the strains with primers aimed at detecting enterocin A, enterocin B, enterocin L50A, enterocin L50B, enterocin Q, enterocin CRL35, or mundticin. In contrast, amplification with primer sets EntP and EntP2 allowed the visualization of 120 and 132 bp PCR products, respectively, indicating the presence of the structural gene coding for enterocin P, a IIa class heat-stable bacteriocin, in both strains (Fig. 2).

The 132 bp PCR product was sequenced, and the nucleotide sequence obtained for strain *E. faecium* LHICA 51, isolated from turbot, exhibited 97.2, 97.2, 96.7, and 87.1% DNA homology with the enterocin P gene of *E. faecium* ATB 197a [31], *E. faecium* P13 [32], *E. faecium* JCM 5804T

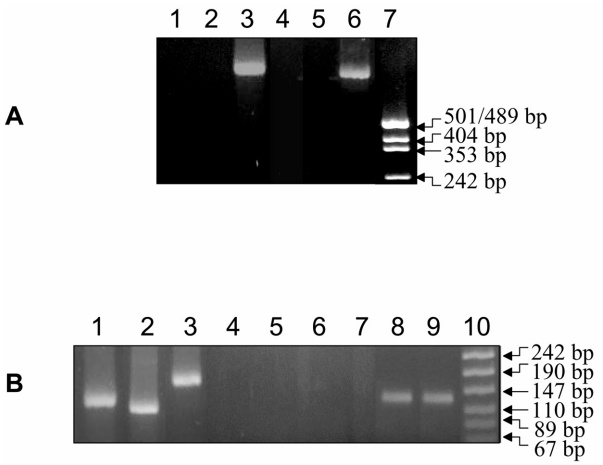

Figure 2. Detection of the 132 bp enterocin P structural gene with EntP2 primers. (A) Lanes 1, 2, and 3: *E. faecium* LHICA 28-4, *E. faecium* LHICA 51, and positive control (3.4 kb) amplified with Mun1 primers, respectively; lanes 4, 5, and 6: *E. faecium* LHICA 28-4, *E. faecium* LHICA 51, and positive control (3.1 kb) amplified with ORF primers, respectively; lane 7: M_r marker consisting of a *Msp*I-digest of plasmid pUC18 (Sigma). (B) Lane 1: enterocin P positive control (132 bp); lane 2: enterocin A positive control (130 bp); lane 3: enterocin B positive control (160 bp); lanes 4 and 5: *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51 amplified with EntA primers, respectively; lanes 6 and 7: *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51 amplified with EntB primers, respectively; lanes 8 and 9: *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51 amplified with EntP2 primers, respectively; lane 10: M_r marker as in (A).

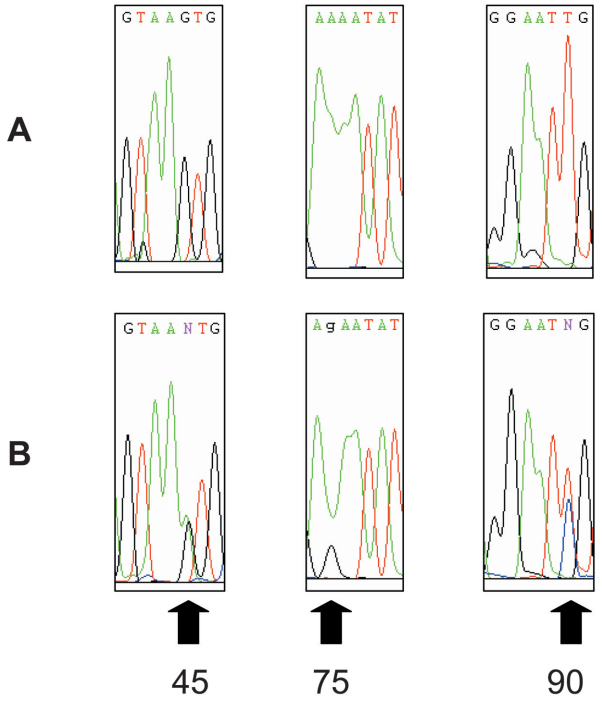

Figure 3. SNPs in the enterocin P structural gene. (A) *E. faecium* LHICA 51; and (B) *E. faecium* LHICA 28-4. The coexistence of two different nucleotides at positions 45 (G/A), 75 (A/G), and 90 (T/C) in strain 28-4 is highlighted with arrows.

[33], and *E. faecium* GM-1 [34], respectively (Table 3), previously described in the scientific literature. The four enterocin P-producing *E. faecium* strains referred above were laboratory strains (strain JCM 5804T) [33], or were isolated from environmental samples (strain ATB 197a) [31], fermented meat (strain P13) [32], or from human feces (strain GM-1) [34], while the enterocin P-producing strain *E. faecium* LHICA 51 constitutes, to our knowledge, the first report of an enterocin-P-producing strain isolated from fish so far.

With respect to the nucleotide sequence of the enterocin P structural gene of strain *E. faecium* LHICA 28-4, this was identical to that found in strain LHICA 51 except for the presence of three SNP events, silent at the amino acid level, detected at positions 45 (G/A), 75 (A/G), and 90 (T/C) (Fig. 3).

The amino acid sequence of the mature enterocin P deduced from the nucleotide sequences consists of 44 amino acids and a theoretical M_r of 4.493 kDa. The sequence: ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH was obtained. The putative amino acid sequence of the enterocin produced by strains *E. faecium* LHICA 28-4 and LHICA 51 resulted to be identical to that reported for *E. faecium* P13 [32] and exhibited similarities in the range of 86.3–95.4% with respect to the other three enterocin P proteins isolated so far [31, 33, 34]. Table 4 compiles the most remarkable differences at the nucleotide and amino acid levels among the enterocin P of *E. faecium* LHICA 28-4, *E. faecium* LHICA-51, and the other four enterocins P previously reported in the scientific literature.

The genetic relationship between the enterocin P genes was investigated, the enterocin P genes from *E. faecium* strains LHICA 28-4 and LHICA 51 clustered with the enterocin P gene from strain *E. faecium* P13. A second cluster included the enterocin P genes from *E. faecium* ATB 197a and *E. faecium* JCM5804T, while the enterocin P gene of *E. faecium* GM-1 diverged considerably with respect to the other five enterocin P genes.

4 Discussion

In our study, the antimicrobial activities exhibited by *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51 were found to be sensitive to proteinase K treatment. Additionally, when subjected to thermal treatment above 100°C, the extracellular extracts of both strains exhibited stable antimicrobial

Table 3. Alignment of the nucleotide sequences of the enterocin P structural genes of *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51, and with respect to previously reported enterocin P genes

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E. faecium LHICA 51 (DQ867125)      GCTACGCGTTCATATGGTAATGGTGTTTATTGTAATAATAGTAAGTGCTG
E. faecium LHICA 28-4 (DQ867124)    GCTACGCGTTCATATGGTAATGGTGTTTATTGTAATAATAGTAARTGCTG
E. faecium P13 (AF005726)           GCTACGCGTTCATATGGTAATGGTGTTTATTGTAATAATAGTAAATGCTG
E. faecium JCM 5804T (AB075741)     -------- CATATGATAATGGTATTTATTGTAATAATAGTAAGTGCTG
E. faecium ATB 197a (AY633748)      GCTACGCGTTCATATGATAATGGTATTTATTGTAATAATAGTAAGTGCTG
E. faecium GM-1 (AY728265)          GCTACGCGTTCATATGGTAATGGTGTTTATTGTAATGATGATAAATGCTG
                                           ****** ******* ********** **  *** *****

E. faecium LHICA 51 (DQ867125)      GGTTAACTGGGGAGAAGCTAAAGAAAATATTGCAGGAATTGTTATTAGTG
E. faecium LHICA 28-4 (DQ867124)    GGTTAACTGGGGAGAAGCTAAAGARAATATTGCAGGAATYGTTATTAGTG
E. faecium P13 (AF005726)           GGTTAACTGGGGAGAAGCTAAAGAGAATATTGCAGGAATCGTTATTAGTG
E. faecium JCM 5804T (AB075741)     GGTTAATTGGGGAGAAGCTAAAGAAAATATTGCAGGAATTGTTATTAGTG
E. faecium ATB 197a (AY633748)      GGTTAACTGGGGAGAAGCTAAAGAAAATATTGCAGGAATTGTTATTAGTG
E. faecium GM-1(AY728265)           GGTGAACTGGAATGAAGCAAATCAACAAATCGCTGGAATTGTGATTAGTG
                                    *** ** ***   ***** **  *  * ** ** ***** ** *******

E. faecium LHICA 51 (DQ867125)      GCTGGGCTTCTGGTTTGGCAGGTATGGGACAT
E. faecium LHICA 28-4 (DQ867124)    GCTGGGCTTCTGGTTTGGCAGGTATGGGACAT
E. faecium P13 (AF005726)           GCTGGGCTTCTGGTTTGGCAGGTATGGGACAT
E. faecium JCM 5804T (AB075741)     GCTGGGCTTCTGGCTTGGCAGGTATGGGACAT
E. faecium ATB 197a (AY633748)      GCTGGGCTTCTGGCTTGGCAGGTATGGGACAT
E. faecium GM-1(AY728265)           GTTGGGCTTCTGGTTTGGCAGGTATGGGACAT
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Accession numbers are indicated between brackets.
Alignment of sequences was carried out using the CLUSTAL W software.

Table 4. Summary of most remarkable nucleotide variations and predicted amino acid substitutions in the enterocin P structural genes of *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51, as compared with the four previously reported enterocin P genes

Producer strain (accession number)	Codon position (predicted amino acid)						
	6	9	15	19	25	30	38
E. faecium LHICA 51 (DQ867125)	GGT (G)	GTT (V)	AAG (K)	AAC (N)	GAA (E)	ATT (I)	GGT (G)
E. faecium LHICA 28-4 (DQ867124)	GGT (G)	GTT (V)	AAR (K)	AAC (N)	GAR (E)	ATY (I)	GGT (G)
E. faecium P13 (AF005726)	GGT (G)	GTT (V)	AAA (K)	AAC (N)	GAG (E)	ATC (I)	GGT (G)
E. faecium JCM 5804T (AB075741)	GAT (D)	ATT (I)	AAG (K)	AAT (N)	GAA (E)	ATT (I)	GGC (G)
E. faecium ATB 197a (AY633748)	GAT (D)	ATT (I)	AAG (K)	AAC (N)	GAA (E)	ATT (I)	GGC (G)
E. faecium GM-1 (AY728265)	GGT (G)	GTT (V)	AAA (K)	AAC (N)	GAA (E)	ATT (I)	GGT (G)

Amino acid abbreviations: D, aspartic acid; G, glycine; V, valine; I, isoleucine; K, lysine; N, asparagine; E, glutamic acid.
E. faecium GM-1 exhibits additional nucleotide differences at codons 13, 14, 18, 21, 23, 24, 25, 26, 27, 28, 31, and 34.

activity, even after 21 days of storage at 4°C. This result, which was confirmed for both the strains, seems to be related to the inactivation of bacterial proteases, and provided further evidence of the proteinaceous nature of the substance responsible for the antimicrobial activity. The antimicrobial activities against *L. monocytogenes* were neither caused by the bacterial acidification of the medium nor were they related to the production of hydrogen peroxide by any of the two LAB strains tested; these results confirming that the antimicrobial activities of *E. faecium* LHICA 28-4 and *E. faecium* LHICA 51 were caused by the biosynthesis and secretion of bacteriocins.

The highest levels of antimicrobial activity were observed during the stationary phase of growth, this being in agreement with other bacteriocinogenic LAB strains [35, 36]. The inhibitory action of the bacteriocins studied here increased as pH decreased, confirming previous studies referring to other LAB strains [37–39].

Little information is available about the isolation and potential role of bacteriocin-producing enterococci in nonfermented foods. In our study, we have described the isolation and characterization of two *E. faecium* bacterocinogenic strains from raw meat and raw fish, respectively. Both strains were isolated after long storage periods under refrigeration conditions – 10 and 28 days, respectively – this indicating their ability to adapt to low temperatures. In the case of *E. faecium* LHICA 28-4, such strain was recovered from vacuum-packed raw beef after 10 days of storage at 4°C. The VSP system included a surface heat-treatment when the upper film descended over the meat surface, this implying a selection of heat-resistant microorganisms, such as certain LAB, over other heat-sensitive bacteria [14]. In the case of *E. faecium* LHICA 51, such strain was isolated from fresh turbot – a flat-fish species of remarkably high commercial value in Europe – after 28 days of refrigerated storage. The high incidence of marine LAB strains able to produce bioactive compounds may be related to the extended shelf-life of fresh refrigerated turbot [17].

A comparison of both strains was performed by phenotypic and genotypic analyses. At least four phenotypic differences – assimilation of L-arabinose, amygdaline, melibiose and gluconate – between both enterococci were observed. The production of acid from melibiose and gluconate had also been described as a variable phenotype for bacteriocin-producing *E. faecium* strains [25]. However, *E. faecium* LHICA 28-4 did not produce acid from L-arabinose while *E. faecium* LHICA 51 did not produce acid from amygdaline. In contrast, the three bacteriocin-producing *E. faecium* strains previously characterized by Du Toit *et al.* [25] fermented L-arabinose but did not ferment amygdaline. Interestingly, both *E. faecium* strains characterized in this study did not ferment glycerol, while the previously described enterocin P-producing strain *E. faecium* P13 was able to ferment such polyalcohol [32]. *E. faecium* P13 and *E. faecium* LHICA 51 fermented L-arabinose, while *E. faecium* LHICA 28-4 did not produce acid from that carbohydrate. However, *E. faecium* LHICA 51, unlike *E. faecium* LHICA 28-4 and *E. faecium* P13, did not ferment amygdaline and melibiose. It should be remarked that strains *E. faecium* LHICA 28-4, isolated from fresh meat, and *E. faecium* P13, isolated from fermented sausage [32] were more similar at the phenotypic level among themselves (2 discordances), than with respect to *E. faecium* LHICA 51, isolated from turbot muscle (4 discordances). With respect to genotypic analysis, the nucleotide sequence of the 533 bp DNA fragment from the 16S rRNA gene of both strains was identical, such sequence also exhibiting similarities above 99% with other type and reference *E. faecium* strains (Fig. 1). The 533 bp fragment of the 16S rRNA amplified with BAL5/BAL6 primers represents a well-conserved region in enterococci and may be useful for the identification of *Enterococcus* spp. with respect to other genera of LAB. In global terms, both *E. faecium* strains characterized in this work were closely related although they were not identical, as determined by phenotypic analysis.

As mentioned above, enterocins are found within class I, class IIa, class IIc, and class III bacteriocins. Several genes found in close proximity, frequently organized in an operon structure, are required for the production of class IIa bacteriocins. Enterocin A, a class IIa bacteriocin, contains a pediocin-like structure that includes a YGNGVXC amino acid motif near the N-terminus of the antilisterial active peptide [40]. Enterocin B does not exhibit a pediocin-like structure, but is quite similar to the class II bacteriocins in its chemical properties, heat resistance, and antilisterial activity [41]. Enterocin P is a class IIc bacteriocin whose secretion occurs through the *sec*-pathway [32]. Enterocin A [40] and enterocin B [41] are chromosomally coded, while both chromosomally coded [42] and plasmid-coded [43] enterocin P genes have been reported. Plasmids including sequences coding for enterocin L50A, L50B [44], and enterocin Q [26] have also been isolated. Enterocin CRL35 and mundticin KS biosynthetic clusters have also been reported to be plasmid-coded and exhibit similar gene organization, including *munA*, *munB*, and *munC* genes [27, 28].

Molecular identification of the bacteriocins produced by both *E. faecium* LHICA strains was also undertaken in our study. The amplification of total DNA with EntP and EntP2 primers allowed the visualization of the 120 and 132 bp PCR products, respectively, confirming the presence of an enterocin P structural gene (Fig. 2). The lack of amplification with EntA, EntB, EntL50, EntL50(2), EntQ, ORF, and mun1 primers also suggests the absence of enterocin A, enterocin B, enterocin L50A, enterocin L50B, enterocin Q, mundticin KS, and enterocin CRL35. The heat resistance of the antimicrobial activity also proved that no enterocin belonging to the class III group was produced by any of the *E. faecium* strains characterized in this study. However, although PCR provided evidence of the presence of enterocin P in both *E. faecium* strains, the absence of antilisterial peptides other than enterocin P in such bacteriocinogenic strains cannot be fully discarded.

The nucleotide sequence of the enterocin P gene of *E. faecium* LHICA 51 was highly homologous (96.7–97.2% range) with the enterocin P gene of *E. faecium* ATB 197a [31], *E. faecium* P13 [32], and *E. faecium* JCM 5804T [33], but exhibited lower homology with the enterocin P gene of *E. faecium* GM-1 [34] (Table 3). Cluster analysis of the enterocin P sequences revealed three main groups. One group included strains ATB 197a and JCM 5804T. A second group included LHICA 51, LHICA 28-4, and P13 strains, while strain GM-1 constituted itself a third group. None of the four enterocin P-producing *E. faecium* strains described so far had been isolated from fish or from nonfermented

food, this study also representing the first report of an enterocin P-producing strain – *E. faecium* LHICA 51 – isolated from fresh fish or from nonfermented food up to now.

The enterocin P structural gene of strain *E. faecium* LHICA 28-4 exhibited three SNPs silent at the amino acid level at positions 45 (G/A), 75 (A/G), and 90 (T/C) (Fig. 3). Interestingly, the coexistence of two nucleotides at such *loci* found in *E. faecium* LHICA 28-4 in this study, may explain the differences reported for nucleotides 75 and 90 for strain *E. faecium* P13 on one hand, and for strains *E. faecium* ATB 197a, JCM 5804T, and GM-1 on the other hand (Table 3). In addition, the G/A SNP event detected in *locus* 45 in strain LHICA 28-4 may also explain the differences reported for such nucleotide among strains P13 and GM-1 on one hand, and strains ATB 197a and JCM 5804T on the other hand (Table 3). The results obtained for *E. faecium* LHICA 28-4 may be explained in terms of the coexistence of two different enterocin P genes in the same enterococcal strain, probably linked to different plasmids. In this sense, Abriouel *et al*. [43] have confirmed the widespread occurrence of enterocin P-coding plasmids in enterococci. Interestingly, they [43] discovered by DNA/DNA hybridization that two copies of the enterocin P structural gene could coexist in two different plasmids of *ca.* 26 and 35–38 kb harbored in *E. faecium* UJA6, UJA8, UJA9, and UJA13 strains [43], although these authors did not provide sequencing data to check whether both enterocin P genes were identical or not. Other approaches such as the separation of PCR amplicons by cleavage with endonucleases specific for one of the amplicons, or individual cloning, might be useful for the individual sequencing of each amplicon. However, the direct visualization of coexisting nucleotides related to the SNP events described in this study was successfully achieved by direct sequencing of the amplification products. Interestingly, the three SNP events analyzed in this work involved nucleotide positions previously described as polymorphic in other enterocin P-producing strains, this supporting the hypothesis that the three SNP events were not artefacts due to a potential incorporation of false nucleotides by the DNA polymerase during the PCR process.

As stated above, the primary structures of the mature enterocin P of strains *E. faecium* LHICA 28-4 and LHICA 51 were identical, since the three SNP events found in the enterocin P structural gene of the former strain were silent at the amino acid level. The sequence includes the pediocin-like consensus sequence YGNGV in positions 5 to 9, two conserved cysteine residues in positions 11 and 16, and other features previously reported for enterocin P [32]. The similarities between the enterocin P predicted for *E. faecium* LHICA 28-4 and LHICA 51 and other enterocin P genes fell in the range of 86.3–95.4% [31, 33, 34]. Remarkably, only two differences were observed between the enterocin P of *E. faecium* LHICA strains and the enterocin P of *E. faecium* JCM 5804T [33] or *E. faecium* ATB 197A [31], these affecting residues 6 and 9 (Table 4). In contrast, the enterocin P of *E. faecium* LHICA strains differed from that described for *E. faecium* GM-1 strain [34] in six amino acid residues at positions 13, 14, 21, 24, 25, and 26.

In summary, the present study constitutes the first report of the isolation of enterocin P-producing *E. faecium* strains from nonfermented foodstuffs, and opens the way to the use of enterocin P-producing enterococci as biopreservation agents in nonfermented animal foods. In the case of *E. faecium* LHICA 51, this strain also represents the first report of an enterocin P-producing strain isolated from fish so far. The presence of three SNPs at *loci* 45, 75, and 90, silent at the amino acid level, in strain *E. faecium* LHICA 28-4 may explain some of the previously published genetic discordances among enterocin P structural genes. Gene disruption studies aimed at confirming the link between the expression of the entP gene and the antilisterial activity of *E. faecium* LHICA 28-4 and LHICA-51 will allow to ellucidate the genetic location of such antimicrobial activity in these strains. This work also provides evidence of the coexistence of two different copies of the enterocin P gene in *E. faecium* LHICA 28-4, isolated from nonfermented meat. The sequence data of 16S rRNA and enterocin P reported in this study are available at the GenBank under the accession numbers DQ867126, DQ867127 for 16S rRNA and DQ867124, DQ867125 for enterocin P.

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5 References

[1] Marth, E. H., *Food Technol.* 1998, *52*, 57 – 62.
[2] Cotter, P. D., Hill, C., Ross, P. H., *Nature Rev.* 2005, *3*, 777 – 788.
[3] Drider, D., Fimland, G., Héchard, Y., McMullen, L. M., Prévost, H., *Microbiol. Mol. Biol. Rev.* 2006, *70*, 564 – 582.
[4] Marrug, J. D., *Food Biotechnol.* 1991, *5*, 305 – 312.
[5] Huss, H. H., Jeppesen, V. F., Johansen, C., Gram, L., *J. Aquat. Food Prod. Technol.* 1995, *42*, 5 – 26.
[6] Tagg, J. R., Adnan, S. D., Wamna-Maker, L. W., *Bacteriol. Rev.* 1976, *40*, 722 – 756.
[7] Yamazaki, K., Suzuki, M., Kawai, Y., Inque, N., Montville, T., *J. Food Prot.* 2003, *66*, 1420 – 1425.
[8] Yang, R., Johnson, M. C., Ray, B., *Appl. Environ. Microbiol.* 1992, *58*, 3355 – 3359.
[9] Foulquié-Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., *Int. J. Food Microbiol.* 2006, *106*, 1 – 24.
[10] Giraffa, G., *Int. J. Food Microbiol.* 2003, *88*, 215 – 222.

[11] Hugas, M., Garriga, M., Aymerich, M. T., *Int. J. Food Microbiol.* 2003, *88*, 223–233.

[12] Holzapfel, W. H., Haberer, P., Snel, J., Schillinger, U., Huis in't Veld, J. H. J., *Int. J. Food Microbiol.* 1998, *41*, 85–101.

[13] Ennahar, S., Deschamps, N., Richard, J., *Curr. Microbiol.* 2000, *41*, 1–4.

[14] Barros-Velázquez, J., Carreira, L., Franco, C. M., Fente, C. A. *et al.*, *J. Food Prot.* 2003, *66*, 2085–2092.

[15] Vázquez, B. I., Carreira, L., Franco, C. M., Fente, C. A. *et al.*, *Eur. Food Res. Technol.* 2004, *218*, 118–122.

[16] Rodriguez, O., Barros Velázquez, J., Ojea, A., Piñeiro, C., Aubourg, S. P., *J. Food Sci.* 2003, *68*, 2764–2770.

[17] Rodríguez, O., Barros-Velázquez, J., Piñeiro, C., Gallardo, J. M., Aubourg, S. P., *Food Chem.* 2006, *95*, 270–278.

[18] Campos, C., Rodríguez, O., Calo-Mata, P., Prado, M., Barros-Velázquez, J., *Food Res. Int.* 2006, *39*, 356–364.

[19] Himelbloom, B., Nilsson, L., Gram, L., *J. Appl. Microbiol.* 2001, *91*, 506–513.

[20] Coventry, M. J., Gordon, J. B., Wilcock, A., Amarc, K. *et al.*, *J. Appl. Microbiol.* 1997, *83*, 248–258.

[21] Phaff, H. J., Starmer, W. T., Lachance, M. A., Ganter, P. F., *Int. J. Syst. Bacteriol.* 1994, *44*, 641–645.

[22] Ben-Gigirey, B., Vieites-Baptista de Sousa, J. M., Villa, T. G., Barros-Velázquez, J., *Int. J. Food Microbiol.* 2000, *57*, 19–31.

[23] Miranda, J. M., Franco, C. M., Vázquez, B. I., Fente, C. A. *et al.*, *Lett. Appl. Microbiol.* 2005, *41*, 153–156.

[24] Niven, C. F., Jeffrey, M. B., Corlett, D. A., *Appl. Environ. Microbiol.* 1981, *41*, 321–322.

[25] Du Toit, M., Franz, C. M. A. P., Dicks, L. M. T., Holzapfel, W. H., *J. Appl. Microbiol.* 2000, *88*, 482–494.

[26] Cintas, L. M., Casaus, P., Herranz, C., Hâvarstein, L. S. *et al.*, *J. Bacteriol.* 2000, *182*, 6806–6814.

[27] Kawamoto, S., Shima, J., Sato, R., Eguchi, T. *et al.*, *Appl. Environ. Microbiol.* 2002, *68*, 3830–3840.

[28] Saavedra, L., Minahk, C., De Ruíz-Holgado, A. P., Sesma, F., *Antimic. Agents Chemother.* 2004, *48*, 2778–2781.

[29] Kumar, S., Tamura, K., Jakobsen, I. B., Masatoshi, N., *MEGA2: Molecular evolutionary genetics analysis software, version 2.1*, Arizona State University, Tempe, Arizona 2001.

[30] Saitou, N., Nei, M., *Mol. Biol. Evol.* 1987, *4*, 406–425.

[31] Klocke, M., in: *ATB Research Report 2004*, Institute of Agricultural Engineering, Bornin, Postdam 2004, pp. 1–56.

[32] Cintas, L. M., Casaus, P., Havarstein, L. S., Hernández, P. E., Nes, I. F., *Appl. Environ. Microbiol.* 1997, *63*, 4321–4330.

[33] Park, S. H., Itoh, K., Fujisawa, T., *J. Appl. Microbiol.* 2003, *95*, 294–300.

[34] Kang, J. H., Lee, M. S., *J. Appl. Microbiol.* 2005, *98*, 1169–1176.

[35] Stoffels, G., Nes, I., Guomundsdottir, A., *J. Appl. Bacteriol.* 1992, *73*, 309–316.

[36] Pilet, M. F., Dousset, X., Barre, R., Novel, G. *et al.*, *J. Food Prot.* 1995, *58*, 256–262.

[37] Muriana, P., *J. Food Prot.* 1996, *Suppl.*, 54–63.

[38] Schillinger, U., Chung, S., Keppler, K., Holzapfel, W. H., *J. Appl. Microbiol.* 1998, *85*, 657–663.

[39] Blom, H., Katia, T., Nissen, H., Holo, H., *Curr. Microbiol.* 2001, *43*, 227–231.

[40] Aymerich, T., Holo, H., Havarstein, L. S., Hugas, M. *et al., Appl. Environ. Microbiol.* 1996, *62*, 1676–1682.

[41] Casaus, P., Nilsen, T., Cintas, L. M., Nes, I. F. *et al.*, *Microbiology* 1997, *143*, 2287–2294.

[42] Parente, E., Brienza, C., Moles, M., Ricciardi, A., *J. Microbiol. Methods* 1995, *22*, 95–108.

[43] Abriouel, H., Ben Omar, N., Lucas, R., Martínez-Cañamero, M., Gálvez, A., *Lett. Appl. Microbiol.* 2006, *42*, 331–337.

[44] Cintas, L. M., Casaus, P., Holo, H., Hernández, P. E. *et al.*, *J. Bacteriol.* 1998, *180*, 1988–1994.

