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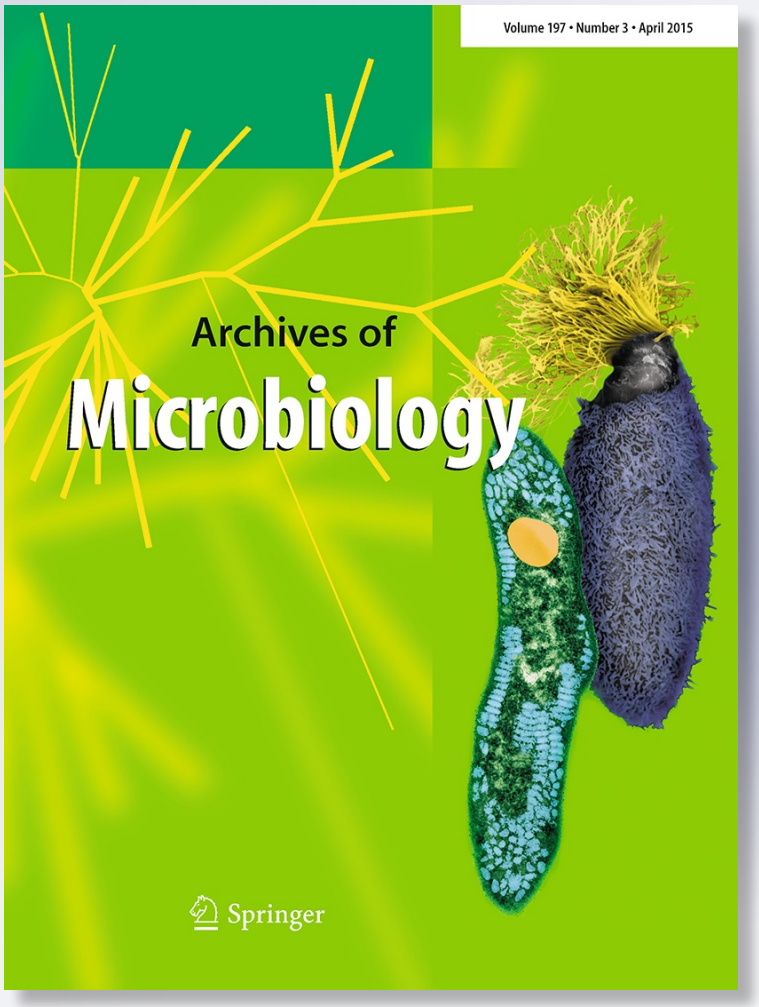

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ORIGINAL PAPER

Potential aquaculture probiont *Lactococcus lactis* TW34 produces nisin Z and inhibits the fish pathogen *Lactococcus garvieae*

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Abstract Bacteriocin-producing *Lactococcus lactis* TW34 was isolated from marine fish. TW34 bacteriocin inhibited the growth of the fish pathogen *Lactococcus garvieae* at 5 AU/ml (minimum inhibitory concentration), whereas the minimum bactericidal concentration was 10 AU/ml. Addition of TW34 bacteriocin to *L. garvieae* cultures resulted in a decrease of six orders of magnitude of viable cells counts demonstrating a bactericidal mode of action. The direct detection of the bacteriocin activity by Tricine-SDS-PAGE showed an active peptide with a molecular mass ca. 4.5 kDa. The analysis by MALDI-TOF-MS detected a strong signal at *m/z* 2,351.2 that corresponded to the nisin leader peptide mass without the initiating methionine, whose sequence STKDFNLDLVSVSKKDSGASPR was confirmed by MS/MS. Sequence analysis of nisin structural gene confirmed that *L. lactis* TW34 was a nisin Z producer. This nisin Z-producing strain with probiotic properties might be considered as an alternative in the prevention of lactococcosis, a global disease in aquaculture systems.

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Keywords Probiotic · Lactic acid bacteria · Bacteriocin · *Lactococcus lactis* · *Lactococcus garvieae* · Fish pathogen

Introduction

Aquaculture is becoming an intensive activity, with fewer but much larger farms. Infectious diseases may cause significant stock losses and problems to animal welfare (Romero et al. 2012). *Lactococcus garvieae* is the etiological agent of lactococcosis, an emerging pathology affecting different wild and farmed fish species all over the world. This is one of the most important diseases that threat the sustainability of the rainbow trout *Oncorhynchus mykiss* (Walbaum) farming industry, causing important economic losses (Vendrell et al. 2006). Outbreaks affecting rainbow trout have been reported in several countries, such as Australia, South Africa, Japan, Taiwan, England, Spain and Italy (Vendrell et al. 2006; Aguado-Urda et al. 2011; Reimundo et al. 2011). In addition, this bacterium is currently considered a potential zoonotic microorganism since it may produce several opportunistic human infections (Chan et al. 2011).

In fish farming, indiscriminate and prophylactic use of antibiotics has led to an increase of antibiotic-resistant bacteria, turning ineffective antibiotic treatment in the outbreaks of some bacterial diseases (Vendrell et al. 2006; Pérez-Sánchez et al. 2011a). Antibiotic misuse in aquaculture could also result in the transfer of antibiotic resistance determinants to bacteria of land animals and to human pathogens (Cabello 2006). Thus, it is necessary a global effort to promote a more controlled prophylactic antibiotic use along with the development of an efficient strategy to contain and manage resistance-gene emergence and spreading (Romero et al. 2012).

The use of probiotics is an environmentally suitable alternative to prevent bacterial infections in aquaculture (Verschuere et al. 2000; Irianto and Austin 2002; Balcázar et al. 2006; Gatesoupe 2008; Pérez-Sánchez et al. 2014). Probiotics can beneficiate the host in multiple ways; some of the proposed mechanisms of probiotics include the inhibition of pathogens by the production of antimicrobial compounds (e.g., organic acids, oxygen peroxide and bacteriocins), the competition for adhesion sites, the competition for essential nutrients and the modulation of the host immune responses (Verschuere et al. 2000; Balcázar et al. 2006; Merrifield et al. 2010). Recently, the disruption of quorum sensing in bacterial pathogens was also suggested as a novel strategy for using in aquaculture (Defoirdt et al. 2004, 2011; Nhan et al. 2010; Chu et al. 2011). Several members of the lactic acid bacteria (LAB) group have been used as probiotics in aquaculture (Gatesoupe 2008; Ferguson et al. 2010; Pérez-Sánchez et al. 2011a). Particularly, most of the species of the genera *Lactococcus* and *Lactobacillus* are generally recognized as safe (GRAS) microorganisms (Salminen et al. 1998), and some strains showed beneficial effects on fish development and health (Pérez-Sánchez et al. 2011a; Carnevali et al. 2004; Avella et al. 2012).

The gastrointestinal tract is one of the most important sites of interaction with the outside world and one of the main routes of pathogenic invasion of fish (Dimitroglou et al. 2011). It is well known that the gastrointestinal tract, by the feco-oral route, is a preferred target for colonization by *L. garvieae* (Vendrell et al. 2006; Pérez et al. 2010). The commensally microbiota is considered the first line of defense. Vendrell et al. (2008) proved that the administration of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* confers protection against *L. garvieae* in rainbow trout. Recent evidence showed that some LAB strains, notably the genera *Lactococcus* and *Carnobacterium*, are important transient or permanent inhabitants in the gastrointestinal tract of fish (Balcázar et al. 2007a; Gatesoupe 2008; Pérez et al. 2010). They exert several beneficial effects there, such as nutritional contribution and protection against pathogens, either by the production of antimicrobial compounds or through the competition for mucosal binding sites (Balcázar et al. 2007b).

An alternative approach to disease prevention in aquaculture is the use of bacteriocin-producing probiotic bacteria (Gillor et al. 2008). Bacteriocins are a heterogeneous group of ribosomally synthesized antimicrobial peptides and proteins that vary in their spectrum of activity, mode of action, molecular mass, genetic origin and biochemical properties (Abee et al. 1995). Only a few bacteriocins from marine microbial sources have been described; hence, the knowledge about these antimicrobial peptides is at the early stage (Desriac et al. 2010; Heo et al. 2012). In a previous work, we isolated *Lactococcus lactis* TW34 from the intestinal tract of a marine patagonian fish and demonstrated that its antimicrobial activity against several fish pathogens was due to a bacteriocin with an optimal temperature for its production as low as 15 °C (Sequeiros et al. 2010). The aim of this study was to isolate and to identify the bacteriocin produced by *L. lactis* TW34 and to determine its mode of action against *L. garvieae*.

Materials and methods

Microorganisms

Lactococcus lactis TW34 was isolated from the intestine of the fish *Odontesthes platensis* (Berg), which was collected on the northeast coast of Chubut Province, Patagonia, Argentina (Sequeiros et al. 2010). The fish pathogen *L. garvieae* 03/8460 was kindly provided by Dr. Jose Francisco Fernandez-Garayzabal (School of Veterinary Medicine, Complutense University of Madrid, Spain). This strain is a clinical specimen isolated from viscera of infected rainbow trout and collected from a site where the infection with *L. garvieae* is endemic, with regular seasonal occurrence (Eyngor et al. 2004).

Antibacterial activity assay

Antibacterial activity was assessed by the agar well diffusion assay as described in Sequeiros et al. (2010). Briefly, MRS agar (Britania, Bs. As., Argentina) was inoculated with a standardized suspension (10^6 CFU/ml) of the indicator strain *L. garvieae* 03/8460 (1 % v/v). The inoculated medium was rapidly dispensed in sterile Petri dishes. After solidification, 6-mm wells were bored in the agar. TW34 cultures were centrifuged (10 min, 10,000×*g*), and the supernatants were neutralized (pH 6.5) and autoclaved. Aliquots (50 µl) of TW34 supernatant prepared as described were dispensed into each well. Plates were allowed to diffuse at 4 °C for 2 h, and then incubated for 24 h at 30 °C, the optimal growth temperature of the indicator strain (Vela et al. 2000; Eyngor et al. 2004). Antimicrobial activity was expressed as the diameter of the inhibition halo surrounding each well. Alternatively, serial twofold dilutions of neutralized and autoclaved TW34 supernatant were dispensed into the wells and the antimicrobial activity expressed as arbitrary units (AU). To obtain the AU per milliliter, the reciprocal of the highest dilution which gave a definite inhibition zone was multiplied by the conversion factor (20 when 50 µl was used).

Effect of the bacteriocin produced by *L. lactis* TW34 on *L. garvieae* growth

Erlenmeyers containing 120 ml of MRS broth were inoculated with 1.2×10^7 CFU/ml of *L. garvieae* 03/8460 and incubated at 30 °C. After 3.5 h of incubation (exponential growth phase), the neutralized (pH 6.5) and autoclaved supernatant of TW34 was added to give a final concentration of 32 AU/ml. *L. garvieae* 03/8460 growth was periodically monitored measuring $A_{600\ nm}$ with an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, Palo Alto, CA) and viable cell counts in MRS plates incubated at 30 °C for 24 h. Erlenmeyers without neutralized and autoclaved TW34 supernatant addition were used as controls. All experiments were performed in triplicate.

Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) of TW34 bacteriocin over *L. garvieae* 03/8460 was performed by the standard broth dilution method according to the British Society for Antimicrobial Chemotherapy guidelines (Andrews 2001). The neutralized (pH 6.5) and autoclaved supernatant of TW34 was twofold diluted in MRS broth to achieve concentrations ranging from 320 to 2.5 AU/ml. A standardized suspension (10^6 CFU/ml) of the indicator strain *L. garvieae* 03/8460 (1 % v/v) was added into each tube. The tubes were incubated at 30 °C for 24 h. The MIC was considered as the lowest concentration of the bacteriocin which inhibited the visible growth of *L. garvieae* (Andrews 2001). Controls were prepared using sterile 10 mM phosphate buffer pH 6.0 instead of neutralized and autoclaved TW34 supernatant, to confirm that the absence of growth was inhibition and not dilution of nutrients. Moreover, after 24 h of incubation, an aliquot of 10 µl of each culture tube was spread over MRS agar plates and further incubated at 30 °C for 24 h to determine the minimum bactericidal concentration (MBC). The lowest concentration showing no revival of *L. garvieae* was considered as the MBC. All the tests were performed in triplicate.

Purification and molecular characterization of the bacteriocin produced by *L. lactis* TW34

Adsorption studies

Adsorption of the bacteriocin to the producer cells was studied as described by Yang et al. (1992). After culturing *L. lactis* TW34 in Trypticase Soy Broth (TSB) at 15 °C for 24 h, the culture was heated at 80 °C to kill the cells. An aliquot was used as a control, and the remaining culture was adjusted to pH 6.5 with 1 M NaOH and incubated with stirring at 25 °C for 3 h. Then, the cell suspension was centrifuged for 15 min at 10,000×*g* in a Sorvall RC 5C centrifuge (Sorvall Instruments, Dupont, USA) and the cell-free supernatant reserved to measure the antimicrobial activity. Cells were washed in an equal volume of sterile 5 mM sodium phosphate buffer (pH 6.5) and re-suspended in 100 mM NaCl (pH 2.0). This cell suspension was incubated with stirring overnight at 25 °C and then centrifuged (15 min, 10,000×*g*). The cell-free supernatant was neutralized to pH 6.0 with sterile 1 M NaOH and tested for antimicrobial activity as described above. All experiments were performed in triplicate.

Ethanolic precipitation

L. lactis TW34 was cultured in TSB at 15 °C for 24 h. The cell-free supernatant was precipitated with a quarter, a half, three quarters, one, two and three volumes of absolute ethanol overnight at −20 °C, and then, the samples were centrifuged at 4 °C for 15 min at 16,000×*g* in a Sorvall RC 5C centrifuge (Sorvall Instruments, Dupont, USA). The precipitated obtained was re-dissolved in sterile water. For each sample thus obtained, a control was also prepared (untreated cell-free supernatant). Samples and controls were tested for antimicrobial activity against *L. garvieae* 03/8460 by the agar well diffusion assay.

Cation exchange chromatography (CEC)

The ethanol partially purified preparation was applied onto a SP-Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden) equilibrated with 25 mM Citric–citrate buffer, pH 4.5. The chromatography was developed in an Äkta basic equip (GE Healthcare, Uppsala, Sweden). The first step consisted in the washing of the column with one volume of the equilibration buffer followed by the elution with a linear salt gradient of 0–1 M NaCl in the start buffer at a flow rate of 0.5 ml/min. The absorbance ($A_{280\ nm}$) and the antimicrobial activity were tested in all fractions. The fractions showing antimicrobial activity were pooled and stored at −20 °C for further studies.

Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine-SDS-PAGE)

The protein profile of different preparations of the cell-free supernatant of *L. lactis* TW34 was examined through denaturing electrophoresis in Tricine gels composed of a stacking gel (4 % T, 3 % C), a spacer gel (10 % T, 3 % C) and a separating gel (16.5 % T, 3 % C). These gels are especially suitable to resolve low mass molecular peptides. The

electrophoresis was performed in a Miniprotean II Cell (Bio-Rad, Richmond, CA, USA) according to Pardo and Natalucci (2002). Low molecular weight standards (Bio-Rad Laboratories, Richmond, CA, USA) were used. After electrophoresis, the gel was cut in two parts, one of them containing the samples and the molecular weight standard was stained with Coomassie Brilliant Blue G-250 (Sigma Chemical Co., St. Louis, MO, USA). The second part was immediately fixed for 1 h in a solution of 40 % methanol and 10 % acetic acid and washed in sterile deionized water for 2 h, replacing the water bath every 15 min. Then, it was assayed for antimicrobial activity by the direct method of Bhunia et al. (1987).

Mass spectrometry analysis by MALDI-TOF-MS

Mass spectrometric (MS) data from Tricine-SDS–polyacrylamide gel bands with and without tryptic digestion were obtained by using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometer, Ultraflex II (Bruker Daltonics, Billerica, MA, USA), in the MS facility CEQUIBIEM (Centro de Estudios Químicos y Biológicos por Espectrometría de Masa; Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). For the identification of some peptides, manual spectrum interpretation was carried out by comparison of the experimental MS and tandem MS (MS/MS) data with the in silico digestion of the unmodified and modified prenisin peptide.

PCR amplification of bacteriocin gene

TW34 DNA was extracted using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). PCR amplifications of the known structural genes of nisin, lactococcin A, and lacticin 481 were performed with specific primers (Online Resource 1) according to Rodriguez et al. 2000. *L. lactis* ATCC 11454 and *L. lactis* CNRZ481 were used as PCR positive controls for nisin and lacticin 481 amplifications, respectively. PCRs were performed in a Multigene Gradient thermal cycler (Labnet International Inc., Woodbridge, NJ, USA). The presence of PCR products was revealed by electrophoresis in 1.5 % agarose gels. Sequencing on both strands of PCR fragments was performed using the dideoxy chain termination method by the commercial services of the CENPAT Molecular Biology Laboratory (Argentina). Bacteriocin gene sequence similarity searches were carried out using BLAST at the NCBI database (Altschul et al. 1990). Deduced amino acid sequences were obtained with the ExPASy Translate tool program (“http://www.expasy.ch/tools/dna.html”). TW34 bacteriocin gene sequence was deposited in the GenBank database under the accession number KJ781408.

Results

Mode of action of TW34 bacteriocin

Addition of TW34 supernatant containing the bacteriocin to *L. garvieae* cultures at the exponential phase of growth (3.5 h) resulted in a stop of $A_{600\ nm}$ curve, which remained constant to the end of the experiment (Fig. 1a). In contrast, the viable cell count of *L. garvieae* dropped very sharply within 1 h (six orders of magnitude). Thereafter, survival of cells and subsequent recovery of viable bacteria was observed after the next 3 h (Fig. 1b).

TW34 bacteriocin inhibited *L. garvieae* growth at 5 AU/ml (MIC), whereas the MBC value was 10 AU/ml.

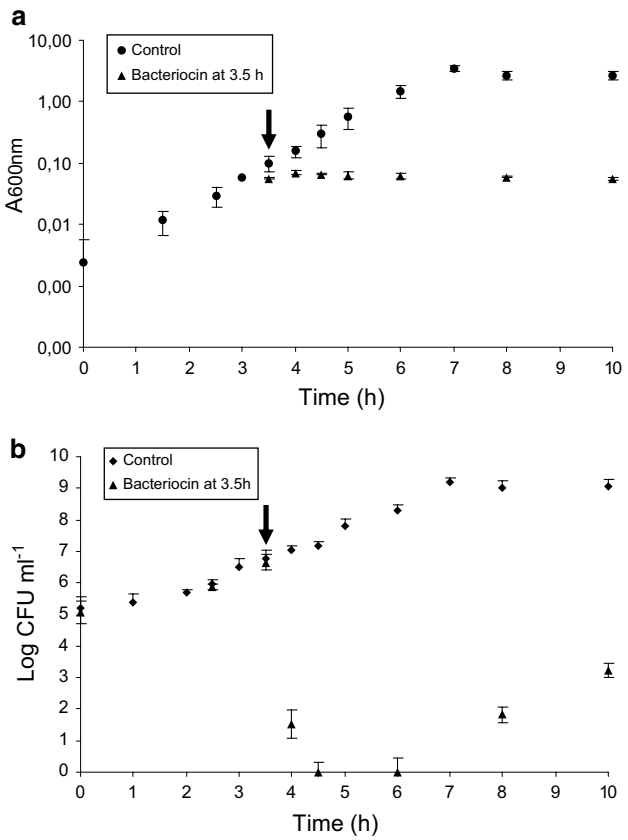

Fig. 1 Effect of *L. lactis* TW34 bacteriocin on the growth of *L. garvieae* 03/8460. $A_{600\ nm}$ (**a**) and colony-forming units (CFU) (**b**) of *L. garvieae* 03/8460 in controls *diomand* and bacteriocin (32 AU/ml) treated cultures at 3.5 h *triangle*

Partial purification and structural characterization of TW34 bacteriocin

Adsorption of bacteriocin to the producer cells

No antimicrobial activity was detected in the neutralized supernatant after treatment of the producer cells with NaCl at pH 2.0. This fact suggests that the bacteriocin did not adhere on the surface of the producer cells.

Ethanolic precipitation and chromatography

The addition of 0.5 volumes of absolute ethanol precipitated virtually all the active bacteriocin (purification factor: 1.5 times), which permitted to concentrate the sample for further chromatographic steps. CEC allowed the adsorption of the bacteriocin onto the immobilized ion exchange groups (SP-Sepharose FF). Desorption was achieved early in the salt concentration gradient giving one active fraction (Online Resource 2).

Electrophoretic comparison of the TW34 supernatant, the ethanol partially purified preparation and the active fraction obtained by CEC is shown in Fig. 2. The TW34 supernatant and the ethanol partially purified preparation (lanes 1 and 2, Fig. 2a) had similar profiles; as expected, the latter was more concentrated. In all samples, the main band was found around 4.5 kDa, this was the only band with antimicrobial activity against *L. garvieae* in the gel (Fig. 2b). The fraction obtained by CEC also contained high molecular mass polypeptides without antimicrobial activity (lanes 5, Fig. 2a).

Analysis by MALDI-TOF-MS

The electrophoretic band of ca. 4.5 kDa was cut off from the gel and processed with and without trypsin before analysis by MALDI-TOF-MS. A signal at *m/z* 2,351.2 appeared in all analyzed mass spectra (with and without tryptic digestion) (Fig. 3a). This peptide was fragmentized and analyzed by MS/MS. The identity of the peak at *m/z* 2,351.16 could be verified and corresponded to the nisin leader peptide sequence without the initiating methionine: STKDFNLDLVSVSKKDSGASPR [2–23 fragment position with a theoretical *m/z* value of 2,351.20] (Fig. 3b). In addition, in the trypsin digested sample, fragments at *m/z* 1,683.74; 1,363.7 and 1,235.29 were individualized and compared with the in silico tryptic digestion of the prenisin A (leader peptide and nisin A) (Table 1). On the contrary to Tricine-SDS-PAGE electrophoresis results, there was no peak of *m/z* ratio near the expected value of 4,500.

Confirmation by PCR of the presence of nisin structural genes in L. lactis TW34

Using nisin primers, an 817-bp PCR product was obtained and sequenced. It was further translated in all six reading frames to identify the amino acid sequence of nisin. One of the amino acid sequence deduced presented the whole sequence of prenisin (Fig. 4). Results indicated that the sequence was 100 % identical to that of nisin Z because an asparagine (N) residue instead of a histidine (H) residue

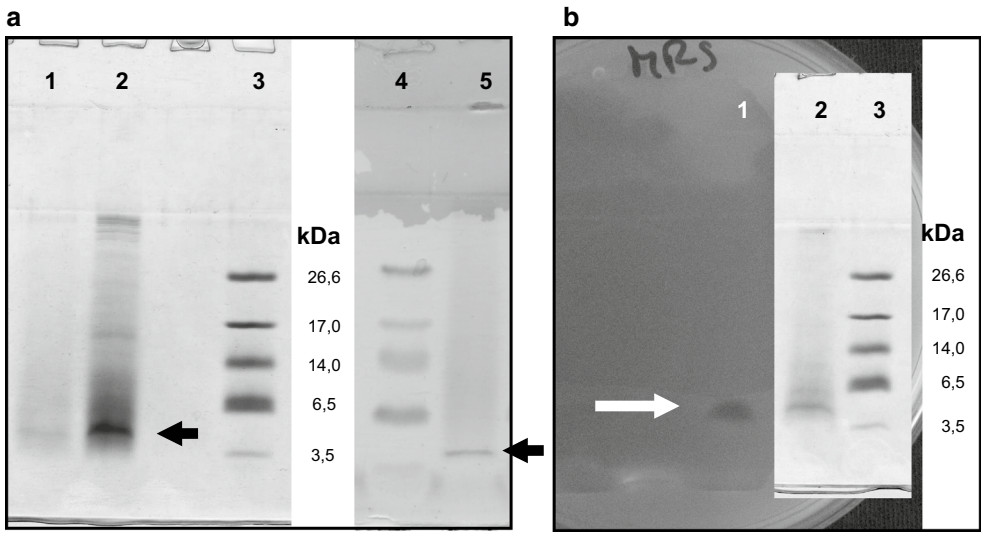

Fig. 2 Tricine-SDS-PAGE profiles of the different preparations of the cell-free supernatant of *L. lactis* TW34. **a** *Line 1* Protein profiles of the cell-free supernatant; *line 2* Ethanol partially purified preparation of the cell-free supernatant; *line 3* and *4* Peptide markers and *line 5* Fraction obtained from cation exchange chromatography. The *arrows* indicate bacteriocin band. **b** Detection of bacteriocin activity in a gel by over-layering the gel with MRS soft-agar medium containing *L. garvieae* 03/8460. *Lanes 1* and *2* Ethanol partially purified preparation of the cell-free supernatant of *L. lactis* TW34 and *lane 3* Peptide markers (26.0, 17.0, 14.4, 6.5 and 3.5 kDa)

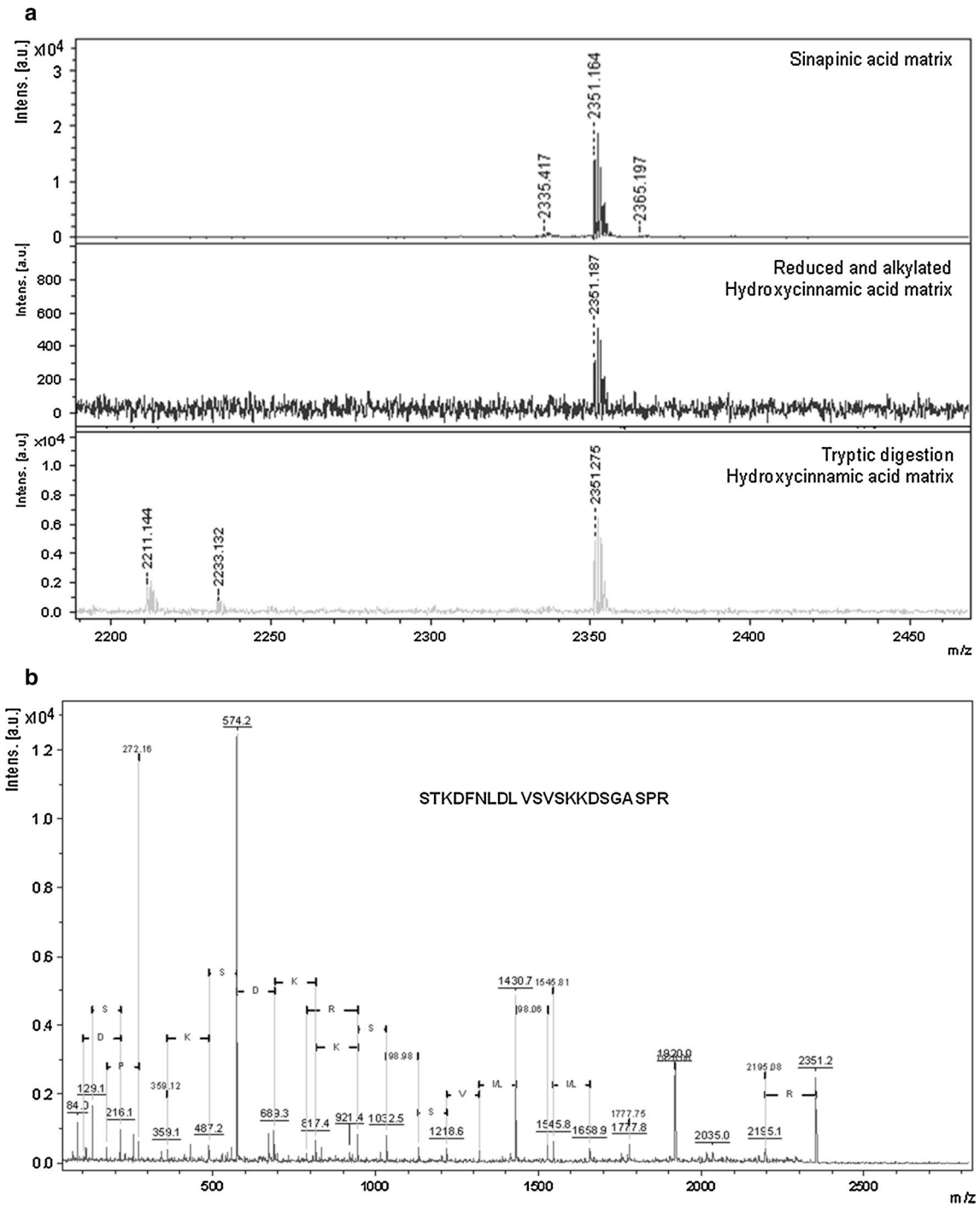

Fig. 3 **a** Detection of nisin leader peptide by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) from SDS-Tricine PAGE gel band (ca 4.5 kDa). Expected average masses for the nisin leader peptide residues 2–23 is 2,351.2 Da. **b** Mass spectrum corresponding to fragmentation of the *m/z* 2,351.2 peptide by MS/MS analysis

was identified at position 27. This indicated that the bacteriocin produced by *L. lactis* TW34 was nisin Z. On the other hand, neither lactococcin A nor lacticin 481 structural genes were detected.

Discussion

The knowledge about LAB strains with antagonistic activity against the fish pathogen *L. garvieae* is scarce and novel

Table 1 Mass comparison of the trypsin digested peptides of TW34 bacteriocin with the in silico digestion of prenisin A (MSTKDFNLDLVSVSKKDSGASPRITS ISLCTPGCKTGALMGCNMKTATCHCSIHVSK)

Experimental mass (*m/z*)	Theoretical mass $[M + H]^+$ (*m/z*)	Fragment position	Peptide fragment sequence
2,351.16	2,351.20	2–23	STKDFNLDLVSVSKKDSGASPR
1,683.74	1,683.90	1–15	MSTKDFNLDLVSVSK
1,364.77	1,364.70	5–16	DFNLDLVSVSKK
1,236.67	1,236.60	5–15	DFNLDLVSVSK

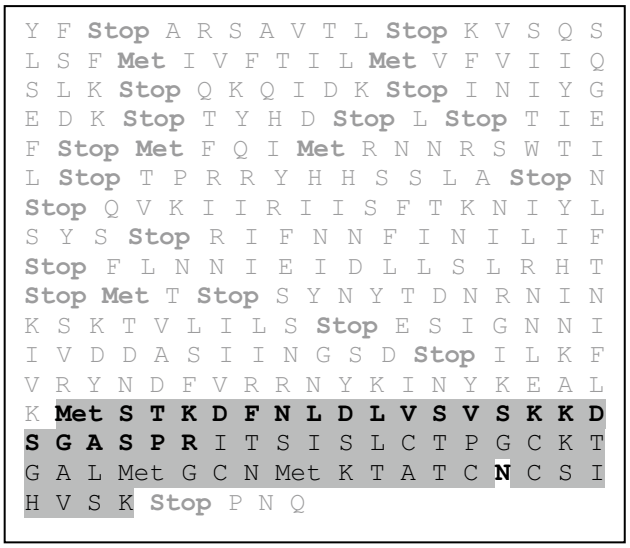

Fig. 4 *L. lactis* TW34 deduced protein sequence from PCR product amplified using nisin-gene specific primers. The sequence *shaded in gray* corresponds to the sequence of prenisin Z, amino acids in *bold* correspond to the leader or signal peptide, followed by the mature nisin sequence, in which the Asn (N) residue characteristic of nisin Z is highlighted in *white*

(Vendrell et al. 2008; Sequeiros et al. 2010; Pérez-Sánchez et al. 2011b; Lin et al. 2013). Even more limited is the information about the bacteriocins from marine bacteria, and only a few of them have been fully characterized (Desriac et al. 2010; Heo et al. 2012). To the best of our knowledge, Enterocin P and another novel bacteriocin with a molecular mass of 6,319 Da produced by *Enterococcus faecium* B3-8 and *E. thailandicus* B3-22, respectively, are the only marine bacteriocins active against *L. garvieae* described to date (Lin et al. 2013). Preliminary studies have shown that *L. lactis* TW34 antimicrobial agent against *L. garvieae* was a bacteriocin-like substance with strong heat stability and high activity in a wide pH range (Sequeiros et al. 2010). In this study, the direct detection of the bacteriocin antimicrobial activity on a Tricine-SDS-PAGE gel revealed an active peptide with a molecular mass ca. 4.5 kDa. Further analysis of the gel active band by MALDI-TOF-MS detected a strong signal at *m/z* 2,351.2 in all analyzed mass spectra. It corresponded to the nisin leader peptide mass without the initiating methionine (2,351.2 Da corresponded to 2–23 residues), whose sequence STKDFNLDLVSVSKKDSGASPR was confirmed by MS/MS analysis. These results suggested that in the range of approximately 4.5 kDa, the leader peptide is present with an electrophoretic mobility similar to the mature bacteriocin. No peak was detected with the *m/z* ratio 3.35 kDa, mass of the mature nisin. This may be due to the fragmentation of the peptide when it was removed from the gel, possibly due to the very small size of the gel pore.

Nisin is synthesized as a two-segment precursor peptide consisting of a leader peptide region (2.48 kDa) and a propeptide region (bacteriocin, 3.35 kDa). The cleavage of the leader peptide sequence from the nisin precursor peptide is not required for its transporting across the cytoplasmic membrane. The presence of the leader peptide attached to the fully modified nisin keeps the bacteriocin in an inactive form (Lubelski et al. 2008). The cleavage of the leader sequence takes place on the outside of cell envelope; thus, nisin A as well as nisin A precursor and leader peptide could be found in the cell-free supernatant of the nisin-producer *L. lactis* strains (van den Berg et al. 2008; van der Meer et al. 1994; Kuipers 2010). Interestingly, our results are in agreement with those reported by Kuipers (2010), who detected in the culture medium of the nisin-producer *L. lactis* NZ9700 two peptides by anti-leader peptide antibodies on a SDS-PAGE gel; one corresponded to a very intense band around 4 kDa and the other to a weaker band below 8.4 kDa. Moreover, the nisin leader peptides (2–23, 2,352.6 Da and 1–23, 2,483.8 Da) and nisin (3,354.2 Da) were accumulated in the supernatant of the overnight *L. lactis* NZ9700 cultures and apparently displayed a similar electrophoretic mobility (Kuipers 2010).

Furthermore, during the purification steps, TW34 bacteriocin did not adsorb to the producer cells when cultures were processed as proposed by Yang et al. (1992). The same was observed for bacteriocins produced by *Pediococcus pentosaceus* ALP57 and *L. lactis* subsp. *lactis* B14 (Ivanova et al. 2000; Pinto et al. 2009).

Sequence analysis of nisin structural gene amplified using specific primers confirmed that *L. lactis* TW34 was a nisin Z producer. Nisin Z is a lantibiotic, which is a peptide antibiotic containing thioether-bridged amino acids:

lanthionines and methyllanthionines (Chatterjee et al. 2006). These thioether rings are responsible for lantibiotic notable properties such as acid tolerance, thermo stability, high antimicrobial activity and a specific bactericidal mode of action (De Vuyst and Vandamme 1994; Kuipers 2010). Coincidently, the decrease in the viable cell counts of *L. garvieae* after the addition of TW34 bacteriocin demonstrated a bactericidal mode of action (MBC 10 AU/ml). Moreover, no reduction in absorbance readings indicated that *L. garvieae* cells were not lysed. The bactericidal action of bacteriocins on sensitive cells usually involves the formation of pores in the membrane. As bacteriocins are positively charged molecules with hydrophobic patches, the initial association is charge dependent, due to the interaction between positively charged peptides and negatively charged phospholipids on the target cell membranes via electrostatic interactions (Breukink et al. 1997; Moll et al. 1999). Then, it is likely that the hydrophobic patches insert into the membrane, forming pores and resulting in the leakage of the cellular materials (Abee et al. 1995; Cleveland et al. 2001). This process finally leads to the cell death that may occur with or without cell lysis.

Recently, Merrifield et al. (2010) proposed a list of 11 criteria for selecting potential probiotics. Among them, TW34 belongs to a species (*Lactococccus lactis*) recognized as safe (GRAS) because it is not pathogenic for aquatic animals and human consumers (Salminen et al. 1998). TW34 strain is sensitive to most of the antibiotics tested (Sequeiros et al. 2010). Moreover, it was isolated from wild healthy fish and exhibits antagonistic properties toward *L. garvieae* and other fish pathogens. Besides we demonstrated that *L. lactis* TW34 produces nisin Z, which is another favorable feature related with its safety. Nisin is the only bacteriocin approved for food applications, considered as safe by the Food and Agriculture Organization/World Health Organization (FAO/WHO) in 1969. It was also included as a biopreservative ingredient (E234) in the European food additive list (Balciunas et al. 2013). Since the European Union regulates the authorization, marketing and use of probiotics in food, a nisin-producing strain with proved probiotic properties would be a promising alternative for application as a feed additive in the prevention of lactococcosis which affects a variety of fish farmed species all over the world. Overall, our results provide a ground for further in vivo experiments to confirm the safety and usefulness of TW34 and its bacteriocin in aquaculture, and also to assess administration strategies to fish.

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