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ORIGINAL PAPER

Inhibitory activity against the fish pathogen *Lactococcus garvieae* produced by *Lactococcus lactis* TW34, a lactic acid bacterium isolated from the intestinal tract of a Patagonian fish

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Abstract After enrichment of *Odontesthes platensis* intestinal contents, 53 lactic acid bacteria (LAB) were isolated. From the four isolates that showed inhibitory activity against *Lactococcus garvieae* 03/8460, strain TW34 was selected because it exerted the strongest inhibition. It also inhibited other Gram-positive bacteria, but not Gram-negative fish pathogens. Phenotypic and 16S rDNA phylogenetic analyses showed that TW34 belongs to *Lactococcus lactis*. In addition, TW34 showed to be sensitive to different antibiotics. The production of the inhibitory agent against *L. garvieae* was growth associated, and it was significantly influenced by the incubation temperature. The optimal temperature for the antimicrobial production was as low as 15°C. Both acidification and hydrogen peroxide production were ruled out as the source of inhibition. In contrast, the antimicrobial activity was completely lost by treatment with proteolytic enzymes, which confirmed that the inhibitory substance was a bacteriocin. The bacteriocin was highly thermostable (121°C for 15 min) and active between pH 3 and 11. It remained stable for up to 2 months when stored at 4°C and up to 6 months at −20°C. Our results suggest that the strain *L. lactis* TW34 could provide an alternative for lactococcosis control and therefore be considered for future challenge experiments with fish.

Keywords Lactic acid bacteria · *Lactoccocus lactis* · Bacteriocin · Antimicrobial peptide · *Lactococcus garvieae* · Fish pathogen

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Introduction

Lactococcosis is an emerging disease caused by *Lactococcus garvieae*, which affects many fish species such as rainbow trout, yellowtail, tilapia, and catfish, in both marine and freshwater aquaculture (Vendrell et al. 2006). This illness produces a hyperacute and hemorrhagic septicemia, which evolves depending on the environmental conditions of the fish habitat with temperature and the microbiological quality of the water being especially important (Vendrell et al. 2006).

Two traditional strategies used to fight against bacterial diseases in farmed fish are vaccination and antibiotic treatment; however, both methods have drawbacks. Commercial vaccines are available for some pathogens (Gomes et al. 2006), but vaccination cannot prevent disease development in neither the young nor the very small specimens which are immature and not fully immunocompetent. In the case of lactococcosis, despite the immunity after vaccination gives to fish a good level of protection, it lasts only for a short period (Bednarska et al. 2007). In addition, the indiscriminate use of antibiotics normally leads to the emergence of antibiotic resistant pathogens (Balcázar et al. 2006), which points out the need to minimize their administration.

As an alternative to traditional disease control treatments, the use of probiotics in aquaculture is a strategy

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which is being further developed (Gatesoupe 1999; Irianto and Austin 2002). Probiotics, which are microorganisms or their products with beneficial effects for the host health, are useful to control diseases of aquatic organisms by supplementing or even replacing the use of antibiotics (Irianto and Austin 2002). The microorganisms studied for their potential as aquaculture probiotics are diverse, including strains from the genera *Vibrio* (Austin et al. 1995), *Bacillus* (Moriarty 1998), *Pseudomonas* (Spanggaard et al. 2001), *Aeromonas* (Brunt and Austin 2005), and from the lactic acid bacteria (LAB) group (Gatesoupe 2008).

The knowledge and experience gained from human and animal LAB probiotics have also encouraged the research on this bacterial group for aquaculture probiotics. LAB produce antimicrobial compounds (e.g., lactic acid, diacetyl, carbon dioxide, hydrogen peroxide, and bacteriocins) as a result of its metabolism; this feature makes them interesting to use as biological control agents or food biopreservatives (Gatesoupe 1999; Calo-Mata et al. 2008). In addition, LAB probiotics can act as growth promoters, thus achieving healthier aquatic organisms and shortening the fish growth time before their marketing (Ringø and Gatesoupe 1998; Vázquez et al. 2005). Most of the aquatic LAB evaluated for their probiotic properties in aquaculture belong to the genera *Lactobacillus* and *Carnobacterium* (Byun et al. 1997; Ringø et al. 2000; Balcázar et al. 2008). Additionally, a few *Enterococcus* (Chang and Liu 2002) and *Lactococcus* strains (Balcázar et al. 2007; Sugita et al. 2007) were also tested.

The information about strains with antagonist activity against *L. garvieae* is very limited. *Aeromonas sobria* GC2 was effective as the probiotic against lactococcosis based on the results from in vivo assays using rainbow trout (Brunt and Austin 2005). *Leuconostoc mesenteroides* and *Lactobacillus plantarum* strains, supplied in the fish feed, produced a decrease in mortality of rainbow trout (Vendrell et al. 2008). Regarding in vitro tests Sugita et al. (2002) found that most of the strains isolated from the intestinal tract of the Japanese flounder (*Paralichthys olivaceus*) with inhibitory activity against *L. garvieae,* belonged to the genus *Vibrio* and, to a lesser extent, to *Flavobacterium*. Lategan et al. (2006) purified a substance produced by *Aeromonas media* A199 with inhibitory activity against *L. garvieae,* whereas Balcázar et al. (2007) isolated a *L. mesenteroides* strain which also produced *L. garvieae* antagonistic substances.

With worldwide distribution, *L. garvieae* has been reported as a cause of important economic losses showing in outbreaks across Europe (Ghittino and Prearo 1992; Bednarska et al. 2007), Asia (Chang et al. 2002), Africa, and Australia (Carson et al. 1993). The aim of this study was to isolate and to characterize aquatic LAB showing antimicrobial activity against *L. garvieae*. We focused on the production and biochemical characteristics of antagonist compounds against *L. garvieae* produced by the selected strain *Lactococcus lactis* TW34, a resident of the *Odontesthes platensis* intestinal tract. We also analyzed its antimicrobial spectrum against other fish pathogens. To our knowledge, this is the first report of a *L. lactis* strain isolated from fish which shows inhibitory activity against *L. garvieae*.

Materials and methods

Sampling and LAB isolation

Coastal fish specimens of *Odontesthes platensis* were collected with standard fishing methods on the northeast coast of Chubut Province, Patagonia, Argentina. Each specimen was aseptically dissected and the content from intestinal tract was homogenized in 1 ml of sterile saline solution. An aliquot of the homogenates was inoculated into De Man, Rogosa and Sharpe (MRS) broth (Biokar) and incubated at 25°C for 6 h. The enrichments were then plated onto the following solid media: (a) MRS adjusted to pH 4.6 and 5.4, (b) MRS without acetate at pH 8, (c) MRS supplemented with 6.5% (w/v) NaCl, and (d) M17 (Biokar). All were then incubated at 25°C for 48 h and at 30°C for 24 h.

Phenotypic tests were based on the methods described by Dykes et al. (1994) using MRS medium. These tests included Gram reaction, cellular morphology, catalase production, carbon dioxide production from glucose, ability to grow at different NaCl concentrations and pH values. All strains identified as LAB were evaluated to determine their antimicrobial activity.

Antimicrobial activity assays

Antibacterial activity was assessed by the agar well diffusion assay described by Parente et al. (1995). Briefly, molten MRS agar (45°C) was first seeded (1% v/v) with a standardized suspension of the indicator strain. The inoculated medium was rapidly dispensed in sterile Petri dishes. After solidification, wells of uniform diameter (6 mm) were bored in the agar. Aliquots (50 µl) of cell-free culture supernatant, adjusted to pH 6.5 with 1 M NaOH, were dispensed into each well. Plates were allowed to diffuse for 2 h at 4°C, and then incubated at the optimum growth conditions for each indicator strain (Table 1) and examined after 24 h. Antimicrobial activity was expressed as the diameter of the inhibition halo surrounding each agar well. Alternatively, serial twofold dilutions of the cell-free 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

Table 1 Antimicrobial activities of cell-free supernatant from *L. lactis* TW34 toward indicator bacteria

Indicator strains	Culture medium	Incubation temperature (°C)	Antimicrobial activity[b]
Lactococcus garvieae 03/8460[a]	MRS	30	++
Lactococcus garvieae 03/8702[a]	MRS	30	++
Lactococcus piscium 23.3.92[a]	MRS	20	++
Carnobacterium piscicola 4020[a]	MRS	30	+
Streptococcus iniae 2378[a]	MRS	30	+
Streptococcus iniae MT 2376[a]	MRS	30	++
Enterococcus faecalis ATCC 29212	MRS	30	+
Vibrio anguilarum 1603A[a]	PCA	30	−
Yersinia ruckeri ATCC 29473	PCA	30	−
Aeromonas salmonicida ATCC 33658	TSA	20	−
Listeria monocytogenes ATCC 7644	BHI	37	+
Listeria inocua ATCC 33090	BHI	37	+

MRS de Man–Rogosa–Sharpe, *PCA* Plate Count Agar, *TSA* Trypticase Soy Agar, *BHI* Brain Heart Infusion

[a] These strains were kindly provided by Dr. Jose Francisco Fernandez-Garayzabal (School of Veterinary Medicine, Complutense University of Madrid)

[b] Inhibition halo: +, ≥10 mm; ++, ≥15 mm; −, no inhibition

a definite inhibition zone was multiplied by the conversion factor (20 when 50 µl was used).

Spectrum of antimicrobial activity

In a first step, antimicrobial activity of LAB isolates against the indicator pathogen *L. garvieae* 03/8460 was determined using the well diffusion assay described above, in MRS agar (Biokar) at 30°C. The cell-free supernatant (adjusted to pH 6.5) of the selected LAB was also tested against the indicator strains *L. garvieae* 03/8702, *Carnobacterium piscicola* 4020, *Streptococcus iniae* 2378, *S. iniae* MT 2376, *Lactococcus piscium* 23.3.92, *Enterococcus faecalis* ATCC 29212, *Aeromonas salmonicida* ATCC 33658, *Vibrio anguilarum* 1603A, *Yersinia ruckeri* ATCC 29473, *Listeria monocytogenes* ATCC 7644, and *L. inocua* ATCC 33090. For each strain, the culture medium and incubation temperature used are indicated in Table 1. Results were examined after 24 h of incubation.

Strain characterization and phylogenetic analysis

Carbohydrate fermentation pattern was determined using API 50 CH gallery and API 50 CHL medium (bioMérieux, France) according to the manufacturer's instructions. Ammonia production from arginine, growth at 10 and 40°C, growth with 4 and 6.5% (w/v) NaCl, and growth at pH 9.2 were performed as described by Teuber (1995). Antibiotic susceptibilities were assessed by the disk diffusion test in Mueller–Hinton agar using the Kirby–Bauer method (Bauer et al. 1966) and the National Committee for Clinical Laboratory Standards (NCCLS 2000) guidelines. The antibiotic sensitivity disks (Laboratorios Britania S.R.L., Buenos Aires, Argentina) included: penicillin (10 U), oxacillin (1 µg), erythromycin (15 µg), clindamycin (2 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), vancomycin (30 µg), levofloxacin (5 µg), gentamicin (10 µg), rifampin (5 µg), minocycline (30 µg), chloramphenicol (30 µg), and teicoplanin (30 µg). Agar plates were incubated at 35°C for 24 h. *E. faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 were used as quality control microorganisms. The diameters of the growth inhibition halos were measured, and the antibiograms interpreted in agreement with the National Committee for Clinical Laboratory Standards recommendations.

The 16S rRNA gene sequence (corresponding to positions 27-1492 in the *Escherichia coli* gene) was PCR-amplified as described by DeLong (1992), using a DNA thermal cycler Multigene Gradient (Labnet International Inc., Woodbridge, NJ). Sequencing on both strands of PCR-amplified fragments was performed using the dideoxy chain termination method by the commercial services of Macrogen Inc. (Seoul, Korea). The 16S rRNA homology searches against the NCBI database were carried out using BLAST program (Altschul et al. 1990). The phylogenetic analysis was done according the maximum-parsimony (MP) method, using the Heuristic Search algorithm (1,000 replicates) with PAUP program version 4.0b10 (Swofford 2001). The stability among clades was assessed with 1000-replication bootstrap analysis. The 16S rRNA gene sequence generated in this

study was deposited at the GenBank database under the accession number GQ845022.

Culture conditions and antimicrobial activity production

Three different culture media were used to evaluate the production of the antimicrobial agent produced by the selected LAB. One was the highly nutrient liquid medium TSB (Difco), and the other two were the special culture media M17 (Biokar) and MRS (Biokar) for lactic streptococci and lactobacilli, respectively. Selected LAB was grown in such media for 20 h at 10, 15, 20, 25, 30, and 35°C. Each titer of antimicrobial activity was assayed by serial twofold dilutions of cell-free supernatant (adjusted to pH 6.5) using the agar well diffusion method.

The kinetics of antimicrobial activity production was performed in MRS broth inoculated at 1% (v/v) with the selected strain and incubated for 24 h at 15 and 30°C. Samples were collected periodically, and the cell growth was determined by absorbance measurements (600 nm) with a Jenway model 6405 spectrophotometer (Bibby Sterling Ltd, London). The titer of antibacterial activity against *L. garvieae* 03/8460 was assessed by the dilution assay previously described.

Characterization of the causative agent of the inhibitory activity

Determination of hydrogen peroxide

The presence of hydrogen peroxide in the cell-free supernatant was tested by two methods. One in which the cell-free supernatant was treated with 2 mg ml^{-1} of catalase (Sigma), incubated at 37°C for 1 h, and this then determined the residual antimicrobial activity against *L. garvieae* 03/8460. The other method was performed according to Gilliland (1969). Briefly, 10 µl of peroxidase (1 mg ml^{-1}) and 20 µl of *o*-dianisidine solutions were added to 1 ml of cell-free supernatant and incubated at 25°C. After 10 min of incubation, 0.2 ml of 4 M HCl was added to stop the reaction and to stabilize the color. The absorbance of each sample was then measured at 400 nm and compared with a calibration curve.

Bacteriocin production

In order to detect bacteriocin production, the cell-free supernatant was processed in three different ways: (a) supernatant neutralized to pH 6.5 with 1 M NaOH, (b) the same as the previous treatment, but heated for 5 min at 100°C, and (c) supernatant without treatment. All were then assessed for inhibitory activity by the agar well diffusion method.

Sensitivity to hydrolytic enzymes

The cell-free neutralized supernatant was treated with hydrolytic enzymes in a final concentration of 1 mg ml^{-1} for lysozyme (Genbiotech) and 1–5 mg ml^{-1} for pronase E (Sigma), proteinase K (Genbiotech), trypsin (ICN Biomedicals), and papain (Sigma). Enzymes were dissolved in the buffers recommended by the suppliers. For papain, cysteine (20 mM) was added to the buffer to keep the—SH group of the enzyme active site reduced. Enzyme solutions alone and an aliquot of cell-free neutralized supernatant were diluted with sterile water and used as negative and positive controls, respectively. The samples were incubated at 30°C for 3 h, and the residual activity against *L. garvieae* 03/8460 was determined by the agar well diffusion assay.

Stability to heat and pH

Aliquots of 500 µl of cell-free neutralized supernatant were adjusted to pH 3.0, 5.0, 7.0, 9.0, and 11.0 with 500 µl of different 0.2 M buffers: Gly-HCl (pH 3), citrate buffer (pH 5), phosphate buffer (pH 7), and Gly-NaOH (pH 9 and 11). The same buffers (0.1 M) were used as negative controls, and an aliquot of 500 µl of neutralized supernatant diluted with 500 µl of sterile water was used as positive control. Samples and controls were incubated at 25, 60, 100, and 121°C for 15 min. After each treatment, samples and controls were tested for antimicrobial activity against *L. garvieae* 03/8460 by the agar well diffusion assay as described above.

Storage at 4 and −20°C

In order to investigate the effect of storage time on antagonist stability, cell-free supernatant was stored at 4°C and −20°C for different periods of time. After the treatments, the residual antimicrobial activity against *L. garvieae* 03/8460 was determined.

Results

Isolation and characterization of isolate TW34

After enrichment of *Odontesthes platensis* intestinal tract contents, 53 LAB were isolated. From the four isolates that showed inhibitory activity against *L. garvieae* 03/8460, strain TW34 was selected because it exerted the strongest inhibition. TW34 was a Gram-positive and catalase-negative coccus. It was able to hydrolyze

arginine and to ferment glucose, but it did not produce gas. This strain grew at 10 and 40°C as well as at pH 9.2. TW34 also grew in a medium containing 4% (w/v) NaCl, whereas growth was undetectable at 6.5% (w/v) NaCl. This isolate was susceptible to penicillin, erythromycin, clindamycin, vancomycin, levofloxacin, gentamicin, minocycline, chloramphenicol, and teicoplanin. It also showed intermediate susceptibility to oxacillin, and resistance to rifampin and trimethoprim/sulfamethoxazole. Carbohydrate fermentation profile showed positive fermentation to ribose, D-xylose, galactose, glucose, fructose, manose, mannitol, *N*-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, saccharose, trehalose, starch, and gentiobiose. API profile identified isolate TW34 as belonging to *Lactococcus lactis* subsp. *lactis.*

To further characterize this strain, a phylogenetic analysis based on 16S rRNA gene sequence (1,416 bp) was performed (Fig. 1). The cladogram placed TW34 in the *Lactococcus lactis* clade, with 100% recovery in bootstrap analysis. However, TW34 was not clustered with any particular *L. lactis* subspecies (Fig. 1). A similar value of 99.2% was obtained when comparing the 16S rDNA sequences of isolate TW34 with those of the *L. lactis* subspecies type strains.

Antimicrobial spectrum

Table 1 shows the inhibitory spectrum of the neutralized supernatant of *L. lactis* TW34. This strain did not inhibit the growth of the Gram-negative fish pathogens studied. In contrast, it showed a strong antimicrobial activity against Gram-positive pathogens (Table 1).

Effects of culture media and temperature on TW34 antimicrobial activity

The results of inhibitory activity of *L. lactis* TW 34 over *L. garvieae* 03/8460 in three culture media at different temperatures are shown in Table 2. A similar inhibitory activity profile versus temperature was observed in the three media. The optimal temperature for inhibitory activity production was 15°C. Such activity decreased as the temperature increased. Further, the maximum antimicrobial activity production was achieved when the strain was grown in TSB medium.

Table 2 Antimicrobial activity against *L. garvieae* 03/8460 produced by cell-free supernatants of *L. lactis* TW34 cultured in different media and temperatures

Temperature (°C)	Inhibitory activity on different media (AU ml^{-1})		
	MRS	M17	TSB
10	80	40	80
15	1280	1280	2560
20	1280	640	640
25	320	320	320
30	320	80	80
35	160	40	80

Data are means of triplicates. Standard errors were less than 5.0% of the means

Kinetics of growth and antimicrobial activity production

Figure 2 shows the growth and antibacterial activity curves of *L. lactis* TW34 cultured at 30°C (Fig. 2a) and 15°C (Fig. 2b). At both temperatures, inhibitory agent production against *L. garvieae* 03/8460 was growth associated. At 30°C, the maximum cell density was reached after 8 h of growth (generation time, gt = 55.8 min), at which point the extracellular inhibitory activity was about 300 AU ml^{-1} (Fig. 2a). It took almost 16 h to reach the same cell density at 15°C (gt = 186.0 min). However, at that point the inhibitory activity was four times higher than that registered at 30°C, accounting for 1,200 AU ml^{-1} approximately (Fig. 2a, b).

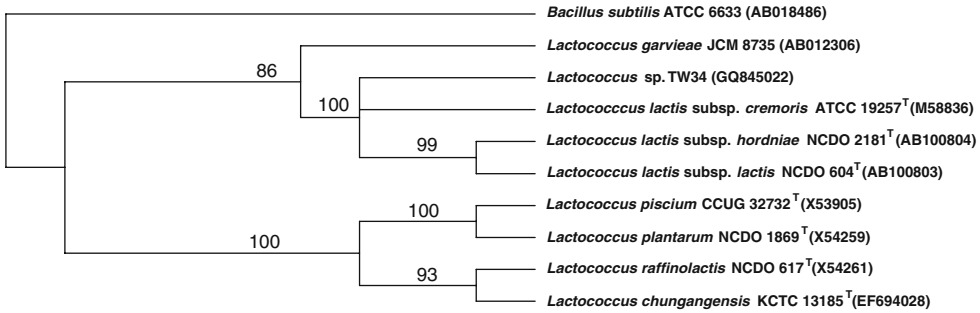

Fig. 1 Most parsimonious phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between isolate TW34 and members of the genus *Lactococcus*. The *numbers* at internal nodes are bootstrap support values. *GenBank accession numbers* are given in parentheses. The 16S rRNA sequence of *Bacillus subtilis* was chosen arbitrarily as the outgroup sequence

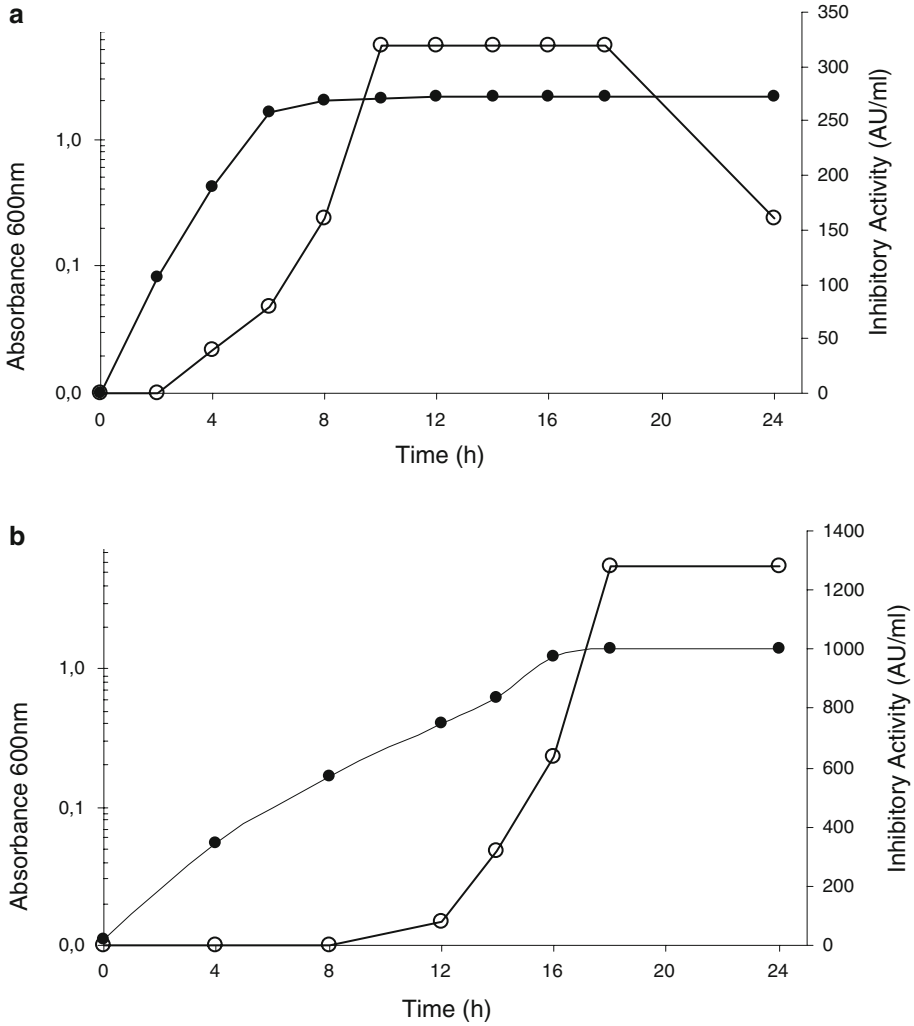

Fig. 2 Growth of *L. lactis* TW34 (*filled circle*) and antimicrobial activity production (*open circle*) in MRS broth at 30°C (**a**) and 15°C (**b**)

Identification and characterization of the causative agent of the inhibitory activity

L. lactis TW34 did not produce hydrogen peroxide. It was also confirmed that the acidity (pH 4.5) produced by TW34 strain was not the antimicrobial agent against *L. garvieae* because a large inhibition halo was kept constant (17 mm) after adjusting cell-free supernatant to pH 6.5. On the other hand, the neutralized supernatant presented the same diameter of inhibitory activity after a short heat treatment (5 min at 100°C), suggesting that the antimicrobial activity could be due to the presence of a small peptide (Fig. 3). The antimicrobial activity was completely inhibited by 1 mg ml^{-1} of the proteolytic enzymes papain, trypsin and pronase E, and by 5 mg ml^{-1} of proteinase K, confirming that the inhibitory substance produced by *L. lactis* TW34 was a bacteriocin (Table 3).

The effects of pH and temperature on the bacteriocin stability are presented in Table 4. The bacteriocin activity was highly thermostable, keeping antimicrobial activity at elevated temperatures (at pH 7) and even after autoclaving (121°C for 15 min). The bacteriocin activity was very stable in a wide pH range from 3 to 7, showing a slight activity

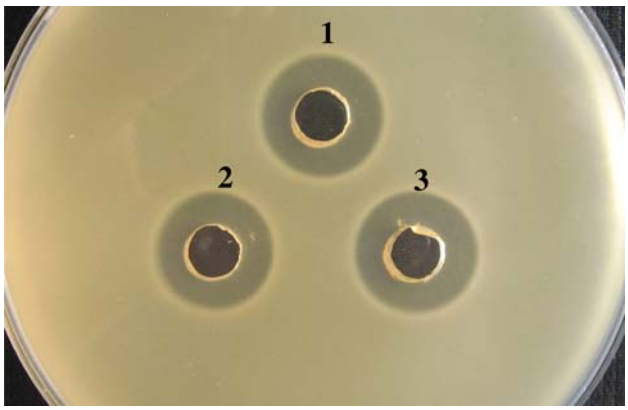

Fig. 3 Agar well diffusion assay of cell-free supernatant of *L. lactis* TW34 against *L. garvieae* 03/8460: 1—supernatant without treatment, 2—supernatant neutralized to pH 6.5, and 3—supernatant neutralized and heated for 5 min at 100°C

Table 3 Effect of enzymes on the antimicrobial activity of cell-free supernatant from *L. lactis* TW34

Treatment	Final concentration (mg ml^{-1})	Residual activity (AU ml^{-1})
Control	–	160
Papain	1	0
Pronase E	1	0
Trypsin	1	0
Proteinase K	1	160
Proteinase K	5	0
Lysozyme	1	160

Data points are means of triplicates. Standard errors were less than 5.0% of the means

Table 4 Stability of the bacteriocin activity produced by *L. lactis* TW34 to pH and heat treatments

Treatment	Residual activity (AU ml^{-1}) at pH				
	3	5	7	9	11
25°C for 15 min	320	320	320	160	80
60°C for 15 min	320	320	320	160	Nd
100°C for 15 min	320	320	320	40	Nd
121°C for 15 min	160	80	20	0	Nd

Nd not determined. Data points are means of triplicates. Standard errors were less than 5.0% of the means

decreasing from pH 9 up to 11. Besides the medium alkalinity, the stability of the bacteriocin was affected by the temperature. At high pH values, greater than nine, the inhibitory activity decreased and remained completely inactive at pH 9 after treatment at 121°C for 15 min (Table 4).

The results of bacteriocin stability throughout the storage time showed that the maximum inhibitory activity remained constant up to 2 months when the supernatant was stored at 4°C, but after 6 months of storage there was a decrease of 50% in the activity. In contrast, 100% of the initial activity was observed after six-month storage at −20°C.

Discussion

L. lactis is frequently isolated from milk, dairy products, and vegetables (Salama et al. 1995), but much less is known about its presence in the aquatic environment (Hagi et al. 2004; Itoi et al. 2008). In this study, we found that some LAB resident from the intestinal tract of the Patagonian fish *Odontesthes platensis* had the ability to inhibit the fish pathogen *L. garvieae*. Particularly, *L. lactis* TW34 showed a strong inhibitory activity over *L. garvieae* 03/8460 as well as on other Gram-positive bacteria. Regarding its phenotypic characteristics and carbohydrate fermentation pattern, TW34 was tentatively assigned to *L. lactis* subsp. *lactis*, even though it shows some phenotypic differences with other strains of this species also isolated from intestinal tracts of fish (Itoi et al. 2008, 2009) and with the type strain *L. lactis* subsp. *lactis* KCTC 3769^{T} (Cho et al. 2008). Such differences agree with the observations of Itoi et al. (2009) who concluded that *L. lactis* subsp. *lactis* strains show a large diversity in phenotypic adaptation to different environments. In addition, most of the members of the genera *Lactococcus* and *Lactobacillus* are generally recognized as safe (GRAS) microorganisms (Salminen et al. 1998). In this regard, TW34 showed to be sensitive to the majority of the antibiotics examined.

LAB inhibitory activity against fish pathogens has been frequently evaluated among a group of probiotic characteristics (hydrophobicity coefficient, competition for attachment sites, adhesion to intestinal mucus and bile resistance) without discriminating whether such activity is caused by the production of organic acids, hydrogen peroxide, or the presence of bacteriocins. *L. lactis* TW34 antimicrobial activity was due to the production of a bacteriocin, as indicated by the results of the treatment with proteolytic enzymes. Such treatment caused the total loss of antibacterial activity, confirming the proteinaceous nature of the inhibitory agent. As well, the high thermostability of the antimicrobial agent demonstrated that it was a low molecular weight bacteriocin. On the other hand, *L. lactis* TW34 antimicrobial activity was not associated with hydrogen peroxide production or acidification of the culture as observed for other LAB (Villamil et al. 2003; Vázquez et al. 2005; Sugita et al. 2007).

TW34 bacteriocin presented a spectrum of inhibitory activity affecting not only closely related LAB, but also other Gram-positive pathogens. The *L. lactis* strains isolated by Balcázar et al. (2007) were unable to inhibit *L. garvieae* but inhibited *A. salmonicida* and *Y. ruckeri*. In contrast, *L. lactis* TW34 did not inhibit any of the Gram-negative strains tested, but it showed a strong antimicrobial activity against the causal agent of lactococcosis. In general, the target of the bacteriocin antimicrobial activity is the cell envelope which can result affected by different bacteriocin mechanisms such as disruption of the cell wall biosynthesis or pore formation (Deegan et al. 2006). For some bacteriocins, such as nisin, the nonsusceptibility of Gram-negative bacteria was associated with the outer membrane which avoids the access of hydrophobic substances to the peptidoglycan layer (Chatterjee et al. 2005).

Bacteriocin production was growth associated in *L. lactis* TW34 cultures, agreeing with the results reported for other strains of this species (Onda et al. 2003; Campos et al. 2006). We found that moderate to cold temperatures

enhanced TW34 bacteriocin production. Regardless of the culture medium, the synthesis of bacteriocin reached the maximum at 15°C, even though the growth rate was lower than at higher temperatures (Fig. 2). De Vuyst et al. (1996) suggested that a low growth rate or unfavorable growth conditions may increase the production of bacteriocins. Other studies have also observed differences between the optimal temperatures for growing and bacteriocin production (Matsusaki et al. 1996; Cheigh et al. 2002; Noonpakdee et al. 2003). However, an optimal temperature for bacteriocin production as low as 15°C is the lowest reported for *L. lactis* strains isolated from marine fish intestinal tract. This feature makes TW34 attractive as a biological control agent for salmonid culture. Particularly rainbow trout, which is a sensitive species to lactococcosis, has an optimum water temperature for culture below 21°C (FAO 2005–2009).

Like other lactococcal bacteriocins (Ryan et al. 1996; Noonpakdee et al. 2003), TW34 bacteriocin was active in a wide pH range. Other interesting biotechnological properties of this bacteriocin were its high thermal stability at acid and neutral pH values. Further, its stability to storage has important implications when it comes to commercial applications.

Although some studies characterizing LAB bacteriocins produced by isolates from fish or aquatic environments have been reported (Brillet et al. 2004; Tahiri et al. 2004; Campos et al. 2006), they explored bacteriocins as potential biopreservers for human food. As well, they are mainly focused on the genera *Carnobacterium*, *Lactobacillus,* or *Enterococcus*. Our results suggest that the strain *L. lactis* TW34 could provide an alternative for lactococcosis control and therefore be considered for future challenge experiments with fish.

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