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Identification and characterization of lactic acid bacteria isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), with inhibitory activity against *Lactococcus garvieae*

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Abstract

A study was conducted to evaluate the probiotic properties of endogenous rainbow trout microbiota against pathogenic *Lactococcus garvieae*. A total of 335 bacterial strains were isolated from rainbow trout and screened for antagonistic activity against *L. garvieae* using an agar spot assay. Antagonistic strains were grouped by PCR amplification of repetitive bacterial DNA elements (rep-PCR) and identified by 16S rRNA gene sequence analysis. The results revealed that the antagonistic strains belonged to the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc*. Further probiotic characteristics, such as specific growth rate, doubling time, resistance to biological barriers, antibiotic resistance, hydrophobicity and production of antimicrobial substances, were also studied. These strains were able to survive low pH and high bile concentrations, showed good adherence characteristics and a broad spectrum of antibiotic resistance. The antagonistic efficacy was maintained after sterile filtration and was sensitive to proteinase K, indicating that proteinaceous extracellular inhibitory compounds were at least partially responsible for pathogen antagonism. Based on these results, these strains should be further studied to explore their probiotic effects in challenge experiments *in vivo*. This study shows clear evidence that the indigenous trout-associated microbiota may provide a defensive barrier against *L. garvieae*.

Keywords: aquaculture, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, probiotics.

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Introduction

Lactococcus garvieae is a Gram-positive pathogen that causes haemorrhagic septicaemia and meningoencephalitis in several species of fish (Vendrell, Balcázar, Ruiz-Zarzuela, de Blas, Gironés & Múzquiz 2006) and mammals (Teixeira, Merquior, Vianni, Carvalho, Fracalanzza, Steigerwalt, Brenner & Facklam 1996). Moreover, this bacterium has also been isolated from humans, suggesting that *L. garvieae* could be classified as a potential zoonotic agent (Fefer, Ratzan, Sharp & Saiz 1998). *Lactococcus garvieae* outbreaks in aquaculture are treated with antibiotics; however, these are often ineffective, and their indiscriminate use has led to an increase in antibiotic resistance (Romalde & Toranzo 2002; Vendrell *et al.* 2006).

The last decade has seen a growing interest in the application of probiotics through the use of beneficial microorganisms to prevent pathogenic micro-

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organisms and reduce the incidence of fish diseases (Irianto & Austin 2002; Balcázar, de Blas, Ruiz-Zarzuela, Cunningham, Vendrell & Múzquiz 2006; Kesarcodi-Watson, Kaspar, Lategan & Gibson 2008). Most probiotics proposed as biological control agents in aquaculture are lactic acid bacteria (LAB), such as *Lactococcus* spp., *Pediococcus* spp. or *Lactobacillus* spp., although other genera (e.g. *Vibrio*, *Bacillus* and *Pseudomonas*) and yeast have also been studied (Merrifield, Dimitroglou, Foey, Davies, Baker, Bøgwald, Castex & Ringø 2010; Pérez, Balcázar, Ruiz-Zarzuela, Halaihel, Vendrell, de Blas & Múzquiz 2010). Several mechanisms have been suggested for the inhibitory action of probiotics towards bacterial pathogens, including a decrease in localized pH, the production of antibacterial substances, competition for nutrients and adhesion sites, and stimulation of the host's immune responses (Balcázar *et al.* 2006; Merrifield *et al.* 2010).

When selecting a new microorganism for testing as an effective probiotic, a number of properties need to be considered. To colonize the gastrointestinal tract, potential probiotics should express high tolerance to acid and bile and have the ability to adhere to intestinal surfaces (Jöborn, Olsson, Westerdahl, Conway & Kjelleberg 1997; Nikoskelainen, Salminen, Bylund & Ouwehand 2001; Balcázar, Vendrell, de Blas, Ruiz-Zarzuela, Múzquiz & Gironés 2008). Because of the serious concerns about the emergence of antibiotic resistance, probiotic strains should also be carefully screened for antimicrobial susceptibility (Vizoso-Pinto, Franz, Schillinger & Holzapfel 2006).

Consequently, the aim of this study was to identify and characterize the properties of the endogenous microbiota of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against pathogenic *L. garvieae* by studying their antibacterial activity, pH and bile tolerances, antibiotic resistance and adherence characteristics to determine their potential use as probiotics.

Materials and methods

Isolation and initial screening

A total of 60 healthy rainbow tout weighing 35–40 g were collected from two fish farms (n = 30) in north-east Spain. The farms were participating in a health-improvement programme and thus underwent regular health monitoring. All fish were killed with tricaine methanesulphonate (MS-222; Syndel Laboratories) at a concentration of 150 mg L^{-1} of water for 15 min. The protocol (PI04/09) was approved by the Ethical Committee on Animal Experimentation of the Universidad de Zaragoza, Spain.

Mucus samples were collected as previously described (Balcázar, de Blas, Ruiz-Zarzuela, Vendrell, Gironés & Muzquiz 2007a; Balcázar, Vendrell, de Blas, Ruiz-Zarzuela, Gironés & Múzquiz 2007b). Briefly, the gill mucus was isolated after removing the gills, and cutaneous mucus was collected from the whole body by scraping the surfaces with a rubber spatula into 1 mL of phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.2). For intestinal mucus, the intestine was separated and mucus was collected and homogenized in 1 mL of PBS. Serial tenfold dilutions were then plated on tryptic soy agar (TSA; Scharlau) and de Man, Rogosa and Sharpe agar (MRS; Pronadisa) with incubation at 22 °C for 48 h. Colonies with different morphological characteristics from each sample were selected, subcultured in tryptic soy broth (TSB; Scharlau) or MRS broth (Pronadisa) and stored in sterile glycerol (15% v/v) at −80 °C.

To assess the growth inhibition of a virulent strain of *L. garvieae* CLFP LG 1, previously isolated during a natural lactococcosis outbreak in rainbow trout (Vendrell, Balcázar, Ruiz-Zarzuela, de Blas, Gironés & Múzquiz 2007), all strains (n = 335) were grown on TSA and MRS at 22 °C for 24–48 h. After incubation, a loop of each strain was spotted onto the surface of TSA or MRS agar previously inoculated with overnight cultures of the target strain (CLFP LG 1). Clear zones after overnight incubation at 22 °C indicated the presence of antibacterial substances.

Phenotypic characterization

All antagonistic strains (n = 11) were initially characterized by determining colony morphology, cell morphology, motility, Gram stain and the production of cytochrome oxidase and catalase. Further biochemical characteristics were determined using API 50 CH and API 20 Strep tests (bioMérieux), according to the manufacturer's instructions.

Rep-PCR genomic fingerprinting

Rep-PCR analysis was used to group the isolates. Genomic DNA of each isolate was extracted and purified following the method previously described

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by Balcázar *et al.* (2007a). Amplification reactions were performed in a total volume of 25 μL containing 0.2 μL of *Taq* polymerase, 1.5 μL of $MgCl_2$, 1 μL of each deoxynucleoside triphosphate, 2 μL of 20 μM $(GTG)_5$ primer (5′-GTG GTG GTG GTG GTG-3′) (Versalovic, Schneider, De Brujin & Lupski 1994) and 5 μL of DNA template. The PCR temperature profile (MJ Mini Gradient Thermal Cycler; Bio-Rad Laboratories) consisted of an initial denaturation at 94 °C for 2 min followed by 40 cycles of 45 s at 93 °C, 1 min at 50 °C, 1 min at 72 °C and a final extension for 6 min at 72 °C. The genetic fingerprints were resolved in a 1.5% agarose gel in Tris–acetate-EDTA buffer.

Genotypic identification

Following DNA extraction, the 16S rRNA gene was amplified using primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) (Lane 1991) in a MJ Mini Gradient Thermal Cycler (Bio-Rad Laboratories). The PCRs were conducting using 0.4 μL of *Taq* polymerase, 1 μL of $MgCl_2$, 1.25 μL of each deoxynucleoside triphosphate, 1.25 μL of 1 μM of each primer and 5 μL of DNA template. The samples underwent an initial denaturation of 10 min at 95 °C, and then 30 cycles of 15 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by 10 min at 72 °C.

The PCR products were purified (Promega Biotech Iberica) and were directly sequenced on a MegaBACE 500 sequencer following the manufacturer's protocols (Amersham Biosciences). The sequences obtained were compared against the sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Center for Biotechnology Information using the BLASTN (Altschul, Gish, Miller, Myers & Lipman 1990).

Growth

Following preculturing at 22 °C in MRS broth (pH 6.2) for 24 h, isolates were diluted and inoculated in MRS broth in triplicate with an initial concentration of 10^7 CFU mL^{-1} (OD600) and stirred gently to maintain a homogeneous bath culture. The optical density was recorded every 4 h for 24 h, and the readings of the profiles were averaged. Growth profiles of the three candidate probiotics, such as specific growth rate (μ) and doubling time (t_d), were determined as described by Vine, Leukes & Kaiser (2004).

Hydrophobicity

The ability of the organisms to adhere to hydrocarbons (as a measure of their hydrophobicity) was utilized as an indicator of their ability to adhere to intestinal epithelial cells (after Pan, Li & Liu 2006). The assay was conducted as described by Vinderola & Reinheimer (2003) with some modifications. The LAB strains were harvested in the stationary phase by centrifugation at 5000 ***g*** for 5 min at 5 °C, washed twice in 50 mM K_2HPO_4 (pH 6.5) buffer and finally resuspended in the same buffer. These cell suspensions were adjusted to 1.0 (OD560) with the buffer, and 3 mL of bacterial suspension was put in contact with 600 μL of toluene (Lab-Scan) and vortexed for 2 min. The two phases were allowed to separate for 1 h at 37 °C. The aqueous phase was carefully removed and optical density measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula $H\% = [(OD_0 - OD)/OD_0] \times 100$, where OD_0 and OD are the absorbance before and after the extraction with toluene, respectively.

pH and bile tolerances

Tolerance to different pH conditions was determined using LAB cultures grown to stationary phase (18 h) in MRS broth and adjusted to 10^8 CFU mL^{-1} (OD between 0.6 and 0.7). These bacterial suspensions were harvested by centrifugation (2500 ***g***, 10 min, 4 °C) and washed once in PBS. Bacterial pellets were resuspended in PBS and used for *in vitro* acid tolerance studies. Five hundred microlitres of the bacterial suspension was added to 4.5 mL of sterile PBS and adjusted to a series of pH values (1.0, 2.0 and 3.0) with HCl (Prasad, Gill, Smart & Gopal 1998). The suspensions were incubated at 22 °C, and after 0, 1, 2 and 3 h, viable counts were determined by plate counting on duplicate MRS agar (24 h at 22 °C).

Bile used in these studies was desiccated ox-bile (Oxoid). The procedure of Klaenhammer & Kleeman (1981) was used to determine the tolerance of the strains to bile (at final concentrations of 0%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0% w/v) on MRS agar in duplicate.

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Sensitivity to antibiotics

Antibiotic susceptibilities were assessed by the disc diffusion test in Mueller-Hinton agar. The antibiotic sensitivity discs included amoxicillin/clavulanic acid (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), chlortetracycline (30 μg), clindamycin (2 μg), doxycycline (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), florfenicol (30 μg), flumequine (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), oxolinic acid (2 μg), penicillin (10 μg), streptomycin (10 μg), tetracycline (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), tylosin (150 μg) and vancomycin (30 μg). Agar plates were incubated at 22 °C for 48 h. The diameters of the growth inhibition halos were measured and the antibiograms interpreted in agreement with the National Committee for Clinical Laboratory Standards recommendations.

Partial characterization of inhibitory substances

Antimicrobial activity was determined as described by Nikoskelainen *et al.* (2001). Briefly, the supernatant from cultures of LAB in 50 mL MRS broth for 48 h at 22 °C was prepared by centrifugation at 2000 ***g*** for 10 min and sterilized by passage through a 0.45-μm Millipore membrane (Millipore). After sterilization, 25 mL of each cell-free culture supernatant was neutralized (pH 6.8) with 5 N NaOH to prevent the inhibitory effect of lactic acid. The other 25 mL was assessed at the *in situ* pH. *Lactococcus garvieae* was grown in MRS broth overnight at 22 °C. The cells were harvested by centrifugation (2000 ***g***), washed twice with PBS and resuspended in 5 mL of PBS. The bacterial suspensions were spread on MRS plates in triplicate, and 6-mm wells were made in each agar plate with a sterile Pasteur pipette and were filled with either 50 μL of neutralized supernatant or 50 μL of untreated supernatant and then air-dried for 10 min. In a further two wells, neutralized MRS and pH 6.0 MRS were added as negative controls. After incubation of *L. garvieae* for 24 h at 22 °C, the clearing zone was determined.

The sensitivity of cell-free culture supernatants to proteinase K (GE Healthcare) at a final concentration of 1.0 mg mL^{-1} was also tested in buffers recommended by the supplier. Samples, with and without proteinase K, were incubated at 37 °C for 2 h, and residual activity was determined by the previously described well plate assay.

Statistical analysis

Data were assessed by one-way analysis of variance (ANOVA) (after checking normal distribution with the Kolmogorov–Smirnov test), with Duncan's *post hoc* test. All statistics were performed using SPSS for Windows version 15.0 (SPSS).

Results

Isolation and identification of the strains

Eight of 198 isolated strains from the intestinal mucosa exhibited inhibitory activity against *L. garvieae* CLFP LG 1, but only two from the 105 gill isolates and one from the 32 cutaneous mucus isolates exhibited inhibitory activity. Of the inhibitory bacteria, six strains showed strong inhibition, causing a clear zone > 20 mm in the agar spot assay (Table 1).

Rep-PCR analysis demonstrated that these strains produced different genetic fingerprints, which could be grouped into three groups (data not shown). Subsequently, this was confirmed by partial 16S rRNA gene sequence analysis, which revealed that the strains showed highest nucleotide alignment identities to one of the three LAB strains: *Lactobacillus plantarum* subsp. *plantarum* ($n = 5$), *Lactococcus lactis* subsp. *cremoris* ($n = 5$) and *Leuconostoc mesenteroides* subsp. *mesenteroides* ($n = 1$) (Table 1). However, the results of the phenotypic characterization differed from the genetic identification for six strains (Table 1). As only three different species were identified as displaying antagonism, the subsequent probiotic assays were conducted with one representative of each species (CLFP 3, CLFP 25 and CLFP 68).

Growth, pH, hydrophobicity, pH and bile tolerances and sensitivity to antibiotics

The resulting growth curves (data not shown) led to the determination of specific growth rate and doubling time (Table 2). *Lactococcus lactis* showed slower growth rates than *Lb. plantarum* and *Leuc. mesenteroides*, whereas *Leuc. mesenteroides* exhibited greater growth rate and doubling time compared to the other two bacteria (Fig. 1). Similarly, the pH of the medium after 24 h was significantly lower ($P < 0.05$) for *Lb. plantarum* and *Leuc. mesenteroides* than for *L. lactis* (Fig. 2).

The hydrophobicity percentages (H%), an indication of adherence ability, were 24.99% for

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Table 1 Antagonistic activity and identification of lactic acid bacteria strains isolated from rainbow trout against *L. garvieae*

Isolate code	Accession number[a]	Source	Pathogen inhibition[b]	Phenotypic identification (% similarity)[c]	Genetic identification (% similarity)
CLFP 3	FR670524	Mucus	++	*Lactobacillus paracasei* subsp. *paracasei* (99.4)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 6	FR670525	Gills	++	*Lactobacillus plantarum* (98.7)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 9	FR670526	Gills	+	*Leuconostoc* spp. (94.8)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 18	FR670527	Intestine	+	*Leuconostoc* spp. (99.1)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 23	FR670528	Intestine	+	*Leuconostoc* spp. (94.8)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 24	FR670529	Intestine	+	*Lactococcus lactis lactis* (85.7)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 25	FR670530	Intestine	++	*Lactococcus lactis lactis* (85.7)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 30	FR670531	Intestine	++	*Leuconostoc* spp. (94.8)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 31	FR670532	Intestine	++	*Lactobacillus pentosus* (98.7)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 52	FR670533	Intestine	+	*Lactobacillus plantarum* (98.7)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 68	FR670534	Intestine	++	*Leuconostoc mesenteroides* (89.3)	*Leuconostoc mesenteroides* subsp. *mesenteroides* (99.5)

[a]Genbank partial sequence.
[b]+, clear zone of 15 mm or more; ++, clear zone of 20 mm or more.
[c]API 50 CH and API 20 Strep (bioMérieux).

Table 2 Growth rate and doubling time of the selected lactic acid bacteria strains

	Bacterial strains		
Growth parameters	*Lactobacillus plantarum*	*Lactococcus lactis*	*Leuconostoc mesenteroides*
μ^a	0.35	0.31	0.57
$t_d{}^b$	0.86	0.97	0.52

[a]The growth rate (μ) was calculated as the slope of the polynomials for the exponential phase of the dispersion curves obtained from the kinetic growth of each strain evaluated, using the method of adjustment.
CLFP 3: $y = 0.350x + 6.454$; $r^2 = 0.999$
CLFP 25: $y = 0.308x + 6.934$; $r^2 = 0.998$
CLFP 68: $y = 0.571x + 6.158$; $r^2 = 0.999$
where y: Log CFU mL^{-1} and x: time.
[b]The doubling time (t_d) was calculated as: $t_d = \ln 2/\mu$.

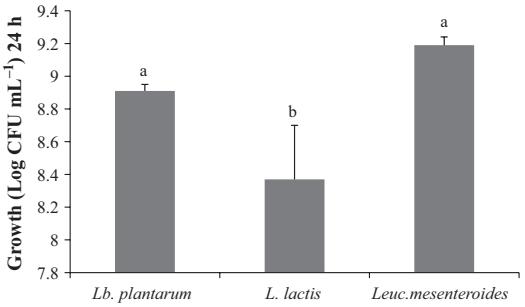

Figure 1 Growth of lactic acid bacteria strains after 24 h. Different letters indicate significant differences ($P < 0.05$).

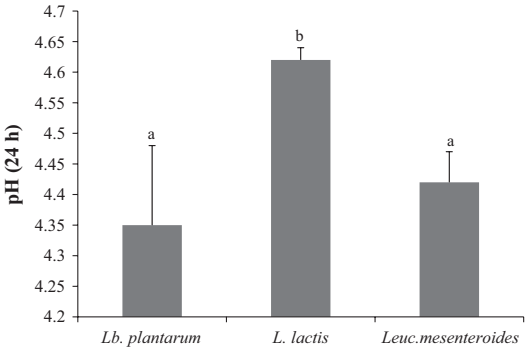

Figure 2 pH of Man, Rogosa and Sharpe broth cultures of lactic acid bacteria after 24-h incubation. Different letters indicate significant differences ($P < 0.05$).

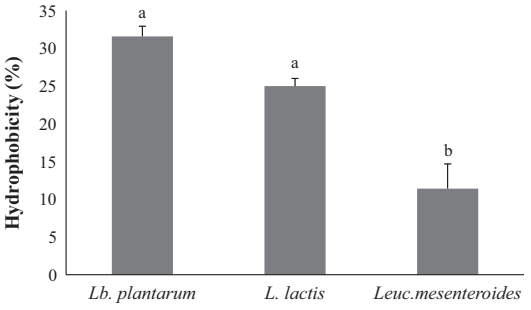

Figure 3 Hydrophobicity percentages of lactic acid bacteria strains. Different letters indicate significant differences ($P < 0.05$).

L. lactis, 31.59% for *Lb. plantarum* and 11.42% for *Leuc. mesenteroides* (Fig. 3). The hydrophobicity of *Leuc. mesenteroides* was significantly lower ($P < 0.05$) than for *Lb. plantarum* and *L. lactis*.

In addition, all LAB strains showed relatively high resistance to bile and low pH. No significant differences ($P > 0.05$) were observed between bile concentration sensitivity for the potential probiotics

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tested. These strains were resistant to 1.0% bile and maintained the original population level through all tested concentrations. There were some indications that *Lb. plantarum* was less resistant to higher levels of bile (> 0.6%) than *L. lactis* and *Leuc. mesenteroides* (Table 3); however, there were no significant differences ($P > 0.05$) at 1.0% bile.

The three LAB strains remained viable after a 3-h exposure to pH values from 2.0 to 3.0, but none could tolerate exposure to pH 1.0 (Table 4). Significant differences ($P < 0.05$) of survival were observed between the LAB strains; *L. lactis* showed higher levels than *Lb. plantarum* and *Leuc. mesenteroides* at pH 2.0, whereas *Leuc. mesenteroides* showed higher levels than the other two bacteria at pH 7.4.

Antibiotic susceptibilities of *L. lactis*, *Lb. plantarum* and *Leuc. mesenteroides* are shown in Table 5. The three LAB strains were resistant to the majority of antibiotics tested. Some variations of susceptibility between strains were observed, but all were resistant to ampicillin, clindamycin, enrofloxacin, flumequine, kanamycin, nalidixic acid, oxolinic acid, penicillin, tetracycline, trimethoprim/sulfamethoxazole and vancomycin; all showed intermediate susceptibility to amoxicillin/clavulanic acid, and all were susceptible to gentamicin.

Table 3 Tolerance of lactic acid bacteria strains to different bile concentrations

	Log CFU mL^{-1} (SD)[1]		
Bile (%)	*Lactobacillus plantarum*	*Lactococcus lactis*	*Leuconostoc mesenteroides*
0.0	7.42 (0.01)	7.54 (0.01)	7.52 (0.08)
0.2	7.33 (0.02)	7.51 (0.08)	7.41 (0.08)
0.4	7.38 (0.03)	7.33 (0.18)	7.52 (0.01)
0.6	7.45 (0.01)^{a}	7.62 (0.04)^{b}	7.57 (0.04)^{b}
0.8	7.31 (0.08)^{a}	7.57 (0.02)^{b}	7.50 (0.04)^{b}
1.0	7.42 (0.04)	7.57 (0.08)	7.59 (0.01)

Different superscripts denote significant differences between the different strains at the respective bile concentration (i.e. between columns). There were no significant differences between the values at different concentrations for the respective probiotics (i.e. within columns).
[1]Data are presented as mean (standard deviations).

Table 4 Tolerance of LAB strains to different pH conditions for 3 h at 22 °C

	Log CFU mL^{-1} (SD)[1]		
pH	*Lactobacillus plantarum*	*Lactococcus lactis*	*Leuconostoc mesenteroides*
1.0	ND	ND	ND
2.0	3.82 (0.03)^{Aa}	4.24 (0.01)^{Ab}	3.80 (0.01)^{Aa}
3.0	6.51 (0.04)^{Ba}	6.48 (0.03)^{Ba}	6.51 (0.02)^{Ba}
7.4	6.46 (0.04)^{Ba}	6.55 (0.06)^{Ba}	6.66 (0.04)^{Bb}

ND, not detected; LAB, lactic acid bacteria.
Lowercase superscripts denote a significant difference between the LAB strains at the respective pH level (i.e. differences between columns).
Capital superscripts denote a significant difference between values for the respective strains at different pH concentrations (i.e. differences within columns).
[1]Data are presented as mean (standard deviations).

Partial characterization of inhibitory substances

After incubation of *L. garvieae* on MRS plates, measurable clearing zones were detected around the wells filled with both untreated and neutralized extracellular supernatants from the respective LAB strains. Additionally, the antimicrobial substances exhibited by the three LAB strains were found to be sensitive to proteinase K.

Discussion

There is no clear evidence that probiotic strains isolated from the host perform better than strains from a different habitat (Merrifield *et al.* 2010), but logically, a good initial site to screen for beneficial bacteria is the host's own microbiota (Fjellheim, Klinkenberg, Skjermo, Aasen & Vadstein 2010). In the present study, we obtained a pool of bacterial isolates from rainbow trout and characterized those with inhibitory activity against *L. garvieae*. Our study demonstrated that 3.28% (11) of the screened bacteria ($n = 335$) were antagonistic towards *L. garvieae*, which supports other studies that have also reinforced the view that antagonism between endogenous gut microorganisms of fish and bacterial pathogens occurs in nature and the establishment of a normal or protective microbiota might constitute a key component of defensive barrier function (Gómez & Balcázar 2008; Cain & Swan 2010). However, it should be noted that no isolates cultured on TSA displayed antagonism, and thus, it is recommended that future screening studies focus on using MRS agar to isolate antagonistic isolates. Several LAB isolated from fish and aquatic animals display antagonistic activity against fish pathogenic agents (Jöborn *et al.* 1997; Ringø, Sepploa, Berg, Olsen, Schilinger & Holzapfel 2002; Kim & Austin 2008; Rengpipat, Rueangruklikhit & Piyatiratitivorakul 2008; Ringø, Løvmo, Kristiansen, Bakken, Salinas, Myklebust, Olsen & Mayhew 2010); however, to our knowledge, this is the first report demonstrating a clear and consistent antagonism of

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Table 5 Antibiotic susceptibilities of lactic acid bacteria strains

Antibiotic	*Lactobacillus plantarum*	*Lactococcus lactis*	*Leuconostoc mesenteroides*
Amoxicillin/clavulanic acid (bioMérieux)	I	I	I
Ampicillin (bioMérieux)	R	R	R
Chloramphenicol (Bio-Rad)	S	S	S
Chlortetracycline (Mast Diagnostics)	R	R	I
Clindamycin (Bio-Rad)	R	R	R
Doxycycline (bioMérieux)	S	I	I
Enrofloxacin (Bio-Rad)	R	R	R
Erythromycin (BBL Sensi-Disc)	S	R	S
Florfenicol (BBL Sensi-Disc)	R	I	I
Flumequine (Bio-Rad)	R	R	R
Gentamicin (Bio-Rad)	S	S	S
Kanamycin (BBL Sensi-Disc)	R	R	R
Nalidixic acid (BBL Sensi-Disc)	R	R	R
Nitrofurantoin (Bio-Rad)	I	R	R
Oxolinic acid (BBL Sensi-Disc)	R	R	R
Penicillin (bioMérieux)	R	R	R
Streptomycin (BBL Sensi-Disc)	S	R	R
Tetracycline (bioMérieux)	R	R	R
Trimethoprim/Sulfamethoxazole (Bio-Rad)	R	R	R
Tylosin (Neo-Sensitabs)	S	R	R
Vancomycin (bioMérieux)	R	R	R

R, resistant; I, intermediate; S, susceptible.

indigenous rainbow trout microbiota against *L. garvieae*, mediated, in part at least, by extracellular antimicrobial peptides.

The ability to adhere to intestinal epithelial cells is thought to be an important property of potential probiotic strains. Pan *et al.* (2006) assessed the ability of LAB strains to adhere to intestinal epithelial cells and concluded that the higher hydrophobic strains displayed stronger adhesive capability. *Lactobacillus plantarum* and *L. lactis* displayed significantly better hydrophobicity values than *Leuc. mesenteroides*. Thus, it is likely that the ability of *Leuc. mesenteroides* to adhere to intestinal epithelial cells is less than that of the other two LAB strains. Tolerance to bile is also important for the probiotic strains to grow and survive in the fish intestine (Nikoskelainen *et al.* 2001; Chabrillón, Arijo, Díaz-Rosales, Balebona & Moriñigo 2006; Fjellheim *et al.* 2010; Lazado, Caipang, Rajan, Brinchmann & Kiron 2010). However, there is still no consensus about the precise concentration to which the selected strain should be tolerant (Balcázar *et al.* 2008). In the present study, the three LAB strains tested showed little or no decrease in viable cell numbers after 3-h incubation at pH 3.0 and at a 1.0% bile. However, there were some indications that *Lb. plantarum* might be less resistant to higher bile acid concentrations than the other two strains. Potential probiotic strains with high hydrophobicity that are less sensitive to acid and bile may be more likely to survive passage through the gastrointestinal tract and potentially colonize the intestinal surfaces of the fish (Nikoskelainen *et al.* 2001; Zhou, Pan, Wang & Li 2007).

In the present study, the three LAB strains showed a broad spectrum of antibiotic resistance. It should be emphasized that antimicrobial compounds are still applied in aquaculture in addition to feed or by immersion (Cabello 2006). Thus, chemotherapy may disturb the homeostasis of gut microecology and physiology, which could cause fish to be vulnerable to infections (Kim & Austin 2008). In this respect, antibiotic-resistant probiotics may be advantageous in the case of administration of antibiotics to fish and in the establishment of the beneficial microorganisms in the intestine for prolonged periods (Kim & Austin 2008). However, in future, it should be ensured that such resistance cannot be transferred via plasmids.

The partial characterization of inhibitory substances suggested that the inhibitory activity was not caused only by organic acid production, as has been observed elsewhere (Brunt & Austin 2005), but also by proteinaceous compounds, which could be bacteriocins or bacteriocin-like inhibitory substances (BLIS). Bacteriocins are ribosomally synthesized, extracellularly released, bioactive peptides or peptide complexes that have a strong inhibitory

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effect against other bacterial species (Dawn & Falkiner 1997; Riley & Wertz 2002) and are known to be produced by a wide range of LAB species, including those from the genera isolated in the present study (Klaenhammer 1993; Nes, Diep, Havarstein, Brurberg, Eijsink & Holo 1996).

Lactic acid bacteria form part of the normal microbiota of the gastrointestinal tract of both hatchery-cultured and wild-caught rainbow trout (González, Encinas, García-López & Otero 2000; Heikkinen, Vielma, Kemiläinen, Tiirola, Eskelinen, Kiuru, Navia-Paldanius & von Wright 2006; Balcázar *et al.* 2007a,b) as well as being found in other marine and freshwater fish species (Bucio, Hartemink, Schrama, Werreth & Rombouts 2006; Itoi, Abe, Washio, Ikuno, Kanomata & Sugita 2008). Thus, these antagonistic LAB may be beneficial for the control of intestinal microbiota by competition with pathogen species, as it has been suggested that the gastrointestinal tract is a possible port of entry for the bacterial fish pathogen *L. garvieae* (Vendrell *et al.* 2006) as well as a target for protective treatments, such as feeds containing probiotic bacteria (Brunt & Austin 2005; Vendrell, Balcázar, de Blas, Ruiz-Zarzuela, Gironés & Múzquiz 2008).

Given the good pH and bile tolerances, the ability to suppress pathogen growth under *in vitro* conditions as well as the positive indications towards epithelial adherence, *L. lactis*, *Lb. plantarum* and *Leuc. mesenteroides* should be further studied in challenge experiments *in vivo* for use as potential probiotics for the control of lactococcosis and may provide an alternative to the current use of antimicrobial compounds.

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