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RESEARCH PAPER

Isolation of *Lactobacillus* Strains from Shellfish for their Potential Use as Probiotics

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Abstract Microorganisms intended for use as probiotics in aquaculture should exert antimicrobial activity and be regarded as safe not only for their aquatic hosts but also for their surrounding environments and humans. The objective of this work was to investigate antimicrobial activity against various pathogens, bile salt tolerance, and acid tolerance of 65 presumptive *Lactobacillus* spp. isolated from shellfish samples. Four strains (HL1, HL12, HL20, and JL28) were selected after qualitatively identifying high levels of antimicrobial activity against bacteria including *Staphylococcus aureus*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Escherichia coli* O157:H7, *Vibrio ichthyoenteri*, *Edwardsiella tarda*, *Streptococcus iniae*, and *V. parahaemolyticus*. The sequence analysis of their 16S rRNA genes revealed that the four strains belong to the *Lactobacillus plantarum* species. In addition, their survivability was tested in bile salt and acidic conditions to show their potential use as probiotics in the gastrointestinal tract.

Keywords: probiotics, lactobacillus, shellfish, pathogen, antimicrobial

1. Introduction

Although antibiotics improve survival, they also alter the intestinal microbiota and induce resistant populations of bacteria, with unpredictable long-term effects on public health [1]. Comparison of animals raised without exposure to microorganisms with those that have been colonized by components of the microbiota has revealed that a range of host functions are affected by indigenous microbial communities [2]. For example, the complex microbial communities of the intestinal tract provide both nutritional benefits and protection against pathogens, and are vital in modulating interactions with the environment and the development of beneficial immune responses [3-5].

A growing concern for the high consumption of chemotherapeutic agents in aquaculture has led to a search for alternative methods of disease control. Probiotics are usually members of the healthy intestinal microbiota, therefore, they may provide an alternative means of reducing the use of antibiotics in aquaculture, since their addition can assist in returning a disturbed microbiota to its normal beneficial composition. Probiotics have been defined as "a viable microbial food supplement that beneficially influences the health of the host" [6]. It is important to point out that probiotic effects in aquaculture can also improve the health of fish and shellfish by controlling pathogens and water quality through modifying the microbial composition of water and sediment [7]. To date, most probiotics proposed as biocontrollers and bioremediation agents for aquaculture belong to the LAB group (lactic acid bacteria, mainly of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, and *Carnobacterium*) [8].

The aim of this study was to identify potential probiotics present in shellfish harvested from a marine environment possessing antibacterial activity against a range of pathogens.

2. Materias and Methods

2.1. Pathogenic bacterial strains and growth conditions

Pathogenic bacterial strains including *Staphylococcus aureus* KCCM12214, *Salmonella typhimurium* KCCM40406,

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Salmonella enteritidis KCCM40763, and *Escherichia coli* O157:H7 KCCM41038 were obtained from the Korean Culture Center of Microorganisms. *Vibrio ichthyoenteri* FP4004, *Edwardsiella tarda* FP5060, and *Streptococcus iniae* FP5228 were obtained from the National Fisheries Research & Development Institute Fish Pathology Division, and pathogenic *V. parahaemolyticus* KCTC2729 were obtained from the Korean Collection for Type Cultures. Pathogenic bacterial strains were grown at 37°C with shaking in Luria-Bertani medium (Difco, MI, USA), with the exception of *Vibrio* spp., *E. tarda,* and *S. iniae*, which were grown at 30°C with shaking in Brain-Heart Infusion medium (Difco) containing 3% NaCl. *Lactobacillus* spp. were grown anaerobically in deMan, Rogosa, and Sharpe medium (MRS, Difco) at 37°C for subculture.

2.2. Isolation of *Lactobacillus* spp.

From 2012 to 2013, we tested 6 shellfish (*Venerupis philippinarum*, *Batillus cornutus*, *Crassostrea gigas*, *Cyclina sinensis*, *Mytilus edulis*, *Mactra veneriformis* Reeve) from shellfish harvesting areas in the West Sea, Korea. Samples were collected in pre-sterilized bottles and were transported on ice to the laboratory to be processed within 6 h. Before homogenizing the shellfish samples, their shells were removed using autoclaved knives. Shellfish meat (200 g) was transferred to an autoclaved beaker and mixed with 200 mL of phosphate-buffered saline (PBS; 0.24 g/L KH_2PO_4, 1.44 g/L Na_2HPO_4, pH 7.2). The mixture was ground in an autoclaved stainless steel blender (7011S, Waring, Torrington, CT, USA) for 90 sec (30 sec at low speed and 60 sec at high speed), and the shellfish juice was serially diluted $10^{-1} \sim 10^{-2}$ in PBS. Next, 100 μL of the shellfish homogenates and the serial 10-fold dilutions (with 0.1% peptone water) of the homogenates were spread on Rogosa agar (BD, Sparks, MD, USA), a medium selective for *Lactobacillus* spp., containing 1.5% agar. The plates were incubated at 37°C for 2 days under anaerobic conditions. After incubation, the catalase-negative, gram-positive isolates were presumptively identified as *Lactobacillus* spp. and subsequently grown in MRS broth or agar. To confirm indirectly whether the isolates produced lactic acid, they were plated on MRS agar containing 0.1% bromocresol purple, which is purple at a neutral pH and becomes yellow as the pH decreases. Only yellow colonies were selected and the isolates were stored in 25% glycerol at -70°C.

2.3. Screening of isolated strains against various pathogens

The antimicrobial activity of isolated strains was tested against 8 pathogens (*S. aureus* KCCM12214, *S. typhimurium* KCCM40406, *S. enteritidis* KCCM40763, *V. parahaemolyticus* KCTC2729, *V. ichthyoenteri* FP4004, *E. tarda* FP5060, *S. iniae* FP5228, and *E. coli* O157:H7 KCCM41038).

The antimicrobial activity of supernatants from isolated strain cultures grown in MRS broth at 37°C for 24 h was determined by an agar well-diffusion test as previously described by Cintas *et al.* [9]. After cultivation, cell-free supernatant was obtained by centrifugation at 4,000 × *g* at 4°C for 10 min. Following this, the supernatant was passed through 0.22 μm filters (Millipore Corp., Bedford, MA, USA) and stored at 4°C until further use. Approximately 70 μL of supernatant was placed into wells of 6 mm in diameter cut into cooled plates of Mueller-Hinton Agar (MHA) or MHA with salt (3% w/v) and previously seeded with pathogenic bacteria (at an OD600 of 1.0). The plates were incubated under the conditions mentioned above to allow for the growth of target microorganisms, and the presence of inhibition zones around the wells was subsequently analyzed.

2.4. Biochemical tests and identification

Selected isolates were identified by Gram staining, conventional biochemical tests [10], and sequencing of the 16S ribosomal RNA gene using universal primers (518F and 800R). PCR and sequencing were performed by Macrogen Co. (Daejeon, Korea). Sequence similarity between strains was analyzed by nucleotide alignment using the Macrogen Alignment program, a web-based tool for identification based on 16S rRNA gene sequences [11]. Basic Local Alignment Search Tool (BLAST) analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to compare the sequences obtained with available DNA sequences registered in the database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Sequences were aligned using the PHYDIT program (http://plaza.snu.ac.kr/~jchun/phydit/), and alignments were manually corrected. A phylogenetic tree was constructed with the neighbor-joining method using MEGA 5.0 software [12].

2.5. Adherence assay

Lactobacillus spp. were characterized based on their ability to form biofilm with a semi-quantitative adherence assay in 96 polystyrene microplates according to Chaieb *et al.* [13], with some modifications. An overnight culture in MRS broth at 37ºC was diluted hundredfold with 2% glucose in MRS solution (wt vol^{-1}). Aliquots of 200 μL of the cell suspensions were transferred to 96-well microtiter plates (SPL Life Sciences Co., Ltd., Korea). Each strain was tested in triplicate. Wells with sterile MRS alone were used as controls. The plates were incubated aerobically at 37ºC for 24 h. The supernatant was discarded, and the wells were washed twice with PBS (7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl; pH 7.4) to remove the non-adherent cells. The plates were dried in an inverted position.

Adherent bacteria were fixed with 95% ethanol and stained for 5 min with 100 μL of crystal violet solution (1%, Showa, Japan). The wells were washed three times with 300 μL of sterile distilled water, and then the plates were air-dried again. The optical density of bacteria at 570 nm (OD570) was measured with UV/Visible Spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech Inc., NJ, USA). Adhesion ability was interpreted as highly positive (OD570 ≥ 1), moderately to weakly positive (1 > OD570 > 0.1), or negative (OD570 ≤ 0.1).

2.6. Bile tolerance

The modified method described by Arihara *et al.* [14] was used to determine the bile tolerance of selected isolated strains. Before testing for bile tolerance, selected strains were grown at 37°C for 24 h in MRS broth without bile. One milliliter of the culture broth was used to inoculate the MRS broth with bile salt concentrations of 1, 2, 3, and 4% (v/v). Bacterial growth was determined after incubation at 37°C for 24 h.

2.7. Acid tolerance

The modified method described by Erkkila and Petaja [15] was applied in this study. The selected strains grown in MRS broth at 37°C for 24 h were collected by centrifugation at 4,000 × *g* for 10 min. The resulting cell pellet was washed twice and resuspended in PBS to an OD_{600} of 1.0 before the addition of sterile PBS with pH values of 2.5, 3.0, and 4.0 (adjusted using 5 M HCl) to achieve an OD_{600} of 1.0. The tubes were incubated at 37°C and viable organisms were counted after exposure to acidic conditions for 0, 1, 2, 3, and 4 h in 0.1 M PBS incubated at 37°C for 24 h. The survival cell count was calculated according to the number of colonies grown on MRS agar, compared to the initial concentrations.

3. Results and Discussion

3.1. Isolation of *Lactobacillus* spp.

A total of 65 *Lactobacillus* spp. strains were isolated from 6 shellfish species. *Venerupis philippinarum* (n = 28), *Batillus cornutus* (n = 14), *Crassostrea gigas* (n = 8), *Cyclina sinensis* (n = 7), *Mytilus edulis* (n = 5), and *Mactra veneriformis* Reeve (n = 3; Fig. 1). In other studies, LAB have sometimes been found to be abundant in the intestines of freshwater fish [8], and lactobacilli isolated from wild or farmed freshwater fish have been reported [16]. It has also been shown that lactobacilli are effective probiotics for several fish species [17,18]. The shellfish from which Lactobacillus spp. were isolated in this study was from the natural marine environment. Therefore, it is expected that the lactobacilli isolates in this study are more adaptable to marine aquaculture conditions than those from freshwater fish.

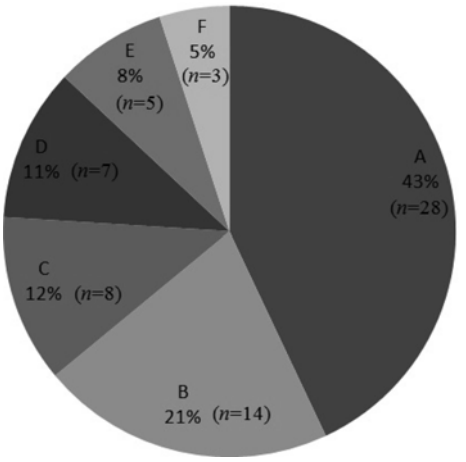

Fig. 1. Frequency of isolates from shellfishes harvested from the west Sea. A = *Tapes philippinarum*, B = *Batillus cornutus*, C = *Crassostrea gigas*, D = *Cyclina sinensis*, E = *Mytilus edulis*, and F = *Mactra veneriformis* Reeve.

3.2. Antimicrobial activity

Isolated strains were screened for their antimicrobial activity against selected pathogens (Table 1). Zones of inhibition were defined as the diameter of the circle formed as a result of the inhibitory activity of isolates against indicator organisms, excluding the diameter of the cork borer used (6 mm). These zones ranged from 0 to 20 mm in diameter. Negative control (filtered MRS broth) did not show any inhibitory activities. The largest inhibition zones (20 mm) were formed by isolate JL28 against *S. iniae*, followed by HL21 against *S. iniae*, and JL28, HL12, HL20, JL26, and HL1 against *V. ichthyoenteri*. *V. ichthyoenteri* showed the highest susceptibility to *Lactobacillus* spp. isolates while *Salmonella typhimurium* demonstrated the lowest susceptibility. Among 65 isolates, four strains (HL1, HL12, HL20, and JL28) were selected based on qualitative determination of high antimicrobial activity, and their biochemical characteristics are shown in Table 2. Such antimicrobial activity has also been observed in the work of Adesokan *et al.* [19], where *Lactobacillus* spp. were tested against *S. aureus, Pseudomonas aeruginosa, Candida albicans, Escherichia coli*, and *Proteus vulgaris.* Obadina *et al*. [20] also showed the antagonistic effects of *Lactobacillus plantarum* on *Salmonella typhi*, *S. aureus*, *E. coli*, and *Bacillus subtilis.* Puttalingamma *et al*. [21] showed the ability of *Lactobacillus* spp. to inhibit pathogenic bacteria using the agar well method from Tagg *et al.* [22] *Lactobacillus* spp. isolates showing antimicrobial activity were found to produce antimicrobial substances such as lactic acid, hydrogen peroxide, and diacetyl, showing

Table 1. Origin and direct antimicrobial activity against pathogens of LAB isolated from shellfishes

Origin	Strain	Pathogens (mm)							
		Staphylococcus aureus	*Salmonella typhimurium*	*Salmonella enteritidis*	*Escherichia coli O157:H7*	*Vibrio parahemolyticus*	*Vibrio ichthyoenteri*	*Edwardsiella tarda*	*Streptococcus iniae*
Shortnek clam (*Tapes philippinarum*)	HL1	14	9	9	11	14	18	11	15
	HL12	11	11.5	10	9	14	18	12	17
	HL15	7	9	10	9	0	13	9	13
	HL16	0	0	7	0	0	10	0	0
	HL18	7	9	8	9	10	14	8	12
	HL2	11	9	9	10	14	17	10	16
	HL20	11	13	12	9	12	18	12	17
	HL22	0	0	8	9	0	8	0	0
	HL25	0	10	7	7	0	10	8	11
	HL26	0	11	9	8	9	13	10.5	13
	HL29	0	11	8	11	9	14	9	13
	HL3	0	0	10	0	10	9	0	0
	HL31	0	10	8	9	0	13	9	12
	HL36	7	11	8	8	8	13	9	14
	HL4	0	0	8	0	0	9	0	0
	HL9	9	9	9	9	11	15	9	13
	JL10	10	9	9	10	9	14	11	14
	JL11	10	10	9	10	9	15	11	14
	JL15	8	10	9	8	9	14	10	13
	JL16	0	7	0	11.5	0	10	0	11
	JL24	10	12	9	9	10	15	10.5	14
	JL25	10	11	9	10	13	15	9	13
	JL26	10	12	9	9	12	18	11	17
	JL27	10	10	8	9	11	13	8	14
	JL29	8	10	8	9	10	12	9	14
	JL30	10	12	9	9	10	15	9	15
	JL31	10	12	9	9	11	16	10	15
	JL32	10	10	8	9	9	15	9	15
Spiny top shell (*Batillus cornutus*)	HL11	0	0	8	0	0	13	9	13
	HL19	9	11	10	9	11	15	11	14
	HL21	0	9	10	11	10	13	8	18
	HL33	0	10	8	8	0	11	8	12
	JL1	8	10	11	9	0	14	9	13
	JL19	11	12	8	10	10	16	13	14
	JL2	8	10	12	9	8	13	9	12
	JL20	10	11	9	11	10	16	10	15
	JL21	10	10	8	10	9	15	10	15
	JL22	10	10	9	11	9	17	10	14.5
	JL23	11	11	8	10.5	10	16	11	15
	JL3	0	10	10	9	7	12	9	11
	JL33	10	10	10	9	10	14	9	15
	JL34	10	10	8	9	11	14	10	14
Oyster (*Crassostrea gigas*)	HL32	0	11	9	8	0	12	9	12
	HL7	7	9	9	0	9	13	8	13
	JL17	11	12	10	10	11	17	13	16
	JL18	11	11	10	10	10	17	13	16
	JL28	11	12	11	12	13	18	13	20
	JL7	0	8	8	9	0	11	0	12
	JL8	8	9	9	10	0	16	0	12
	JL9	8	9	8	11	0	12	9	11
Corb shell (*Cyclina sinensis*)	HL10	7	0	9	8	9	12	8	13
	HL13	7	11	8	9	8	12	9	7
	HL14	0	0	0	0	0	0	0	0
	HL17	0	0	0	0	0	9	0	10
	HL23	0	0	8	8	0	10	0	11
	HL27	7	10	0	9	0	12	0	12
	HL28	7	11	8	8	11	13	10	14
Mussel (*Mytilus edulis*)	HL34	7	10	8	8	0	10	8	14
	HL8	9	10	10	10	12	14	9	13
	JL4	8	9	10	9	7	14	10	12
	JL5	8	10	9	9	8	14	10	13
	JL6	8	11	8	10	8	14	10	13
Surf clam (*Mactra veneriformis* Reeve)	JL12	11	9	8	10	8	13	10	13
	JL13	0	10	9	9	9	13	9	12
	JL14	0	11	8	9	10	14	10	14

Zones of inhibition were defined as the diameter of the circle formed as a result of the inhibitory activity of isolates against indicator organisms, excluding the diameter of the cork borer used (6 mm).

Table 2. Biochemical characteristics of isolated strains

Biochemical Tests	Isolated strains			
	HL1	HL12	HL20	JL28
Gram reaction	+	+	+	+
2-nitrophenyl-βD-galactopyranoside (ONPG)	-	-	-	-
L-arginine (ADH)	-	-	-	-
L-lysine (LDC)	-	-	-	-
L-ornithine (ODC)	-	-	-	-
Trisodium citrate (CIT)	-	-	-	-
Sodium thiosulfate (H_2S)	-	-	-	-
Urea (URE)	-	-	-	-
L-tryptophane (TDA)	+	+	+	+
L-tryptophane (IND)	-	-	-	-
Sodium pyruvate (VP)	+	+	+	+
Gelatin (GEL)	-	-	-	-
D-glucose (GLU)	+	+	+	+
D-mannitol (MAN)	+	+	+	+
Inositol (INO)	-	-	-	-
D-sorbitol (SOR)	+	-	+	+
L-rhamnose (RHA)	-	-	-	-
D-sucrose (SAC)	+	+	+	+
D-melibiose (MEL)	-	-	-	-
Amygdalin (AMY)	+	+	+	+
L-arabinose (ARA)	-	-	+	+
Adhesion score	0.014 ± 0.004	0.162 ± 0.067	1.139 ± 0.085	0.313 ± 0.023
Accession number	KP260630	KP230423	KP230424	KM970021

that their ability to inhibit other microorganisms is directly related to their capacity to produce these substances.

3.3. Identification and adhesion of *Lactobacillus* spp.

The four isolates were further analyzed by sequencing their 16S rRNA genes. BLAST analysis revealed that selected strains showed sequence similarities to *Lactobacillus plantarum*. The 16S rRNA gene sequences determined in this study were deposited in the NCBI GenBank database under the accession numbers KP260630 (HL1), KP230423 (HL12), KP230424 (HL20), and KM970021 (JL28).

The selected strains were also checked for adhesion. One of the strains was highly adhesive to polystyrene, with a score of 1.058 (HL20, Table 2). Two strains were moderately adherent, and the remaining strain was weakly adherent. The selection process was a test of adhesion to an abiotic surface which may indicate the potential of lactic acid bacteria to colonize the gut and to further antagonize pathogens [23].

3.4. Bile salt tolerance

Four strains (HL1, HL12, HL20, and JL28) were tested for their ability to grow at bile salt concentrations from 1 to 4% (v/v), in order to select bile-tolerant strains (Fig. 2). We

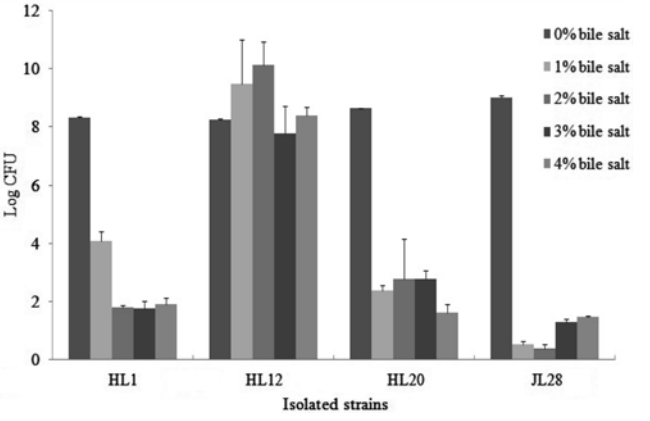

Fig. 2. Bile salt tolerance of *Lactobacillus* spp. isolates HL1, HL12, Hl20, and JL28 from shellfish.

determined that HL12 shows high resistance to all concentrations tested based on an increase in bacterial count. This is similar to the result obtained by Erkkila and Petaja [15] whereby strains of *Pediococcus acidilactici*, *Lactobacillus curvatus*, and *Lactobacillus sake* demonstrated the highest resistance to 0.3% bile salt at pH 6. Pennacchia *et al.* [24] reported that the bile salt tolerance of *Lactobacillus* strains allows them to grow in MRS agar supplemented

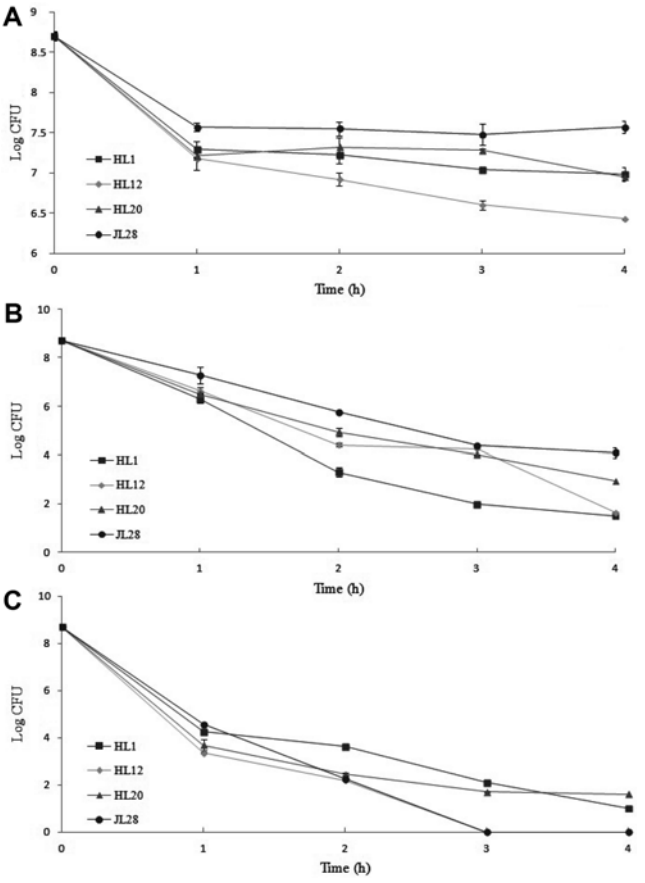

Fig. 3. Acid tolerance of *Lactobacillus* spp. isolates HL1, HL12, Hl20, and JL28 from shellfish: (A) pH 4.0; (B) pH 3.0; and (C) pH 2.5.

with 0.3% bile salt. It has also been reported that certain strains of *Lactobacillus* are able to reduce the detergent effect of bile salts due to their ability to hydrolyze the salts using the enzyme bile salt hydrolase [15]. The products are then readily excreted from the gastrointestinal tract [25].

3.5. Acid tolerance

The strains were further tested for their ability to tolerate acidic conditions at pH values of 2.5, 3.0, and 4.0, respectively. Only strains HL1 and HL20 were able to tolerate pH 2.5, with a 2.08 and 1.70 log CFU/mL reduction after 3 h of exposure, respectively (Fig. 3C). Four strains (HL1, HL12, HL20, and JL28) survived at pH 3.0 for 4 h, with counts of 1.51, 1.64, 2.94, and 4.10 log CFU/mL, respectively (Fig. 3B) and these were also the only strains to have survived exposure to pH 4.0 for 4 h (Fig. 3A). These results are in agreement with those of Succi *et al*. [26], who isolated *Lactobacillus rhamnosus* strains from Parmigiano Reggiano cheese based on their survival after 2 and 4 h of incubation at pH 3.0. Pennacchia *et al.* [24] reported that *Lactobacillus* spp. showed survival rates of 60 ~ 80% after 3 h incubation at 37°C in a PBS buffer of pH 2.5. Some *Lactobacillus* strains even retained their viability after exposure to pH 1 for 1 h [25]. In the present study, strains HL1 and HL20 were selected based on their survival at pH 2.5 for 4 h. None of the strains were able to survive exposure to pH 2 for 1 h (data not shown). Acid tolerance is a fundamental property of probiotic micro-organisms, reflecting their ability to survive passage through the stomach. Prasad *et al.* [27] obtained four acid tolerant strains from 200 *Lactobacillus* spp. isolates based on their 80% survival rate after exposure to pH 3 for 3 h.

4. Conclusion

This work shows that antimicrobial activity against pathogens is a widespread probiotic property amongst *Lactobacillus* spp. isolated from shellfish. Sixty-five strains of *Lactobacillus* spp. were isolated from 6 shellfish species. Strains HL1, HL12, HL20, and JL28, identified as *L. plantarum,* were tested for their survival at low pH values and high bile salt concentrations, indicating their potential for survival in the gastrointestinal tract. Furthermore, they also showed inhibitory activity against pathogens including *S. aureus*, *S. typhimurium, S. enteritidis*, *E. coli* O157:H7, *V. ichthyoenteri*, *E. tarda*, *S. iniae*, and *V. parahaemolyticus*. The present work outlines a valuable strategy for the preliminary selection of putatively safe *Lactobacillus* spp. intended for use as probiotics in aquaculture while avoiding the dissemination of bacterial cultures with harmful traits into the aquatic environment.

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