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Isolation and Characterization of a *Bacillus* spp. Against *Vibrio Parahaemolyticus* from Shrimp Culture Ponds

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Abstract: Pathogenic *Vibrio* species is one of the major factors affecting the development of aquaculture and the safety of seafood. Using the antagonistic activity of probiotics against pathogens offers a promising alternative to fish and shrimp aquaculture. In the present study, nine strains of bacteria were isolated from the shrimp culture ponds and screened for their directly antimicrobial activity against pathogenic *Vibrio parahaemolyticus* Vp1. Strain G, showing significant antimicrobial and non-hemolytic activity, was selected for further assays. The results of biochemical and 16S rRNA sequence analysis indicated that strain G highly related to *Bacillus licheniformis*. The present study also evaluated the *in vitro* and *in vivo* antagonistic effect of strain G against the *Vibrios*. Strain G exhibited significant inhibitory activity of *Vibrio fluvialis* FX-2, *Vibrio parahaemolyticus* K, and *V. parahaemolyticus* Vp1 *in vitro*. The inhibition diameter of strain G against *Vibrio* spp. ranged from 16 to 20 mm on Nutrient Agar. Under *in vivo* conditions, strain G was non-toxic to zebrafish and effectively protected zebrafish against *V. parahaemolyticus* Vp1. The non-toxicity of strain G showed final survival rate of 100% in zebrafish at inoculation densities up to 5.6×10^{10} CFU/ml at 96 h postchallenge. A significant reduction in mortality ($P<0.001$) was found by addition of 1.5×10^{8} CFU/ml or 1.5×10^{7} CFU/ml strain G in zebrafish against *V. parahaemolyticus* Vp1. In conclusion, the present study result reveals that strain G is a promising probiotic candidate and has potential applications for controlling pathogenic *Vibrios* in aquaculture practices.

Keywords: Antagonist, Aquaculture, *Bacillus.* spp, *Vibrio parahaemolyticus*, Probiotic

1. Introduction

With the increase in seafood consumption around the world, the occurrence of seafood safety issues presents a rising trend in recent years [1, 2]. Human pathogen *Vibrio parahaemolyticusis* is widely distributed in the marine environments and frequently isolated from a variety of raw seafood, particularly shellfish. In the recent years, it is recognized as a main causative agent of human gastroenteritis associated with seafood consumption in many coastal countries, including China, Japan, India, and the United States [3-7]. *V. parahaemolyticus* infections were found in almost all the cultured marine animals such as crustacean, mollusks, and fish, and serious infections often led to mass mortality. The *V. parahaemolyticus* infected animals, including farmed aquatic animals, are the principal vehicle in the transmission of the pathogenic bacteria to human [5].

Currently, the use of various antibiotics to control vibriosis in farmed aquatic animals has a serious negative impact on environment caused by rapid increase of antibiotic resistance in pathogenic bacteria. Many pathogenic *Vibrio* strains isolated from fish show resistance to a variety of antibiotics [8]. In addition, the overuse of antibiotics as prophylactic agents in feed results in high levels of drug residues in aquaculture products, which may cause toxicity, allergic reactions and alteration of normal microflora of consumer, and

stimulate the development of resistance in bacterial pathogen [9-11]. Recently, biocontrol agents are used as environmental friendly countermeasure to the diseases in aquaculture. Bacterial antagonistic activity against *Vibrio* have been reported in *Carnobacterium* spp. [12], *lactic acid bacteria* (LAB) [13], *Pseudomonas* sp. PS-1 [14], and *Roseobacter* strain 27-4 [15]. Hence, antagonistic activity of probiotics is used as an alternative strategy to antibiotics for controlling the food-borne pathogen *V. parahaemolyticus* in aquaculture [16]. However, there is little work to isolate an antagonistic bacterial against a broad range of *Vibrio*.

Bacteria of the genus *Bacillus* are widely distributed in the nature, useful in agriculture and industry, and occasionally directly harmful to humans [17]. *Bacillus* has been definitively classified by the sequence analysis of 16S rRNA genes. Many *Bacillus* species have been proven safe in humans, and used as fermentation strains for food production or as probiotics drugs for oral consumption. The use of a few *Bacillus* isolates as biological control agents also reported in previous studies, which suggests that bacteria of the genus *Bacillus* should be a potential source of probiotics [18]. However, recent studies indicated that *Bacillus* might contain toxin producing genes [19]. Some screened *Bacillus* spp. lack safety assessments and therefore have an adverse effect on subsequent actual production applications in aquaculture [20]. Consequently, these results have given rise to concern about the safety of *Bacillus* products. A more rigorous selection process is thus required for *Bacillus* probiotic candidates.

In order to develop biological control agents against pathogenic *V. parahaemolyticus* in aquaculture, we isolated several antibiotic-producing bacterial strains from the shrimp culture ponds. One of the isolates, strain G exhibited significant antibacterial activity against broad range of fish pathogenic *Vibrio*. In this paper we describe characters, phylogenetic analysis based on 16S rDNA sequence, and antagonistic activity of strain G against *V. parahaemolyticus* Vp1 of pathogenic *Vibrio* species. We also describe the pathogenic property of strain G to evaluate the safety for marine aquaculture application.

2. Materials and Methods

2.1. Bacterial Strains and Media

Antibiotic-producing strain G was isolated from the water samples collected from high-intensive shrimp culture ponds in Cixi, Zhejiang, China. Pathogenic *Vibrio* species, *V. fluvialis* FX-2, *V. parahaemolyticus* K and *V. alginolyticus* SH-1 used in this study were kindly provided by National Pathogen Collection Center for Aquatic Animals (NPCCAA), Shanghai Ocean University. *Vibrio parahaemolyticus* Vp1 (MF943220) was isolated from local sewer of aquatic products wholesale market in Zhejiang province and conserved by NPCCAA.

The media used in this study were listed as following: (1) 2216E agar media for isolating bacteria against *V. parahaemolyticus* Vp1: 5 g of peptone, 1 g of yeast extract, 0.1 g of Ferric citrate, and 15 g of agar per 1,000 ml ddH_2O; (2) Tryptic Soy Broth (TSB) for growth of *Vibrio* species: 15 g of tryptone, 5 g of soy peptone, and 5 g of NaCl per 1,000 ml ddH_2O; (3) Nutrient Agar (NA) for growth of isolated strain G: 5 g of peptone, 3 g of beef extract, 5 g of NaCl, and 15 g of agar per 1,000 ml ddH_2O; (4) Luria-Bertani broth (LB) for growth of isolated strain G: 5 g of yeast extract, 10 g of peptone, and 10 g of NaCl per 1,000 ml ddH_2O. All media used in this study were supplied by LuQiao Co. Ltd (Beijing, China) and adjusted to pH 7.2±0.2.

2.2. Isolation of Bacteria

The water samples collected from shrimp culture ponds were serially diluted ten-fold (to 10^{-4}) with 0.85% sterile saline solution. 100 µl of each dilution was spread-plated in triplicate on 2216E agar plates. All plates were incubated at 28°C until the morphology of the colony could be distinguished (24–48 h). The growth well, single-irregular, rough surface colonies were picked out from the each sample and plated-streaking individually. Those purified colonies were incubated on NA medium plates at 28°C for 24 h, the single colonies were picked up for further assay.

2.3. Antimicrobial Activity Assay

The antimicrobial activity of all isolates against Vp1 was assayed by using a spot inoculation method [21]. The indicator bacteria *V. parahaemolyticus* Vp1 were activated from dormant status and transferred to TSB medium. Following incubation at 28°C with vigorous shaking for 20 h, the concentration of *V. parahaemolyticus* Vp1 culture was calculated by dilution plating. *V. parahaemolyticus* Vp1 cultures were ultimately diluted to 1×10^5 CFU/ml for the direct assay. All isolates obtained in this study were spotted in triplicate onto NA plates pre-inoculated with indicator bacteria *V. parahaemolyticus* Vp1. Following incubation at 28°C for 24–48 h, the inhibition zone diameters were measured with a Vernier caliper.

2.4. Morphological, Biochemical, and Physiological Characterization of Strain G

The morphological, biochemical and physiological tests of strain G were carried out based on the methods described in Bergey's Manual of Systematic Bacteriology. The colony morphology was determined after 24 h incubation at 28 °C on NA medium. The temperature range for growth was tested at 10°C, 20°C, 30°C, 40°C, 50°C and 60°C in LB medium. The pH range for growth was examined at pH 2, 4, 6, 8, 10 and 12 in LB medium. Tolerance to sodium chloride (2, 4, 6, 8 and 10%) was tested using LB medium. Various biochemical tests were carried out using micro-biochemical tubes (Hangzhou Tianhe Micro-organism Reagent, China). The results of biochemical tests were interpreted by referring to the identification code book of Hangzhou Tianhe Micro-organism Reagent Co. (China). The inoculation of micro-biochemical tubes used the protocol as described by the manufacturer and was carried out at 28°C for 24 h.

2.5. Molecular Identification of the Bacterial Strain G

The total genomic DNA was extracted from 24 h-broth culture of strain G by using Bacterial genomic DNA extraction kit (TianGen) according to the procedure described by the manufacturer. 16S rDNA sequence was amplified with universal primers: 16S-27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S-1492R (5'-TACGGCTAC CTTGTTACGACTT-3'). The PCR reaction mixture (total volume 50 µl) consisted of 25 µl 2×PCR Master Mix, 1 µl DNA template, 2 µl Primer F, 2 µl Primer R and 20 µl sterile ddH_2O. PCR reactions were carried out in a mastercycler thermocyclers (Eppendorf, Germany) with three-step cycling programmed as following: one cycle of 94°C for 5 min; 25-30 cycles of 94°C for 20 s, 50°C for 60 s, and 72°C for 1.5 min; a final extension at 72°C for 10 min. The PCR product was observed by 1% agarose gel electrophoresis. The sequencing of 16S rRNA gene was performed by Sangon Biotech Co. Ltd, Shanghai, China. The sequences which shared over 98% similarity with currently available sequences were considered to be the same species. The multiple sequence alignment of the representative sequences was performed by using the ClustalX 2.0.6 [22]. A neighbour-joining analysis [23] and bootstrap analysis of 1,000 data re-samplings were performed to determine the robustness of each topology. A phylogenetic tree was constructed by using MEGA 5.05 [24].

2.6. Antagonistic Spectrum Activity Test of Strain G

The antagonistic spectrum of strain G were investigated using the same method described in Antimicrobial activity assay. After 24 h incubation at 28°C on 5 ml LB medium, culture of strain G was adjusted at the absorbance of 0.8 (UV/VIS Spectrophotometer, 1650-PC, Shimadzu, Japan). Twenty microliter of the bacterial suspension was spotted in triplicate onto NA plates pre-inoculated with different pathogenic *Vibrio*, including *V. alginolyticus* SH-1, *V. parahaemolyticus* K, or *V. fluvialis* FX-2. Following incubation at 28°C for 24 h, the results were determined by measuring the inhibition zone diameters.

2.7. Pathogenicity of Potentially Probiotic Strain G

The healthy and energetic zebrafish (~3 months old) used throughout this study were obtained from institute of life sciences, Chinese academy of sciences, all fishes were maintained in recirculating aquarium systems. Fish husbandry followed the methods of Westerfield [25]. Seventy zebrafish were randomly divided into five experimental, one positive control, and one negative control groups, each comprising 10 fish. Strain G and *V. parahaemolyticus* Vp1 was cultured in LB and TSB respectively, incubated at 28°C for 24 h. The negative and positive control groups were injected intraperitoneally (i.p.) with 10 µl of 0.85% sterile saline solution and 3.5×10^9 CFU/ml live *V. parahaemolyticus* Vp1, respectively. The five experimental groups were injected i.p. with 10 µl of 5.6×10^6, 5.6×10^7, 5.6×10^8, 5.6×10^9, or 5.6×10^{10} CFU/ml live strain G. The injected fish were then kept at 28°C for 4 d and the mortality was recorded. The pathogenicity of strain G to the zebra fish was evaluated using the methods described in GB/T 13267-91 [26]. The procedures have been approved by the Authors' Institution's Ethic Committee.

2.8. Virulence of Pathogenic V. parahaemolyticus Vp1

Ninety zebrafish were randomly divided into eight experimental and one negative control groups, each comprising 10 fish. The negative control group was injected i.p. with 10 µl of 0.85% sterile saline solution. The eight experimental groups were injected i.p. with 10 µl of 1.2×10^9, 4×10^8, 1.3×10^8, 4.4×10^7, 1.5×10^7, 4.9×10^6, 1.6×10^6, or 5.5×10^5 CFU/ml live *V. parahaemolyticus* Vp1. The injected fish were then kept at 28°C for 4 d and the mortality were recorded. The mean percentage mortality was plotted against the logarithm of dose (CFU/ml), and the dose killing fifty percent of the zerafish (LD_{50}) was calculated using the method described by [27].

2.9. Virulence Assays Mixing Potentially Probiotic and Pathogenic Strains

The virulence of mixed infection with potentially probiotic strain G and pathogenic *V. parahaemolyticus* Vp1 in zebrafish was assayed using the method described by Ravi [28]. Fifty zebrafish were randomly divided into five groups, each comprising 10 fish. Infection experiments were carried out by intraperitoneal co-injection of strain G and *V. parahaemolyticus* Vp1. Treatments for all five groups were listed in Table 1. The group exposed only to 0.85% sterile saline or pathogenic strain Vp1 were also used as negative and positive controls, respectively. The injected fish were then kept at 28°C for 4 d and the mortality were recorded.

Table 1. *The experimental groups (injection volume 20 µl /fish).*

Groups	Injection (i.p.)
Group 1	Vp1: 2.1×10^8 CFU/ml (10 µl) + 0.85% sterile saline (10 µl)
Group 2	Vp1: 2.1×10^8 CFU/ml (10 µl), strain G: 1.5×10^9 CFU/ml (10 µl)
Group 3	Vp1: 2.1×10^8 CFU/ml (10 µl), strain G: 1.5×10^8 CFU/ml (10 µl)
Group 4	Vp1: 2.1×10^8 CFU/ml (10 µl), strain G: 1.5×10^7 CFU/ml (10 µl)
Group 5	0.85% sterile saline (20 µl)

3. Results

3.1. Antagonistic Activity of Bacterial Isolates Against V. parahaemolyticus Vp1

A total of nine bacteria, strain A to I were isolated from the shrimp culture ponds and screened for their directly antimicrobial activity against *V. parahaemolyticus* Vp1 with spot inoculation (Figure 1). The 16S rDNA sequences indicated that all antagonistic isolates belonged to *Bacillus* species (data not shown). As showed in Figure 1, strain A, C, G and H had significantly higher antagonistic activity than the rest isolates. Considering the hemolytic activity of strain A, C and H, only strain G was selected for further assay in this study.

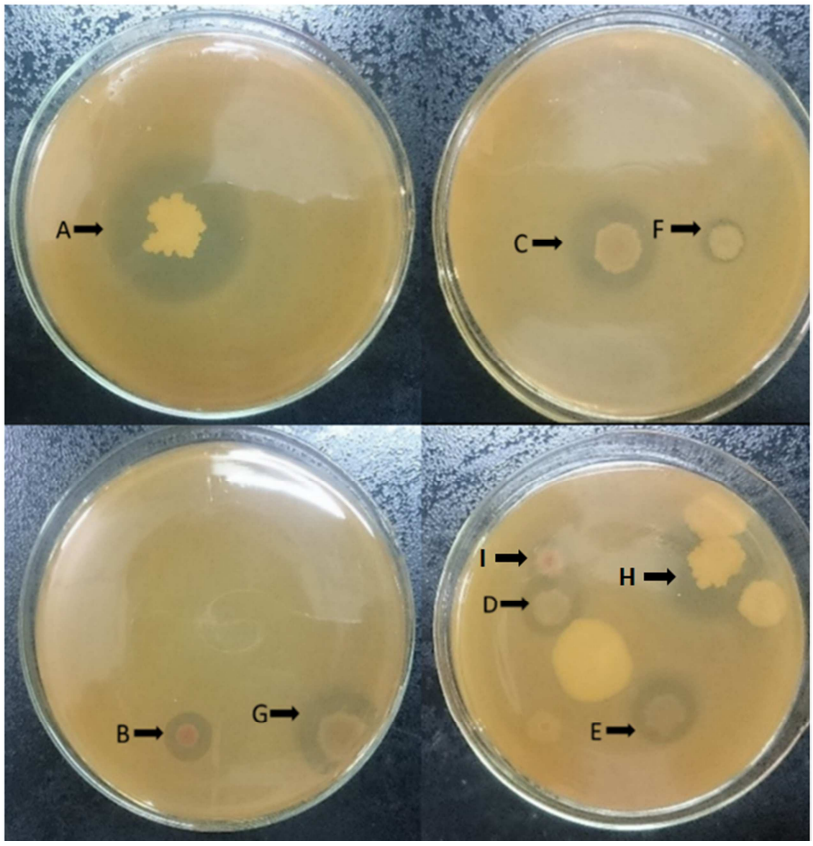

Figure 1. *Antagonistic activity of potential strains against V. parahaemolyticus Vp1 in vitro.*

3.2. Identification of Potential Probiotics Strain G

3.2.1. Preliminary Morphological and Physiological Identifications

The colony morphology of strain G was (light yellow, circular, opaque, flat, with a rough surface, and irregular edges around the colony on Nutrient Agar). This potential probiotic was Gram-positive, *Bacillus* spp. The spores of strains G were (located in the middle of the cell, and underwent no swelling). Strain G could not grow in the presence of sodium chloride at concentrations above 8%. The temperature range for growth was 20-40°C and the pH range for growth was 4.0–10.0. The results of biochemical analyses for strain G were summarized in Table 2. The morphological, cultural, and physiological characteristics indicated that strains G was closely related to *Bacillus* spp. which is well known for having the widely antimicrobial activity [2, 29, 30, 31].

Table 2. *Physiological characters of the isolated strain G.*

Parameter	Characters
Colony morphology	light yellow, circular, opaque, flat, with a rough surface, and irregular edges around the colony on nutrient agar
Gram strain	Gram positive
Growth in temperature	
10°C	-
20°C	+
30°C	+
40°C	+
50°C	+
60°C	-
Growth in pH	
2	-
4	+
6	+
8	+
10	+
12	-
Growth in NaCl	
2%	+
4%	+
6%	+
8%	+
10%	-
Nitrate reduction	+
Citrate utilization	+
Nitrite reduction	+
Amylohydrolysis	+
Urea hydrolysis	-
Casein hydrolysis	+
Adonitol	+
Arabinose	+
Fructose	+
Sorbitol	-
Lactose	-
Galactose	+
Sorbitol	-
V-P test	+
Indole test	+
Catalase test	+
Gelatin test	+
Lecithin test	+
Methyl red test	+

3.2.2. Molecular Biological Identifications Based on 16S rRNA Gene Sequences

The 16S rRNA sequences of strain G (1414 bp) was submitted to NCBI GenBank with accession number MF871790. The sequence of strain G and 16S rRNA sequences obtained from other species within the same genus, and downloaded from GenBank at NCBI, were aligned to construct the phylogenetic tree. Homology searches through BLAST server of the NCBI revealed that strain G had similarity of 99% with DNA sequence of *Bacillus licheniformis* (NC_006270). Furthermore, phylogenetic analysis based on 16S rDNA sequence showed that strain G was grouped in the same clade with *B. licheniformis* (Figure 2).

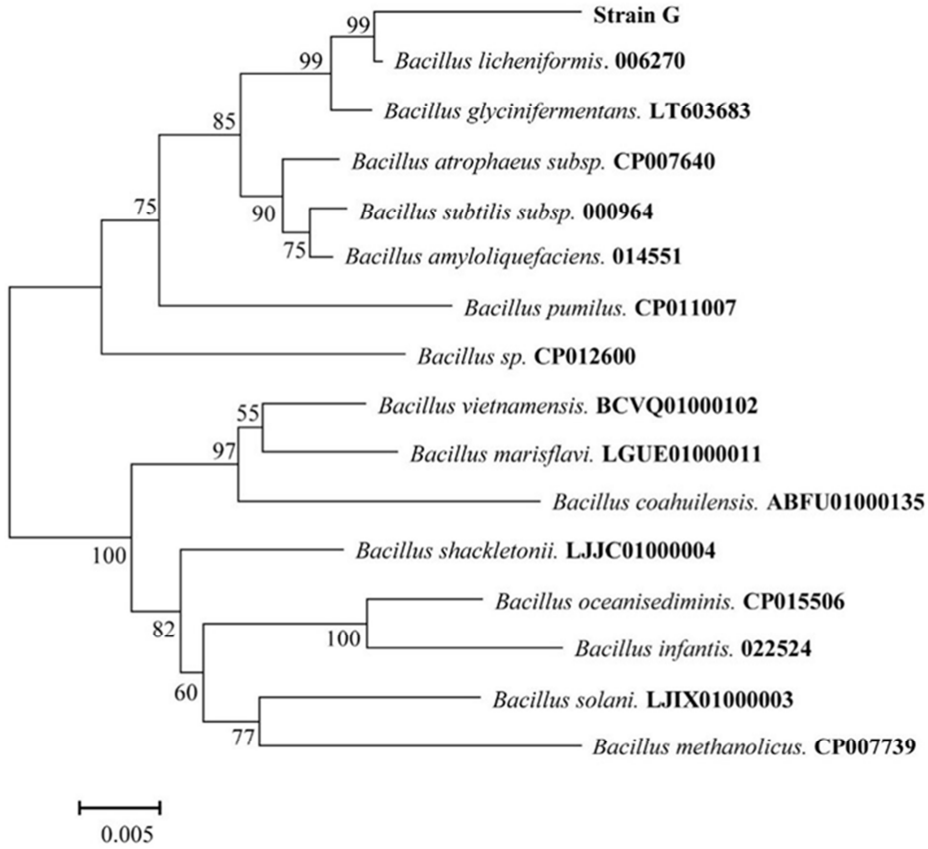

Figure 2. *Neighbour-joining tree exhibiting the phylogenetic position of strain G and representatives of other related strains within Bacillus genus based on 16S rRNA gene sequence.*

3.3. Antagonistic Spectrum Activity of Strain G

The culture of strain G was further investigated against four indicator bacteria, common pathogens in aquaculture using a spot inoculation method. Strain G inhibited the growth of *V. fluvialis* FX-2, *V. parahaemolyticus* K, and *V. parahaemolyticus* Vp1, but it was inactive against *V. alginolyticus* SH-1 (Table 3). The inhibition diameter of strain G against *Vibrio* spp. ranged from 0 to 20 mm.

Table 3. *The inhibitory zone diameter (mm) of direct antimicrobial activity assay with spot inoculation.*

Active isolate	Pathogens	Inhibition diameter (mm)
Strain G	*V. alginolyticus* SH-1	0
	V. fluvialis FX-2	16
	V. parahaemolyticus K	18
	V. parahaemolyticus Vp1	20

3.4. The Absence of Pathogenicity in Potentially Probiotic Strain G

The pathogenicity of strain G against zebrafish was determined by intraperitoneal injection. No mortality of zebrafish was observed in the experimental groups (5.6×10^6, 5.6×10^7, 5.6×10^8, 5.6×10^9, or 5.6×10^{10} CFU/ml live strain G) and the negative control group (0.85% sterile saline solution) up to 4 d in the experiment. Injection of strain G also did not induce any pathological signs. The positive control, 3.5×10^9 CFU/ml live *V. parahaemolyticus* Vp1 did cause 100% mortality of zebrafish within 4 d (Table 4). The results revealed that the 50% lethal dose (LD_{50}) of strain G in zebrafish should be much higher than 5.6×10^{10} CFU/ml, which was considered as non-pathogenic.

Table 4. *Acute toxicity of the strain G on Zebra fish.*

Group	Dosage mg/l	Concentration CFU/ml	Death number									Mortality (%)
			1h	2h	4h	6h	8h	24h	48h	72h	96h	
Control group	normal saline	0.85%	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	
1	0.233	5.6×10^{6}	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	
2	2.33	5.6×10^{7}	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	
3	23.3	5.6×10^{8}	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	
4	233	5.6×10^{9}	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	
5	2330	5.6×10^{10}	0	0	0	0	0	0	0	0	0	0
			0	0	0	0	0	0	0	0	0	
			0	0	0	0	0	0	0	0	0	
Vp1	23.3	3.5×10^{9}	0	0	0	0	0	3	6	8	9	100%
			0	0	0	0	0	4	5	7	10	
			0	0	0	1	3	4	7	8	10	

∗ 10 zebra fish of each group

3.5. Virulence of Pathogenic V. parahaemolyticus Vp1

The virulence of pathogenic strain *V. parahaemolyticus* Vp1 against zebrafish was determined on the basis of LD_{50}. According to the mortality caused by various concentrations (5.5×10^{5}-1.2×10^{9} CFU/ml), Vp1 strain had an LD_{50} of 4.4×10^{7} CFU/ml. The Vp1 strain did cause 100% mortality in the groups challenged with 1.2×10^{9} or 4×10^{8} CFU/ml per fish within 4 d. No mortality was observed in the negative control group challenged with 0.85% sterile saline solution up to 4 d.

3.6. Virulence Assays Mixing Potentially Probiotic and Pathogenic Strains

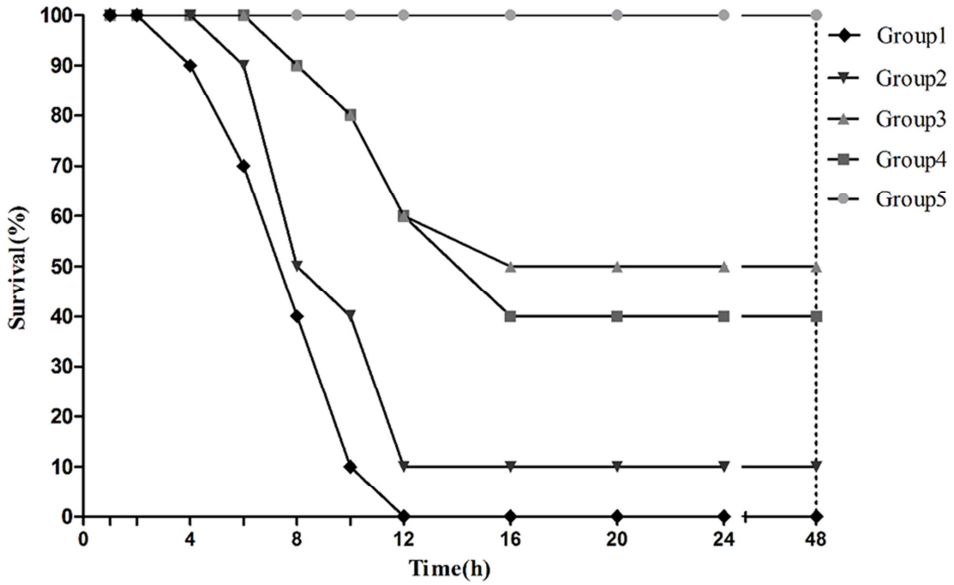

Figure 3. *Protective capability of strain G to zebra fish infected with V. parahaemolyticus Vp1 Group1:2.1×10^{8} CFU/ml (10 μl) Vp1 + 0.85% sterile saline (10 μl); Group2: 2.1×10^{8} CFU/ml (10 μl) Vp1+1.5×10^{9} CFU/ml (10 μl) strain G; Group 3: 2.1×10^{8} CFU/ml (10 μl) Vp1+ 1.5×10^{8} CFU/ml (10 μl) strain G; Group4: 2.1×10^{8} CFU/ml (10 μl) Vp1+ 1.5×10^{7} CFU/ml (10 μl) strain G; Group 5: 0.85% sterile saline (20 μl).*

The co-injection of potentially probiotic strain G and pathogenic strain *V. parahaemolyticus* Vp1 was used to analyze the antibacterial potential of the former against vibriosis in zebrafish. Survival curves for the five treatments analyzed jointly were shown in Figure 3. The comparisons of the survival curves revealed that there were significant differences between the negative control (group 5) and fish treated with the pathogen alone (group 1) or with probiotics in

the mixed challenges (group 2-4). Negative control treated with 0.85% sterile saline solution (group 5) showed survival rate of 100% within 48 h after treatment. Fish infected with *V. parahaemolyticus* Vp1 alone (group 1) showed survival rate of 0 at 12 h postchallenge. The analyses also showed that the survival of zebrafish was significantly increased by the addition of potentially probiotic strain G. The final survival rate in the presence of strain G was up to 50% (group 3) at 16 h postchallenge. Significant differences between fish treated with varied dose of probiotics in the mixed challenges were found as well. The final survival rates in the presence of 1.5×10^9 CFU/ml, 1.5×10^8 CFU/ml, or 1.5×10^7 CFU/ml strain G were 10% (group 2), 50% (group 3), or 40% (group 4), respectively. The results of virulence assays indicated that strain G provided significant protection against the pathogenic action of *V. parahaemolyticus* Vp1 under the given experimental conditions.

4. Discussion

The concept of using probiotics as biological control against pathogens has received widespread attention during the last few decades [32]. In this study, we demonstrated that the growth of fish pathogenic *Vibrio* was controlled by non-pathogenic strain G isolated from the highly intensive shrimp culture ponds, *in vitro* and *in vivo* conditions (Figure 1-3 & Table 1-3). Besides of the morphological and physiological characters, the homology search based on 16s rDNA sequence showed that strain G was the number of *Bacillus* spp. and highly related to *Bacillus licheniformis* with 99% sequence similarity. *In vivo* examination carried out in this study confirmed that the *Bacillus* isolate strain G was non-hemolytic activity and nonpathogenic bacteria for zebrafish, suggesting that strain G could be a good candidate for probiotics in aquaculture. In addition, recent studies indicated that *Bacillus* might contain toxin producing genes [19], might conduct to the insecurity for food and environment and limits it's clinical application. Hence, the strain G might have the potential possibility to be used as a safe probiotic in aquaculture.

In the present study, zero survival was observed in zebrafish treated with *V. parahaemolyticus* Vp1 alone, which confirmed that the *Vibrio* strain Vp1 is highly pathogenic. When the fish were exposed to the mixture of pathogenic strain Vp1 and potential probiotic strain G, their survival rate was significantly (P<0.001) increased (Figure 3). *In vivo* antagonistic activity results were well correlated with our *in vitro* observations (Table 2). This result finds support of other workers. Kennedy [33] had recorded that the inoculation of a probiotic *B. subtilis* isolate into the rearing water resulted in the apparent elimination of *Vibrio* spp. from the snook larvae. A similar effect has been observed with another probiotic *B. cereus* isolate at 10^5 CFU/ml that protects shrimp larvae of *P. monodon* against *V. harveyi* with 10^4 CFU/ml by increasing the survival rate to 60% [28]. Co-injection experiments showed that the final survival rate of *Vibrio* infected zebrafish increased from 40% to 50% with increasing concentration of strain G (antagonist) from 1.5×10^7 to 1.5×10^8 CFU/ml. To act as probiotics, strain G must be present at significantly high levels and the degree of probiotic protect increased with the level of strain G. However, the final survival rate dropped to 10% when the *Vibrio* infected zebrafish was treated with higher concentration of strain G (1.5×10^9 CFU/ml). This result suggested that the optimal concentration for strain G to exhibit the best probiotic effect against *Vibrio* infection in zebrafish should be close to 1.5×10^8 CFU/ml.

Several species are known to be antibiotic-producing bacteria, such as *Carnobacterium* spp. [12], lactic acid bacteria (LAB) [13], *Pseudomonas* sp. [14], and *Roseobacter* [15], and *Bacillus* spp. [17, 35, 36]. Especially *Pseudomonas* S2V2 inhibited *V. alginolyticus*, *V. anguillarum*, *V. fluvialis*, *V. harveyi*, *V. metschnikovii*, *V. splendidus*, *V. ordalii*, *V. parahaemolyticus*, and *V. vulnificus* but inactive against *V. campbellii* [34], Susceptibility variations to strain S2V2 were exhibited among species and among strains in the same species of *Vibrio* sp. However, there is a lack of broad-spectrum antibacterial activity against *Vibrio* in *Bacillus such as B. pumilus* B16, *Bacillus mojavensis* J7 [17], *Bacillus licheniformis* DAHB1 [35], *Bacillus subtilisb* [36], but for strain G, it exhibited significant inhibitory activity of *V. fluvialis* FX-2, *V. parahaemolyticus* K, and *V. parahaemolyticus* Vp1 *in vitro*. The inhibition diameter of strain G against *Vibrio* spp. ranged from 16 to 20 mm on Nutrient Agar. The spectrum activity implies that the nature of antibiotic produced by strain G is different to antibiotics produced by other *Baccillus* species reported before.

The mechanism of antagonistic effects could be the growth inhibition of bacterial pathogens due to the bioactive compounds produced by probiotics. Previous studies also showed that *Bacillus* species produce various bioactive compounds such as subtilin [37], subtilomycin [38], cerecidin [39], and haloduracin [40], which display inhibitory activity against a broad spectrum of bacteria, including *Staphylococcus*, *Listeria*, *Aeromonas*, *Vibrio*, *Pseudomonas*, and *Alteromonas* species, etc [41]. In the present study, strain G exhibited zone of clearance against pathogenic *Vibrios* on Nutrient Agar plates (Figure 1 & Table 3). It could be considered that the growth of pathogenic *Vibrios* is inhibited by the diffusion of antibacterial compounds produced by strain G. Thus, purification and characterization of the antibacterial compounds produced by strain G may contribute to a better understanding of the mechanism of the antagonistic effect.

5. Conclusion

From this study, it can be concluded that the *Bacillus* isolate, strain G is non-toxic to zebrafish and can effectively inhibit the growth of pathogenic *Vibrios* on Nutrient Agar plates and protect zebrafish against *V. parahaemolyticus* Vp1 by significantly (P<0.001) increasing the survival rate in culture systems. Therefore, the ability of strain G to suppress pathogen growth *in vitro* and *in vivo* conditions suggests that it is a promising probiotic candidate that may be a good

alternative to antibiotics in aquaculture. Further study is need to provide valuable insight into the exact mode of action of observed probiotic effects and the possibilities and limitations of bacterial disease control in situations directly relevant to aquaculture conditions.

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