

ORIGINAL ARTICLE

Selection and identification of non-pathogenic bacteria isolated from fermented pickles with antagonistic properties against two shrimp pathogens

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In this study, potential probiotic strains were isolated from fermented pickles based on antagonistic activity against two shrimp pathogens (*Vibrio harveyi* and *Vibrio parahaemolyticus*). Two strains L10 and G1 were identified by biochemical tests, followed by 16S ribosomal RNA gene sequence analysis as *Bacillus subtilis*, and characterized by PCR amplification of repetitive bacterial DNA elements (Rep-PCR). Subsequently, *B. subtilis* L10 and G1 strains were tested for antibacterial activity under different physical conditions, including culture medium, salinity, pH and temperature using the agar well diffusion assay. Among the different culture media, LB broth was the most suitable medium for antibacterial production. Both strains showed the highest level of antibacterial activity against two pathogens at 30 °C and 1.0% NaCl. Under the pH conditions, strain G1 showed the greatest activity against *V. harveyi* at pH 7.3–8.0 and against *V. parahaemolyticus* at pH 6.0–8.0, whereas strain L10 showed the greatest activity against two pathogens at pH 7.3. The cell-free supernatants of both strains were treated with four different enzymes in order to characterize the antibacterial substances against *V. harveyi*. The result showed considerable reduction of antibacterial activity for both strains, indicating the proteinaceous nature of the antibacterial substances. A wide range of tolerance to NaCl, pH and temperature was also recorded for both strains. In addition, both strains showed no virulence effect in juvenile shrimp *Litopenaeus vannamei*. On the basis of these results and safety of strains to *L. vannamei*, they may be considered for future challenge experiments in shrimp as a very promising alternative to the use of antibiotics. *The Journal of Antibiotics* (2012) 65, 289–294; doi:10.1038/ja.2012.17; published online 11 April 2012

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INTRODUCTION

The development of aquaculture industry has been accompanied by the emergence of a large variety of pathogens. Bacterial species such as *Vibrio alginolyticus*, *Vibrio anguillarum*, *V. harveyi*, *V. parahaemolyticus* and *Vibrio vulnificus* have been associated with high mortalities in fish and shellfish facilities.^{1–3} Prevention and control of diseases has led to a substantial increase in the use of chemotherapeutic agents. However, the utility of these agents as a preventive measure has been questioned, given the increasing concern about antibiotic resistance genes in bacteria.^{4,5} Thus, the use of probiotic is receiving considerable attention as an alternative to control infections.^{6–8} Probiotics are defined as 'live microorganisms, which when administered in adequate amounts confer a health benefit to the host'.⁹

The range of probiotic examined for use in aquaculture encompasses both Gram-negative and Gram-positive bacteria, although the use of other organisms such as bacteriophages, yeasts and unicellular algae has also been explored.^{5,10} The genus *Bacillus* has been reported as beneficial bacteria for aquaculture. In this regard, administration of

Bacillus has shown beneficial effects on growth performance, survival rate and immune response of black tiger shrimp, *Penaeus monodon*.^{11,12} For instance, *B. subtilis* UTM 126 isolated from shrimp culture pond, produced antibacterial activity against *Vibrio* species.¹³ Another study showed that *B. subtilis* E20, isolated from fermented soybean, improved the immunity of white shrimp (*L. vannamei*) and resistance to the pathogen *V. alginolyticus*.¹⁴

A common way to select probiotics is to perform identification of candidate bacteria and *in vitro* antagonism tests, in which pathogens are exposed to the candidate probiotics or their extracellular products.^{5,15} However, antagonist activity is highly dependent on environmental factors and could be disturbed by physical conditions. Furthermore, resistance to acidic conditions is important for potential probiotics to survive and grow in the gastrointestinal tract.

Given this, the aim of this study was to isolate and identify beneficial bacteria from fermented pickles for potential application as probiotics in shrimp facilities. Several tests of probiotic efficacy were used, including antagonistic activity against two shrimp pathogens

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and different physical conditions for maximum antibacterial production. In addition, the sensitivity of antibacterial substances to different enzymes and the safety of candidate strains for juvenile shrimp, *L. vannamei*, were investigated.

MATERIALS AND METHODS

Isolation and screening for antibacterial activity

Garlic, cabbage, carrot and vinegar were purchased from a local market at Serdang, Selangor, Malaysia. They were washed with distilled water and heated at 60 °C for 10 min. After drying for 10 min, all the ingredients were mixed and covered with liquid containing 30% vinegar and 70% salt water (1% NaCl). The container was sealed and kept for fermentation for 3 months. After the fermentation, bacteria were isolated from the ingredients and liquids. Briefly, the ingredients were separated, homogenized, and serially 10-fold diluted in phosphate-buffered saline solution (PBS; pH 6.6). One hundred microliters of each dilution was then plated on nutrient agar (NA) supplemented with 2% NaCl and incubated at 30 °C for 24–48 h. Single colonies were replica-plated⁴ onto plates containing 10 ml Muller Hinton agar supplemented with 1% NaCl and *V. harveyi*, which was previously grown overnight in LB broth (Difco, Spark, NV, USA). Plates were then incubated for 24 h at 30 °C. Clear zones around replica-plated bacteria were interpreted as antagonistic activity against the pathogen and they were picked from the original plates. Antagonistic strains were stored in glycerol at –80 °C for further investigation.

Agar well diffusion assay

Antagonistic strains were re-examined against *V. harveyi* (ATCC 14126, ATCC, Manassas, VA, USA) and *V. parahaemolyticus* (ATCC 43996) using the agar well diffusion assay to confirm the antagonistic ability. Previous studies have demonstrated that both species are important pathogenic bacteria for aquaculture.^{16,17} Antagonistic strains were grown in LB broth at 30 °C for 48–96 h. After incubation, the bacterial suspensions were removed by centrifugation and culture supernatants were sterilized by passage through 0.20 µm-pore-size filters (Sartorius, Goettingen, Germany). The target *Vibrio* strains were grown overnight in 10 ml of LB broth at 30 °C, and 10 µl of each *Vibrio* was mixed into 10 ml of melted Muller Hinton agar supplemented with 2% NaCl. Wells were then punched (5 mm) into the agar and 25 µl of sterile-filtered supernatants were added. Plates were incubated at 30 °C and observed for clearing zones around the wells after 24 h. Sterile LB broth was used as control.

Identification of bacterial strains

In order to identify and differentiate both strains, conventional and molecular identification were performed. After the Gram-staining and morphological characteristics, strains were subjected to phenotypic tests for conventional identification, including catalase, oxidase, motility, urease, ONPG, citrate, indole, Voges–Proskauer, H₂S production, glucose, inositol, mannitol and reduction ability of nitrate to nitrite.

Extraction and amplification of genomic DNA for 16S rRNA sequence analysis were carried out as described previously.¹⁸ 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.¹⁹

To differentiate and characterize both strains, Rep-PCRs with Rep, Eric and BOX primers were performed. The DNA amplification was followed according to the previous study.²⁰ The annealing temperatures were selected based on the oligonucleotide primers, Rep-PCR (40 °C), Eric-PCR (51 °C) and BOX-PCR (53 °C). PCR reaction for each type of primers without DNA template was performed as control. All PCR reactions were performed in triplicate. A total of 12 µl of PCR amplification products was verified by 2.0% (w/v) agarose gel electrophoresis stained with ethidium bromide for 3.5 h with 65 V, and photographed under UV illumination. Both 1 kb and 100 bp ladders were used as markers.

Effect of culture medium composition on the antibacterial activity

Antibacterial production was estimated using four different culture media including LB broth, marine broth (MB), nutrient broth (NB) and Muller

Hinton broth (MHB) (Difco), supplemented with 1% glucose and without glucose. To evaluate the antibacterial production, 50 µl of a bacterial suspension was inoculated into each culture medium and then incubated at 30 °C for 72 h. All culture media had an initial pH of 6.6. Antibacterial activity of bacterial strains in the different culture media was determined using the agar well diffusion assay, as described above.

Effect of pH, salinity and temperature on the antibacterial activity

The optimal condition for maximum antibacterial production was assessed by growing the bacterial strains into LB broth at different pH (6, 6.6, 7.3, 8 and 8.8), salinity (0, 1, 2, and 3%) and temperature (25, 30, 35 and 40 °C) levels for 96 h. Two milliliters of each grown culture was collected every 24 h, and the antagonistic activity against *V. harveyi* and *V. parahaemolyticus* was determined using the agar well diffusion assay.

Spectrophotometric assay upon antibacterial activity

Bacterial strains were grown in 50 ml LB broth at the optimal condition obtained by the previous assays. The inhibitory activity of sterile-filtered supernatants from each bacterial strain was tested by adding 50 ml overnight cultures of *V. harveyi* or *V. parahaemolyticus*, previously grown in MH broth. Samples were collected every 6 h, and the growth of two shrimp pathogens was monitored by measuring optical density at 600 nm (OD₆₀₀) with a spectrophotometer. Each treatment was tested in triplicate. Penicillin and LB broth were used as positive and negative control, respectively.

Characterization of the antibacterial substances

Sterile-filtered supernatants of both strains were treated with proteinase K (1 mg ml⁻¹, 37 °C), trypsin (50 mg ml⁻¹, 37 °C), α-amylase (1 mg ml⁻¹, 25 °C), and lysozyme (1 mg ml⁻¹, 25 °C) in order to characterize the antibacterial substances. After incubation for 1 h, antibacterial activity of both strains was tested against *V. harveyi*, using well-diffusion agar assay. The residual activity was measured. Sterile-filtered supernatants without enzyme treatment were considered as control. The assay was repeated two times to ensure the reproducibility.

Tolerances of antagonistic strains under different conditions

Tolerance to different pH, salinity and temperature levels was assessed by culturing the antagonistic strains in NA. Tolerance to temperature was determined by incubating the inoculated NA at 4, 10, 20, 30, 40, 50, 60 and 70 °C. Tolerance to salinity was determined with or without the addition of NaCl at different concentrations (from 1 to 10%) to NA cultures, and examining the growth after 24–48 h. To determine the pH tolerance, the antagonistic strains were inoculated on NA at pH 3, 4, 5, 6, 7, 8, 9, 10 and 11 adjusted with 1 N NaOH or 1 N HCl. All treatments were carried out in triplicate.

Safety of candidate strains to *L. vannamei*

In order to demonstrate the safety of both strains to shrimp, an experiment was conducted using juvenile shrimps *L. vannamei*, at Marine Science Research Station, UPM, Port Dickson, Malaysia. Shrimp had not been exposed to shrimp diseases and were deemed pathogen-free by appearances and standard microbiological techniques. Healthy juveniles weighing approximately 3 g were acclimatized for 4 days in 20 ppt seawater. Shrimps were then distributed into 20-l glass tanks of 10 animals each with a constant aeration. Two groups of shrimp were injected with 0.1 ml of 10¹⁰ cfu ml⁻¹ of *B. subtilis* strains L10 and G1. The third group was shrimp injected with 0.1 ml of sterile PBS as control. All shrimps were injected at the third abdominal segment using 1 ml sterile insulin syringe (29 G). In addition, all experiments were performed in triplicate. One shrimp was collected randomly from each tank at the beginning and the end of the experiment to evaluate the concentration of *B. subtilis*. Shrimps were collected in cooled-sterile PBS and homogenized in a glass homogenizer immediately after they were killed. Pooled technique was performed for each treatment. Homogenized and pooled samples were serially diluted in 10-fold steps in PBS, and 100 µl of each dilution spread on Mannitol Egg Yolk Polymyxin agar (MYP agar, Difco) plates to estimate the

concentration of *B. subtilis* species (cfu g⁻¹). All yellow colonies were counted in plates containing the total amount of 30–300 cfu. Shrimps were fed with the commercial pellet feed (BLANCA, Berangan, Malaysia) and 50% of the water was exchanged daily. Shrimps were monitored daily for any mortality or signs of weakness for up to 15 days.

Statistical Analysis

In order to obtain the best physical conditions for maximum antibacterial production against two shrimp pathogens, the data were analyzed by repeated measure analysis of variance and the means were compared by least significant difference (LSD) test. All statistics were performed using the SPSS 14.0 for Windows (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

In selecting a potential probiotic strain for beneficial health effects on the host, many criteria must be considered. In order to colonize the gastrointestinal tract, potential probiotics should produce antibacterial substances against pathogens and express high tolerance to acidic conditions. Although isolation and screening of potential probiotics is usually a long process and time-consuming, the replica-plating method could be used successfully for the isolation of bacterial strains with antibacterial properties.⁴ In the present study, the isolation process of antagonistic strains using replica-plating method was quick and successfully performed.

Isolation and identification of antagonistic strains

A total of 89 colonies were isolated from fermented pickles, including 43, 25 and 21 colonies from vinegar, garlic and cabbage, respectively. They were tested for their possibility of antibacterial activity against *V. harveyi* using the replica-plating method. Overall, 14 bacterial strains produced clearing zones of different size. Out of 14 antagonistic strains, only two strains displayed strong inhibitory activity against *V. harveyi* and *V. parahaemolyticus* in the agar well diffusion assay.

The same pattern of biochemical reaction was observed for both antagonistic strains. Both strains were Gram-positive, rod-shaped cells, motile, oxidase, catalase, urease, ONPG, Voges–Proskauer, citrate and mannitol positive. Ability to reduce nitrate to nitrite and starch hydrolysis were recorded for both strains. Indole, inositol and glucose fermentation was negative and unable to produce H₂S.

PCR amplification using universal primers amplified a fragment of the expected size (900 bp) from the 16S rRNA gene. PCR product were purified and sequenced to identify the antagonistic strains. Strain G1 (GenBank accession number HQ731482) was identified with 100% similarity as *B. subtilis* subsp. *spizizenii* NRRL B-23049^T. Strain L10 (GenBank accession number HQ731481) exhibited 99.89, 99.77 and 99.73% similarity to *B. subtilis* subsp. *spizizenii* NRRL B-23049^T, *Brevibacterium halotolerans* LMG 21660^T and *Bacillus tequilensis* NRRL B-41771^T, respectively (Supplementary material, Table S1).

Genomic DNA fingerprinting based on Rep-PCR characterizes and distinguishes bacterial strains rapidly. In this study, DNA fingerprint produced by Rep-PCR method (REP, ERIC and BOX) distinguished *B. subtilis* strains. Although Rep-PCR revealed a very similar pattern for both strains, the results of BOX-PCR and ERIC-PCR showed higher discriminatory power (Figure 1).

Optimal physical conditions for antibacterial production

The culture conditions influence the formation and production of antibacterial substances. Antibacterial production was not detected when two *B. subtilis* strains were grown in culture media

supplemented with 1% glucose. Similar results were reported²¹ with addition of high level (>0.5%) of glucose, which might be due to catabolite repression of the production of *Bacillus licheniformis* by glucose. In addition, the greatest inhibition zones against *V. harveyi* were recorded in LB broth, followed by MHB, MB and NB (Table 1). LB broth was, therefore, selected as the best culture medium for further assays.

The two *B. subtilis* strains were tested under different salinity levels. The maximum antibacterial production ($P < 0.05$) against *V. harveyi* and *V. parahaemolyticus* was observed at 1% NaCl after 72 h for strain L10 and after 96 h for strain G1 (Figures 2 and 3). Alteration of pH had a significant effect ($P < 0.05$) on antibacterial production. Antagonistic activity of *B. subtilis* strain L10 was higher ($P < 0.05$) at pH 7.3 after 72 and 96 h against *V. harveyi* and *V. parahaemolyticus*, respectively. *B. subtilis* strain G1 showed the greatest antagonistic activity at pH 7.3 and 8.0 ($P < 0.05$) after 72 to 96 h against *V. harveyi*, whereas the highest activity ($P < 0.05$) against *V. parahaemolyticus* was exhibited at pH 6 and 8 after 96 h of incubation (Figures 2 and 3).

At the final stage of the experiment, LB broth was prepared based on the optimal conditions of salinity and pH, and the inoculated bacteria were tested at different temperatures. The highest value ($P < 0.05$) of clearing zone for *B. subtilis* L10 and G1 strains against two pathogens was at 30 °C after 96 h of incubation (Figures 2 and 3) and (Supplementary material, Figure S1).

In terms of pH and temperature, the maximum antibacterial activity in this study was found in accordance with the suitable range for shrimp culture suggested by previous studies.^{22,23} Appropriate NaCl concentration for shrimp culture specially *L. vannamei* has been suggested previously at 10–15 ppt.²³ In this study, both strain showed the highest antibacterial activity at 1% salinity. However, the existence of antibacterial potential at 1% salinity might be efficient to suggest both candidate bacteria for *in vivo* experiment in culture with *L. vannamei*.

Spectrophotometric assay

Antibacterial activity of the strains was spectrophotometrically tested in MH broth prepared at the optimal conditions for antibacterial production. The two *B. subtilis* strains showed a stable activity to control the proliferation of both pathogens, *V. harveyi* and *V. parahaemolyticus*, ranging from approximately 0.6 to 0.8 OD after 24 h. The population of both pathogens increased in the negative controls for 24 h of challenge, ranging from 0.6 to 1.4 OD. However, bacterial pathogens treated with penicillin as positive control showed significant decreasing of population, ranging from 1 to 0.4 OD and 1.1 to 0.4 OD for *V. harveyi* and *V. parahaemolyticus*, respectively (Figure 4). Considering the optimal condition for antibacterial production which is in accordance with shrimp culture condition, both strains might be successful when administered as probiotic for *in vivo* experiment.

Characterization of the antibacterial substances

Bacteria are able to produce variety of antibacterial substances with a wide range of activities. Antimicrobial peptides such as bacteriocins or bacteriocin-like substances are considered among the most essentials because of being inexpensive, effective and non-toxic to animals and human.^{24–26} In fact, *Bacillus* spp. has been reported as one of the major producers of proteinaceous substances.²⁷ In this study, the antibacterial activity of sterile-filtered supernatants obtained from both strains was considerably reduced after being treated with enzymes (Table 2). These results indicate the

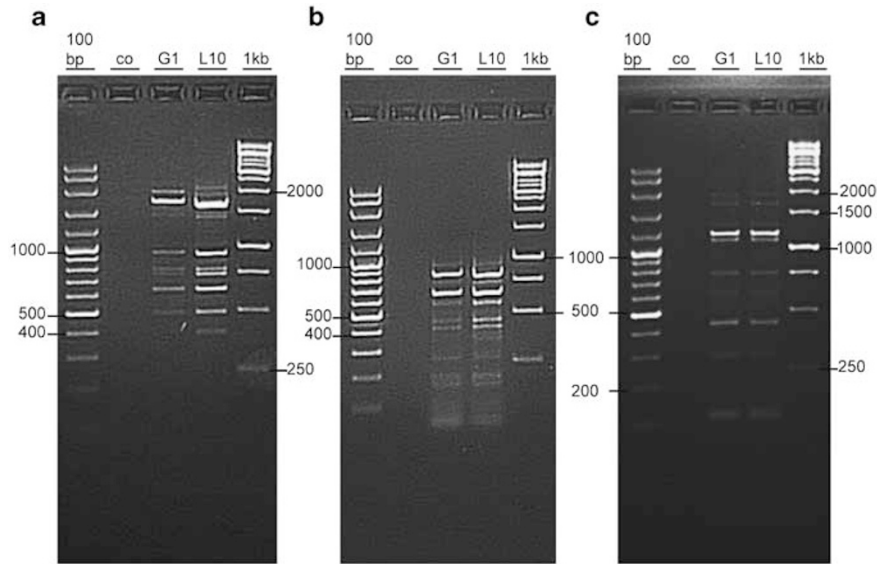


Figure 1 Rep-PCR patterns of both *B. subtilis* strains, L10 and G1, (a) BOX, (b) ERIC and (c) REP. 100 bp and 1 kb ladders were used as markers. Co denotes control for each PCR reaction (without DNA template).

Table 1 Antagonistic activity of the strains in different culture media against *V. harveyi*

Antagonism activity in different culture media (mm)								
Incubation period (h)	LB broth		MHB		MB		NB	
	L10	G1	L10	G1	L10	G1	L10	G1
48	ND	ND	ND	ND	ND	ND	ND	ND
72	8	8	6	6	6	6	6	6
96	9	9	8	7	7	6	6	6

Abbreviations: MB, marine broth; MHB, Muller Hinton broth; NB, nutrient broth; ND, Not detected

proteinaceous nature of the antibacterial substances produced by both strains. Therefore, bacteriocins or bacteriocin-like inhibitory substances might be responsible for controlling or inhibiting the two shrimp pathogen bacteria, *V. harveyi* or *V. parahaemolyticus* (Supplementary material, Figure S2).

Tolerances of antagonistic strains under different conditions

The sustainability of probiotic growth under different laboratory conditions is a common practice in order to evaluate the probiotic bacteria. For instance, the tolerance of *B. subtilis* to temperature has been reported ranging from 11 to 52 °C.²⁸ Another evidence demonstrated that *B. subtilis* E20, isolated from fermented soybean, has a broad tolerance for NaCl levels of 0–9%, pH value of 5–10, and temperatures of 10–50 °C.²⁹ In addition, an *in vitro* study showed a wide range tolerance of *Shewanella algae* to pH, NaCl and temperature, which introduced a good potential probiotic for shrimp aquaculture.³⁰ In the present study, the growth of *B. subtilis* L10 and G1 strains were tested on NA under different culture conditions, including temperature, pH and salinity. Both strains showed an optimum growth at temperatures between 20 and 50 °C, but not at 4, 10, 60 and 70 °C. A wide range of tolerance to pH was

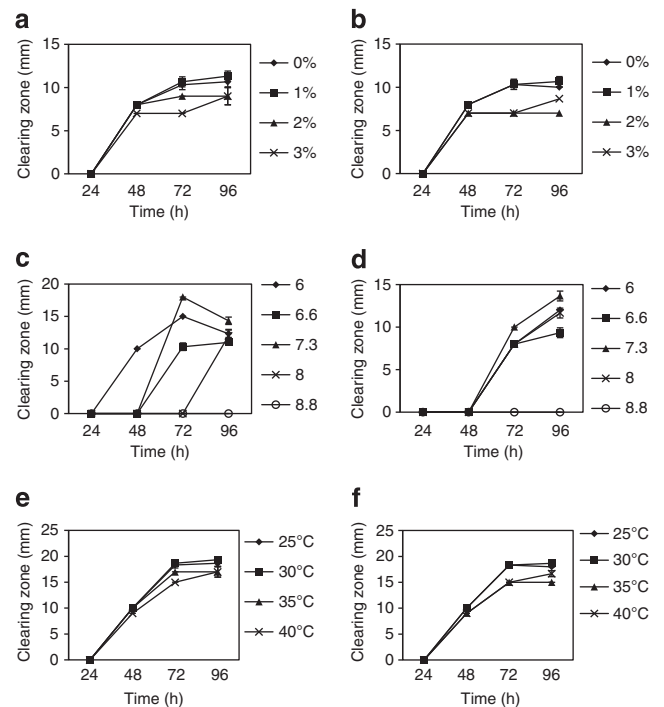


Figure 2 Antagonistic activity of *B. subtilis* L10 at different; (a) salinity against *V. harveyi*; (b) salinity against *V. parahaemolyticus*; (c) pH against *V. harveyi*; (d) pH against *V. parahaemolyticus*; (e) temperature against *V. harveyi*; (f) temperature against *V. parahaemolyticus*.

observed for both strains, *B. subtilis* L10 and G1, at pH 4 to 9. In addition, *B. subtilis* L10 and G1 strains were able to grow on all tested NA with NaCl up to 10%.

Safety of candidate strains to *L. vannamei*

Both strains, L10 and G1, were harmless to juvenile shrimp as no mortality was observed in experimental groups, and there was a total

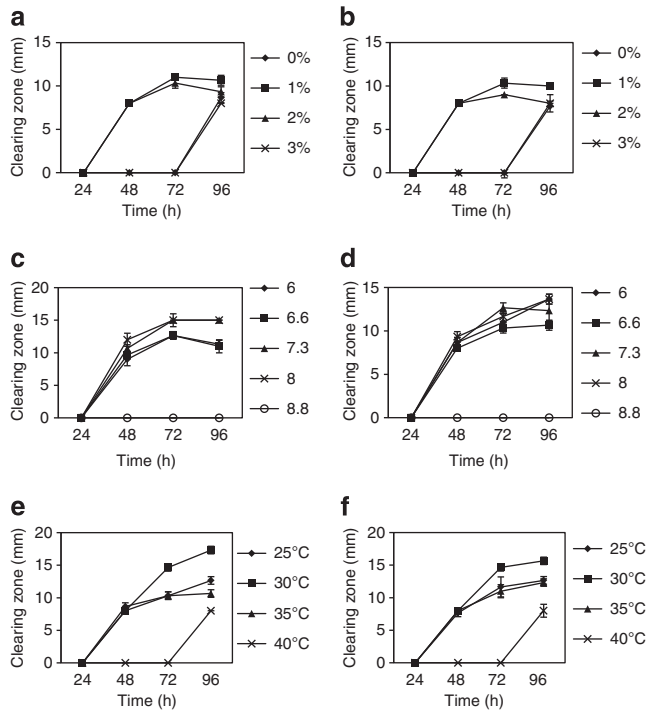


Figure 3 Antagonistic activity of *B. subtilis* G1 at different; (a) salinity against *V. harveyi*; (b) salinity against *V. parahaemolyticus*; (c) pH against *V. harveyi*; (d) pH against *V. parahaemolyticus*; (e) temperature against *V. harveyi*; (f) temperature against *V. parahaemolyticus*.

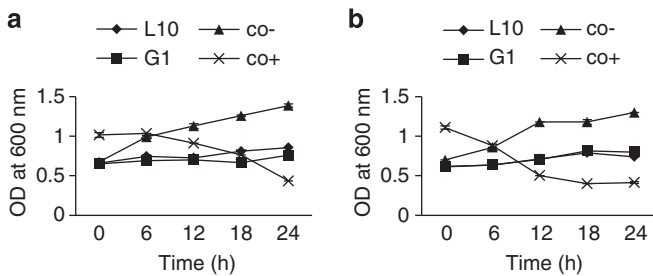


Figure 4 The inhibitory activity of sterile-filtered supernatant from each candidate bacterium at the optimal condition against; (a) *V. harveyi*; (b) *V. parahaemolyticus*. Co- presents as negative control (no addition of antibacterial in the broth culture), Co+ presents as positive control (addition of Penicillin in the broth culture). Bars are presented as s.d. generated from replicates.

absence of any disease signs. At the first day of experiment, bacterial counts on MYP agar showed a great number of *Bacillus* spp. in the body of shrimps treated with both strains compared with control (Figure 5). At day 15 of the experiment, the amount of *Bacillus* species in the shrimp body were considerably reduced (Figure 5) and no mortality or weakness was recorded. Moreover, the activity and appetite of shrimp treated with candidate strains were not affected as compared with the control group.

On the basis of these results and considering the increasing resistance to antibiotic treatment, *B. subtilis* L10 and G1 strains might be promising candidate for potential applications against vibriosis in shrimp, as it has been suggested that the gastrointestinal tract is a possible port of entry for *V. harveyi* and *V. parahaemolyticus*^{31,32} as well as a target for protective treatments, such as feeds containing

Table 2 Effect of different enzymes on antibacterial activity of the cell-free supernatants

Strain	Treatment	Residual activity (%)
L10	Untreated	100
	Proteinase K	50
	α -Amylase	71.43
	Trypsin	42.85
	Lysozyme	0
G1	Untreated	100
	Proteinase K	77.77
	α -Amylase	83.33
	Trypsin	55.55
	Lysozyme	44.44

The cell-free supernatants were treated with enzymes for 1 h and the residual antibacterial activity (%) was tested toward *V. harveyi* using well diffusion agar. Untreated indicates the cell-free supernatant without enzyme treatment.

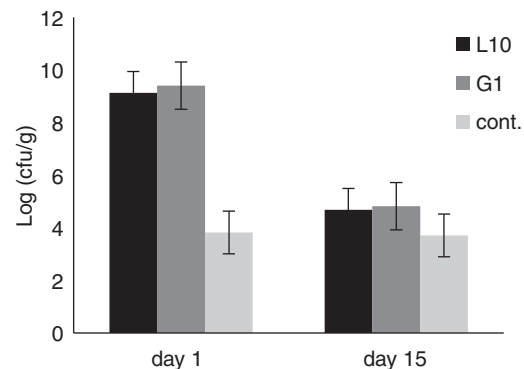


Figure 5 Total *B. subtilis* count using MYP agar at day 1 and 15 of safety experiment. L10, shrimps injected with strain L10 (10^{10} cfu ml⁻¹); G1, shrimps injected with strain G1 (10^{10} cfu ml⁻¹); Cont., shrimp injected with sterile PBS as control.

probiotic bacteria.^{33,34} Further experiments, including the application of both strains and shrimp challenged with pathogens, could provide valuable information of probiotic potential abilities of these candidates for shrimp aquaculture facilities.

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