Int. J. Aquat. Biol. (2022) 10(1): 32-44
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Inhibitory activity of probiotic Bacillus subtilis BF12 against *Vibrio parahaemolyticus* infection and its growth-promoting effects on juvenile *Penaeus monodon*

Efren E. Temario[1,3], Jerwin G. Baure[2], Emelyn Joy G. Mameloco*[3], Rowena E. Cadiz[3], Rex Ferdinand M. Traifalgar[3]

[1]Office of the City Agriculturist, Local Government Unit Kidapawan City, Cotabato, Philippines.
[2]Marine Science Institute, University of the Philippines Diliman, Quezon, Philippines.
[3]Institute of Aquaculture, College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miag-ao, Iloilo, Philippines.

Abstract: In an attempt to elucidate the Vibrio inhibitory activity of saline tilapia green water, we have isolated *Bacillus subtilis* BF12, exhibiting potent secreted antibiotic effects against *Vibrio parahaemolyticus.* We tested *B. subtilis* BF12 pathogenicity to *Penaeus monodon* and its efficacy to protect the shrimp against *V. parahaemolyticus* infection. The results indicate that *B. subtilis* BF12 is not pathogenic to shrimp since no mortalities was observed in all treatment groups. The feeding trial shows that shrimp in the treated group exhibited higher survival and improved growth performance. The infection challenge test with pathogenic *V. parahaemolyticus* administered orally indicates that the group receiving the probiotic has significantly higher survival rates. Lower counts of *V. parahaemolyticus* in the gut of the probiotic treated group were also recorded. Collectively our results indicate that the application of probiotic *B. subtilis* BF12 is an effective, practical and applicable means to prevent *V. parahaemolyticus* infection in *P. monodon* culture.

Article history:
Received 3 December 2021
Accepted 14 January 2022
Available online 25 February 2022

Keywords:
Pathogenicity
Shrimp
Probiotic
Disease

Introduction

The black tiger shrimp *Penaeus monodon* is among the important species in aquaculture. However, *P. monodon* production is constantly challenged by bacterial disease outbreaks mostly *Vibrio* spp., particularly *V. harveyi* and *V. parahaemolyticus* (FAO, 2018). Recently, a virulent strain of *V. parahaemolyticus* has been identified as the causative agent of Acute Hepatopancreatic Necrosis Disease (AHPND), a shrimp disease that first emerged in southern China in 2010 (Tran et al., 2013), and caused major setbacks in shrimp production in mainland Asia (Zorriehzahra et al., 2015). The disease was first documented to affect the early stages of *P. vannamei.* It can also infect all farmed shrimp species, including *P. monodon*, at every developmental stage (De la Pena et al., 2015).

The successful control and prevention of bacterial disease outbreaks in aquaculture, specifically in finfish farming using probiotics, is well-documented (Ringo, 2010). However, no probiotics are known to be a solution to all types of bacterial diseases. For emerging new bacterial diseases, there is a need to discover new and effective probiotics as a control agent. Also, rigorous testing of a presumptive probiotic to know its pathogenicity potential to the host is an important aspect of developing potent probiotics against novel disease agents. AHPND causing *V. parahaemolyticus* is a novel disease agent affecting the shrimp industry. There have been few or limited works published on developing bacterial probiotics against this pathogen. *Lactobacillus plantarum* T13 improved the resistance of *P. vannamei* against AHPND-causing *V. parahaemolyticus* (Nguyen et al., 2018). *Bacillus* species from *Fenneropenaeus penicillatus* and *P. monodon* gut exhibited antimicrobial activity against AHPND-causing *V. parahaemolyticus.* Moreover, a combination of these *Bacillus* strains with red seaweed *Gracilaria* sp. showed *in vitro* inhibition against *V. parahaemolyticus* through competitive exclusion (Lim et al., 2020).

* Correspondence: Emelyn Joy G. Mameloco
E-mail: egmameloco@up.edu.ph

The tilapia green water culture system in shrimp farming utilizes water from the rearing ponds of saline-tolerant tilapia at a salinity of 20-35 ppt. This culture system supports several species of green microalgae, including *Chlorella* spp. and *Nanochloropsis* spp., considered a mature microbial ecosystem, and documented to prevent and control the growth of pathogenic *Vibrio* species (Lio-Po et al., 2005; Wibowo et al., 2015; Sampollo et al., 2018). Further, the bacterial inhibitory activity of the tilapia green water system has been associated with the presence of bacteria, fungi, and algae that secretes inhibitory compounds to Vibrios. In the present work, we isolated *Bacillus* sp. from saline tilapia green water culture system and investigated its efficacy as a probiotic to inhibit the growth of pathogenic *V. parahaemolyticus in vitro* and *in vivo* conditions.

Materials and Methods

Isolation and identification of probiotic bacteria: Water samples (Temp: 32°C; salinity: 27 g L^{-1}; pH: 8.1) were collected from the shrimp ponds in the saline tilapia green water culture system. The samples were serially diluted up to the 9^{th} dilution, and 100 µL aliquot was spread-plated on Luria Bertani (LB) culture media. After 24 hours of incubation at 30°C, the bacterial colonies growing on the culture media were individually isolated based on their morphology (Ruangpan and Tendencia, 2004). To test the antagonistic activity against AHPND-causing *V. parahaemolyticus* (Dabu et al., 2017), the spot-on-the-lawn method was used. Individual colony of the test bacterial isolate was spotted on to the lawn of *V. parahaemolyticus* on LB media containing 2% NaCl. Zones of inhibition were measured after 24 hours of incubation at 30°C (Barcenal et al., 2015). The isolate exhibiting the largest zone of inhibition (Isolate BF12) was selected, and further tests were performed for its probiotic potential against *V. parahaemolyticus.*

Identification of the isolate BF12 was made through morphological and biochemical characterization and 16s rDNA gene sequence analysis. For the 16s rDNA gene analysis, the bacterial genomic DNA was extracted using Wizard® Plus SV Minipreps DNA Purification System (Promega Corporation) according to the manufacturer's protocol. The 16s rDNA gene was amplified by PCR (T100^{TM}; Bio-Rad Laboratories, Inc.) using universal primers 27F: 5'-AGAGTTTGATCCTGGCTCAG (Lane, 1991) and 1492R:5'-TACCTTGTTACGACTT (Turner et al., 1999). Sequencing of the 16s rDNA gene was performed by Macrogen (South Korea,) and the resulting sequences were compared with other microorganisms in Genbank using the National Center for Biotechnology Information-Basic Local Alignment Search Tool (NCBI-BLAST) program.

Optimal salinity and pH for the antagonistic activity against *V. parahaemolyticus*: The optimal conditions for the antagonistic activity and growth of the isolated bacteria were assessed. LB agar was prepared at different pH (5, 6, 7, and 8) and salt concentrations (0, 5, 15, 25, and 35 ppt) (Vijayan et al., 2005). Phosphate buffer was used to prepare LB agar at different pH levels. The spot-on-the-lawn antibacterial assay evaluated the *V. parahaemolyticus* inhibitory activity of the tested probiotic at different pH and salt concentrations (Barcenal et al., 2015). The optimal salinity and pH for the growth of the presumptive probiotic bacteria were assessed using the spread plate method. A 24-hour broth culture of the isolate was serially diluted and spread into LB agar at different salinities and pH. The number of colonies was counted after 24 hours of incubation and the total bacterial count was calculated.

Pathogenicity test of BF12 to *P. monodon*: Assessment of the pathogenicity of BF12 to shrimp was done by immersion method (Chythanya et al., 2002; Tran et al., 2013). 24-hour culture of BF12 was harvested using a sterile loop and increasing levels of probiotic suspension (0, 10^6, 10^8 and 10^{10} cfu ml^{-1}) were prepared. Bacterial concentration was estimated using McFarland Standards.

Healthy shrimp without prior antibiotic treatment were used in the experiment and were initially

evaluated to be free from Vibrio pathogens. Sixty shrimp per treatment were immersed in 3 L of the different levels of probiotic suspension for 1 hour. Following the immersion, 10 shrimps per treatment were collected from the pathogen immersion tanks and distributed into twelve 50 L tanks filled with UV filtered seawater and supplied with aeration. The animals were fed with commercial formulated shrimp feeds (Oversea Feeds Corporation, Philippines, Vannamei Feeds No. 1 (VF1) and were observed for three weeks for disease manifestation and mortalities. Monitoring of water parameters and recording of shrimp mortality was performed on a daily basis.

Gut colonization assay: To test the inhibitory property of BF12 against *V. parahaemolyticus* in shrimp gut, 10 juvenile *P. monodon* (1.1±0.1 g) were randomly distributed into six 30 L tanks containing 25 L UV-treated seawater with three tanks designated as a treated group and the other three tanks served as the control group. The treated group was fed a BF12-supplemented diet, and the other group was fed a control diet (extruded pelletized diet). Both groups were fed *V. parahaemolyticus* supplemented diets. For the preparation of BF12 supplemented diet, 1 ml of 10^9 cfu ml^{-1} probiotic suspensions was sprayed and thoroughly mixed in 1 g commercial *P. monodon* feed (Oversea Feeds Corporation, Philippines, Vanname Feeds No. 1) followed by air drying under aseptic conditions for 15 min (Panigrahi et al., 2004). Similarly, the diet containing the pathogen *V. parahaemolyticus* was prepared by mixing 1 ml of 10^6 cfu ml^{-1} *V. parahaemolyticus* suspension in 1 g of the commercial feed (Dabu et al., 2017). Sterile normal saline solution (NSS; 8.5 g NaCl L^{-1} distilled water) served as the diluent of the bacterial suspensions. Commercial feed sprayed with sterile NSS served as the control diet. Shrimp in the probiotic group were fed *V. parahaemolyticus* diet in the morning and BF12-supplemented diet in the afternoon, while shrimp in the control group were fed *V. parahaemolyticus* diet in the morning and a control diet in the afternoon. Optimal levels of physicochemical parameters were maintained throughout the trial. Shrimp have randomly sampled from each tank replicate for bacterial enumeration daily. The feeding experiment lasted for six days.

Bacterial enumeration followed the method described by Thakur et al. (2004) with some modifications. Shrimp were surface disinfected by swabbing with 70% alcohol. The gut was aseptically removed and was placed in a pre-weighed 1.5 ml Eppendorf tube. The sample was homogenised in sterile NSS (0.1 g sample: 900 µL NSS). One-hundred µL of 10^{-1} and 10^{-2} dilutions were spread onto Vibrio Chromogenic Agar (CONDA Pronadisa) for *V. parahaemolyticus* enumeration, and 10^{-3} and 10^{-4} dilutions were spread onto NA (CONDA Pronadisa) for the viable probiotic count. The plates were incubated at 30°C, and colonies were counted 24-48 hours after incubation. The bacterial counts were reported as cfu g^{-1} gut tissue.

Shrimp feeding trial for growth response evaluation: *Penaeus monodon* PL-10 were purchased from a local hatchery and reared to PL-30 stage (0.1±0.02 g) in laboratory conditions at the facilities of UPV Multi-Species hatchery. One hundred twenty *P. monodon* were stocked in six 50L capacity containers filled with 40 L filtered seawater. The shrimp were divided into two treatments administered with test diets described in the gut colonization assay. Post-larvae in group 1 were fed BF12-supplemented diet and the other group with the control diet. Shrimp were fed three times daily at 15% of average body weight and was adjusted every after sampling. Uneaten feeds were removed 2 hours every after feeding and 50% water change was done daily. Water quality was maintained in optimal conditions (salinity: 30 ppt; pH: 8.0-8.2; Dissolve Oxygen: 5-7ppm; temp 26-28°C) as recommended by Ramanathan et al. (2005). The feeding trial was conducted for 30 days, and mortality was recorded daily. On days 0, 5, 10, 15, 20, 25, and 30, shrimp were randomly sampled from each replicate to monitor and quantify the *V. parahaemolyticus* and the probiotic bacteria in the shrimp gut. Bacterial

enumeration was done following the method described earlier. At the end of the experiment, shrimp under the different treatment groups were weighed to evaluate their growth performance. Growth parameters were measured using the following formula (Hardy and Barrows, 2002).

***Vibrio parahaemolyticus* infection:** Following the feeding trial, 30 shrimp were collected per treatment and subjected to an infection challenge test with the pathogenic *V. parahaemolyticus.* The pathogen bacterial concentration used in the challenge test was previously optimized to determine the LC_{50} dose of the pathogen (Dabu et al., 2017). The shrimp were stocked in tanks containing 30 L UV filtered seawater. Water parameters were maintained in optimal condition (salinity: 30 ppt; temp: 28°C) (Ramanathan et al., 2005). Shrimp in all treatments were fed *V. parahaemolyticus* at 10^6 cfu g^{-1} of commercial feed in the morning and a control diet in the afternoon. Mortality was recorded daily. At the end of the experiment, shrimp were collected and *V. parahaemolyticus* in shrimp gut was quantified following the method described earlier.

Data analysis: All statistical analyses were performed using SPSS17 with a significance level set at 0.05. One-Way Analysis of Variance (ANOVA) was done to compare differences in the means of zones of inhibition, total bacterial counts in vitro, and survival during the pathogenicity test. The results with significant differences were subjected to post-hoc analysis using Duncan's test. To compare differences in the survival, growth performance, and bacterial count during the gut colonization assay, feeding trial, and *V. parahaemolyticus* infection, independent samples t-test was used. Percent survival and growth rate were arcsin transformed while bacterial counts were log-transformed before analyses.

Results

Based on the results, out of the ten isolates, only 2 were antagonistic against *V. parahaemolyticus.* These bacteria were isolates BF12 and GW4 (Fig. 1). Among the isolates, BF12 displayed the highest zone of inhibition indicating a higher antagonistic activity against *V. parahaemolyticus* and then selected for further evaluation. BF12 was found to be a spore-forming, gram-positive, rod-shaped bacterium that is oxidase negative and capable of gelatin liquefaction and sugar fermentation which indicates that the isolate is a member of the genus *Bacillus*. The 16s rDNA sequence identified BF12 as *Bacillus subtilis* with 99.16% homology with other *B. subtilis* strains (Fig. 2) The sequence of *B. subtilis* BF12 has been deposited in Genbank under accession number OK614112.

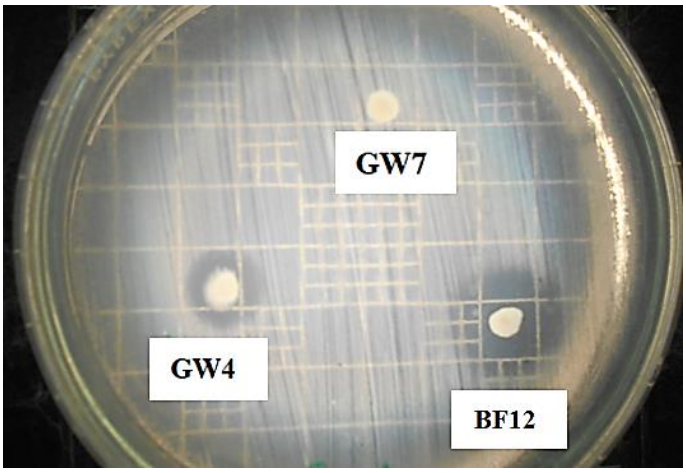

Figure 1. Antagonistic activity of isolated bacteria against *Vibrio parahaemolyticus.*

BF12 was able to exhibit antagonism against *V. parahaemolyticus* at all salinities tested, but the highest antagonistic activity was documented at 0 ppt (Fig. 3). Antagonistic activity decreased significantly when salinity was increased to 5 ppt but showed no significant difference when salinity was increased further. The probiotic isolate BF12 could grow at all salinities with optimal growth observed at 0 to 5 ppt (Fig. 4). Growth decreased significantly when salinity was increased to 15 ppt but showed no significant difference when salinity was increased further. Moreover, BF12 exhibited strong antagonism against *V. parahaemolyticus* at all pH tested except at pH 5 (Fig. 5). The highest antagonistic activity was found at a pH of 7. *Bacillus subtilis* BF12 is able to grow at different pH except pH 5 (Fig. 6).

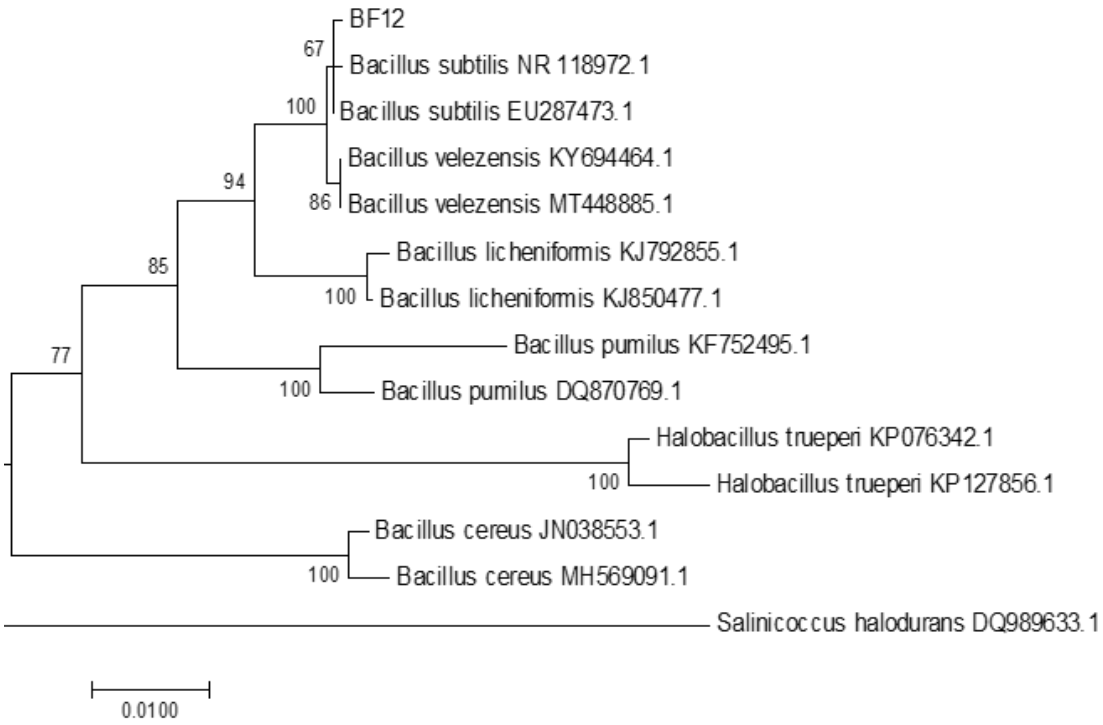

Figure 2. Phylogenetic tree based on partial 16S ribosomal RNA sequences showing evolutionary relationships between strain BF12, other members of Bacillus and halophilic strains from other groups. The tree was constructed using the neighbor-joining method. Names of the different groups along with the accession numbers are shown next to the taxa. Bootstrap values (2000 replications) are indicated at the interior branches.

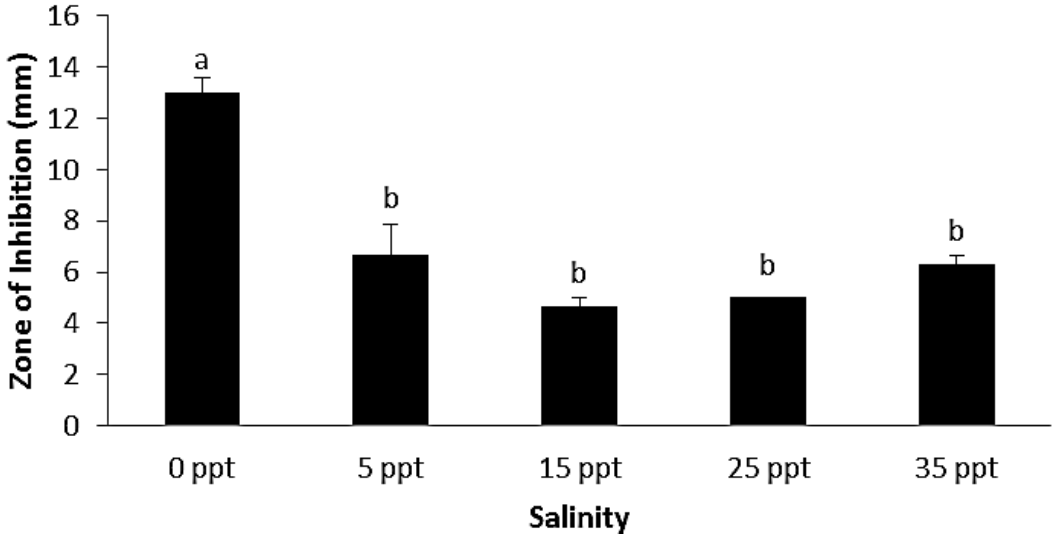

Figure 3. Antagonistic activity against *Vibrio parahaemolyticus* of BF12 at different salinities. Bars with different labels are significantly different at $P<0.05$.

Survival of *P. monodon* was not affected when they were exposed to increasing levels of BF12. No mortality was recorded in all groups even in the highest level of BF12 at 10^{10} cfu ml^{-1}. During the colonization assay, a significantly higher *V. parahaemolyticus* count was recorded in the control group from day-2 through the end of the colonization assay (Fig. 7). Furthermore, viable BF12 colonies from the gut of the probiotic-supplemented shrimp ranged from $2.91\pm0.64\times10^{7}$ cfu g^{-1} to $7.84\pm0.70\times10^{7}$ cfu g^{-1}.

Significantly enhanced growth performance of shrimp fed the BF12-supplemented diets shrimp was recorded (Fig. 8). Moreover, the SGR and PER of

Table 1. Performance of *Penaeus monodon* under different treatment during feeding trial. All values were presented as mean ±SEM.

	Control	BF12
Final Weight (g)	0.42±0.03[b]	0.51±0.03[a]
Weight Gain (g)	0.32±0.30[b]	0.41±0.28[a]
Specific Growth Rate (SGR)	3.81±0.12[b]	4.90±0.04[a]
Feed Conversion Ratio (FCR)	2.31±0.11[b]	2.05±0.05[a]
Protein Efficiency Ratio (PER)	0.02±0.00[b]	0.06±0.00[a]

*Values within a column with different superscripts are significantly different at $P<0.05$.

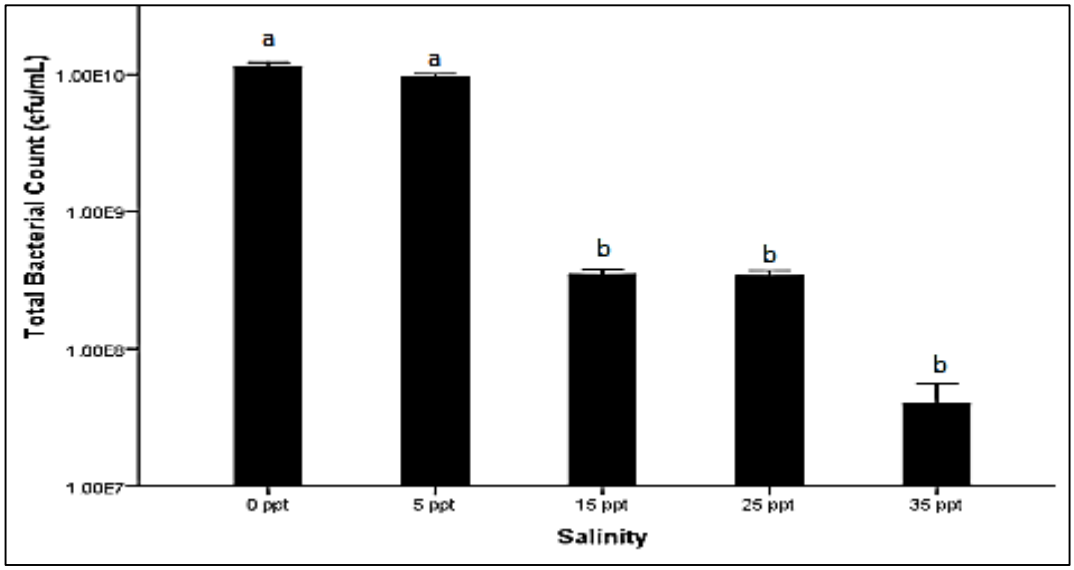

Figure 4. Growth of BF12 at different salinities. Bars with different labels are significantly different at $P<0.05$.

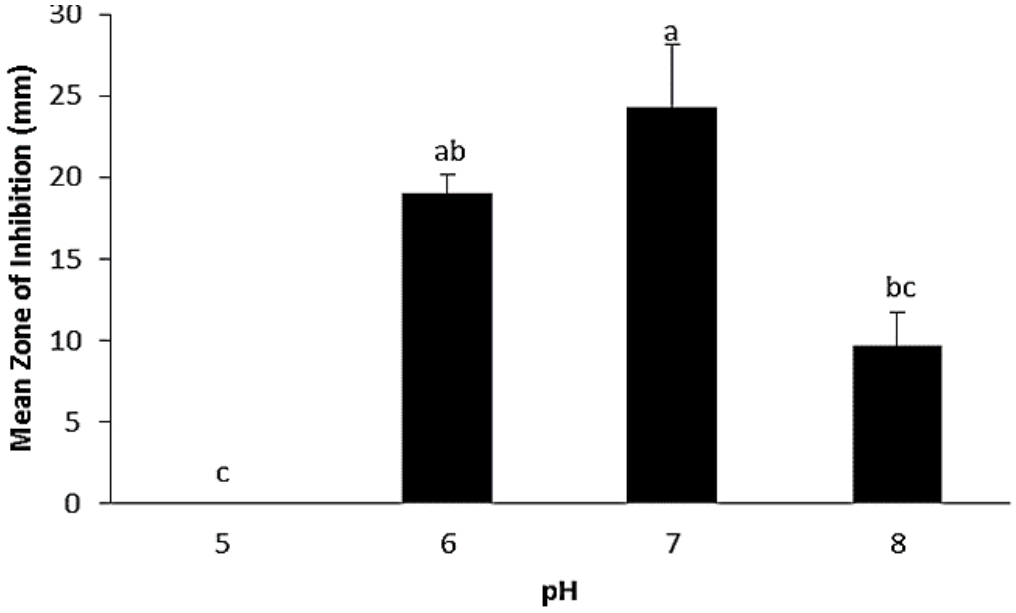

Figure 5. Antagonistic activity against *Vibrio parahaemolyticus* of BF12 at different pH (mean±SEM). Values with different labels are significantly different at $P<0.05$. No antagonistic activity was observed in pH 5, while zone of inhibition at pH 6, 7 and 8 are 19.00±1.15, 24.33±3.84, and 9.67±2.03 mm, respectively.

BF12-supplemented *P. monodon* were significantly higher while the FCR was significantly better than the control shrimp (Table 1). The *V. parahaemolyticus* counts in shrimp gut were significantly higher in the control group as compared to the BF12-supplemented group (Fig. 9). *Vibrio*

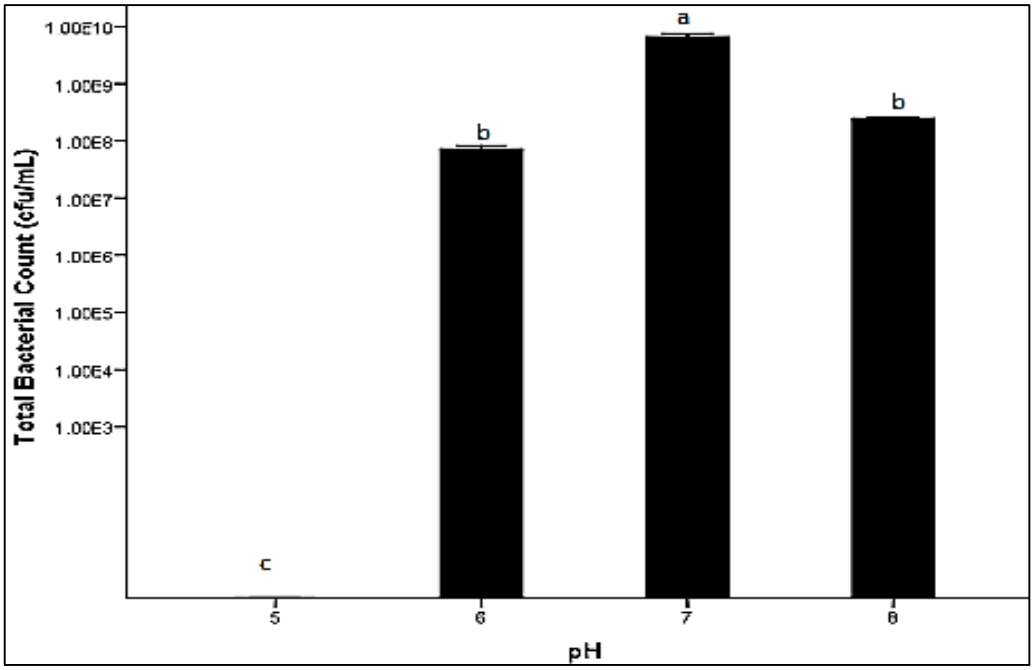

Figure 6. Growth of BF12 at different pH (mean±SEM). Bars with different labels are significantly different at $P<0.05$.

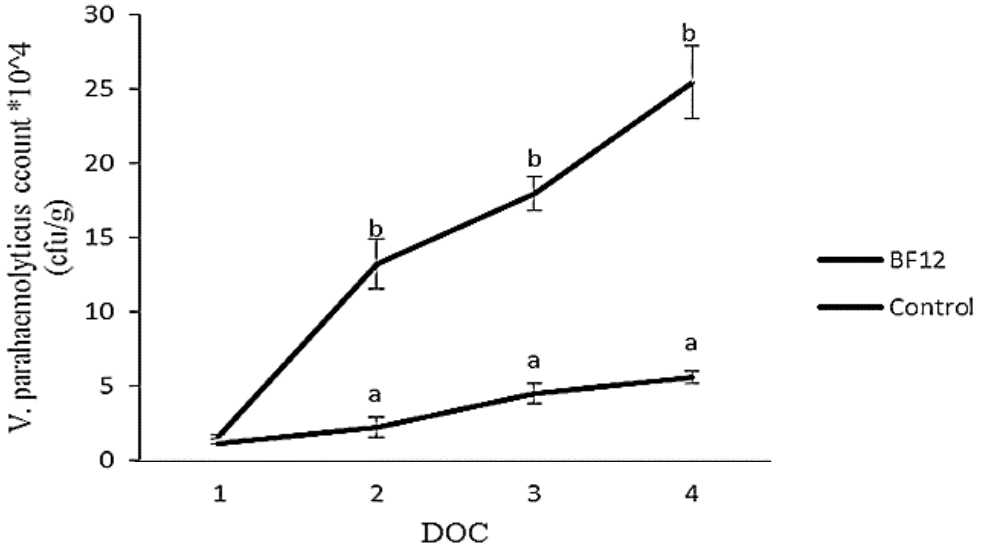

Figure 7. *Vibrio parahaemolyticus* count during the gut colonization assay (mean±SEM). Values with different labels are significantly different at $P<0.05$.

parahaemolyticus count decreases in the probiotic treated group as the trial period progresses. *Vibrio parahaemolyticus* were no longer present, with zero counts, in the probiotics treated shrimp starting at DOC 10 until the end of the trial. Moreover, viable probiotic BF12 colonies recovered from the gut of the shrimp fed with BF12-supplemented diet ranged from $1.27\pm1.40\times10^{7}$ cfu g^{-1} to $6.36\pm0.92\times10^{8}$ cfu g^{-1}. Higher survival was recorded in shrimp fed withBF12-supplemented diet compared to the control group (Fig. 10) ($P<0.05$).

During the infection test, the survival of the BF12-supplemented shrimp was significantly higher than the control group (Fig. 11). *Vibrio parahaemolyticus* count in shrimp gut was significantly reduced in the BF12-supplemented group compared to the control group (Fig. 12). The *V. parahaemolyticus* count in the gut of probiotic-

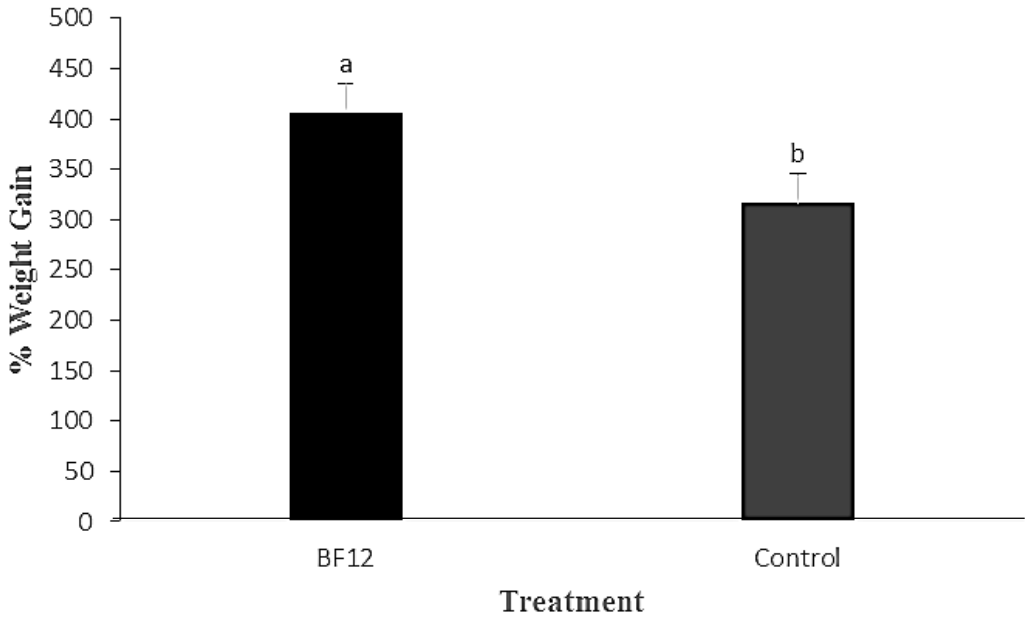

Figure 8. Percent weight gain of *Penaeus monodon* during the feeding trial (mean±SEM). Values with different labels are significantly different at $P<0.05$.

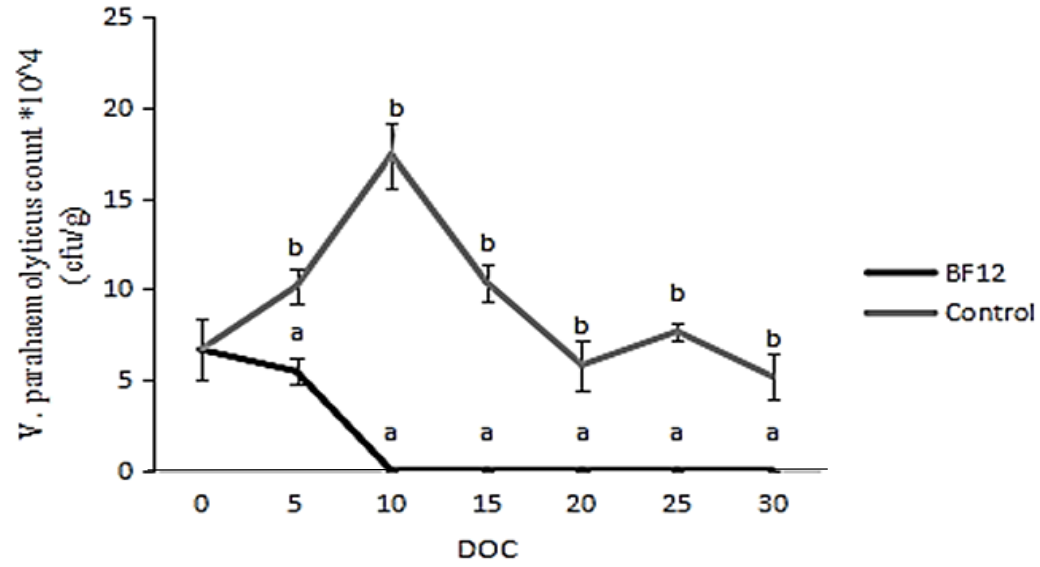

Figure 9. *Vibrio parahaemolyticus* count during the feeding trial (mean±SEM). Values with different labels are significantly different at $P<0.05$.

supplemented shrimp was 1 log lower than the control group at the end of the experiment.

Discussion

The present study proves the efficacy of *B. subtilis* BF12 isolated from saline tilapia green water against pathogenic *V. parahaemolyticus* both *in vitro* and *in vivo*. Antagonism assays show that *B. subtilis* BF12 is able to antagonize *V. parahaemolyticus* in freshwater conditions, but is also able to perform antagonism in brackish and seawater. *Bacillus subtilis* BF12 grows best at a neutral pH but can tolerate the acidic environment of the gut and the alkaline environment in the ponds (Barcenal et al., 2015). The growth and antagonistic activity against *V. parahaemolyticus* of *B. subtilis* BF12 over a wide range of pH and salinities have confirmed its probiotic potential in shrimp culture. The present findings are in agreement with the results of Vaseeharan and Ramsamy (2003) wherein *B. subtilis*

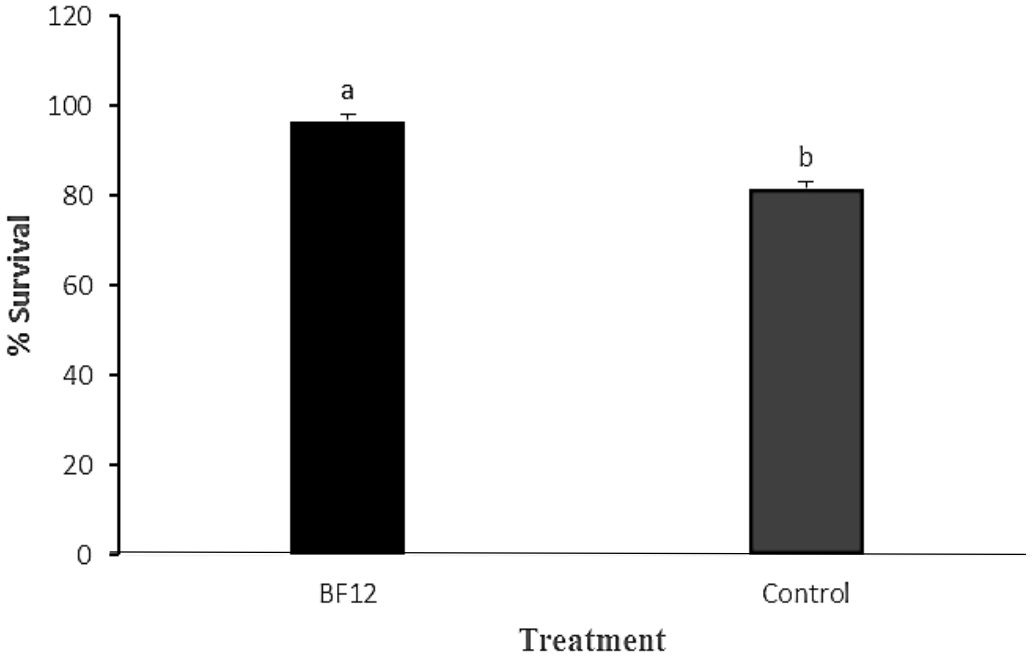

Figure 10. Percent survival of *Penaeus monodon* during the feeding trial (mean±SEM). Values with different labels are significantly different at $P<0.05$.

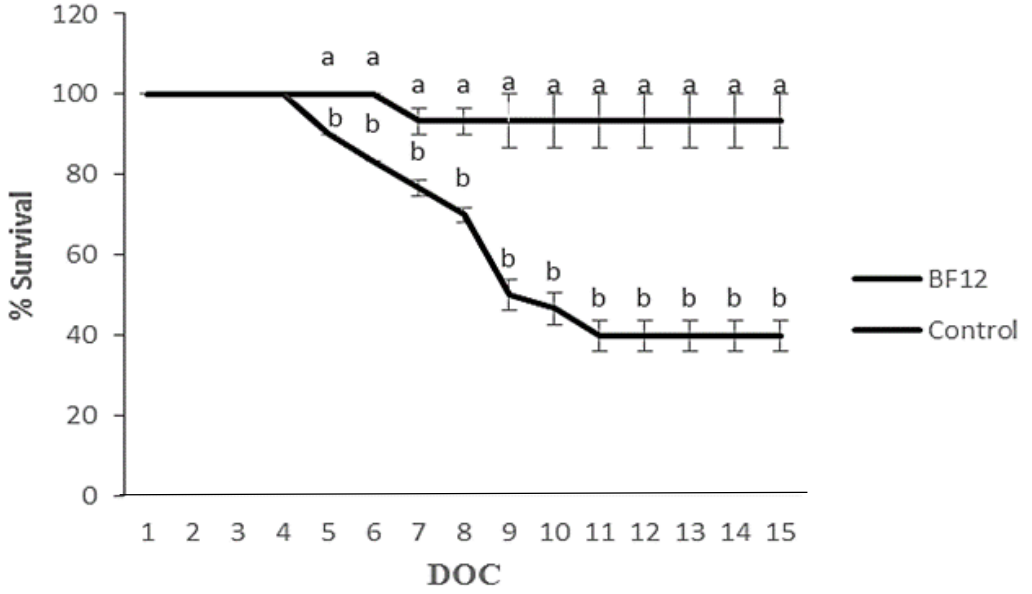

Figure 11. Percent survival of *Penaeus monodon* during the infection challenge.

BT23 showed inhibitory activity against *V. anguillarium*, *V. harveyi* and *V. vulnificus* obtained from *P. monodon* culture ponds. In addition, filtered supernatant of *B. subtilis* UTM126 exhibited inhibitory activity against *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus* (Balcázar and Rojas-Luna, 2007). *Bacillus subtilis* IPA S5.1 isolated from *P. vannamei* also showed inhibitory activity against *V. alginolyticus* and *V. parahaemolyticus* (Interaminense et al., 2018).

The pathogenicity test has confirmed that *B. subtilis* BF12 is not pathogenic to shrimp as no mortality and other disease symptoms were observed in all shrimp exposed to the different levels of *B. subtilis* BF12. In earlier reports, *Bacillus* sp. has been shown to cause no harmful effects on the fairy shrimp in an immersion challenge test for 72 hours (Purivirojkul, 2013). Also, 100% survival of

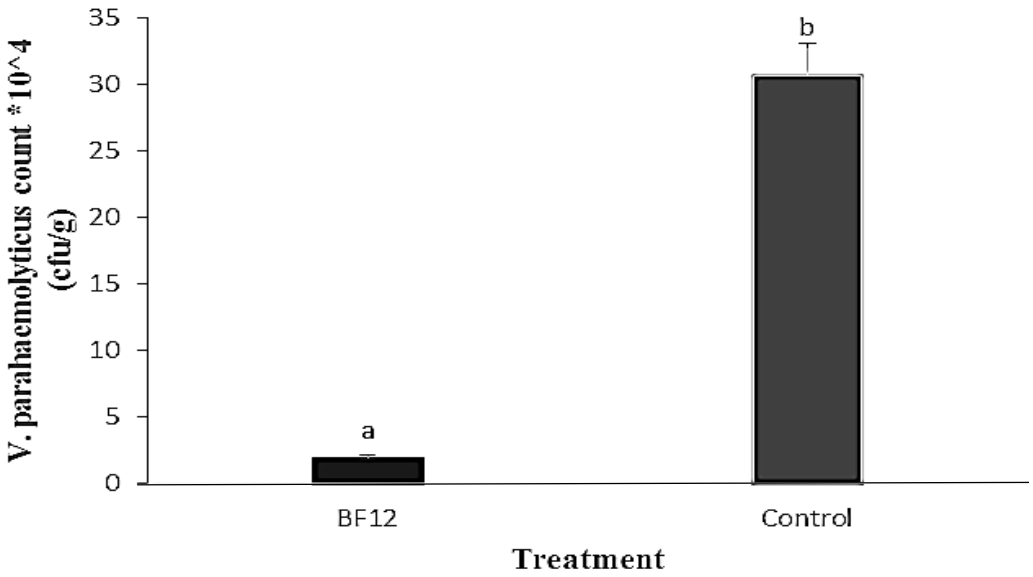

Figure 12. *Vibrio parahaemolyticus* count during the infection challenge. Values with different labels are significantly different at $P<0.05$.

P. vannamei was obtained after feeding with diet containing *B. subtilis subtilis* (Sanchez-Ortiz et al., 2015). Pathogenicity of *Bacillus* spp. has also been tested in finfishes. Newaj- Fyjul et al. (2007) reported that *B. subtilis* AB1 has not caused any harmful effect on rainbow trout following administration via injection or feeding. Moreover, another *Bacillus* sp. was tested in *Labeo rohita* without any harmful effects, disease symptoms, or mortality to the fish (Ramesh and Souissi, 2018).

Virulent strains of *V. parahaemolyticus* manifest their pathogenicity by colonizing and multiplying in the shrimp gut (Boonthai et al., 2011). This poses a major concern as *Vibrio* spp. infection has been reported to cause slow growth and low survival in shrimp (Lafferty et al., 2015). Elimination of this pathogenic bacterium in the gut would benefit the host animal. The present study showed strong evidence that *B. subtilis* BF12 could eliminate and prevent the growth of pathogenic *V. parahaemolyticus* in the gut by colonizing the gastrointestinal tract of the shrimp. This result could explain the enhanced weight gain, SGR, PER, FCR and survival of *B. subtilis* BF12-supplemented shrimp as reduced *V. parahaemolyticus* in the gut was recorded during the feeding trial. Similarly, Balcazar et al. (2007) have shown that administration of *B. subtilis* UTM 126 in shrimp has prevented *V. parahaemolyticus* infection thereby improving the weight gain, feed utilization, and survival of the experimental animals. Rengpipat et al. (2003) have also demonstrated that *Bacillus* sp. BS11 mixed in the commercially formulated feed has significantly reduced *V. harveyi* in the gut of *P. monodon*, enhancing their growth and survival. Moreover, strains of *B. subtilis* (L10 and G1) isolated from fermented pickles have improved the water quality, growth performance, and resistance against *V. harveyi* of the shrimp (Zokaeifar et al., 2014).

The low *V. parahaemolyticus* in the gut of *P. monodon* supplemented with *B. subtilis* BF12 during the infection challenge confirms the inhibitory activity of the probiotic bacterium against the pathogenic *V. parahaemolyticus.* The antibacterial effect of *B. subtilis* BF12 could be due to strong cellular lytic activity resulting from the release of chemical substances such as antibiotics, proteases, siderophores, and bacteriocins with bactericidal or bacteriostatic effects on pathogenic bacterium (Verschuere et al., 2000). This mode of probiotic action has been proven by Xu et al. (2013) who reported that *B. subtilis* NT6 isolated from a Chinese traditional fermented-soybean paste can produce antimicrobial peptides (AMP's) with high inhibitory activity against *V. parahaemolyticus.*

Additionally, outer membrane protein (OMP) of *B. subtilis* AN11 has produced antibiotics exhibiting inhibitory effects against major aquaculture bacterial pathogens, including *V. parahaemolyticus* (Das et al., 2014). Recently, Doroteo et al. (2018) demonstrated that *Bacillus* spp. isolated from tilapia mucus have exhibited extracellular protease enzyme capable of inhibiting the growth of pathogenic *V. harveyi.* Supplementation of *B. subtilis* BF12 in the diet improved the survival of *P. monodon* against *V. parahaemolyticus* infection. The protective effects of BF12 to *P. monodon* against *V. parahaemolyticus* infection could be attributed to the strong gut colonization property of the probiotics and its ability to secret bacteriostatic compounds against *V. parahaemolyticus.* Administration of *B. subtilis* BT23 reduced the mortality of *P. monodon* when exposed to *V. harveyi* (Vaseeharan and Ramsamy, 2003). A reduction in mortality against *V. harveyi* was also observed when *L. vannamei* was fed *B. subtilis* UTM 126 (Balcázar and Rojas-Luna, 2007). *Penaeus vannamei* fed *B. subtilis* AQAHBS001 significantly increased resistance against AHPND-causing *Vibrio parahaemolyticus* (Kewcharoen and Srisapoome, 2019).

Conclusion

The present study has provided evidence that bacterial isolate from saline tilapia green water could be used as a probiotic to inhibit the growth of the pathogenic *V. parahaemolyticus* in the shrimp gut. Furthermore, better growth and survival of juvenile shrimp occurred due to the reduced *V. parahaemolyticus* in the gut of *P. monodon.* The application of *B. subtilis* BF12 could be an effective strategy in controlling and preventing *V. parahaemolyticus* infection in *P. monodon* culture.

Acknowledgments

This study was funded by the Department of Science and Technology- Philippine Council for Agriculture, Aquatic and Natural Resources Research and Development (DOST-PCAARRD)- Shrimp Project 8.

References

Balcazar J.L., Rojas-Luna T. (2007). Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against vibrio species confers protection against Vibriosis in juvenile shrimp (*Litopenaeus vannamei*). Current Microbiology, 55: 409-412.

Balcazar J.L., Rojas-Luna T., Cunningham D. (2007). Effect of the addition of four potential probiotic strains on the survival of Pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus.* Journal of Invertebrate Pathology, 96: 147-150.

Barcenal A.R., Traifalgar R.F., Corre V. Jr. (2015). Anti-*Vibrio harveyi* property of *Micrococcus luteus* isolated from rearing water under biofloc technology culture system. Current Research in Bacteriology, 8(2): 26-33.

Boonthai T., Vuthiphandchai V., Nimrat S. (2011). Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (*Penaeus mondon*). Aquaculture Nutrition, 17: 634-644.

Dabu I.M., Lim J.J., Arabit P.M., Orense S.J.A., Tabardillo J.A., Corre V.L., Maningas M.B. (2017). The first record of acute hepatopancreatic necrosis disease in the Philippines. Aquaculture Research, 48(3): 792-799.

Das B.K., Neha Nidhi R., Roy P., Muduli A.K., Swain P., Mishra S.S., Jayasankar P. (2014). Antagonistic activity of cellular components of *Bacillus subtilis* AN11 against bacterial pathogen. International Journal of Current Microbiology and Applied Sciences, 3(5): 795-809.

De la Peña L.D., Cabillon N.A., Catedral D.D., Amar E.C., Usero R.C., Monotilla W.D., Calpe A.T., Fernandez D.D., Saloma, C.P. (2015). Acute hepatopancreatic necrosis disease (AHPND) outbreaks in *Penaeus vannamei* and *Penaeus monodon* cultured in the Philippines. Diseases of Aquatic Organisms, 116(3): 251-254.

Doroteo A., Pedroso F., Lopez J.D., Apines-Amar M.J. (2018). Evaluation of potential probiotics isolated from saline tilapia in shrimp aquaculture. Aquaculture International, 26: 1095-1107.

Food and Agriculture Organization of the United Nations (FAO) (2006-2018a). *Penaeus monodon* (Fabricius,

1798). In: Cultured Aquatic Species Information Programme. Retrieved 14 May 2018 at FAO Fisheries and Aquaculture Department (http://www.fao.org /fishery/culturedspecies/Penaeus_monodon/en).

Food and Agriculture Organization of the United Nations (FAO) (2006-2018b). *Penaeus vannamei* (Boone, 1931). In: Cultured Aquatic Species Information Programme. Retrieved 14 May 2018 at FAO Fisheries and Aquaculture Department. (http://www.fao.org /fishery/culturedspecies/Penaeus_vannamei/en).

Hardy R. Barrows F. (2002). Diet formulation and manufacture. In: J. Halver, R. Hardy (Eds.). Fish Nutrition 3rd Edition. Academic Press, United States of America. pp: 505-600

Interaminense J.A., Vogeley J.L., Gouveia C.K., Portela R.W.S., Oliveira J.P., Andrade H.A., Peixoto S.M., Soares R.B., Buarque D.S., Bezerra R.S. (2018). In vitro and in vivo potential probiotic activity of *Bacillus subtilis* and *Shewanella algae* for use in *Litopenaeus vannamei* rearing. Aquaculture, 488: 114-122.

Kewcharoen W., Srisapoome P. (2019). Probiotic effects of *Bacillus* spp. From Pacific white shrimp (*Litopenaeus vannamei*) on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains). Fish and Shellfish Immunology, 94: 175-189.

Lane D.J. (1991) 16S/23S rRNA sequencing. In: E. Stackebrandt, M. Goodfellow (Eds). Nucleic acid techniques in bacterial systematics. New York, NY: John Wiley & Sons, Inc. pp: 115-175.

Lim S.Y., Loo K.W., Wong W.L. (2020). Synergistic antimicrobial effect of a seaweed-probiotic blend against acute hepatopancreatic necrosis disease (AHPND)-causing *Vibrio parahaemolyticus*. Probiotics Antimicrob Proteins. 12(3): 906-917.

Lio-Po G.D., Leano E.M., Penaranda M.D., Villa-Franco A.U., Sombito C.D., Guanzon N.G. (2005). Anti-luminous *Vibrio* factors associated with the green water grow-out culture of the tiger shrimp *Penaeus monodon*. Aquaculture, 250: 1-7.

Lafferty K.D., Harvell C.D., Conrad J.M., Friedman C.S., Kent M.L., Kuris A.M., Powell E.N., Rondeau D., Saksida S.M. (2015). Infectious diseases affect marine fisheries and aquaculture economics. Annual Review of Marine Science, 7: 471-496.

Nguyen T.T.G., Nguyen T.C., Leelakriangsak M., Pham T.T., Pham Q.H., Lueangthuwapranit C., Nguyen V.D. (2018). Promotion of *Lactobacillus plantarum* on growth and resistance against acute hepatopancreatic necrosis disease pathogens in white-leg shrimp (*Litopenaeus vannamei*). The Thai Journal of Veterinary Medicine, 48(1): 19-28

Panigrahi A., Kiron V., Kobayashi T., Puangkaew J., Satoh S., Sugita H. (2004). Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. Veterinary immunology and immunopathology, 102: 379-388.

Purivirojkul W. (2013). Application of probiotic bacteria for controlling pathogenic bacteria in fairy shrimp *Branchinella thailandensis* culture. Turkish Journal of Fisheries and Aquatic Science, 13: 187-196.

Ramanathan N., Padmavathy P., Francis T., Athithian S., Selvaranjitham N. (2005). Manual on polyculture of tiger shrimp and carps in freshwater. Tamil Nadu Veterinary and Animal Sciences University, Fisheries College and Research Institute, Thothukudi. 161 p.

Ramesh D., Souissi S. (2018). Effects of potential probiotic *Bacillus subtilis* KADR1 and its subcellular components on immune responses and disease resistance of *Labeo rohita*. Aquaculture Research, 49: 367-377.

Rengpipat S., Tunyanun A., Fast A., Piyatiratitivorakul S., Menasveta P. (2003). Enhanced growth and resistance to Vibrio challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. Diseases of Aquatic Organisms, 55: 169-173.

Ringo E. (2010). Prebiotics in aquaculture: A review. Aquaculture Nutrition, 16 (2): 117-136.

Ruangpan L., Tendencia E.A. (2004). Laboratory manual of standardized methods for antimicrobial tests for bacteria isolated from aquatic animals and environment. Tigbauan, Iloilo, Philippines: Southeast Asian Fisheries Development Center, Aquaculture Department. 63 p.

Sampollo L., Traifalgar R.F., Corre V.J., Felarca K.G. (2018). Use of Tilapia Green Water to Eliminate *Vibrio parahaemolyticus* and *Vibrio cholerae* in Cultured Green Mussel, *Perna viridis* (Linnaeus 1758). Asian Fisheries Science, 31: 101-112.

Sanchez-Ortiz A.C., Luna-Gonzales A., Campa-Cordova A.I., Escamilla-Montes R., Flores-Miranda M., Mazon-Suastegui J.M. (2015). Isolation and characterization of potential probiotic bacteria from

pustulose ark (*Anadara tuberculosa*) suitable for shrimp farming. Latin American Journal of Aquatic Research, 43(1): 123-236.

Thakur A.B., Vaidya R.B., Suryawanshi S.A. (2004). Pathogenicity and antibiotic susceptibility of Vibrio species isolated from moribund shrimps. Indian Journal of Geo-Marine Sciences, 32(1): 71-75.

Tran L., Nunan L., Redma, R., Mohney L., Patoja C., Fitzsimmons K., Lightner D. (2013). Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of Aquatic Organisms, 105: 45-55.

Turner S., Pryer K.M., Miao V.P., Palmer J.D. (1999). Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. Journal of Eukaryotic Microbiology, 46: 327-338.

Vaseeharan B., Ramasamy P. (2003). Control of pathogenic *Vibrio* spp. By *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon.* Letters in Applied Microbiology, 36: 83-87.

Verschuere L., Rombaut G., Sorgeloos P., Verstraete W. (2000). Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews, 64(4): 655-671.

Vijayan K.K., Bright Singh I.S., Jayaprakash N.S., Alavandi S.V., Somnath Pai S., Preetha R., Rajan J.J.S., Santiago T.C. (2005). A brackishwater isolate of *Pseudomonas* PS-102, a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. Aquaculture, 251: 192-200.

Wibowo A., Fadhar M., Maftuch M. (2015). Utilization of *Tilapia* mucus to inhibit *Vibrioharveyi* on *vannamei (Litopenaeus vannamei).* Journal of Life Sciences and Biomedicine, 5: 141-148.

Xu D., Wang Y., Sun L., Liu H., Li J. (2013). Inhibitory activity of a novel antibacterial peptide AMPNT-6 from *Bacillus subtilis* against *Vibrio parahaemolyticus.* Food Control, 30: 58-61.

Zokaeifar H., Babaei N., Saad C.R., Kamarudin M.S., Sijam K., Balcazar J.L. (2014). Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp, *Litopenaeus vannamei.* Fish and Shellfish Immunology, 36: 68-74.