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Research Article
Probiotic Potential of an Indigenous Marine *Bacillus thuringiensis* on Shrimp (*Penaeus monodon*) Culture Infected with *Vibrio mimicus*

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Abstract

Background and Objective: Antibiotic resistance problem has led to the search for alternate approaches for disease management in aquaculture systems. Probiotic potential of indigenous marine *Bacillus thuringiensis* was carried out on shrimp (*Penaeus monodon*) culture infected with *Vibrio mimicus* in a non-flow through system. **Materials and Methods:** *Bacillus* species (potential probiotic candidates) were isolated from healthy shrimp intestine while pathogenic *Vibrio mimicus* was previously isolated from moribund shrimp intestine. Agar well diffusion assay was employed for *in vitro* antibacterial assay. Acute pathogenicity test followed by histopathological examination of shrimp hepatopancreas were carried out. Challenge test was carried out with *Vibrio mimicus* for 12 days after addition of the antagonistic bacterium. Water quality parameters such as temperature, pH, salinity, ammonia, nitrite, nitrate and dissolved oxygen were monitored. **Results:** *Bacillus* sp., B1 amongst the investigated potential probiotic candidates proved antagonistic to *Vibrio mimicus* with inhibition zone of 16.0±0.32 mm. Histopathology result proved safety of *Bacillus* sp., B1 when it neither cause any mortality on the test shrimp, nor cause any detrimental effects on their internal organs while *Vibrio mimicus* proved lethal to the postlarvae. Molecular identification of *Bacillus* sp., B1 revealed its close relatedness to *Bacillus thuringiensis* strain G5-8-3T02. **Conclusion:** Challenge test revealed that shrimp with the probiotic addition exhibited resistance to the pathogen. This was expressed by the weight and length increase in the experimental shrimp. Water quality parameters were also at optimum levels throughout the culture period. Indigenous *Bacillus thuringiensis* strain G5-8-3T02 could be a good probiotic bacterium for shrimp culture against vibriosis.

Key words: Shrimp culture, pathogenicity, hepatopancreas, marine *Bacillus*, *Vibrio mimicus*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aquaculture is the farming of aquatic organisms in the artificial bodies of water to enhance high yield of production and private ownership of the stock being cultivated. It has been found to play a crucial role in the development of the economy. Shrimp farming remains a viable industry for quite some time now. Considered an important source of high nutritive value and cheap animal proteins, aquaculture becomes an important economic activity in many countries including Nigeria[1].

Aquaculture is seen as one of the food-producing sector which is growing rapidly[2]. Apart from such societal gain, this industry is not without its own problems. Occupational hazards and safety concerns are paramount in the aquaculture industry as well as environmental degradation caused by some practices among others. Nevertheless, much higher natural and man-made toxic substances such as antibiotics, pesticides, disease infections and persistent organic pollutants have been found in some farmed fish than wild fish. The most alarming among these is the threat caused by *Vibrio* species. This group of bacterial pathogens has been associated with diseased shrimp worldwide[3]. They are widely reported in hatchery facilities all over the globe. These contaminants in fish pose health concerns even to unsuspecting consumers of the product[4].

Vibriosis syndrome is one main problem of shrimp culture. Although antibiotics have been lifesaving and their use exponentially increased in curbing vibriosis syndrome, the problem of antibiotic resistance and its epidemiological consequences cannot be overemphasized. This led to the need for exploration of several alternate approaches for disease management in aquaculture systems. Amongst them is the exploration of probiotics as prophylactics.

There is scarcity of information on marine indigenous probiotic bacteria commercially viable for large scale shrimp aquaculture in Nigeria, especially against the shrimp pathogen *Vibrio* species. This may be due to the scarcity, at present, of indigenous shrimp rearing practice in Nigeria. This study was therefore designed for probiotic trial of indigenous antagonistic marine bacterium, *Bacillus thuringiensis*, on shrimp (*Penaeus monodon*) culture infected with *Vibrio mimicus*. To the best of our knowledge, this is the first time an indigenous, non-pathogenic marine *Bacillus thuringiensis* with ability to protect shrimp culture against *Vibrio* infection is reported.

MATERIALS AND METHODS

***Bacillus* spp. isolation:** Healthy shrimp were cleaned externally with 70% ethanol. Dissections of their gastrointestinal tracts were carried out in sterile conditions. Then, 1 g of pooled gut content was removed and transferred to sterile 9 mL physiological saline. The suspension was heated for 10 min at 80°C in a water bath. Afterward, 10-fold serial dilution up to 10^{-3} was prepared. Then, 0.1 mL of each sub-diluted sample was cultured on sterile nutrient agar plates via spread plate method. The plates were incubated at 30°C for 24-48 h. Isolates with distinct colony morphology were picked and streaked repeatedly on nutrient agar plates to obtain pure isolates. The purified isolates were identified to generic level using their characteristics in terms of morphology, physiology and biochemistry with reference to Bergey's Manual of Determinative Bacteriology[5].

Antibacterial activity: Agar well diffusion assay was employed for *in vitro* examination of the purified cultures of *Bacillus* spp. for their inhibitory effects against previously isolated *Vibrio* sp. V1[6] which was molecularly identified as *Vibrio mimicus*[7]. Wells were punched with a cork borer (6 mm diameter) in Mueller Hinton agar (TM Media) plates freshly seeded with 0.1 mL of 24 h broth culture of *Vibrio mimicus*. Then, 0.1 mL of a 24 h broth culture of different isolates of *Bacillus* spp. including a control (nutrient broth containing 1.0% sodium chloride) were placed into separate wells. The plates were then incubated for 24 h at 37°C and the diameter of the zones of inhibition measured and expressed as antimicrobial activity[8].

Determination of safety of antagonistic *Bacillus* sp. (B1): Determination of the safety of the antagonistic bacterium was done using a method described by Chau *et al.*[8]. Healthy shrimp post-larvae were collected from Elechi Creek in Port Harcourt and acclimatized for 2 days prior to experimental analysis. A stock concentration of the *Bacillus* sp., B1 suspension grown on nutrient agar for 24 h was prepared by diluting the bacterial suspension and adjusting to McFarland No. 1 standard turbidity which is equivalent to 3×10^8 CFU mL^{-1}. A ten-fold serial dilution was carried out in order to obtain other concentrations (3×10^7, 3×10^6, 3×10^5, 3×10^4 and 3×10^3 CFU mL^{-1}). Six different tanks of 4 L capacity, each containing 2 L of habitat water were set up. These tanks were buffered using sterile bicarbonate powder. Maximum oxygen supply was ensured

in each of the tanks with electric aerator. The shrimp were daily fed throughout the culture period with a commercial shrimp feed (coupens, 0.5 mm). About 50 of healthy shrimp were stocked into each of the tanks. The safety of each prepared concentration (3×10^7-3×10^3 CFU mL^{-1}) of *Bacillus* sp., B1 suspension was tested by immersing 0.1 mL of each concentration into each of the tanks containing the healthy shrimp. Shrimp post-larvae grown in habitat water alone served as control. The larval mortalities, if any was monitored and recorded daily for 4 days post immersion with bacterial suspension. The shrimp exposed to bacterial suspension were observed for pathogenic signs. At the end of culture period, the number of shrimp that remained was calculated in order to know the mortality rate. The experiment was carried out in duplicate.

Hepatopancreatic histopathology of the experimental shrimp: The experimental shrimp were subjected to a hepatopancreatic histopathology after the safety test of the antagonistic bacterium. The paraffin technique was adopted[9]. The resultant micrographs of the tissue cross-sections of the test animal were compared with a control[7].

Challenge experiment: Zero water discharged method as adopted by Ariole and Anyanwu[7] was used in this experiment. Healthy shrimp post-larvae were collected from Elechi Creek in Port Harcourt and acclimatized for 2 days prior to experimental analysis and were daily fed throughout the period with commercial shrimp feed (0.5 mm coupens). The initial weight and length of the shrimp post-larvae measured before the start of the experiment were 1.5 g and 5.1 cm, respectively. Three experimental groups comprising three different tanks of 4 L capacity, each containing 2 L of habitat water were set up. These tanks were buffered using sterile bicarbonate powder. Maximum oxygen supply was ensured in each of the tanks with aerator. About 50 of healthy shrimp were stocked into each of the tanks. Shrimp culture tank 1 was infected with 0.1 mL 3×10^5 CFU mL^{-1} fresh culture of *Bacillus* sp., B1 for 2 days and challenged with 0.1 mL pathogenic *Vibrio mimicus* in the 3rd day. Shrimp culture tank 2 was non-infected and contained only habitat water (non-infected control). While Shrimp culture tank 3 was challenged with 0.1 mL 3×10^5 CFU mL^{-1} fresh culture of pathogenic *Vibrio mimicus* without any antagonistic bacterium treatment. All infections were done by immersion and experimental tanks were left under observation for 12 days. The experiment was conducted in duplicate. Measurements of water quality parameters such as pH, temperature, DO, salinity, ammonia, nitrate and nitrite were carried out during the culture period. Observation as culture period elapsed was based on survival of shrimp, increase in weight and length of shrimp, re-isolation of the bacterial suspension in pure culture from the dead shrimp.

Determination of water quality: Temperature, pH level, dissolved oxygen (DO) and salinity were measured daily. Thermometer was used to measure the temperature, water quality test kit (multi feed Type) was used for determining the pH level, Tyspe 51B DO meter was used to determine the DO and salinity was determined using a refractometer. The methods described in APHA[10] were employed for nitrate, nitrite and ammonia concentrations at 2 days interval.

Molecular identification of *Bacillus* sp. (B1): This involved chromosomal DNA extraction (Zymo Research Bacterial DNA MiniPrep Kit), 16S rRNA amplification (using the 27F:5"AGAGTTTGATCMTGGCTCAG3" and 1492R:5"TACGGY TACCTTGTTACGACTT 3" primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 µL for 35 cycles), Gel electrophoresis (electrophoresis in order to ascertain if the PCR exercise was successful), DNA (16S rRNA) sequencing (amplified 16S products were sequenced on a 3500 genetic analyzer using the Bigdye-Termination technique by Inqaba South Africa) and finally, phylogenetic analysis where the sequences were edited using the bioinformatics algorithm Bioedit. Similar sequences were downloaded from the National Biotechnology Information Center (NCBI) data base using BlastN and these sequences were aligned using Clustal X. Neighbor-Joining mechanism was used to infer the history of evolution[11] in MEGA 6.0. Jukes and Cantor[12] method was used to compute the distances in evolution[12].

Statistical analysis: All obtained data from this study were statistically analyzed. One way ANOVA of the SPSS version 20 Statistical Package was used to analyze the difference in shrimp mortality rates and Duncan's multiple range test was used to analyze significant differences among means ($p = 0.05$). Two sample t-test of SPSS version 20 Statistical Package was used for water quality analysis, while LC_{50} of *Vibrio mimicus* was calculated using Probit Analysis of SPSS version 20.

RESULTS

Antibacterial activity: Table 1 shows the zone of inhibition of antagonistic test of the *Bacillus* spp., against *Vibrio mimicus*. The result showed that the isolate *Bacillus* sp., B1 was active (inhibition zone: 16.0±0.34) against *Vibrio mimicus*. This isolate was then selected for subsequent studies.

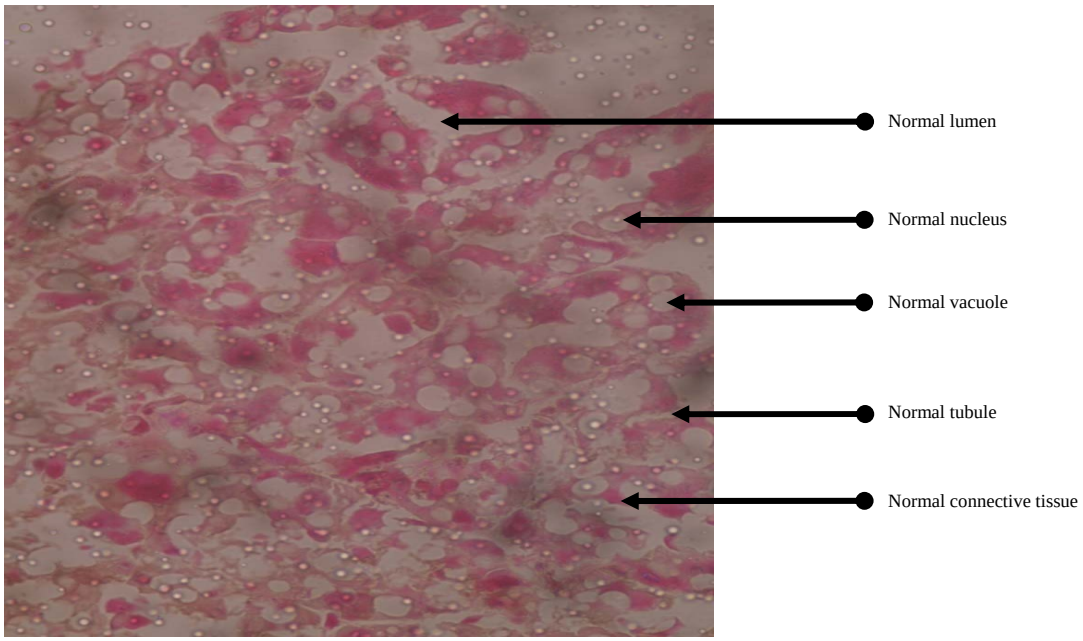

Fig. 1: Hepatopancreas of experimental shrimp infected with *Bacillus* sp. (B1) Mag.×400 H and E (Hemolysin and Eosin). Imp: Healthy hepatopancreas

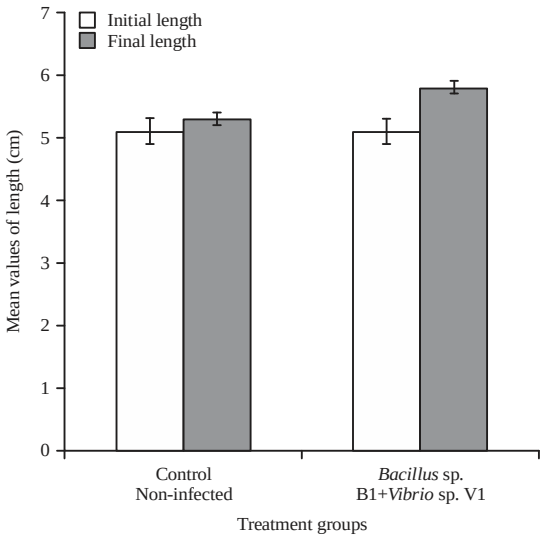

Fig. 2: Mean values of shrimp length before and after the culture period

Table 1: Antagonistic effect of *Bacillus* spp. against *Vibrio mimicus*

Isolates	Zone of inhibition (mm)±SD
Bacillus sp., B1	16.0±0.34
Bacillus sp., B2	-
Bacillus sp., B3	-
Bacillus sp., B4	-

- : No inhibition

Determination of safety of antagonistic *Bacillus* sp., B1: Table 2 shows the effect of the antagonistic *Bacillus* sp., B1 on test-organism *Penaeus monodon* post-larvae using five different concentrations (3×10^3, 3×10^4, 3×10^5, 3×10^6 and 3×10^7 CFU mL^{-1}). No mortality was observed in the tanks. Mortality of 24% was recorded in non-infected control tank.

Hepatopancreatic histopathology of the experimental shrimp: The result from the histopathology test of the experimental shrimp after safety experiment revealed the presence of a normal vacuole and normal nuclei, no degeneration of the epithelial tissues and tubule lumen of the hepatopancreas (HP) of the shrimp infected with *Bacillus* sp., B1 (Fig. 1).

Challenge experiment: In Table 3, result of *in vivo* antagonistic effect of *Bacillus* sp., B1 obtained during 12 days post challenge of shrimp (*Penaeus monodon*) post-larvae with *Vibrio mimicus*. No mortality was recorded in experimental post-larvae infected with *Bacillus* sp., B1 and challenged with *Vibrio mimicus*. Increase in length (Fig. 2) and weight (Fig. 3) in the probiotic treated shrimp culture, in comparison with control group were observed.

Table 2: Safety test of *Bacillus* sp., B1 on shrimp (*Penaeus monodon*) post-larvae

	No. of dead post-larvae/No. tested (with time intervals)					
Bacillus sp., B1 (CFU mL^{-1})	0 h	24 h	48 h	72 h	96 h	Mortality (%)
3×10^3	0/50	0/50	0/50	0/50	0/50	0
3×10^4	0/50	0/50	0/50	0/50	0/50	0
3×10^5	0/50	0/50	0/50	0/50	0/50	0
3×10^6	0/50	0/50	0/50	0/50	0/50	0
3×10^7	0/50	0/50	0/50	0/50	0/50	0
Control (Non-infected)	0/50	2/50	4/50	8/50	12/50	24

Table 3: Probiotic trial of *Bacillus* sp., B1 on shrimp (*Penaeus monodon*) post-larvae challenged with *Vibrio mimicus*

		No. of dead postlarvae/No. tested (with time intervals)					
Treatment groups (Batches)	Bacterial cell (3×10^5 CFU mL^{-1})	0 day	3rd day	6th day	9th day	12th day	Mortality (%)
1	*Bacillus* sp., B1+*Vibrio mimicus*	0/50	0/50	0/50	0/50	0/50	0
2	Non-infected control (habitat water)	0/50	2/50	4/50	8/50	12/50	24
3	Infected control (*Vibrio mimicus*)	0/50	7/50	13/50	20/50	28/50	56

Table 4: Water quality parameters of shrimp culture observed within 12 days challenge test

		Culture range			
Parameters	Optimum range*	Day 3	Day 6	Day 9	Day 12
pH	7.0-8.3	7.00	7.30	7.40	7.60
Temperature (°C)	28-32	28.00	29.00	28.00	29.00
NH_3 (mg L^{-1})	<0.03	0.022	0.023	0.024	0.028
Nitrate (mg L^{-1})	<60	0.90	0.90	1.00	1.00
Nitrite (mg L^{-1})	<1.0	0.02	0.01	0.02	0.04
Salinity (mg L^{-1})	0.5-35	21.10	21.10	21.10	21.20
DO (mg L^{-1})	5.0-9.0	7.52	7.52	7.53	7.54

*Source: Suantika *et al.*[20]

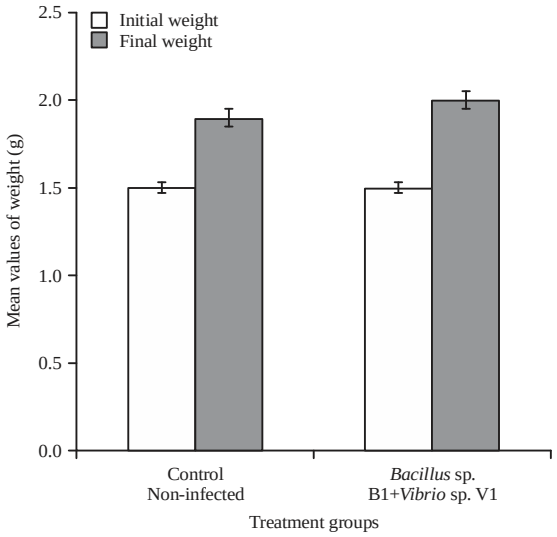

Fig. 3: Mean values of shrimp weight before and after the culture period

Determination of water quality: The result of the water quality parameters observed during the 12 days shrimp culture showed that the culture pH, temperature, ammonia, nitrite, nitrate and dissolved oxygen were at optimum range within the culture period (Table 4).

Molecular identification of *Bacillus* sp., B1: The 16S rRNA of the isolate *Bacillus* B1 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Bacillus* and revealed closely relatedness to *Bacillus thuringiensis* strain G5-8-3T02 (gi:585284320) (Fig. 4).

DISCUSSION

In this study, a marine bacterium, molecularly identified as *Bacillus thuringiensis* was isolated from healthy shrimp (*Penaeus monodon*) intestine and found to possess antibacterial activity against a pathogenic bacterium, *Vibrio mimicus* (Table 1). *Bacillus* spp. with antibacterial effects have been isolated from marine environment[13-15]. The inhibition of *Vibrio mimicus* by *Bacillus thuringiensis* as observed in this study, may be by altering the pH of the growth medium or by producing antibiotic polypeptides such as bacitracin, tyrotricidin, polymyxin or gramicidin S as also reported by Balcazar and Rojas-Luna[16].

The safety test of *Bacillus thuringiensis* did not cause any detrimental effects to the test shrimp and no stress sign even upon infection with 10^7 CFU mL^{-1} whereas,

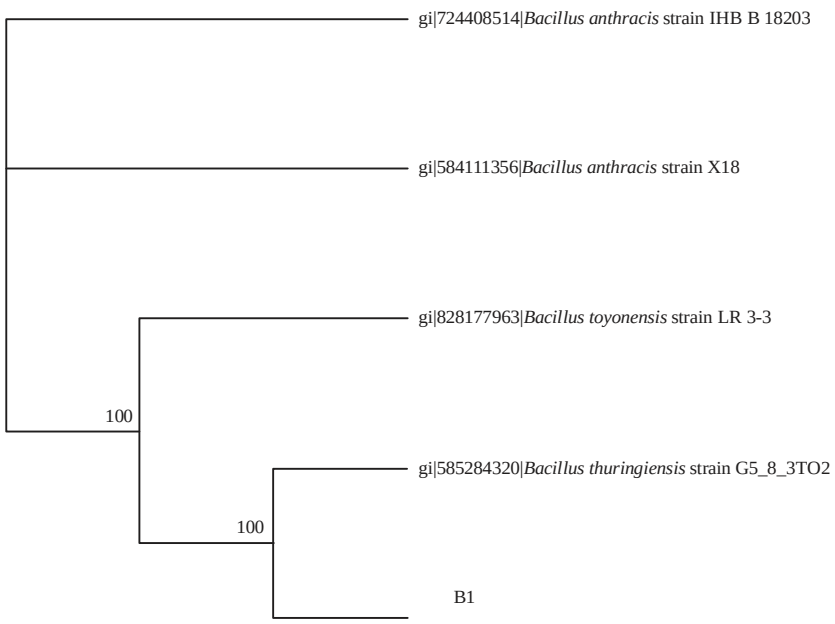

Fig. 4: Phylogenetic tree showing species relatedness of isolate (B1)

the control group (non-infected) showed 24% mortality (Table 2). This affirmed that *Bacillus thuringiensis* strain G5-8-3T02 is a beneficial bacterium and has no pathogenic effects on shrimp. The histopathology result of the experimental group treated with *Bacillus thuringiensis* revealed normal vacuoles, tubule epithelial cells, normal E, F and R epithelial cells (Fig. 1). These results showed that the shrimp internal organs were not affected by the probiotic candidate. The challenge experiment also revealed that *Bacillus thuringiensis* supplemented *Penaeus monodon* resisted the pathogenic impact of *Vibrio mimicus* when compared with the control groups (Table 3). This finding agrees with that of Rengpipat *et al.*[17], who observed that fed probiotic may improve appetite and growth performance as well as hinders infections by pathogens of shrimp. El-Haroun[18] also reported positive impact of diets supplemented with probiotic on growth of Nile Tilapia and African catfish.

During the experiment the probiotic inclusion might have produced some bioactive and inhibitory substances against the pathogenic organism thus providing protection on the shrimp against the pathogenic bacterium (*Vibrio mimicus*). This is supported by Nayak[19], who reviewed the immune modulatory activity of probiotics and evaluated the factors that regulate the optimum induction of immune responses in shrimp. This is also in agreement with the findings of Suantika *et al.*[20], who reported an increased rate of survival and lack of pathogenicity by supplemented probiotic bacteria in white shrimp hatchery against pathogenic *Vibrio harveyi* during their 12 day experiment. Far *et al.*[21] also reported reduced cumulative mortality after a probiotic feed on white shrimp challenged with some *Vibrio* species.

The result of the water quality parameters tested within the culture period revealed that pH, temperature, ammonia, nitrate, nitrite, DO (dissolved oxygen) and salinity were within the optimum range (Table 4). This result is similar to that reported by Suantika *et al.*[20]. The t-test carried out showed that standard optimum and culture difference are not significant, since the p-value (0.65 for pH, 0.34 for temperature, 0.433 for nitrate, 0.615 for nitrite, 0.902 for salinity and 0.151 for DO) is >0.05 level of significance. The stability of these parameters could be attributed to the bioremediation ability of probiotic bacteria in aquaculture and also the buffering capacity of the calcium carbonate ($CaCO_3$) used as substrate at the bottom of the aquarium[20].

The one-way ANOVA which compared the data from the different samples of the analyzed shrimp after challenge experiment showed that experimental group supplemented with *Bacillus thuringiensis* strain G5-8-3T02 was significantly different from the other control group with reference to improved length (Fig. 2) and weight (Fig. 3). This could be attributed to the fact that probiotic *Bacillus thuringiensis* (Fig. 4) increased their length and weight as a result of growth stimulation. The result of this finding is also in agreement with Irianto and Austin[22], who found increase in the length and weight in shrimp treated with probiotic bacteria than the control group.

CONCLUSION

A marine bacterium, *Bacillus thuringiensis*, isolated from healthy shrimp (*Penaeus monodon*) intestine was found to possess antibacterial activity against a pathogenic bacterium, *Vibrio mimicus*. The challenge experiment conducted revealed that *Bacillus thuringiensis* supplemented shrimp (*Penaeus monodon*) culture resisted the pathogenic impact of *Vibrio mimicus*. The indigenous probiotic bacterium, *Bacillus thuringiensis* strain G5-8-3T02, could contribute to the improvement of a healthy and commercial aquaculture production.

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