Enhancement of Vibriosis Resistance in *Litopenaeus vannamei* by Supplementation of Biomastered Silver Nanoparticles by *Bacillus subtilis*

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Abstract

Shrimp production is devastated by Vibriosis in many aquacultures around the world. These pathogens are highly virulent and most dreadful viral outbreaks in shrimp culture. The potential antibacterial effects of *Bacillus subtilis* silver nanoparticles (AgNPs) from the gut of *Litopenaeus vannamei* were studied. The AgNPs has ~ 420 nm in UV-visible spectrum, diameter 5-25 nm with smooth spherical shape (characterized by transmission electron microscopy) and 29 values corresponding to the presence of silver crystal (X-ray diffraction spectrum). The AgNPs showed promising activity against *Vibrio paraenemolyticus* (21.25 ± 2.55 mm) and *V. harveyi* (19.27 ± 1.36 mm) as compared with *B. subtilis*, silver nitrate (AgNO3) and control. Four different experiments were conducted using different feeding behavior of *L. vannamei*. In comparison to untreated control group, final weight (14.89 ± 0.03 g), weight gain (9.36 ± 0.01), specific growth rate (SGR) 14.41 ± 0.09, feed conversion ratios (FCR) 1.47 ± 0.12, higher survival and haemocyte counts were significantly greater in shrimp fed with AgNPs. The gill of entire experimental animal showed morphological alteration in histopathological investigation. The AgNPs were then tested for shrimp challenged with the *V. paraenemolyticus*. Statistical analysis revealed significant differences in shrimp survival between AgNPs, *B. subtilis* and control group. In the infective experimental study, cumulative survival of the control group (10 ± 0.321%) whereas the shrimp with AgNPs (90.66 ± 0.523%) and (71 ± 0.577%) with *B. subtilis*. Subsequently, real-time PCR was observed for immune related genes to determine the mRNA levels of prophenoloxidase (proPO), anti lipopolysaccharide factor (ALF2 and 4), peroxinectin (PE), superoxide dismutase (SOD), 18S, lipopolysaccharide and β -1,3-glucan-binding protein (LGBP and serine protein (SP). The expression of all immune related genes (mRNA levels) studied was significantly up-regulated in the AgNPs diet shrimp in contrast to the *B. subtilis* and control. This study discovers that the biomastered AgNPs give a promising potential new tool for inhibiting vibriosis in shrimp culture.

Keywords: *Bacillus subtilis*, Silver nanoparticles; Antibioviro activity; Growth performances; *Litopenaeus vannamei*

Introduction

The aquaculture industry continues to expand as a crucial segment of the global seafood market [1]. The pacific white shrimp, *L. vannamei* is one of the most prominent marine aquaculture species [2,3]. It was first introduced in China in the late 1980’s. By 2010 and since it has become one of the world’s three major farmed shrimp, which accounted for 85% of the total shrimp production in China [2]. The world total production of *L. vannamei* is raised from less than 10,000 metric tons in 1970 to more than 3,000,000 metric tons in [4,5].

The development of the commercial culture of shrimp has generally been accompanied by increasing problems with diseases, which are mostly caused by opportunistic pathogens, such as viruses, bacteria and fungi [6]. *Vibrioaceae* represent the most dreadful pathogenic bacteria causing diseases in the aquatic organisms especially in shrimp in all seasons causing mass mortality [7-9]. The term vibrios refers to the bacteria of the genus *Vibrio* which causes disease associated to shrimp. They are the natural inhabitants of estuarine and marine environments, well known for causing vibriosis in fish worldwide and the prevention has become a major challenge in aqua farming [10,11].

Infectious disease remains a significant problem to aquaculture, worldwide and many approaches are available to mitigate the effect of pathogens in farmed aquatic animals to cure the disease and improve the production performance [12]. However, the antibiotics to control vibrios in shrimp aquaculture are not allowed in most of the countries and so it is necessary to develop an alternative pathogen control method for shrimp production [13]. Probiotics have been found as the most effective dietary supplements which are living microbial cells with fewer side effects in animals [14]. Although, the probiotics have been used in these culture animals, the bioavailability of these immunomodulators are still a matter of debate.

Therefore, the approaches of ‘Nanotechnology’ have become the best solution these issue. The development of nanotechnology science has increased considerably since the beginning of the 21st Century [15]. The silver nanoparticles become a part of our daily life [16] mainly in cosmetics and bio-remediation [17,18], because of their antimicrobial effect [19]. Moreover, they may delivery anti-cancer drugs on proper place [20], or can binding the HIV gp 120 protein [21]. They can also be used as anti-fouling agents [22], coating of catheter and surgery material, to produce synthetic compounds for odontology, and in homeopathic medicine [23], aquaculture [24], or in various other water treatments [25]. The application of the nanotechnology to aquaculture in infant stage may have the potential to solve many puzzles related to animal health, production, reproduction, as well as prevention and treatment of disease [26].

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AgNPs have been historically used to control microbial proliferation [27]. The antifungal and antibacterial effect of AgNPs, even against antibiotic-resistant bacteria has been demonstrated in *in vitro* conditions [28,29]. It is well known that silver ion and silver-based compounds are highly toxic to microorganisms [30] and they are used as antibacterial compounds [31-33]. A number of research group have demonstrated superior performance of AgNPs over Ag⁺ in controlling the growth and activity of various microorganisms [34].

Microbial source to produce the silver nanoparticles shows the great interest towards the precipitation of nanoparticles due to its metabolic activity [35]. The precipitation of nanoparticles in external environment of a cell, it shows the extracellular activity of organisms. Extracellular synthesis of nanoparticles using cell filtrate could be beneficial to intracellular synthesis, the microbes being extremely good candidates for extracellular process [36]. Since, biological methods are regarded as safe, cost-effective, sustainable and environment friendly processes [37]. In view of that, the present study aimed to test the effect of *B. subtilis* synthesized silver nanoparticles on the growth, survival, antibioactivity and immune gene expression in *L. vannamei* against shrimp pathogens.

**Materials and Methods**

**Isolation and identification of bacterial strain**

Shrimps were collected from the culture lab (IOCAS) and starved for 24 h in order to clear the contents in the alimentary canal. The intestine was removed and homogenized. The homogenate was serially diluted and plated on to LB (Luria-Bertani) agar medium and incubated at 24 h at 37°C and the isolates were identified by biochemical methods and the results were cross-checked with Bergey’s Manual of systematic bacteriology [38] and were confirmed through molecular identification.

**Molecular identification**

Bacterial genomic DNA was isolated (TIANamp bacterial DNA kit; Cat. No. DP302) and the quality of the isolated DNA was checked by 1% agarose gel electrophoresis and was further quantified using NanoQuant (Infinite M200PRO), TECAN. The DNA was analyzed with routine PCR with primers, 27-F and 1492-R. The 50 µl PCR amplification system consisted of 1.0 µl genomic DNA, 5.0 µl 10x Buffer, 1 µl dNt (10 mM), 1.5 µl of forward and reverse primers (10 µM), 1.0 µl Ex Taq DNA polymerase (5 µl/µL, TAKARA, Japan) and 40.5 µl ddH₂O. The PCR programs were: denaturation at 95°C for 5 min; 45 cycles of 95°C, 30 s; 55°C, 45 s; 72°C, 1 min and 30 s; extension at 72°C for 7 min and termination at 16°C. Products were analyzed by electrophoresis on 1% agarose gel. The PCR products were sequenced using ABI 3730 Genetic Analyzer. The 16s rRNA gene sequences were aligned and compared with the closely related neighbor sequences retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI), via BLAST search program.

**Biosynthesis of silver nanoparticles**

*B. subtilis* strain inoculated in to 250-ml Erlenmeyer flask containing 100 ml of sterile LB broth. The cultured flasks were incubated in a rotating shaker (200 rpm) for 24 h at room temperature and the culture was centrifuged at 12,000xg for 10 min. The biomass and supernatant were separated and used separately for the synthesis of silver nanoparticles. The (*B. subtilis*) supernatant was used for studying extracellular production of silver nanoparticles by mixing it with filter-sterilized AgNO₃ (Sinopharm chemical, China, 99.9% pure) solution at 1 mM final concentration. The mixtures were incubated on rotating shaker (200 rpm) at room temperature and we took samples each 6 hours up to 36 hours. The most nanoparticles were produced after 24 h. The reduction of Ag⁺ ions was monitored by visual observation for the nanoparticle formation as described by Velmurugan et al. [33].

**Characterization of silver nanoparticles**

The optical characteristics of the synthesized silver nanoparticles were analyzed using UV–Vis spectrophotometer. Briefly, nanoparticles containing samples were subjected to absorption at 300–700 nm range using UV–Vis spectrophotometer (Hitachi U5100). The size and morphology of the silver nanoparticles synthesized by the supernatant were analyzed by TEM preparing with carbon-coated copper grids. The grids were allowed to stand for 2 min, then extra solution was removed and the grid was allowed to dry prior to measurement and the TEM measurements were recorded using a JEOL-JEM-1200 EX model. The culture supernatant embedded with AgNPs was freeze dried, powdered and used for XRD X-ray diffraction analysis using Rigaku ULTIMA IV X-ray powder diffractometer using Cu-Kα radiation operating between 10° - 80° at 20 angle. The AgNPs were distributed over a glass slide and form the thin film of AgNPs for XRD analysis and the instrument operating at a voltage of 40 kV and current of 40 mA.

**Bacterial activity**

The bactericidal activities were tested by the disc-diffusion method [39]. The *V. parahaemolyticus* and *V. harveyi* were aseptically inoculated in nutrient broth and incubated at 37°C for 24 h. Later, the antibacterial sensitivity profile of each strain was tested by spreading in Muller Hinton Agar (MHA) plates. The treated (50 µl of *B. subtilis*, AgNPs and AgNO₃) sample discs (5 mm) were placed on plates spread with each bacterial culture and incubated at 30°C for 24 h and the sterile paper disc without any treatment was used as a control. After incubation, the plates were examined for possible clear zones of formation. The presence of a clear zone around the samples was recorded as an inhibition against the bacterial strains. The diameters of such zones of inhibition were measured, and the mean value for each organism were recorded and expressed in millimeters.

**Shrimp rearing and *in vivo* antibacterial studies**

Four different experiments were conducted to examine the effect of biosynthesized AgNPs administered to the shrimp and these experiments were conducted in triplicate. Shrimp with average body weight (6.82 ± 2.16 g) were collected from Nan Jiang Biotechnology Company (Hainan). Before starting the experiments, the shrimps were kept in our aquarium for 3 days in order to adapt to the laboratory conditions and acclimatized in 50 L capacity rectangular tanks. The major physic-chemical parameters such as salinity, temperature and pH were monitored regularly and were maintained at optimal levels along with water exchange. The selected healthy shrimps were divided into four groups. Each group contained 20 shrimp the experimental setup and the feed preparations are shown in the Table 1. The animals were kept starved for a day prior to start of the experiment, and the experiment was conducted for 65 days. All shrimps were fed daily at 8:00 h and 20:00 h. Each day, the remaining feed was siphoned out before feeding. Every third day, each tank was cleaned and the water was partially changed (about 50%). The percentage of survival rate was monitored daily during days of the experiment by counting the number of the animal died.

**Total haemocyte count**

At the end of the experimental period, haemolymph of all the experimental and control group of shrimp was collected individually

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from rostral sinus of *L. vannamei* using 1 ml syringe containing 0.1 ml of 10% precooled sodium citrate as an anticoagulant. A drop of haemolymph along with anticoagulant (10% sodium citrate) was placed on a haemocytometer and stained using giemsa stain. Haemocytes were counted using a neubauer haemocytometer using a light microscope (Nikon ECLIPS TS 100-F, Japan) at 400 magnification and they were expressed as number of cells/ml.

**Histopathological investigation**

In order to study histopathological investigation, gill tissues were dissected out and immersed in Davidson’s fixative for 48 h and transferred to 70% ethanol. Further, sampled tissues were embedded with paraffin and the sections were stained with Ehrlich’s hematoxylin and eosin stain and were observed under microscope (Nikon ECLIPS TS 100-F, Japan).

**Survival and growth rate analysis**

At the end of the experiment, the final weight, survival rate, weight gain, feed conversion ratio (FCR), and specific growth rate (SGR) of different experiment were calculated according to [40,41].

- Weight gain (g/shrimp) = Final weight (g) - Initial weight (g)
- FCR = Total Feed Given (g) / Weight gain (g)
- SGR; % / day = ((Final weight – Initial weight) / Days) × 100
- Survival rate (%): (Final numbers/Initial numbers) × 100

**Experimental infection**

The 15 days experiment was conducted for *V. parahaemolyticus* infection. The pathogenic bacterium, *V. parahaemolyticus* was grown overnight in 2216E medium and the concentration was adjusted 10^6 CFU/ml. The shrimps were collected and injected with 20 µl of the bacterial suspension into the last abdominal segment. Immediately after injection, 10 shrimp each were transferred into the 30 litter capacity tanks. The experiment was conducted in triplicates and the water was supplied from the previous tanks in order to minimize the stress. In Exp I, animals were fed with unflavored gelatin binder and biosynthesized AgNPs mixing with standard feed. In Exp II, animals fed with standard feed with unflavored gelatin binder added with *B. subtilis* culture mixture. In the control, the shrimp were fed with standard feed devoid of any addition. Subsequently, a group of untreated shrimp with standard feed and injected with 20 µl of PBS, served as a negative control. During the experimental infection, shrimp were fed their specific diets as previously described. The mortality was monitored daily for up to 15 days. After the completion of the experiment, AgNPs, *B. subtilis* and positive control groups of animals was used in relative mRNA expression of immune-related genes.

**Relative mRNA expression of immune-related genes**

The expression of immune related genes of shrimp following challenge with *V. parahaemolyticus* was determined by real-time RT-PCR (RT-qPCR). One shrimp from each replicate was randomly collected and dissect the different tissue samples gut, gill, hepatopancreas (HP), stomach, and muscle for RNA extraction [42]. All the tissue samples were stored at -80°C and freeze-dried using ample amounts of liquid nitrogen and homogenized using RNase free mortar and pestle. RT-qPCR assays were conducted with six specific primers and 18s as the house keeping gene was used to determine immune-related gene expression (Table 2). Total RNA was extracted with Unizol reagent (Boxing Company, Shanghai, China). By NanoDrop 1000 spectrophotometer detection (Thermo Fisher Scientific Inc., USA) (A260/A280) and electrophoresis on 1% agarose gel, RNA concentration, purity and integrity were verified. Before cDNA synthesis, RNA was treated with RNase-free DNase (TAKARA, Japan) to remove contaminating DNA. The reaction system of cDNA synthesis contained 1 µg RNA, 1×M-MLV buffer, 0.125 mmol/L dNTP, 10 µM HEX random primers (Sangon, China), 20 U RNasin (TARAKA, Japan), 200 U M-MLV (TARAKA, Japan) in a total volume of 25 µL. The cDNA synthesis was subjected to reverse transcription at 37°C for 1 h and 35 min.

**Results**

**Isolation and identification of silver synthesized bacteria**

Specifically 7 presumptive silver producing strains were isolated from the gut of *L. vannamei*. Of this one strain was selected for synthesis of AgNPs based on the biochemical characters. Further, 16S rRNA gene sequence analysis of candidate bacterium revealed that, it has 100% BLAST similarity with the *Bacillus subtilis* (J812207) in the NCBI database. Hence, AgNPs synthesized bacteria-16S rRNA gene sequence was deposited in the NCBI Gene Bank under the name of *B. subtilis* 1725505EJ1 (Accession number: J812207).

**Synthesis of silver nanoparticles**

The color change of extracellular bacterial culture has been reported by reaction with silver nitrate. Control (without silver nitrate) showed no changes in color of the bacterial culture (Figure 1a). The bacterial culture with silver nitrate (AgNO₃) showed a gradual change of color from yellowish brown to intense dark brown, after 24 h of incubation (Figure 1b).
**Characterization of silver nanoparticles**

The color intensity was confirmed by UV-Vis spectral analysis at different wavelengths ranging from 300 to 700 nm. AgNPs have an intense absorption peak in UV absorption spectra due to its surface plasmon excitation. For the bacterial culture of AgNPs was observed the absorption peak at 420 nm (Figure 2), which proving formation of AgNPs. These absorption spectra for the AgNPs were obtained within 24 h. The color change and UV absorption data analysis thus confirm the reduction of AgNO₃ to AgNPs by the culture supernatant of E.coli. For further confirmation of nanoparticles formed by bacterial supernatant, the samples were subjected to TEM analysis. The microscope showed NPs with variable size and shape, most of them were spherical in the size range of 10-25 nm (Figure 3). The crystalline nature of NPs was confirmed by XRD (X-ray diffraction) analysis (Figure 4). The XRD pattern showed intense peaks in the whole spectrum of 2θ (31.82°, 45.47°, 56.46° and 66.25°) value ranging from 10° to 80° with four intense peaks (110), (111), (200) and (220) sets of planes of the face centered cubic (fcc) structure (with reference to JCPDS File no. 04-0783).

**Antibacterial activity of AgNPs against Vibriosis**

Antibacterial tests were performed against *V. paraaemolyticus* and *V. harveyi* on MHA plates treated with different samples AgNPs, *B. Subtilis* and AgNO₃ by zone of inhibition with appropriate controls (Table 3). The each sample showed different levels of antibacterial activity against vibriosis. The discs impregnated with AgNPs inhibited significantly the growth of *V. paraaemolyticus* and *V. harveyi* with inhibition zone of 21.25 ± 2.55 and 19.27 ± 1.36 at the same time *B. subtilis* showed 12.65 ± 1.71 and 13.29 ± 1.58 mm, respectively. The lowest inhibitions were noted in AgNO₃ against *V. paraaemolyticus* and *V. harveyi* with the zones of 4.6 ± 0.33 and 3.9 ± 0.53 mm. The presence of nanoparticles at a certain level inhibited vibriosis growth by more than *B. subtilis* and AgNO₃. In contrast, there was no antibacterial activity in control. Each experiment was performed for three times. The data shown represent the mean ± SE. The data were analyzed statistically using SPSS (version-11.5) software (Table 3).

**Growth responses and survival of shrimp *L. vannamei***

After 65 days, the effect of different diets on the growth and survival rate was calculated. Analyzed data on the growth performance of shrimp in different treatments and control, including initial weight, final weight, weight gain, specific growth rate (SGR), feed conversion ratio (FCR), and survival rate are showed in Table 4.

There was no significant difference (P>0.05) for initial weight between experiment and control at the beginning of the experiment. The shrimp responses revealed that the production was higher in experiment II (Exp. II) diet fed shrimp with the highest production of 9.36 ± 0.01 g against the control group (6.33 ± 0.012 g). The other experiment groups (Exp I, III and IV) also showed significant production (P<0.05) (7.19 ± 0.01 - 3.90 ± 0.04 and 3.04 ± 0.04) but lower than the Exp II. The specific growth rate (SGR) showed the same trend. Better FCR (1.47 ± 0.12) was discovered in Exp II diet fed shrimp compared to that of control (1.74±0.002) and other treated groups Exp I (1.58 ± 0.15), III (1.71 ± 0.22) and IV (1.88 ± 0.03). Survival of *L. vannamei* was recorded at the end of the experiments and the results indicate that the survival was significantly higher (P<0.05) in Exp I and II treated groups ranging from 89.2 ± 0.88 to 100 ± 0.00%, but it was only 83.8 ± 1.42% in control diet fed shrimp. The lowest survival rate was regarded in Exp III and IV (40.6 ± 1.20 and 25.15 ± 0.59) (Table 4).

**Table 2: Specific primers used to evaluate immune status of shrimp, *L. vannamei***

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALF2</td>
<td>Forward</td>
<td>GGAAGATCTCCGTGACCCCAAGC</td>
<td>59</td>
</tr>
<tr>
<td>ALF2</td>
<td>Reverse</td>
<td>TCCCGAGGGATTTCCAGAGCAGT</td>
<td></td>
</tr>
<tr>
<td>ALF4</td>
<td>Forward</td>
<td>ACTAACCCTTCCCTCACCCAC</td>
<td>59</td>
</tr>
<tr>
<td>ALF4</td>
<td>Reverse</td>
<td>CCCACCGAGGAGTAAATGTTG</td>
<td></td>
</tr>
<tr>
<td>LGBP</td>
<td>Forward</td>
<td>CATGTCGCAACTTCCGGTTTCA</td>
<td>57.3</td>
</tr>
<tr>
<td>LGBP</td>
<td>Reverse</td>
<td>ATCCACCCGGTGGACTTTT</td>
<td></td>
</tr>
<tr>
<td>ProPo</td>
<td>Forward</td>
<td>GCGTGGCAGACGCGTCTTA</td>
<td>57.8</td>
</tr>
<tr>
<td>ProPo</td>
<td>Reverse</td>
<td>CGCGGATCGATTTAGTTGTG</td>
<td></td>
</tr>
<tr>
<td>SP</td>
<td>Forward</td>
<td>CGTCGTTAAGTTAAGTGCTTCTT</td>
<td>56.3</td>
</tr>
<tr>
<td>SP</td>
<td>Reverse</td>
<td>TTTACGCCATAAGCAGGTGTT</td>
<td></td>
</tr>
<tr>
<td>18s</td>
<td>Forward</td>
<td>TATACGCTAGTGGAGCTGGA</td>
<td>55</td>
</tr>
<tr>
<td>18s</td>
<td>Reverse</td>
<td>GGAGAGTATGAGCAGAAAAAT</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3: Antibacterial activity of *B. subtilis*, silver nanoparticles and AgNO₃.**

<table>
<thead>
<tr>
<th>Treatments</th>
<th><em>V. Parahaemolyticus</em> (mm)</th>
<th><em>V. harveyi</em> (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>12.65 ± 1.71a</td>
<td>13.29 ± 1.58b</td>
</tr>
<tr>
<td>Silver nanoparticles</td>
<td>21.25 ± 2.55a</td>
<td>19.27 ± 1.36a</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>4.6 ± 0.33a</td>
<td>3.9 ± 0.53a</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values mean ± SD with different superscript letters in a row show significant differences (P < 0.05).
and 185.16 ± 1.249 × 10^5 cells/ml) the control diet group showed a lower THC value of 48.52 ± 1.408 × 10^5 cells/ml was observed in Exp III group shrimp, gill filaments are heavily fused (e) Vibriosis as a feed additives in Exp 3 as a feed additives in Exp II shrimp, different multiple gross structure appearance of the gill filaments (d) AgNO_3 as a feed additives in Exp III group shrimp, gill filaments are heavily fused (e) Vibriosis as a feed additives in Exp IV, the shrimp gill are completely damaged.

### Total haemocyte count and Histopathological investigation

The total haemocyte counts were higher in the shrimp fed with AgNPs (Exp II), exhibiting 204.57 ± 1.28 × 10^5 cells/ml whereas the lower THC value of 48.52 ± 1.408 × 10^5 cells/ml was observed in Exp IV diet fed shrimp (Figure 5). Compared to Exp II and I (204.57 ± 1.28 and 183.16 ± 1.249 × 10^5 cells/ml) the control diet group showed a low count 167.85 ± 1.352 × 10^5 cells/ml. In the diet fed group Exp III, the THC was 102.43 ± 1.208 × 10^5 cells/ml.

The histopathological investigations were carried out in gill samples from all experimental groups. In Exp II group, gills surface of each side of the epipodite and filament has a thin cuticle and an underlined epithelial monolayer. The epithelium is made up of high columnar cells with nucleus in the apical part of the cell under a thin cuticle and each filament tip has a hemolymphatic lacuna and the connective tissues are present in the septum of filaments and in the axis of gills. Epithelial pillar cell processes, span the distance between opposite cuticular walls. The epithelium of the branches and filaments was generally thin, about 0.7-2 µm in transverse section; it is thicker near the nuclei (5-7 µm) and at the tip of the filaments (1-2 µm) which are widened to form a distal lacuna. Furthermore branches were also observed in Exp II shrimp gills in terminal areas of some filaments. The secondary laminae were attached to cephalothoracic wall via a tubular structure. Primary filaments branch from the central axis and which in turn divides into secondary filaments. Similar morphology was noted between control and Exp I group filaments along the length of the gill. Filaments are elongated and have a delicate shape in contrast to control and other groups. In Exp III and IV treatment shrimp, gills are totally damaged (Figure 6).

### Survival and immune-related gene expression

After 15 days of experiment, all experimental groups were challenged with *V. parahaemolyticus* (Figure 7). After being administered with a high dose (10^8 CFU shrimp^-1^) of *V. parahaemolyticus* showed significant difference (P<0.05) in cumulative survival were found in the AgNPs fed shrimps (85.5±0.52%) in contrast to the *B. subtilis* (65.6 ± 0.57%) and control groups 11.6 ± 0.32% (Figure 7). No mortality was observed in shrimp from the negative control which had been injected with PBS. The gene expression study shows that no significant relationship between the different diet groups (AgNPs, *B. subtilis* and control) and the different tissue samples (Gut, Gill, Stomach, Hepatopancreatic (HP) and Muscle). The expression analysis of the different tissue of the shrimp shows that all five genes were expressed. Interestingly, anti-lipopolysaccharide factor (ALF2 and 4), β-1,3-glucan-binding protein (LGBP), prophenoloxidase (ProPo) and serine protein (SP), were expressed at quite high levels in all tissues of AgNPs fed shrimp. In contrast, the ALF4 and ProPo transcription level appeared to be highly expressed in the gut and ALF2, LGBP and SP genes expressed in gill.

### Table 4: Data of growth performance and survival of *L. vannamei* treated with different treatment.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Experimental duration</th>
<th>Control</th>
<th>Exp I</th>
<th>Exp II</th>
<th>Exp III</th>
<th>Exp IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial weight (g)</td>
<td>5.63 ± 0.06^c</td>
<td>5.44 ± 0.01^d</td>
<td>5.53 ± 0.01^b</td>
<td>5.71 ± 0.07^a</td>
<td>5.68 ± 0.01^a</td>
</tr>
<tr>
<td>2</td>
<td>Final weight(g)</td>
<td>11.96 ± 0.09^c</td>
<td>12.63 ± 0.01^c</td>
<td>14.89 ± 0.03^b</td>
<td>9.37 ± 0.05^a</td>
<td>8.74 ± 0.03^a</td>
</tr>
<tr>
<td>3</td>
<td>Weight gain (g)</td>
<td>8.33 ± 0.01^c</td>
<td>7.19 ± 0.01^c</td>
<td>10 ± 0.01^c</td>
<td>3.90 ± 0.04^b</td>
<td>3.04 ± 0.04^b</td>
</tr>
<tr>
<td>4</td>
<td>SGR (%)</td>
<td>7.93 ± 0.03^c</td>
<td>11.06 ± 0.07^c</td>
<td>10 ± 0.01^c</td>
<td>6.0 ± 0.02^c</td>
<td>4.67 ± 0.01^c</td>
</tr>
<tr>
<td>5</td>
<td>FCR</td>
<td>1.74 ± 0.002^c</td>
<td>1.58 ± 0.15^c</td>
<td>1.47 ± 0.12^d</td>
<td>1.71 ± 0.22^c</td>
<td>1.88 ± 0.03^c</td>
</tr>
<tr>
<td>6</td>
<td>Survival (%)</td>
<td>83.8 ± 1.42^c</td>
<td>89.2 ± 0.88^c</td>
<td>100 ± 0.00^c</td>
<td>40.6 ± 1.20^c</td>
<td>25.1 ± 0.59^c</td>
</tr>
</tbody>
</table>

Values (mean ± SD) with different letters are statistically significant from each other (t- test; P<0.05)
Figure 7: Survival (%) of white shrimp 15 days challenged with V. parahaemolyticus and diet as a AgNPs and B. subtilis without any addition fed on control. Each value is the mean ± SD of three replicates; bar with different letters are statistically significant from each other (t-test; P < 0.05).

Figure 8: The mRNA expression of five immune-related genes of white shrimp for 15 days and challenged with V. parahaemolyticus.
of AgNPs experiment animal. Significantly, all genes are up-regulated (P<0.05) in bio synthesized AgNPs shrimp fed supplemented diets compared to the B. subtilis and control groups (Figure 8).

Discussion

The B. subtilis synthesized AgNPs are ecofriendly and have significant advantages over other processes since it takes place at relatively ambient temperature and pressure [43-46]. As the size and shape of nanoparticles can also be controlled in microbial synthesis [44], screening of unexplored microorganisms for AgNPs synthesizing property is very important. Previous studies reported that AgNPs size [47,48] shape [49] surface charge [50] surface coating [51] and solubility [52] affect the AgNPs’ toxicity.

Similar results were reported by Kathiresan et al. [53], investigated the Vibrio sp. in shrimp P. monodon through AgNPs synthesized by a coastal plant Prosopis chilensis. Furthermore, previous studies have shown that the nanoparticles may serve as antimicrobial agent in gram negative bacteria [54,55].

In the present study, biosynthesized AgNPs act as an antimicrobial agent against vibriosis in infected shrimp. The AgNPs is effective in controlling pathogenic Vibrio sp. in vitro and invivo condition [53,56]. Similar results were observed in our study by B. subtilis synthesized AgNPs against V. parahaemolyticus (21.25 ± 2.55 mm) and V. harveyi (19.27 ± 1.36 mm) in contrast to B. subtilis, AgNO3, and control tested in the invito studies. Also, Klabunde and co-workers demonstrated that reactive metal oxide nanoparticles show excellent bactericidal effects [57]. Biosynthesized AgNPs produce digestive enzyme and required growth nutrients such as vitamins and amino acids thereby improving the feed absorption resulting in an enhanced growth rate in host. Earlier report has shown that the AgNPs supplemented with shrimp diet significantly (P<0.05) increased the weight gain at the end of 30 days of culture [53].

Haemocyte are the main component in the defense mechanism of the invertebrate system. These cells are circulated in the invading pathogens as well as prevention of haemolymph loss by coagulation, immobilization of pathogens by clotting, phagocytosis of foreign material, and hardening of cuticle [58,59]. The non-specific immune system can be triggered by the application of probiotics. B. cereus boosted up selected immune parameters in P. monodon [60] and further they also concluded that the increased growth rate of P. monodon might have associated with the elevated immune responses [14]. In accordance with these result, the present study also showed elevated immune responses in shrimp fed with AgNPs Exp II diet. Also, there was a decline in haemocyte counts of shrimp group challenged with V. parahaemolyticus. Accordingly, the total haemocyte count was higher in AgNPs treated groups with a greater value of 205 ± 4.5 cells/ml and 169 ± 7.4 x 10^6 cells/ml, respectively in control diet fed shrimp. Earlier report stated that the haemocyte count in P. monodon were high in which treated with AgNPs than the control group [53].

To further investigate the impact of the AgNPs on the gill morphology, the histopathological investigations were carried out effectively to detect changes in gill health in shrimp L. vannamei. The changes were obtained in gill filament in all the treatment groups. Among those, AgNPs (Exp II) fed shrimp gills are dendrobranchiate, with an axis that supports numerous secondary laminae giving rise, at left angle, to filament divided into two branches near their terminai [8,61]. Only few reports were conducted on the impact of bacteria and metal, on the shrimp gills and some crustacean species [8,62,63]. Compared to gills, the epithelium of epipodites is thicker and more differentiated. The structure of the epipodite cells is very similar to the thick cell [64].

In the present study, the survival and immune status of treated shrimp have also been investigated through an experimental infection with V. parahaemolyticus. AgNPs fed diet showed higher resistance. On the other hand, lower survival was recorded in shrimp fed B. subtilis diet. In control, there was 10 ± 0.321% survival of the shrimp challenged with V. parahaemolyticus at the end of the culture period. Increasing Vibrio population in the animal had reduced the survival rate [65,66]. This revealed that the application of AgNPs resulted in decreased counts of vibrios and hence the higher survival rate of the shrimp [53,65]. Most previous studies on bacterial infections in shrimp have been conducted using injection of different bacteria directly into the body cavity to monitor expression changes of different immune genes. The expressions of five immune-related genes were investigated using the real-time PCR in order to evaluate the immune status of shrimp after being injected with V. parahaemolyticus. The infection experiment studies to evaluate the AgNPs and B. subtilis administration had an effect on the expression of immune-related genes; both fed additives were administrated at five different shrimp tissues (Gut, Gill, Stomach, Hepatopancreatic (HP) and Muscle). Significantly, all genes ALF2, ALF2, LGPB, ProPO and SP was up-regulated (P<0.05) in biosynthesized AgNPs shrimp fed supplemented diets compared to the B. subtilis and control groups. Among decapod crustacean ALF2, ALF4 [67,68], LGPB [69] ProPO [70] and SP [71] and their tissue distribution have been studied in some detail [4,72]. According to the tissue distribution analysis made in this study, all the genes were detected in all the tissues and expressed higher in the AgNPs fed shrimp in contrast to the control.

From the result, B. subtilis could be used for the production of AgNPs from AgNO3. The synthesis of metal NPs by microbes depends on the localization of the reductive components of the cell. The enzyme involved in the synthesis of NPs may be the nitrate reductase present in B. subtilis. If the cell wall secreted reductive enzymes are involved in the reductive process of metal ions, then we can find the NPs extracellularly [73]. The nitrate reductase is induced by nitrate ions and reduces Ag+ to Ag. The reduction of Ag+ ion may occur through electron shuttle enzymatic process which is already proposed for AuNPs [74]. The NADH and NADH dependent reductase enzyme are important in biosynthesis of AgNPs. Bacillus sp. may secrete the co-factor NADH and NADH dependent enzymes importantly nitrate reductase which may be the key for bioreduction of Ag+ to Ag and the formation of AgNPs. This reduction process may be carried out by NADH and NADH dependent reductase as an electron carrier. A similar phenomenon has been hypothesized for magnetotactic bacteria. In these kinds of bacteria the incoming Fe^3+ species is immediately converted to FeO and an invagination of the cytoplasmic membrane may occur forming a magnetosome [75]. This magnetosome might have a disturbed membrane surface, allowing the growth of the crystal only in a particular direction [76].

This major issue of AgNPs induced toxicity and interaction between NPs and cells are cellular uptake and toxic response of the cell. The cellular uptake is processed by endocytosis and it is proportional to time, dose and energy. Endosome and lysosome are majorly affected by AgNPs. The AgNPs may serve as a vehicle to deliver Ag+ more effectively, being less susceptible to binding and reduced bioavailability by common natural ligands to the bacteria cytoplasm and membrane. The proton motion force would therefore decrease the local pH and enhance Ag+ to release. In contrast, the less soluble AgNPs may cause direct toxicity to bacteria via oxidative stress [77,78]. The transformation and releasing of Ag+ ongoing by turnover of oxidase to reductase and vice-versa due to change in pH. This mechanism might have taken place at two levels, at cell membrane level, or in the membrane of endoplasmic reticulum [79,80]. Oxidase gets activated.
at lower pH whereas reductase gets activated at higher pH in the cell membrane. AgNPs affects alterations in the membrane of the bacteria and also affect the cell in areas such as the respiratory chain and cell division, ultimately resulting in cell death [81] (Figure 9). In conclusion, the modernization and intensification of aquaculture industry lead to outbreak of vibriosis resulting in huge loss of aquaculture industry. The administration of biosynthesized silver nanoparticles improved the growth performances, and immune response against the pathogenic bacterium, V. *parahaemolyticus*. In addition, a better survival rate was obtained in shrimp fed silver nanoparticles diets after challenge with *V. parahaemolyticus*. The biosynthesized AgNPs feed will gradually increase and the success of aquaculture in future. It has been synonymous with the success of antimicrobial compound that, if validated through rigorous scientific investigation and used wisely, may prove to be a boon for the aquaculture industry.

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