

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

Elayaraja Sivaramasamy^{1,2}, Wang Zhiwei^{1,3}, Fuhua Li¹ and Jianhai Xiang^{1*}

¹Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China

²Central Research Laboratory, Sree Balaji Medical College and Hospital, Bharath University, Chrompet, Chennai-600 044, Tamil Nadu, India

³University of Chinese Academy of Sciences, Beijing 100049, China

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 2 θ values corresponding to the presence of silver crystal (X-ray diffraction spectrum). The AgNPs showed promising activity against *Vibrio parahaemolyticus* (21.25 \pm 2.55 mm) and *V. harveyi* (19.27 \pm 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 \pm 0.03 g), weight gain (9.36 \pm 0.01), specific growth rate (SGR) 14.41 \pm 0.09%, feed conversion ratios (FCR) 1.47 \pm 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. parahaemolyticus*. 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 \pm 0.321%) whereas the shrimp with AgNPs (90.66 \pm 0.523%) and (71 \pm 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; Antivibrio 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]. *Vibrionacea* 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 vibriosis 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 vibriosis 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].

***Corresponding authors:** Jianhai Xiang, Institute of Oceanology, Chinese Academy of Sciences 7, Nanhai Road, Qingdao 266071, China, Tel: + 86-532-82898568; Fax: + 86-532-82898578; E-mail: jhxiang@qdio.ac.cn

Received January 14, 2016; **Accepted** February 05, 2016; **Published** February 12, 2016

Citation: Sivaramasamy E, Zhiwei W, Li F, Xiang J (2016) Enhancement of Vibriosis Resistance in *Litopenaeus vannamei* by Supplementation of Biomastered Silver Nanoparticles by *Bacillus subtilis*. J Nanomed Nanotechnol 7: 352. doi:10.4172/2157-7439.1000352

Copyright: © 2016 Sivaramasamy E, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

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, antivibrio activity 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 µ/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 30s; extension at 72°C for 7 min and termination at 16°C. Products were analyzed by electrophoresis on 1% agarose gels. 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 12,000×g 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 2θ angle. The AgNPs were distributed over a glass slid 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* was 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

S.No	Experimental setup	Feed preparation (using gelatin as a binder)
1	Exp I	<i>B. subtilis</i> (10 ⁵ counts/ml) + 10g standard feed
2	Exp II	0.1 mg of AgNPs in 10 mg standard feed
3	Exp III	0.1 mg of Pure silver solution (AgNO ₃) + 10 g standard feed
4	Exp IV	<i>V. parahaemolyticus</i> (10 ⁶ counts/ml) + 10 g standard feed
5	Control	Without any treatment

Table 1: Feed preparation of the experimental setups.

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].

$$\text{Weight gain (g/shrimp)} = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$\text{FCR} = \text{Total Feed Given (g)} / \text{Weight gain (g)}$$

$$\text{SGR; \% / day} = ([\text{Final weight} - \text{Initial weight}] / \text{Days}) \times 100$$

$$\text{Survival rate (\%)} = (\text{Final numbers} / \text{Initial numbers}) \times 100$$

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 10⁶ CFU/ml¹. 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 liter 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 the 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).

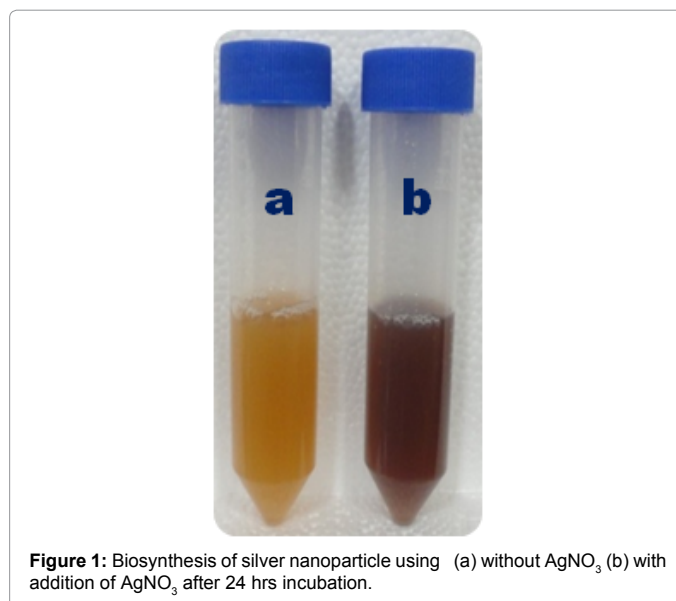


Figure 1: Biosynthesis of silver nanoparticle using (a) without AgNO₃ (b) with addition of AgNO₃ after 24 hrs incubation.

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 proofing 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 EJ1. 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 (101), (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. parahaemolyticus* 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. parahaemolyticus* and *V. harveyi* with inhibition zone of 21.25 ± 2.55 and 19.27 ± 1.36 at the same time *B.*

Gene	Primer	Sequence (5'-3')	Temp (°C)
ALF2	Forward	GGAAAGTACTCCGTGACCCCAAAGC	59
ALF2	Reverse	TCCCAGGAGTTCAGTTCAGCAGTT	
ALF4	Forward	ACTAACCTTTTCGCTCCACCCAC	59
ALF4	Reverse	CCCACCGAGAGGTAGTAAATGCTT	
LGBP	Forward	CATGTCCAACCTTCGCTTTCAGA	57.3
LGBP	Reverse	ATCACCGCGTGGCATCTT	
ProPo	Forward	GCCTTGGCAACGCTTTCA	57.8
ProPo	Reverse	CGGCATCAGTTCAGTTTGT	
SP	Forward	CGTCGTTAGGTTAAGTGCCTTCT	56.3
SP	Reverse	TTTCAGCGCATTAAGACGTGTT	
18s	Forward	TATACGCTAGTGGAGCTGGAA	55
18s	Reverse	GGGGAGGTAGTGACGAAAAAT	

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

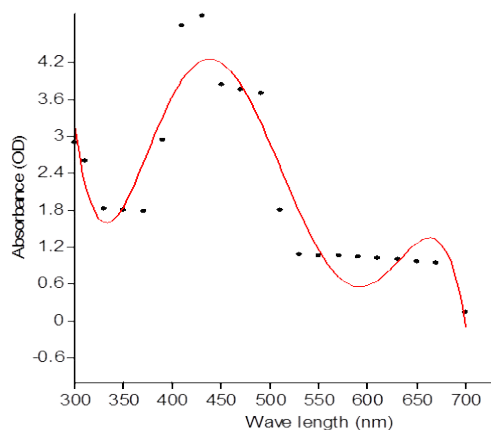


Figure 2: UV-Vis spectrum of biosynthesis of silver nanoparticle by using *B. subtilis*.

Treatments	<i>V. Parahaemolyticus</i> (mm)	<i>V. harveyi</i> (mm)
<i>B. subtilis</i>	12.65 ± 1.71 ^b	13.29 ± 1.58 ^b
Silver nanoparticles	21.25 ± 2.55 ^a	19.27 ± 1.36 ^a
AgNO ₃	4.6 ± 0.33 ^c	3.9 ± 0.53 ^c
Control	0	0

Values mean ± SD with different superscript letters in a row show significant differences (P < 0.05).

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

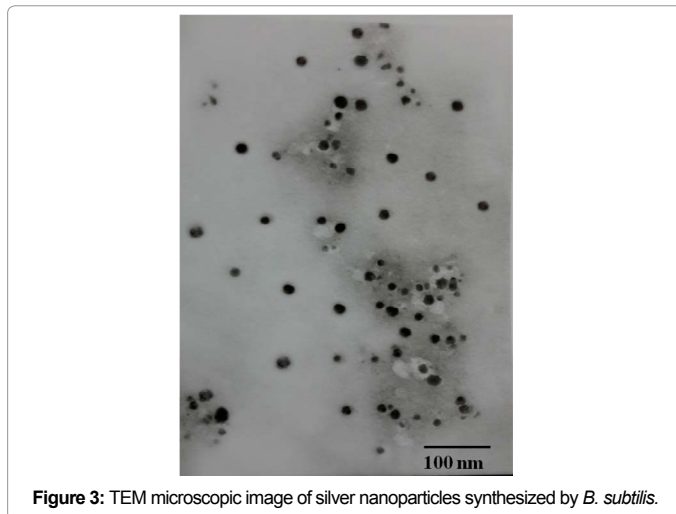


Figure 3: TEM microscopic image of silver nanoparticles synthesized by *B. subtilis*.

subtilis showed 12.65 ± 1.71 and 13.29 ± 1.58 mm, respectively. The lowest inhibitions were noted in AgNO₃ against *V. parahaemolyticus* 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 ± 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).

S. No	Experimental duration	Control	Exp I	Exp II	Exp III	Exp IV
1	Initial weight (g)	5.63 ± 0.06 ^a	5.44 ± 0.01 ^d	5.53 ± 0.01 ^c	5.71 ± 0.07 ^a	5.68 ± 0.01 ^b
2	Final weight(g)	11.96 ± 0.09 ^c	12.63 ± 0.01 ^b	14.89 ± 0.03 ^a	9.37 ± 0.05 ^d	8.74 ± 0.03 ^e
3	Weight gain (g)	6.33 ± 0.01 ^c	7.19 ± 0.01 ^b	9.36 ± 0.01 ^a	3.90 ± 0.04 ^d	3.04 ± 0.04 ^e
4	SGR (%)	9.73 ± 0.03 ^c	11.06 ± 0.07 ^b	14.41 ± 0.09 ^a	6.0 ± 0.02 ^d	4.67 ± 0.01 ^e
5	FCR	1.74 ± 0.002 ^c	1.58 ± 0.15 ^d	1.47 ± 0.12 ^e	1.71 ± 0.22 ^b	1.88 ± 0.03 ^a
6	Survival (%)	83.8 ± 1.42 ^c	89.2 ± 0.88 ^b	100 ± 00 ^a	40.6 ± 1.20 ^d	25.1 ± 0.59 ^e

Values (mean ± SD) with different letters are statistically significant from each other (t-test; P<0.05)

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

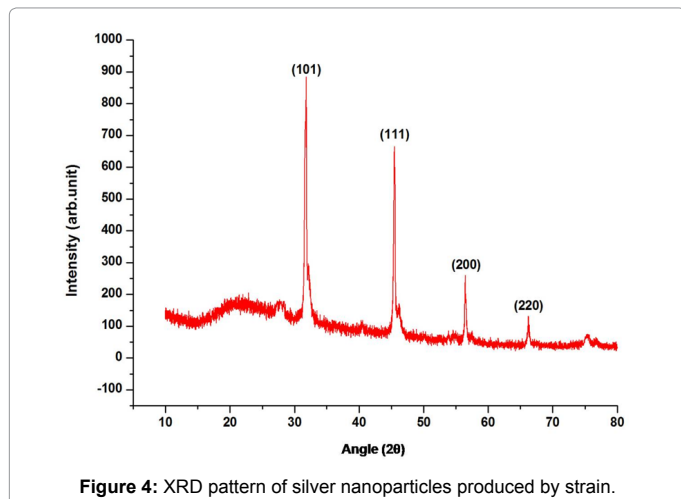


Figure 4: XRD pattern of silver nanoparticles produced by strain.

Total haemocyte count and Histopathological investigation

The total haemocyte counts were higher in the shrimp fed with AgNPs (Exp II), exhibiting $204.57 \pm 1.28 \times 10^5$ cells/ml, whereas the lower THC value of $48.52 \pm 1.408 \times 10^5$ cells/ml was observed in Exp IV diet fed shrimp (Figure 5). Compared to Exp II and I (204.57 ± 1.28 and $185.16 \pm 1.249 \times 10^5$ cells/ml) the control diet group showed a low count $167.85 \pm 1.352 \times 10^5$ cells/ml. In the diet fed group Exp III, the THC was $102.43 \pm 1.208 \times 10^5$ 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 hemolympathic 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 (10^6 CFU shrimp⁻¹) of *V. parahaemolyticus* showed significant difference (P<0.05) in cumulative survival were found in the AgNPs fed shrimps ($85.5 \pm 0.52\%$) in contrast to the *B. subtilis* ($65.6 \pm 0.57\%$) and control groups $11.6 \pm 0.32\%$ (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 (LGPB), 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, LGPB and SP genes expressed in gill

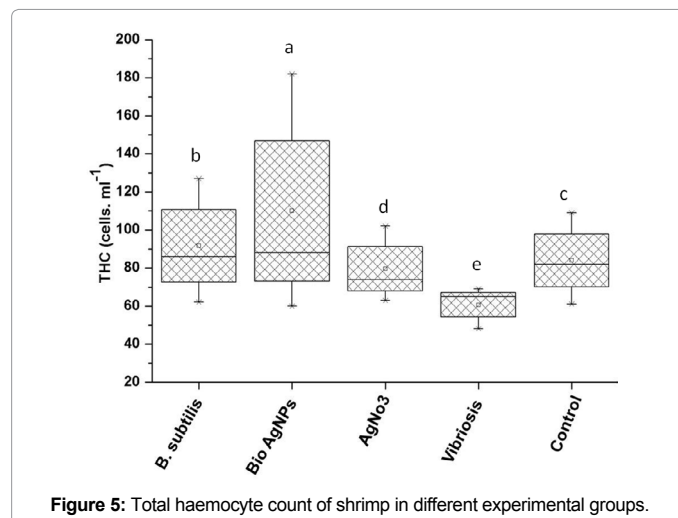
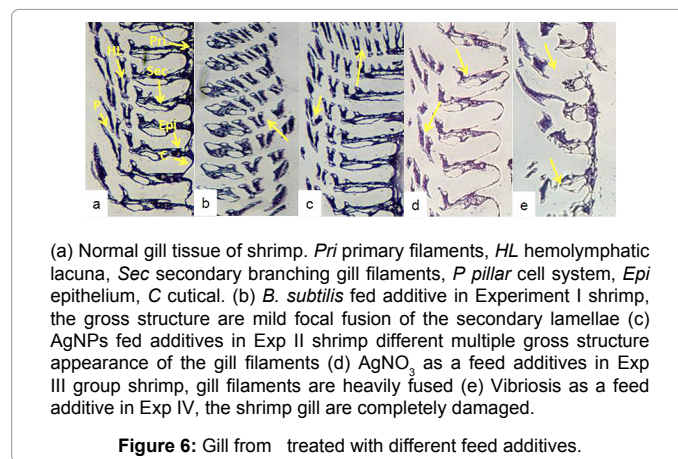
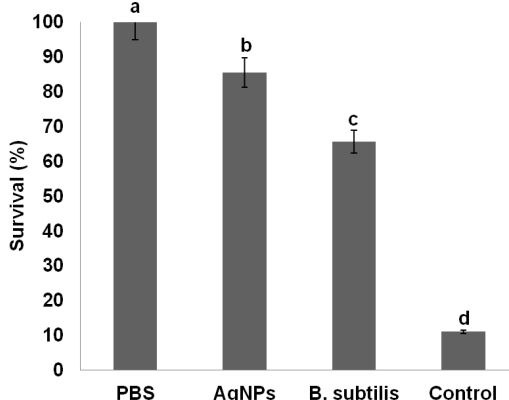


Figure 5: Total haemocyte count of shrimp in different experimental groups.



(a) Normal gill tissue of shrimp. Pri primary filaments, HL hemolympathic lacuna, Sec secondary branching gill filaments, P pillar cell system, Epi epithelium, C cutical. (b) *B. subtilis* fed additive in Experiment I shrimp, the gross structure are mild focal fusion of the secondary lamellae (c) AgNPs fed additives in Exp II shrimp different multiple gross structure appearance of the gill filaments (d) AgNO₃ as a feed additive in Exp III group shrimp, gill filaments are heavily fused (e) Vibriosis as a feed additive in Exp IV, the shrimp gill are completely damaged.

Figure 6: Gill from treated with different feed additives.



(PBS)-without treatment but injected with (20μl) PBS (served as positive control): Other groups are treated with *V. parahaemolyticus* and diet as a AgNPs and *B. subtilis* and 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 7: Survival (%) of white shrimp 15 days challenged with.

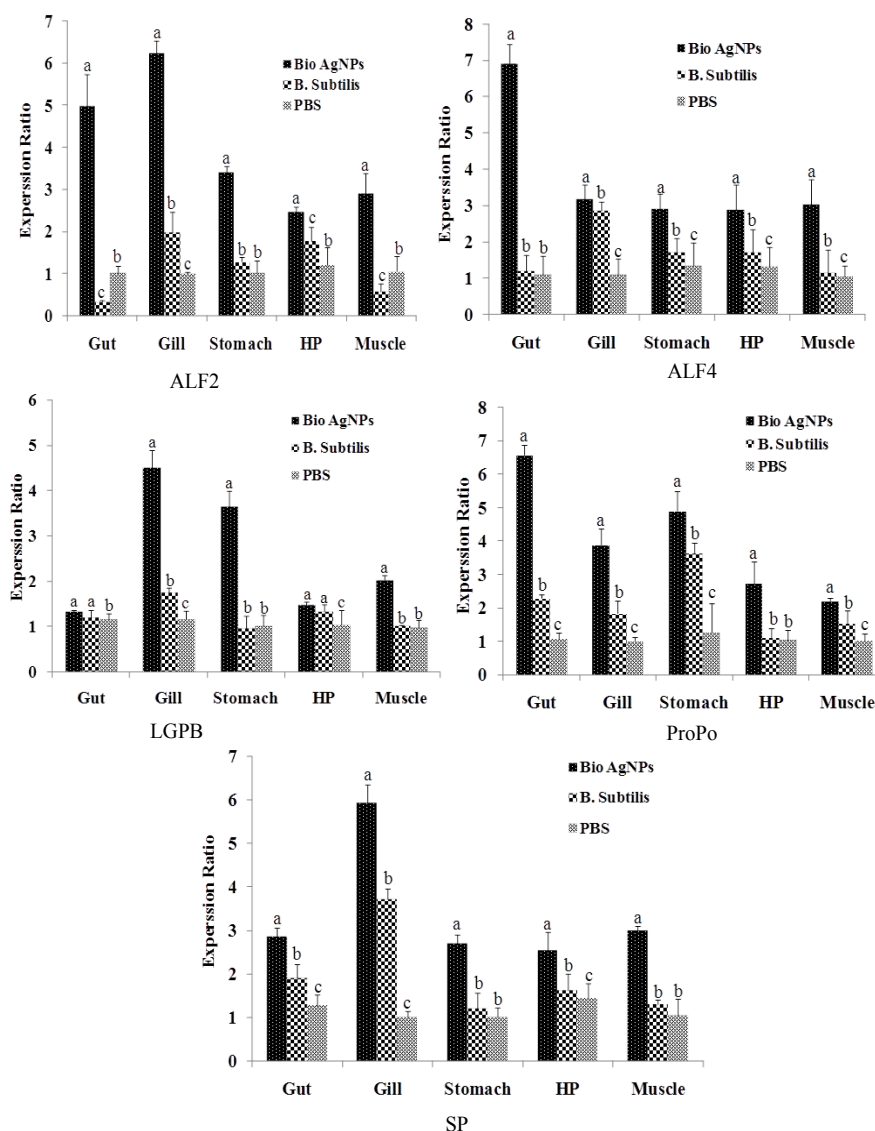


Figure 8: The mRNA expression of five immune-related genes of white shrimp for 15 days and challenged with *V.*

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 AgNP 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 *in vitro* and *in vivo* 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*, AgNO_3 and control tested in the *in vitro* 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 greatest value of 205 ± 4.5 cells/ml and $169 \pm 7.4 \times 10^5$ 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 terminal [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 $10 \pm 0.321\%$ 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 been 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 AgNO_3 . 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^0 . 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 buiosynthesis 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^0 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 Fe_3O_4 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

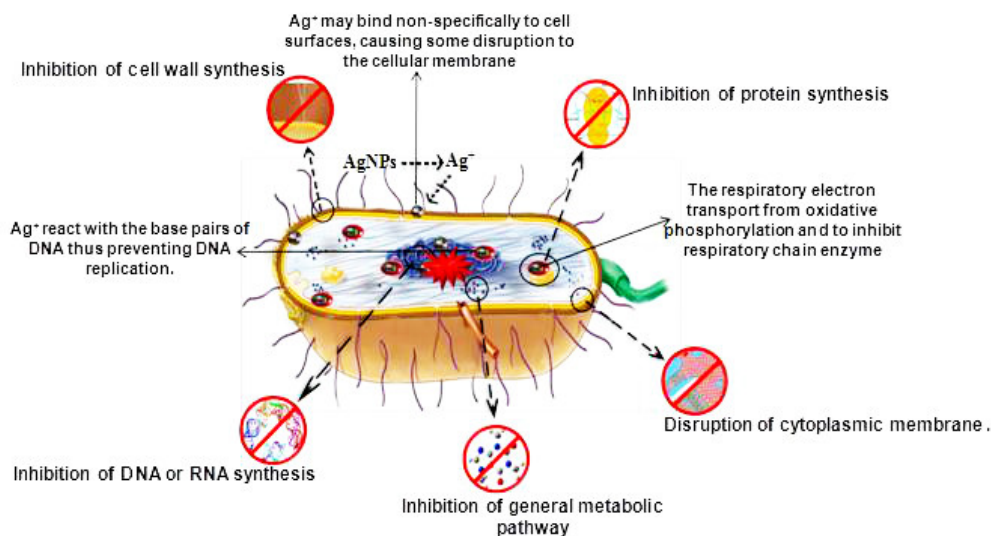


Figure 9: Schematic of AgNPs, Ag⁺ and cell intraction

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 industries. 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.

Acknowledgements

We would like to thank Dr. Yu Yang, Dr. Li Shihao and Liu Jing Wen (IOCAS, Qingdao) helping us in performing experiments and Dr. Sun Yu Miao (IOCAS, Qingdao) and doc. Dr. Kamil Zagorsek (Technical University of Liberec, Czech Republic) for critically reviewing the former version of the manuscript. The work was supported by grants of the Major State Basic Research Development Program of China (Grant number: 973 program, 2012CB114403); National High Technology Research and Development Program (Grant Number: 863rd Program, 2012AA10A404) and CAS President's International Fellowship Initiative (PIFI). Our thanks go also to the anonymous reviewers, who, with their valuable comments, improve the paper and made them easier readable.

References

- Hoagland P, Powell HK, Schumacher DJM, Katz L, Klinger D (2007) Economic sustainability of marine aquaculture. A report to the marine aquaculture task force, Takoma, Park.
- Clarke JL, Waheed MT, Lössl AG, Martinussen I, Daniell H (2013) How can plant genetic engineering contribute to cost-effective fish vaccine development for promoting sustainable aquaculture? *Plant Mol Biol* 83: 33-40.
- Poveda CM, Lucas M, Jover M (2015) Utilization of corn gluten meal as a protein source in the diet of white shrimp *Litopenaeus vannamei*. *Aquacult Nutr* 21: 824-834.
- Li F, Xiang J (2013) Recent advances in researches on the innate immunity of shrimp in China. *Dev Comp Immunol* 39: 11-26.
- Xia MH, Huang XL, Wang HL, Jin M, Li M, et al. (2015) Dietary niacin levels in practical diet for *Litopenaeus vannamei* to support maximum growth. *Aquacult Nutr* 21: 853-860.
- Lightners DV (1985) A review of the diseases of cultured penaeid shrimps and prawns with emphasis on recent discoveries and developments. Aquaculture Department, Southeast Asian Fisheries Development Center. Iloilo City, Philippines.
- Lavilla-Pitogo CR, Leano EM, Paner MG (1998) Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent vibrios in the rearing environment. *Aquaculture* 164: 337-349.
- Soonthornchai W, Rungrasamee W, Karoonthaisiri N, Jarayabhand P, Klinbunga S, et al. (2010) Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Dev Comp Immunol* 34: 19-28.
- De Schryver P, Defoirdt T, Sorgeloos P (2014) Early mortality syndrome outbreaks: a microbial management issue in shrimp farming? *PLoS Pathog* 10: e1003919.
- Schaperclaus W (1986) Fish disease. Amerind Publishing Co. Pvt. Ltd, New Delhi, India.
- FAO (2013) Report of the FAO/MARD technical workshop on Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPND) of cultured shrimp (under TCP/VIE/3304). Hanoi, Fisheries and Aquaculture Rome, Italy.
- Ringo E, Song SK (2016) application of dietary supplements (synbiotics and probiotics in combination with plant products and β -glucans) in aquaculture. *Aquacult Nutr* 22: 4-24.
- Selvin JH, Manilal A, Sujith S, Kiran GS, Lipton AP (2011) Efficacy of marine green alga *Ulva fasciata* extract on the management of shrimp bacterial diseases. *Lat Am J Aquat Res* 39: 197-204.
- NavinChandran M, Iyapparaj P, Moovendhan S, Ramasubburayan R, Prakash S (2014) Influence of probiotic bacterium *Bacillus cereus* isolated from the gut of wild shrimp *Penaeus monodon* in turn as a potent growth promoter and immune enhancer in *P. monodon*. *Fish Shellfish Immunol* 36: 38-45.
- Moreno-Garrido I, Pérez S, Blasco J (2015) Toxicity of silver and gold nanoparticles on marine microalgae. *Mar Environ Res* 111: 60-73.
- Pourjavadi A, Soleyman R (2011) Silver nanoparticles with gelatin nanoshells: photochemical facile green synthesis and their antimicrobial activity. *J Nanopart Res* 13: 4647-4658.
- Gong N, Shao K, Feng W, Lin Z, Liang C, et al. (2011) Biototoxicity of nickel oxide nanoparticles and bio-remediation by microalgae *Chlorella vulgaris*. *Chemosphere* 83: 510-516.
- Arulvasu C, Jennifer SM, Prabhu D, Chandhirasekar D1 (2014) Toxicity effect of silver nanoparticles in brine shrimp *Artemia*. *Scientific World Journal* 2014: 256919.

19. Devi JS, Bhimba BV (2014) Antimicrobial potential of silver nanoparticles synthesized using *Ulva reticulata*. Asian J Pharm Clin Res 7: 82-85.
20. El-Kassas HY, El-Sheekh MM (2014) Cytotoxic activity of biosynthesized gold nanoparticles with an extract of the red seaweed *Corallina officinalis* on the MCF-7 human breast cancer cell line. Asian Pac J Cancer Prev 15: 4311-4317.
21. Jimnez-Fernández E, Zuasti E, Ruyra A, Roher N, Infante C, et al. (2013) Nanoparticles as a novel delivery system for vitamin C administration in aquaculture. Commun Agric Appl Biol Sci 78: 202-203.
22. Ren J, Han P, Wei H, Jia L (2014) Fouling-resistant behavior of silver nanoparticle-modified surfaces against the bioadhesion of microalgae. ACS Appl Mater Interfaces 6: 3829-3838.
23. Atiyeh BS, Costagliola M, Hayek SN, Dibo SA (2007) Effect of silver on burn wound infection control and healing: review of the literature. Burns 33: 139-148.
24. Torkamani S, Wani SN, Tang YJ, Sureshkumar R (2010) Plasmon-enhanced microalgal growth in miniphotobioreactors. Appl Phys Lett.
25. Foldberg RB, Autrup H (2013) Mechanisms of silver nanoparticle toxicity. Arch Basic Appl Med 1: 5-15.
26. Fondevila M (2010) Potential Use of Silver Nanoparticles as an Additive in Animal Feeding. Nanotechnology and Nanomaterials.
27. Wright JB, Lam K, Burrell RE (1998) Wound management in an era of increasing bacterial antibiotic resistance: a role for topical silver treatment. Am J Infect Control 26: 572-577.
28. Elechiguerra JL, Burt JL, Morones JR, Camacho-Bragado A, Gao X, et al. (2005) Interaction of silver nanoparticles with HIV-1. J Nanobiotechnology 3: 6.
29. Asharani PV, Hande MP, Valiyaveetil S (2009) Anti-proliferative activity of silver nanoparticles. BMC Cell Biol 10: 65.
30. Slawson RM, Van Dyke MI, Lee H, Trevors JT (1992) Germanium and silver resistance, accumulation, and toxicity in microorganisms. Plasmid 27: 72-79.
31. Kathiresan K, Alikunhi NM, Pathmanaban S, Nabikhan A, Kandasamy S (2010) Analysis of antimicrobial silver nanoparticles synthesized by coastal strains of *Escherichia coli* and *Aspergillus niger*. Can J Microbiol 56: 1050-1059.
32. Knetsch MLW, Koole LH (2011) New strategies in the development of antimicrobial coatings: The example of increasing usage of silver and silver nanoparticles. Polymers 3: 340-366.
33. Velmurugan P, Iydroose M, Mohideen MH, Mohan TS, Cho M, et al. (2014) Biosynthesis of silver nanoparticles using *Bacillus subtilis* EWP-46 cell-free extract and evaluation of its antibacterial activity. Bioprocess Biosyst Eng 37: 1527-1534.
34. Lok CN, Ho CM, Chen R, He QY, Yu WY, et al. (2006) Proteomic analysis of the mode of antibacterial action of silver nanoparticles. J Proteome Res 5: 916-924.
35. Li X, Xu H, Chen ZS, Chen G (2011) Biosynthesis of nanoparticles by microorganisms and their applications. J Nanomater.
36. Natarajan K, Selvaraj S, Murty VR (2010) Microbial production of silver nanoparticles. Dig J Nanomater Bios 5: 135-140.
37. Mohammadian A, Shojaosadati SA, Rezaei MH (2007) *Fusarium oxysporum* mediates photogeneration of silver nanoparticles. Sci Iran 14: 323-326.
38. Garrity G, Boon DR, Castenholz RW (2001) Bergeys manual of systematic bacteriology: The archaea and the deeply branching and phototrophic bacteria. (2nd edn.), Springer-Verlag, New York, USA.
39. Singh B, Sahu PM, Sharma MK (2002) Anti-inflammatory and antimicrobial activities of triterpenoids from *Strobilanthes callosus* nees. Phytomedicine 9: 355-359.
40. Felix N, Sudharsan M (2004) Effect of glycine betaine, a feed attractant affecting growth and feed conversion of juvenile freshwater prawn *Macrobrachium rosenbergii*. Aquac Nutr 10: 193-197.
41. Zokaeifar H, Luis Balcazar J, Saad CR, Kamarudin MS, Sijam K, et al. (2012) Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 33: 683-689.
42. Chi Y, Li S, Wang D, Sun Y, Li F (2013) Expression and function analysis of Akt/PKB in pathogens challenged chinese shrimp *Fenneropenaeus chinensis*. Oceanol Limnol Sin 44: 611-617.
43. Mukherjee P, Roy M, Mandal BP, Dey GK, Mukherjee PK (2008) Green synthesis of highly stabilized nanocrystalline silver particles by a nonpathogenic and agriculturally important fungus *T-asperellum*. Nanotechnology.
44. Jain N, Bhargava A, Majumdar S, Tarafdar JC, Panwar J (2011) Extracellular biosynthesis and characterization of silver nanoparticles using *Aspergillus flavus* NJP08: a mechanism perspective. Nanoscale 3: 635-641.
45. Wei X, Luo M, Li W, Yang L, Liang X, et al. (2012) Synthesis of silver nanoparticles by solar irradiation of cell-free *Bacillus amyloliquefaciens* extracts and AgNO₃. Bioresour Technol 103: 273-278.
46. Narayanan KB, Sakthivel N (2010) Biological synthesis of metal nanoparticles by microbes. Adv Colloid Interface Sci 156: 1-13.
47. Carlson C, Hussain SM, Schrand AM, Braydich-Stolle LK, Hess KL, et al. (2008) Unique cellular interaction of silver nanoparticles: size-dependent generation of reactive oxygen species. J Phys Chem B 112: 13608-13619.
48. Sotiriou GA, Pratsinis SE (2010) Antibacterial activity of nanosilver ions and particles. Environ Sci Technol 44: 5649-5654.
49. Pal S, Tak YK, Song JM (2007) Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the Gram-negative bacterium *Escherichia coli*. Appl Environ Microbiol 73: 1712-1720.
50. El Badawy AM, Silva RG, Morris B, Scheckel KG, Suidan MT, et al. (2011) Surface charge-dependent toxicity of silver nanoparticles. Environ Sci Technol 45: 283-287.
51. Yang X, Gondikas AP, Marinakos SM, Auffan M, Liu J, et al. (2012) Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*. Environ Sci Technol 46: 1119-1127.
52. Ma R, Levard C, Marinakos SM, Cheng Y, Liu J, et al. (2012) Size-controlled dissolution of organic-coated silver nanoparticles. Environ Sci Technol 46: 752-759.
53. Kathiresan K, Nabeel MA, Gayathridevi M, Asmathunisha N, Gopalakrishnan A (2013) Synthesis of silver nanoparticles by coastal plant *Prosopis chilensis* (L.) and their efficacy in controlling vibriosis in shrimp *Penaeus monodon*. Appl Nanosci 3: 65-73.
54. Rai M, Yadav A, Gade A (2009) Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv 27: 76-83.
55. Sondi I, Salopek-Sondi B (2004) Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. J Colloid Interface Sci 275: 177-182.
56. Xiu ZM, Zhang QB, Puppala HL, Colvin VL, Alvarez PJ (2012) Negligible particle-specific antibacterial activity of silver nanoparticles. Nano Lett 12: 4271-4275.
57. Stoimenov PK, Klinger RL, Marchin GL, Klabunde KJ (2002) Metal oxide nanoparticles as bactericidal agents. Langmuir 18: 6679-6686.
58. Soderhall K, Smith VJ (1986) the prophenoloxidase activating system: the biochemistry of its activation and role in arthropod cellular immunity, with special reference to crustaceans. In: Immunity in invertebrates. Brehelin M (ed). Springer, Berlin Heidelberg New York, USA.
59. Lightner DV (1996) Disease of cultured penaeid shrimp. In: Handbook of Mariculture. Mcvey JP. (2nd edn), Crustacean Aquaculture CRC Press, Boca Raton, USA.
60. Balcázar JL, de Blas I, Ruiz-Zarzuola I, Cunningham D, Vendrell D, et al. (2006) The role of probiotics in aquaculture. Vet Microbiol 114: 173-186.
61. Mitchell SO, Baxter EJ, Holland C, Rodger HD (2012) Development of a novel histopathological gill scoring protocol for assessment of gill health during a longitudinal study in marine farmed Atlantic salmon (*Salmo salar*). Aquacult Int 20: 813-825.
62. Soegianto A, Charmantier-Daures M, Trilles J, Charmantier G (1999) Impact of copper on the structure of gills and epipodites of the shrimp *Penaeus japonicus* (Crustacea: Decapoda). J Crust Biol 19: 209-223.
63. Wu JP, Chen HC, Huang DJ (2009) Histopathological alterations in gills of white shrimp, *Litopenaeus vannamei* (Boone) after acute exposure to cadmium and zinc. Bull Environ Contam Toxicol 82: 90-95.
64. Barra JA, Pequeux A, Humbert W (1983) A morphological study on gills of a crab acclimated to fresh water. Tissue Cell 15: 583-596.
65. Singh B (1986) Studies on the bacteria associated with *Penaeus indicus* in a culture system. Cochin University of Science and Technology, India.
66. Hameed ASS (1993) A study of the aerobic heterotrophic bacterial flora of

- hatchery reared eggs, larvae and postlarvaeo *Penaeus indicus*. Aquaculture 117: 195-204.
67. Somboonwivat K, Marcos M, Tassanakajon A, Klinbunga S, Aumelas A, et al. (2005) Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*. Dev Comp Immunol 29: 841-851.
68. de la Vega E, O'Leary NA, Shockey JE, Robalino J, Payne C, et al. (2008) Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection. Mol Immunol 45: 1916-1925.
69. Cheng W, Liu CH, Tsai CH, Chen JC (2005) Molecular cloning and characterization of a pattern recognition molecule, lipopolysaccharide and b-3-glucan binding protein (LGBP) from the white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 18: 297-310.
70. Lai CY, Cheng W, Kuo CM (2005) Molecular cloning and characterisation of prophenoloxidase from haemocytes of the white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 18: 417-430.
71. Jiménez-Vega F, Vargas-Albores F, Söderhäll K (2005) Characterisation of a serine proteinase from *Penaeus vannamei* haemocytes. Fish Shellfish Immunol 18: 101-108.
72. Zokaeifar H, Babaei N, Saad CR, Kamarudin MS, Sijam K, et al. (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 Shellfish Immunol 36: 68-74.
73. Rey MW, Ramaiya P, Nelson BA, Brody-Karpin SD, Zaretsky EJ, et al. (2004) Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. Genome Biol.
74. He S, Zhang Y, Guo Z, Gu N (2008) Biological synthesis of gold nanowires using extract of *Rhodospseudomonas capsulata*. Biotechnol Prog 24: 476-480.
75. Dennis AB, Richard BF (2004) Magnetosome formation in prokaryotes. Nat Rev Microbiol 2: 217-230.
76. Kumar SA, Abyaneh MK, Gosavi SW, Kulkarni SK, Pasricha R, et al. (2007) Nitrate reductase-mediated synthesis of silver nanoparticles from AgNO₃. Biotechnol Lett 29: 439-445.
77. Koch AL (1986) The pH in the neighborhood of membranes generating a protonmotive force. J Theor Biol 120: 73-84.
78. Kemper MA, Urrutia MM, Beveridge TJ, Koch AL, Doyle RJ (1993) Proton motive force may regulate cell wall-associated enzymes of *Bacillus subtilis*. J Bacteriol 175: 5690-5696.
79. Klasen HJ (2000) Historical review of the use of silver in the treatment of burns. Renewed interest for silver. Burns 26: 117-130.
80. Russell AD, Hugo WB (1994) Antimicrobial activity and action of silver. Prog Med Chem 31: 351-370.
81. Mijndonckx K, Leys N, Mahillon J, Silver S, Van Houdt R (2013) Antimicrobial silver: uses, toxicity and potential for resistance. Biometals 26: 609-621.