Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*)

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**Abstract**

The objective of this study was to evaluate the effect of dietary supplementation of a probiotic bacterium, *Bacillus subtilis*, on the growth, immune response and antioxidant activities of shrimp (*Litopenaeus vannamei*). Shrimps with an average initial weight of 2.11 ± 0.17 g were randomly assigned to four groups with three replicates. The control group was fed a basal diet, and three treated groups were fed diets supplemented with *B. subtilis* at doses of $1 \times 10^7$, $5 \times 10^7$, and $10 \times 10^7$ colony-forming unit (CFU) g$^{-1}$ feed respectively. After 40 days of culture, 10 shrimps from each replicate were taken randomly for the determination of immune response and oxidation resistance indices. The results showed that the shrimps fed with *B. subtilis* at a dose of $1 \times 10^7$, $5 \times 10^7$ CFU g$^{-1}$ feed showed significantly better growth than that of the control diet. The phenoloxidase activities showed a tendency to increase with an increased dose of *B. subtilis* in diets but there was no significant difference among the three treated groups. Shrimps treated with $5 \times 10^7$ CFU g$^{-1}$ feed probiotic bacterium showed the highest lysozyme activity and it was significantly higher ($P < 0.05$) than the other groups. However, there was no significant difference in acid phosphatase and alkaline phosphatase activity across all the groups. The total antioxidant capacity, superoxide dismutase and glutathione peroxidase activities in the probiotic-treated groups were significantly increased ($P < 0.05$) as compared with the control groups. Both maleic dielode concentration and superoxide anion activities in the probiotic-treated groups were significantly lower ($P < 0.05$) than those of the control. The probiotic did not affect the nitric oxide synthase and the catalase activity in any of the control and treated groups. These results indicated that the probiotic *B. subtilis* could significantly promote the growth rate of shrimp by increasing the immune function and antioxidant capacity. The most effective dose of *B. subtilis* in the diet was $5 \times 10^7$ CFU g$^{-1}$ feed.

**Keywords:** *Bacillus subtilis*, *Litopenaeus vannamei*, growth, immune response, antioxidant activities

**Introduction**

During the last decade, shrimp production has been affected severely by infectious diseases caused by bacteria and viruses. With the expansion and increased intensification of *Litopenaeus* shrimp farming, there has been a negative impact on the pond environment, leading to the outbreak of infectious diseases (Gatesoupe 1999). A wide range of antimicrobial drugs, pesticides, disinfectants and chemicals...
are routinely used to control disease (Gomez-Gil, Roque & Turnbull 2000). The excessive and inappropriate use of such antimicrobials in disease prevention and growth promotion not only caused a deterioration in the pond environment, affecting the health status of shrimp, but has also led to the development of resistant strains of bacteria in shrimp culture (Weston 1996; Esiobu, Armenta & Ike 2002). A number of preventive approaches such as the use of vaccines (Gudding & Evesen 2005), immunostimulants (Sakai 1999) and probiotics (Rengpipat, Rukpratanporn, Piyaratitivorakul & Menasaveta 2000) have been explored in order to reduce the losses due to diseases and mortality of culture stock.

The use of probiotics in the culture of aquatic organisms is increasing, with the demand for more environment-friendly aquaculture practices (Gatesoupe 1999). Probiotics in aquaculture have been shown to have several modes of action: competitive exclusion of pathogenic bacteria through the production of inhibitory compounds; improvement in water quality; enhancement in the immune response of the host species to resist against infectious agents; and enhancement in the nutrition and growth of host species (Thompson, Abreu & Cavalli 1999; Verschuere, Rombaut, Sorgeloos & Verstraete 2000). Bacteria that have been used successfully as probiotics include *Bacillus* lactic acid bacteria, yeasts, *Pseudomonas* and *Vibrio* (Moriarty 1998; Rengpipat et al. 2000; Alavandi, Vijayan & Santiago 2004; Wang, Tian, Yao & Li 2008). Studies have shown that administration of these bacteria as probiotics in the shrimp *Litopenaeus vannamei* and *Fenneropenaeus indicus* as well as in the fish *Labeo rohita* led to improved growth and survival rate were improved, as well as enhancement in immunity and disease resistance (Rengpipat, Rukpratanporn, Piyaratitivorakul & Menasaveta 1998; Rengpipat et al. 2000; Saeed, Mehran & Ghobad 2006; Nayak, Swain & Mukherjee 2007). Among these, *Bacillus* bacteria have been used widely as putative probiotics. Especially *B. subtilis*, a Gram-positive non-pathogenic, spore-forming bacterium, is now being used for oral bacterial therapy, prophylaxis of gastrointestinal disorders, improving pond water quality and the survival of animals in aquaculture (Irene, Alberto, Ángeles & José 2005). Because ingestion of appropriate quantities of *B. subtilis* is anticipated to revive the normal microbiota of animal after antibiotic use or critical illness (Mazza 1994) or produce a wide spectrum of antibacterial substances (Sugita, Hirose, Matsuo & Deguchi 1998). In addition, the clinical effects of *B. subtilis* as an immunomodulatory agent in a variety of diseases have been documented (Green, Wakeley, Page, Barnes, Baccigalupi, Ricca & Cutting 1999). A number of microbial cell components such as muramyl dipeptide, lipopolysaccharides, β-glucans and heat-killed bacterial preparations are reported to possess immunostimulatory properties (Sakai 1999). However, studies on the use of live bacterial cells as probiotics to improve the immune system and antioxidant capacity are scarce (Rengpipat et al. 2000). Therefore, the present investigation was carried out to study the effect of a dietary probiotic, *B. subtilis*, on the growth, immune response and antioxidant capacity of the shrimp *Litopenaeus vannamei*.

**Material and methods**

**Bacteria and diet preparation**

The probiotic bacterium, *B. subtilis*, isolated and identified from the gut contents of farm-reared shrimp (*Litopenaeus vannamei*), was grown for 48 h at 30 °C in shaken bottles with Luria–Bertani media. The culture was centrifuged at 2795 g for 15 min at 4 °C. The supernatant was discarded, while the pellet was re-suspended in a sterile 10% NaCl solution. The suspension was similarly washed and re-centrifuged three times. The cell density was calculated from the OD$_{600}$ value and also correlated to the colony-forming unit (CFU) count using serial dilution and spread plate techniques. Purified and quantified bacteria were maintained at 4 °C in a suspended form and were used for feed preparation as required.

Four different types of feed were prepared for the experiment. Supplements and their composition are shown in Table 1. All the ingredients and chemicals used were purchased from Sangon and East China Pharmaceuticals Company, Shanghai, China. The control feed was a basal diet without any probiotics. In the other three treatments, the probiotic bacterium *B. subtilis* suspension was added at a final dose of $1 \times 10^4$, $5 \times 10^4$ and $10 \times 10^4$ CFU g$^{-1}$ feed respectively. In order to achieve accurate final concentrations of feed, the bacterial suspension was added slowly to the feed, with gradual mixing in a drum mixer. The amount of *B. subtilis* in each feed was determined by plate counting. The experimental diets were dried in a drying cabinet using an air blower at 38 °C until the moisture levels were around 10%. After drying, the finished pellets were stored at 4 °C until used.
Table 1 The percentage composition of the basal diet used in the experiment

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Composition (%)</th>
<th>Proximate composition (% wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>30</td>
<td>Crude protein 39.22</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>20</td>
<td>Crude fat 6.25</td>
</tr>
<tr>
<td>Peanut meal</td>
<td>9</td>
<td>Crude fiber 3.67</td>
</tr>
<tr>
<td>Bread flour</td>
<td>23.2</td>
<td>Crude ash 13.28</td>
</tr>
<tr>
<td>Beer yeast</td>
<td>5</td>
<td>Gross energy (kcal kg⁻¹) 4339</td>
</tr>
<tr>
<td>Shrimp head meal</td>
<td>7</td>
<td>Moisture 9.39</td>
</tr>
<tr>
<td>Fish oil</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Soybean</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>phospholipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin*</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Mineral mixture†</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

*Vitamin composition: Vitamin A 14 000 IU, vitamin D 500 IU, vitamin E 44 mg, vitamin K 1.6 mg, vitamin Bl 20 mg, vitamin B2 20 mg, vitamin B5 40 mg, vitamin B6 14 mg, vitamin B12 0.002 mg, calcium pantothenate 10 mg, t-biotin 0.04 mg, inositol 400 mg kg⁻¹ feed.
†Composition (mg kg⁻¹ feed): Cu 53, Fe 60, Zn 100, Mn 60, Se 0.49, Co 50, I 30.
Vitamin and mineral mixture: commercially obtained from Zhanjiang Yuehai Feed (China).

Experimental design
Six hundred healthy shrimps of average weight 2.11 ± 0.17 g obtained from a commercial shrimp farm (Shaoxing Shuijiang Aquaculture, Shaoxing County, Zhejiang Province) were fed with a basal diet and acclimatized for 7 days before conducting the experiment, and then randomly assigned to four groups with three replicates. Each replicate included 50 shrimps, which were maintained in an aquarium of 400 L capacity, with a daily one-third water exchange and fed a diet at 5% of body weight twice daily. During a 40-day feeding experimental period, different physico-chemical parameters such as temperature, dissolved oxygen (DO) and pH were routinely monitored. The temperature, salinity and pH of the rearing aquaria varied from 25 °C to 30 °C, 5 to 8% and 7.4 to 7.8 respectively. The DO level was maintained above 6 mg L⁻¹ by setting the air pump.

Sampling for analysis
At the end of the experiment, the survival rate and weight gain rate (WGR) of the different groups were calculated according to the following equations:

Survival rate (%)  = 100 × N_t/N_0
WGR (%)  = 100 × (W_t - W_0)/W_0

where N_t and W_t are the number and the average weight of shrimp at the termination of the experiment, N_0 and W_0 are the number and average weight of shrimp at the start of the experiment respectively.

On the 40th day, ten shrimps from each replicate of groups were taken to assay the immune response and oxidation resistance indices. The haemolymph was collected from each of the experimental shrimps from the ventral sinus, using a sterile disposable 26 G needle fitted to a tuberculin syringe containing 100 μL anticoagulant solution (30 mM trisodium citrate, 388 mM sodium chloride, 115 mM glucose and 10 mM ethylene diaminetetraacetic acid). The haemolymph test samples were centrifuged at 4600 g for 10 min at 4 °C. The supernatant was collected in a fresh sterile tube and used to determine the activities of lysozyme (LSZ), phenoloxidase (PO), acid phosphatase (ACP) and alkaline phosphatase (AKP).

The liver samples were dissected from the cephalothorax of each shrimp using the autopsy technique on the 40th day, rinsed in 0.9% NaCl and stored at -80 °C before analysis. The liver samples were divided into two parts for determination of antioxidative enzymatic activity and lipid peroxidation. The samples for enzyme analysis were homogenized in PBS buffer (pH 7.4) using a glass homogenizer. Samples were then centrifuged at 15 099 g for 10 min, and the supernatants were used to determine the total antioxidant capacity (T-AOC), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), nitric oxide synthase (NOS) and superoxide anion (O₂⁻) activities. The second part of the liver tissue was homogenized in 1.15% KCl and assayed for maleic dialdehyde (MDA) content, the final product of lipid peroxidation.

Analytical methods
Immune enzyme assays
Phenoloxidase activity was measured spectrophotometrically using I-3,4-dihydroxyphenylalanine (Sigma, Shanghai, China) as the substrate (Soderhall 1981) in a flat-bottomed microtitre plate. The dopachrome formed was measured by reading the absorbance at 490 nm using a MULTISCAN ELISA reader (Thermo Multiskan MK3, Labsystems, Helsinki, Finland) for 1 and 3 min respectively. Phenoloxidase
activity was expressed in units defined as the amount of enzyme yielding an increase in absorbance of 0.001 min$^{-1}$.

The LSZ activity in the serum was measured according to Parry, Chandan and Shahani (1965), with a slight modification. A turbidometric assay utilizing lyophilized *Micrococcus flauus* NCIMB8166 was used as the substrate. Lyophilized *M. flauus* cells were re-suspended in 2.0 mL of 0.05 M phosphate buffer (pH 6.2) at a concentration of 0.25 mg mL$^{-1}$ and incubated at 30 °C for 5 min, and then 100 μL of serum was added to 2.0 mL of the suspension. The transmittance was measured after 5 and 125 s at 540 nm (Thermo Multiskan MK3, Labsystems). Lysozyme activity = ($T_{5} - T_{125}$)/($S_{5} - S_{125}$) where $T$ is the sample transmittance and $S$ is the standard transmittance.

The ACP and AKP activities were measured using a disodium phenyl phosphate method (Liu 2006). One unit of ACP activity is defined as the production of 1 mg phenol 15 min$^{-1}$ mg$^{-1}$ protein, and AKP activity is defined as the production of 1 mg phenol 60 min$^{-1}$ mg$^{-1}$ protein.

Antioxidative assays

The T-AOC was determined using TPTZ reagent. This method measures the ability of the antioxidants present in the sample to reduce ferric-tripiridyltriazine (Fe$^{3+}$ TPTZ) to a ferrous form (Fe$^{2+}$), which absorbs light at 593 nm (Thermo Multiskan MK3, Labsystems). The T-AOC levels of the sample were calculated by plotting a standard curve of absorbance against μmol L$^{-1}$ concentration of an Fe$^{2+}$ standard solution (Benzie & Strain 1996).

The SOD level was measured spectrophotometrically using the xanthine oxidase–cytochrome c method (McCord & Fridovich 1969) at a wavelength of 550 nm (Thermo Multiskan MK3, Labsystems). The SOD levels of the sample were calculated by following the rate of disappearance of H$_2$O$_2$ at 240 nm (Thermo Multiskan MK3, Labsystems) (Bergmeyer 1963). One unit of CAT activity is defined as the amount of enzyme catalysing the degradation of 1 μmol of H$_2$O$_2$ min$^{-1}$ and specific activity corresponding to μmol transformation of substrate (H$_2$O$_2$) min$^{-1}$ mg$^{-1}$ protein.

The analysis of lipid peroxidation was carried out as described, with minor modifications (Beuge & Aust 1978). The reaction mixture was prepared by adding 1 mL homogenate to a 4 mL reaction solution (15% trichloro acetic acid0.376% thiobarbituric acid0.25 N NaOH, 1:1:1, w/v), the mixture was heated to 100 °C for 10 min, then cooled to room temperature and centrifuged at 11 180 g for 10 min. The absorbance of the supernatant was recorded at 532 nm (Thermo Multiskan MK3, Labsystems). The MDA concentration was expressed as nmol mg$^{-1}$ protein in the supernatant.

The superoxide anion (O$_2$·$^-$) was calculated by reduction of nitro blue tetrazolium as described (Johnson 1984). The NOS was measured using the enzymatic double cycle method as described, with minor modifications (Wang, Inoue & Nakayama 2000).

Protein assay

The protein contents in the serum and the supernatants of liver tissue for biochemical analysis were determined using a Coomassie brilliant blue staining method with bovine serum albumin as a standard. All analyses were performed in duplicate.

Statistical analysis

Data are reported as means ± standard deviation. All data means were compared using Duncan’s multiple range test (Statistical Package Social Science, SPSS, version 13.0). Statistical analyses of the data were performed using a one-way analysis of variance. A value of $P < 0.05$ was considered to be statistically significant.

Results

Growth performance

Data on the growth performance of the shrimp, including the final weight, WGR and survival rate, are given in Table 2. After 40 days of culture, there were no significance differences ($P > 0.05$) in the survival rate between all the treated and control groups. Shrimp treated with *B. subtilis* at dose of 5 × 10$^8$ CFU g$^{-1}$ feed showed the highest final weights and WGR (7.26 ± 0.15 g and 247.37 ± 4.97), respectively, followed by the shrimp treated at a dose...
of 1 × 10^4 CFU g⁻¹ feed (6.23 ± 0.42 g and 191.12 ± 13.98 respectively). The final weight and WGR in both groups were significantly higher (P < 0.05) than the other groups, while there was no significant difference. Clearly, feed supplemented with B. subtilis at a dose of 5 × 10^3 and 1 × 10^4 CFU g⁻¹ feed appeared to enhance the growth of shrimp.

**Immune response**

The effects of addition of B. subtilis in feed on shrimp serum immune factors including phenoloxidase. 

**Table 2** Growth performance of shrimp treated with different doses of Bacillus subtilis

<table>
<thead>
<tr>
<th>Group (CFU g⁻¹ feed)</th>
<th>Final weight (g)</th>
<th>WGR (%)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.50 ± 0.48a</td>
<td>160.66 ± 16.12a</td>
<td>92.0 ± 1.3a</td>
</tr>
<tr>
<td>1 × 10^4</td>
<td>6.23 ± 0.42b</td>
<td>191.12 ± 13.98b</td>
<td>94.2 ± 2.0b</td>
</tr>
<tr>
<td>5 × 10^4</td>
<td>7.26 ± 0.15c</td>
<td>247.37 ± 4.97c</td>
<td>93.1 ± 1.0c</td>
</tr>
<tr>
<td>10 × 10^4</td>
<td>5.58 ± 0.57d</td>
<td>164.45 ± 28.55d</td>
<td>94.0 ± 2.5d</td>
</tr>
</tbody>
</table>

Results are presented as means ± SD of triplicate observations. Means in the same column with different superscripts are significantly different (P < 0.05).

WGR, weight gain rate.

**Table 3** Effect of probiotic Bacillus subtilis on serum immune factors after 40 days

<table>
<thead>
<tr>
<th>Group</th>
<th>LSZ (U mL⁻¹)</th>
<th>PO (U mg⁻¹ prot)</th>
<th>ACP (U mg⁻¹ prot)</th>
<th>AKP (U mg⁻¹ prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1447.62 ± 367.37b</td>
<td>1.38 ± 0.20b</td>
<td>297.02 ± 0.34</td>
<td>311.11 ± 18.67</td>
</tr>
<tr>
<td>1 × 10^4</td>
<td>1638.10 ± 287.61b</td>
<td>2.23 ± 0.06b</td>
<td>298.32 ± 1.41</td>
<td>314.89 ± 12.74</td>
</tr>
<tr>
<td>5 × 10^4</td>
<td>2590.48 ± 237.91a</td>
<td>2.40 ± 0.06a</td>
<td>302.46 ± 0.56</td>
<td>332.92 ± 2.49</td>
</tr>
<tr>
<td>10 × 10^4</td>
<td>1409.53 ± 147.57b</td>
<td>2.67 ± 0.26b</td>
<td>300.15 ± 3.34</td>
<td>307.04 ± 7.40</td>
</tr>
</tbody>
</table>

Results are presented as means ± SD of triplicate observations. Means in the same column with different superscripts are significantly different (P < 0.05).

PO, phenoloxidase; LSZ, lysozyme; ACP, acid phosphatase; AKP, alkaline phosphatase.

**Table 4** Effect of probiotic Bacillus subtilis on serum antioxidation factors after 40 days (U mg⁻¹ protein)

<table>
<thead>
<tr>
<th>Group</th>
<th>T-AOC</th>
<th>GSH-Px</th>
<th>SOD</th>
<th>CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.61 ± 0.38c</td>
<td>15.19 ± 0.57b</td>
<td>11.29 ± 3.55b</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>1 × 10^4</td>
<td>7.12 ± 2.00a</td>
<td>143.18 ± 54.34a</td>
<td>28.18 ± 5.92a</td>
<td>1.05 ± 0.06</td>
</tr>
<tr>
<td>5 × 10^4</td>
<td>4.40 ± 1.95b</td>
<td>139.37 ± 19.98b</td>
<td>20.41 ± 2.91b</td>
<td>1.29 ± 0.21</td>
</tr>
<tr>
<td>10 × 10^4</td>
<td>8.34 ± 2.50a</td>
<td>120.47 ± 46.41a</td>
<td>27.34 ± 5.51a</td>
<td>1.05 ± 0.03</td>
</tr>
</tbody>
</table>

Group | INOS | MDA | O₂⁻
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>21.19 ± 1.76</td>
<td>8.67 ± 0.81a</td>
</tr>
<tr>
<td>1 × 10^4</td>
<td>21.08 ± 1.01</td>
<td>7.81 ± 0.15a</td>
</tr>
<tr>
<td>5 × 10^4</td>
<td>20.41 ± 2.02</td>
<td>5.71 ± 0.72b</td>
</tr>
<tr>
<td>10 × 10^4</td>
<td>23.50 ± 0.32</td>
<td>4.83 ± 0.67b</td>
</tr>
</tbody>
</table>

Results are presented as means ± SD of triplicate observations. Means in the same column with different superscripts are significantly different (P < 0.05).

T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; iNOS, nitric oxide synthase; O₂⁻, superoxide anion; MDA, malonic dialdehyde.
probiotic-treated groups were significantly lower ($P<0.05$) than the control. The maleic dialdehyde concentrations in $5 \times 10^4$ and $10 \times 10^4$ CFU g$^{-1}$ treated groups were significantly lower ($P<0.05$) than the other treated groups and the control group. There was no significant difference in the nitric oxide synthase and CAT activity ($P>0.05$) among all the treated and control shrimp.

**Discussion**

In this present study, it was shown that the maximum growth of shrimp (*P. vannamei*) was obtained by dietary supplementation of *B. subtilis* after a 40-day feeding trial, and appropriate doses of *B. subtilis* were $5 \times 10^4$ feed and $1 \times 10^5$ CFU g$^{-1}$ feed. Similar results were obtained in *Pseudomonas mondon*, *Fenneropenaeus indicus* and *P. vannamei* using *Bacillus* as a probiotic in the feed or in the culture water (Rengpipat et al. 1998; Mariel, Fabiano & Jenny 2004; Li, Zheng, Tian, Yuan, Zhang & Hong 2007; Wang 2007). However, Shariff, Yusoff, Devaraja and Srinivasa Rao (2003) and McIntosh, Samocha, Jones, Lawrence, McKee, Horowitz and Horowitz (2000) found that treatment of *P. mondon* and *P. vannamei* with a commercial *Bacillus* probiotic did not increase either survival or growth significantly ($P>0.05$). Hence, the effect of a probiotic *Bacillus* strain is determined by its characteristics, including enzymatic activity-secreted inhibition of pathogen growth, ability to improve immune response and antioxidant activities of host and so on. Administration of the *Bacillus* bacteria may enhance the digestion and nutrient absorption of shrimp by the synthesis of vitamins, cofactors or enzyme (Gatesoupe 1999), resulting in an increase in weight (Saeed et al. 2006; Li et al. 2007). *Bacillus*, when used as a probiotic, was able to inhibit pathogens by colonizing both the culture water and the shrimp digestive tract to exclude other harmful bacteria in *P. mondon* and *P. vannamei*, producing an anti-bacterial substance or activating both celluar and humoral immune defences in shrimp (Rengpipat et al. 1998; Mariel et al. 2004), which in turn contributed to improved growth and survival ability in the shrimps. In this study, the shrimp treated with *B. subtilis* at the highest dose of $10 \times 10^4$ CFU g$^{-1}$ feed showed a growth response similar to that of the control group, which indicated that the effects of probiotic bacteria are not directly dose-dependent. The results of the present study are in accordance with earlier reports (Epifario 1979; Uma, Abraham, Jeyaseelan & Sundararaj 1999; Murthy & Naik 2002). The reduced growth caused by the higher proportion of probiotics was due to poor digestion (Epifario 1979), a catabolic effect (Murthy & Naik 2002) and excessive faecal loss (Uma et al. 1999). Most microbes in the gastrointestinal tract are transients in aquatic animals and may change rapidly with the intrusion of microbes emerging from water and food. Thus, the reduced growth of shrimps treated with the highest dose of *B. subtilis* might also be related to the imbalance of microbial flora in the gastrointestinal tract.

The pro-phenoloxidase system was involved in encapsulation and melanization, which functions as a non-self-recognition system (Johansson & Soderhall 1989). The PO and LSZ activities in all the treatments were significantly higher than in the control, while ACP and AKP activities in the treatments remained the same as in the control treatment value. The results indicated that the immune response of shrimp was strongly stimulated by ingestion of *B. subtilis*. Similar results were obtained by Rengpipat et al. (2000) with *Bacillus S11 in P. mondon* and by Mariel et al. (2004) with *Bacillus P64 in P. vannamei*. Tseng, Ho and Huang (2009) also found that shrimp fed at a higher concentration of the probiotic ($10^5$ CFU g$^{-1}$ feed) exhibited a significant increase in phenoloxidase activity, phagocytic activity and clearance efficiency compared with the control shrimp. The bacterial biomass mixed with feed might help in colonization of the intestine to perform competitive exclusion mechanism in shrimp (Rengpipat et al. 2000), and colonization with specific microbiota may play a role in balancing the intestinal mucosal immune system, which may contribute towards the induction and maintenance of immunological tolerance or inhibition of the deregulated responses induced by pathogens in the host (Gatesoupe 1999).

The effectiveness of T-AOC can be evaluated using two approaches. Firstly, its inhibitory effects on the formation of lipid peroxidation products, and secondly, its relationship with each of the enzymatic and nonenzymatic antioxidant factors (Benzie & Strain 1996). Assaying of antioxidant enzymes can provide an indication of the antioxidant status of the organisms and can serve as biomarkers of oxidative stress (Kohen & Nyska 2002). One of the most important biochemical parameters for antioxidant effects is the SOD level of tissues. SOD is an enzyme-metabolizing superoxide radical, and its level is directly related to GSH-Px and CAT activities. Studies of the enhancement of the antioxidant ability of shrimps arising from the administration of *Bacillus* are scarce. In the present study, a promising antioxidative response sti-
mulation of *P. vannamei* was shown with probiotic *B. subtilis* treatment. The *B. subtilis* probiotic significantly increased T-AOC, SOD, GSH-Px and reduced superoxide anion (O$_2^-$) generation. A probable explanation could be that the expression of antioxidants such as SOD and GSH-Px can neutralize reactive oxygen metabolites to avoid self-damage.

The superoxide anion (O$_2^-$) can react with NO to generate peroxynitrite, which may lead to the formation of hydroxyl (OH) radicals (Hogg, Darley-Usmar, Wilson & Moncada 1992; Koppenol, Moreno, Pryor, Ischiropoulos & Beckman 1992). Both peroxynitrite and hydroxyl radicals are good oxidants and can damage protein. In this study, the nitric oxide synthase remained unchanged in treated and control shrimps, indicating that supplementation with probiotic *B. subtilis* did not affect the generation of NO.

Lipid peroxidation, considered to be a complex process that is self-propagating and causes destruction, increases the rigidity of cellular membranes. The widely used assay for lipid peroxidation is MDA formation, which represents the final product of lipid peroxidation. The concentration of MDA is direct evidence of the toxic processes caused by free radicals and the MDA level was considered to be suitable indicator of the extent of lipid peroxidation (Nogueira, Quinhones, Jung, Zeni & Rocha 2003). The decrease in the MDA level might be an indicator of an increase in the enzymatic and nonenzymatic antioxidants of defence mechanisms. During the present investigations, a significantly lower MDA concentration in the shrimp fed with $5 \times 10^4$ CFU g$^{-1}$ probiotics was observed compared with the control group. The results revealed that dietary supplementation of *B. subtilis* increased the activities of SOD and GSH-Px, and consequently reduced lipid peroxidation and superoxide anion (O$_2^-$) in the liver of shrimp (*P. vannamei*).

**Conclusion**

The results of this study showed that probiotic *B. subtilis* at a dose of $1 \times 10^8$ and $5 \times 10^3$ CFU g$^{-1}$ in shrimp (*P. vannamei*) diets can significantly improve the growth by enhancing the immune response (PO and LSZ) and antioxidative activity (T-AOC, SOD and GSH-Px activity). However, the related mechanism remains to be tested in further studies.

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**References**


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