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# Effects of potential probiotic *Bacillus subtilis* T13 on growth, immunity and disease resistance against *Vibrio splendidus* infection in juvenile sea cucumber *Apostichopus japonicus*

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ABSTRACT

A feeding experiment was conducted to determine influences of potential probiotic *Bacillus subtilis* T13 (isolated from intestine of healthy sea cucumbers) on growth, immunity and disease resistance against *Vibrio splendidus* infection in juvenile sea cucumbers *Apostichopus japonicus*. Animals were fed with diets containing *B. subtilis* T13 at 0, $10^5$, $10^7$ and $10^9$ CFU/g for 30 days, respectively. At the end of the growth trial, fifteen sea cucumbers from each aquarium were sampled for immune indices measurement. Then twenty sea cucumbers from each replicate were challenged with *V. splendidus*. Results showed that administration of *B. subtilis* T13 had significant effect on the specific growth rates (SGR) of sea cucumbers ($P < 0.05$). Phagocytosis, respiratory burst activity and total nitric oxide synthase (T-NOS) activity were significantly improved in coelomocytes of sea cucumbers fed with T13 at $10^9$ CFU/g diet ($P < 0.05$). The highest values of the total coelomocytes counts (TCC) and superoxide dismutase (SOD) activity were found in sea cucumbers fed diet containing T13 at $10^9$ CFU/g. The cumulative mortality after *V. splendidus* challenge decreased significantly in sea cucumbers fed with T13 at dose of $10^9$ CFU/g ($P < 0.05$). The present study confirmed the potential beneficial effects of *B. subtilis* T13 as dietary probiotic in juvenile *A. japonicus*.



## 1. Introduction

Sea cucumber *Apostichopus japonicus* (Selenka) is one of the commercially important holothurian species in China. During the last decade, sea cucumber farming has developed rapidly and become a vigorous industry in northern China [1,2]. However, it has suffered from intractable problems of infectious diseases. For example, the skin ulceration syndrome is severe and causes considerable economic losses [3–7]. Many methods have been taken to increase the aquaculture animal's immunity and disease resistance, such as the use of vaccines [8], immunostimulants [9] and probiotics [10–12]. Probiotics are live microorganisms which can confer a health benefit on the host, when administered in adequate amounts [13]. The genus *Bacillus* is one of dominant probiotics, which are commonly used in aquaculture. Numerous studies have found that endogenous or exogenous *Bacillus* strains could be effective in improving growth, immunity and disease resistance in fish [14–16] and shrimp [17–20]. As for sea cucumber, the application of *Bacillus* is still in its infancy, especially endogenous *Bacillus* strains. In addition to researches conducted by Zhou et al. [21] and Luo [22], there is little published data on application of endogenous *Bacillus* sp. in sea cucumbers. In present study, a new endogenous potential probiotic *Bacillus* strain T13 was isolated from healthy sea cucumber intestine and was identified as *B. subtilis* by cluster analysis on the sequence of 16S rDNA. The *in vitro* antimicrobial assay showed that *B. subtilis* T13 inhibited the growth of *V. splendidus* which causes skin ulceration syndrome of sea cucumbers. However, the beneficial effects of probiotic *in vitro* could not guarantee the efficiency *in vivo*. Therefore, this work was also conducted *in vivo* to evaluate the potential of endogenous *B. subtilis* T13 as dietary probiotics on growth, immunity and disease resistance against *V. splendidus* in sea cucumber *A. japonicus*.

## 2. Materials and methods

### 2.1. Bacteria

The bacteria strain T13 used in this study was isolated from the intestine of healthy sea cucumber. It was identified as *B. subtilis* by cluster analysis on the sequence of 16S rDNA [23,24]. Using spot-

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on-lawn method [25], T13 was found to be inhibitory against *V. splendius* which caused the skin ulceration syndrome disease in sea cucumber. The safety of T13 was tested by immersing sea cucumbers in the suspension of T13 at final concentrations of $10^3$, $10^5$, $10^7$ CFU/ml [24,26]. Fresh suspension of T13 was added into each aquarium again after seawater was changed every 24 h. The disease symptoms and mortality of sea cucumbers were monitored for 7 days. The safety test showed that T13 did not induce disease symptoms and mortality of sea cucumbers.

### 2.2. Experimental diets

Seaweed (*Sargassum thunbergii*) powder is the palatable food for sea cucumbers and was applied universally in the juvenile sea cucumbers nursing. Therefore, the *S. thunbergii* powder was used as the basal diet in the present study. The experimental diets were prepared by supplementing graded doses of T13 at 0, $10^5$, $10^7$, $10^9$ CFU/g feed, respectively. *S. thunbergii* powder was sifted through a 149 μm mesh. Preparation of T13 was according to Aly et al. [27] with slight modification. Briefly, T13 isolates was inoculated in trypticase soy broth (TSB) with 1.5% NaCl and incubated for 24 h at 30 °C. They were then centrifuged at 5000 g for 10 min at 4 °C. After centrifugation, the bacteria were washed twice with sterile saline and the concentration of the final suspension was adjusted to $1 \times 10^{10}$ bacteria per ml in sterile saline. Graded doses of T13 were supplemented into the *S. thunbergii* powder followed by mixing thoroughly. The experimental diets were prepared every day in order to guarantee the vitality of T13.

### 2.3. Feeding experiment

Juvenile sea cucumbers were obtained from a commercial farm in Qingdao, China. Prior to the initiation of this feeding trial, sea cucumbers were acclimated to the rearing conditions for 2 weeks. Then selected sea cucumbers of similar sizes (0.204 ± 0.009 g, means ± SE) were randomly distributed into 12 aquaria. Each aquarium (20 l) was stocked with 40 sea cucumbers. Each diet was assigned to three aquaria as a treatment. During the 30-day feeding trial, all experimental animals were fed diets twice (06:00 and 18:00) daily, and 50% water in each aquarium was replaced with fresh seawater every day. Water temperature was maintained at 20–22 °C, salinity 28–30 and pH 7.8–8.3. Low pressure electrical blowers provided aeration via air stones and maintained dissolved oxygen levels at or near air-saturation.

### 2.4. Sample collection

At the termination of the feeding trial, animals were not fed for 24 h. After animals were dissected, the coelomic fluid was collected immediately, and then was thoroughly mixed with equal volume anticoagulant (0.02M EGTA, 0.48M NaCl, 0.019M KCl, 0.068M Tris–HCl, pH 7.6). The coelomic fluid from fifteen sea cucumbers in each aquarium was pooled for immunological analyses. After an aliquot of the coelomic fluid sample was taken for total coelomocytes counts (TCC), phagocytosis activity test and respiratory burst analysis, the left was centrifuged at 3000 g, 4 °C for 10 min to collect coelomocytes. Coelomocytes were resuspended in 600 μl cold 0.85% saline and then sonicated at 22 kHz for 25 s at 0 °C followed by centrifugation at 4000 g, 4 °C for 10 min. After centrifugation, the cells lysate supernatant (CLS) was frozen in liquid nitrogen and stored at −80 °C for superoxide dismutase (SOD) activity, total nitric oxide synthase (T-NOS) activity and acid phosphatase (ACP) activity assay.

### 2.5. Immune assays

#### 2.5.1. TCC

Coelomocytes were counted and calculated as cells per ml using a hemocytometer (Qiujing Inc., Shanghai, China) under light microscope at 400 × magnification.

#### 2.5.2. Phagocytic assay

Coelomocytes phagocytosis was evaluated by neutral red method [28,29] with slight modifications. Three replicates of 100 μl sea cucumber coelomic fluid from each aquarium were added to a 96-well plate and incubated at 25 °C for 30 min. The supernatant was removed and 100 μl 0.029% neutral red (Shanghai, China) was added to each well. The plates were then incubated at 25 °C for 30 min. Cells were then washed with PBS for 3 times and incubated with cell lysis buffer (acetic acid: ethanol = 1:1) for 20 min. The results were recorded with a universal microplate spectrophotometer (Thermo, Waltham, MA, USA) using a test wavelength of 540 nm. The absorbance of $10^6$ cells represents the capability of coelomocytes phagocytosing neutral red.

#### 2.5.3. Respiratory burst activity

Production of superoxide anion was evaluated using nitroblue tetrazolium (NBT, Amresco, Solon, OH, USA) following the method of Song and Hsieh [30] with slight modifications. The wells of 96-well plate were coated with 100 μl 0.2% poly-L-lysine (Sigma, St. Louis, MO, USA) solution to increase coelomocytes adhesion. Three replicates of 100 μl aliquot of coelomic fluid from sea cucumbers in each aquarium were added to wells and centrifuged at 300 g for 10 min at 4 °C. The supernatant was discarded and 100 μl phorbol 1, 2-myristate 1, 3-acetate (PMA, Calbiochem, La Jolla, CA, USA) (1 μg/ml) was added to each well. Then plates were incubated at 37 °C for 30 min. The cells in each well were then stained with 100 μl 0.3% NBT at 37 °C for 30 min. Absolute methanol was added to terminate the staining. Each well was washed three times with 70% methanol and air-dried. Then 120 μl 2M KOH and 140 μl dimethyl sulfoxide (DMSO, Amresco, Solon, OH, USA) were added and the colour was subsequently measured at 630 nm with a universal microplate spectrophotometer (Thermo, Waltham, MA, USA) using KOH/DMSO as a blank. The absorbance of $10^6$ cells represents the capability of coelomocytes respiratory burst activities.

#### 2.5.4. SOD activity

SOD activity was determined according to Ōyanagui [31] with the assay kit of Nanjing Jiancheng, Bioengineering Institute, China. The reaction was based on its inhibitory effect on the rate of superoxide anion generating by xanthine and xanthine oxidase reaction system. The colour was measured at 550 nm with a universal microplate spectrophotometer (Thermo, Waltham, MA, USA). One unit of SOD activity was defined as the amount of enzyme required for inhibiting superoxide-induced oxidation by 50%. The specific SOD activity was expressed as SOD unit per ml CLS.

#### 2.5.5. T-NOS activity

T-NOS activity was determined according to Green et al. [32] with the assay kit of Nanjing Jiancheng, Bioengineering Institute, China. The reaction was based on its catalytic ability to convert L-Arginine into NO. NO was oxidized to nitrite ($NO^{2-}$) and nitrate ($NO^{3-}$). The nitrate ($NO^{3-}$) was converted to nitrite ($NO^{2-}$) utilizing nitrate reductase. Then the addition of Griess reagents converts all the nitrite ($NO^{2-}$) into a colour compound. The optical density was measured at 530 nm with a universal microplate spectrophotometer (Thermo, Waltham, MA, USA). One unit of T-NOS activity was defined as the amount of T-NOS producing 1 nmol NO/min. The T-NOS specific activity was expressed as T-NOS unit per ml CLS.

#### 2.5.6. ACP activity

ACP activities were determined by the method of King [33] using disodium phenyl phosphate as substrate with a chemical detection kit (Nanjing Jiancheng, Bioengineering Institute, China). The unit definitions of ACP enzymatic activity corresponded to the degradation of 1 mg phenol per 100 ml CLS at 37 °C within 30 min.

### 2.6. V. splendidus immersion challenge

A virulent strain of *Vibrio splendidus* was provided by Yellow-sea Fishery Research Institute, Chinese Academy of Fishery Sciences (Qingdao, China). The strain was originally isolated from sea cucumbers diagnosed with skin ulceration disease [34]. The LD50 was determined prior to challenge and the result showed that the LD50 for 7 days by immersion was $6 \times 10^7$ CFU/ml. *V. splendidus* was grown in TSB medium with 1.5% NaCl at 28 °C for 24 h. At the end of the feeding trial, twenty sea cucumbers from each aquarium were immersed with live *V. splendidus* at the final concentration of $6 \times 10^7$ CFU/ml. The seawater was totally exchanged every 24 h and then fresh *V. splendidus* was added into the aquarium again. The mortality was monitored for 7 days.

### 2.7. Calculations and statistical analysis

$$\text{Specific growth rate(SGR)} = (\text{Ln}\, W_t - \text{Ln}\, W_0) \times 100/t$$

Where $W_t$ and $W_0$ are final and initial weights of sea cucumbers respectively, and $t$ is duration of experimental days-30 days.

Statistical analysis was performed using the SPSS 13.0 for windows. The results were presented as means ± SE (standard error of the means). All percentage data were arcsine transformed and subjected to one-way analysis of variance (ANOVA). When overall differences were significant at less than 5% level, Tukey's multiple range tests were used to compare the means between individual treatments.

## 3. Results

### 3.1. Growth performance

Effects of the dietary treatments on SGR of juvenile sea cucumbers were showed in Fig. 1. There were no significant differences in SGR of sea cucumbers among treatments with 0, $10^5$ and $10^7$ CFU/g dietary T13. However, sea cucumbers fed with $10^9$ CFU/g dietary T13 had the significant highest SGR ($P < 0.05$) among all the treatments.

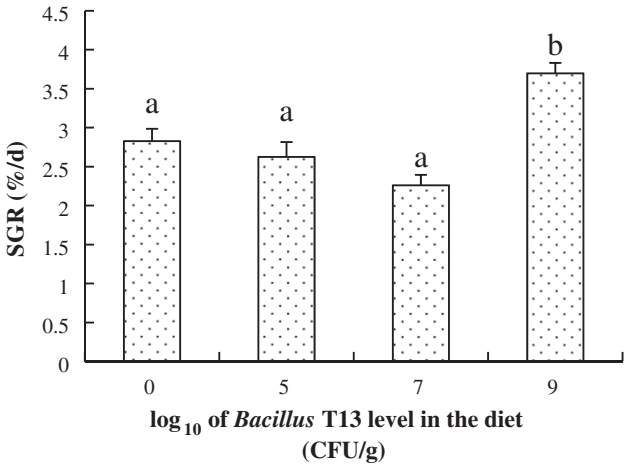


**Fig. 1.** Effects of dietary *Bacillus* T13 on the specific growth rate (SGR) of juvenile *Apostichopus japonicus*. Values represent means and standard errors of three replicates (means ± SE; $n = 3$). Different letters represent significant difference in the growth of sea cucumbers fed different level of *Bacillus* T13 ($P < 0.05$).

### 3.2. Immune response

#### 3.2.1. TCC

The highest value of the TCC in sea cucumbers was found in the treatment with $10^9$ CFU/g dietary T13 supplement. However, influences of dietary T13 on the TCC were not significant ($P > 0.05$) (Fig. 2A).

#### 3.2.2. Phagocytosis

Phagocytic activities of sea cucumbers were increased with dietary T13 supplements (Fig. 2B). The highest value of phagocytic activity was found as 0.864 (OD540/$10^6$cells) in the group with $10^9$ CFU/g dietary T13 supplement. Furthermore, it was significant higher than those in control and the group with $10^5$ CFU/g dietary T13 supplement, but not in that with $10^7$ CFU/g dietary T13.

#### 3.2.3. Respiratory burst activity

Respiratory burst activity of sea cucumbers fed diet containing T13 at $10^9$ CFU/g was significantly higher than those of sea cucumbers fed basal diet and diets containing T13 at $10^5$–$10^7$ CFU/g ($P < 0.05$) (Fig. 2C).

#### 3.2.4. SOD activity

The SOD activity in coelomocytes of sea cucumbers fed with T13 at $10^9$ CFU/g diet was the highest one among all the treatments. However, influences of dietary T13 on SOD activities in coelomocytes were not significant ($P > 0.05$) (Fig. 2D).

#### 3.2.5. T-NOS activity

The T-NOS activity of coelomocytes was significantly improved in sea cucumbers fed with T13 at $10^9$ CFU/g diet ($P < 0.05$) (Fig. 2E). It was significantly higher than that of sea cucumbers fed basal diets and diets containing T13 at $10^5$–$10^7$ CFU/g.

#### 3.2.6. ACP activity

The ACP activity in coelomocytes of sea cucumbers ranged from 3.71 U/100 ml CLS to 4.54 U/100 ml CLS. Dietary supplementation of T13 did not significantly influence these activities ($P > 0.05$) (Fig. 2F).

### 3.3. V. splendidus immersion challenge

The immersion challenge test showed that administration of T13 enhanced disease resistance of sea cucumbers against *V. splendidus* infection (Fig. 3). The cumulative mortality rate of sea cucumbers fed with $10^9$ CFU/g dietary T13 was 20.0%, which was significantly lower than those of sea cucumbers fed with basal diet (56.2%) and diet containing T13 at $10^5$ CFU/g (50.0%) or $10^7$ CFU/g (50.0%).

## 4. Discussion

The present study showed that feeding *B. subtilis* T13 at $10^9$ CFU/g diet significantly increased the SGR of sea cucumbers. Growth-improving effect of *Bacillus* sp. was observed in tilapia Nilotica, which was fed with *B. pumilus* at $10^6$ CFU/g diet for 60 days [14] and with *B. subtilis* at $10^7$ CFU/g diet for 30 days or 60 days [35]. This growth-improving effect was also found in grouper fed *B. clausii* at $10^8$ CFU/g diet for 60 days [16], and in shrimp *Litopenaeus vannamei* fed *B. subtilis* at $5 \times 10^4$ CFU/g diet for 40 days [36]. In addition, Zhang et al. [29] demonstrated that a commercial *B. subtilis* could significantly increase growth of *A. japonicus* at $1.82 \times 10^7$ CFU/g diet

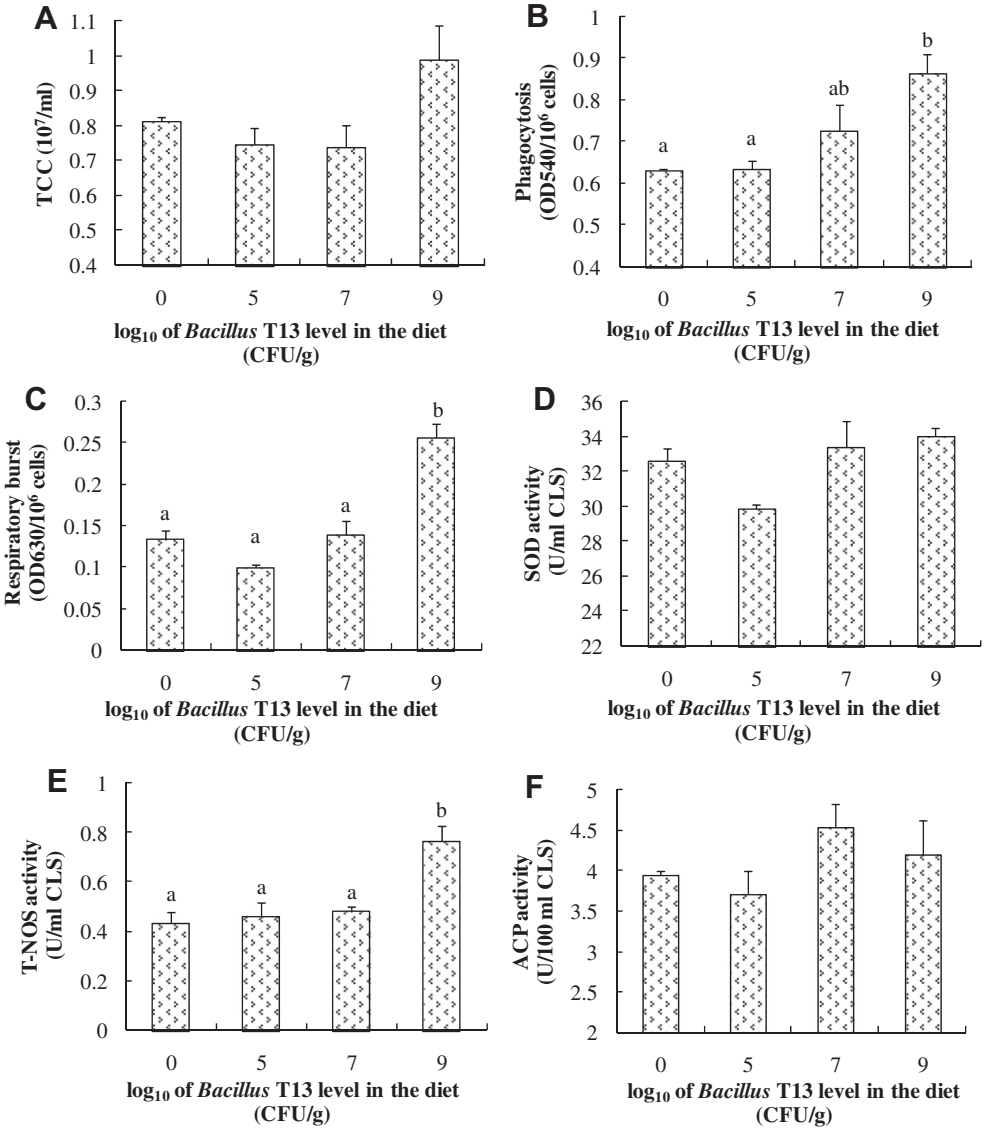


**Fig. 2.** Total coelomocytes counts (TCC) (A), phagocytosis (B), respiratory burst (C), superoxide dismutase (SOD) activity (D), total nitric oxide synthase (T-NOS) activity (E), acid phosphatase (ACP) activity (F) of juvenile *Apostichopus japonicus* fed with graded doses of dietary *Bacillus* T13. Values are means and standard errors of three replicates (means ± SE; $n = 3$). Treatments with different letters are significantly different ($P < 0.05$).

for 56 days. Compared to those reports, in the present study, the dietary level of *B. subtilis* T13, which could significantly improve the SGR of sea cucumbers, was much higher ($10^9$ CFU/g). It was suggested that although *Bacillus* sp. could improve the growth of aquatic animals, suitable doses in diet are needed. It could be attributed to the differences in *Bacillus* strains, animals (species and sizes) and experimental conditions, such as manufacturing operation of diet containing probiotics and the methods of feeding. In the present study, *B. subtilis* T13 was directly mixed with the seaweed powder and then the mixture was dissolved in the seawater and spray evenly into the aquaria, so the bacteria in the powder could leak out. This could be one of the reasons why the dietary level of *B. subtilis* T13 for improving the growth of sea cucumbers was much higher. Level of $10^9$ CFU/g dietary *B. subtilis* T13 could not be the optimal content for the growth of sea cucumbers. Micropellet feed may be effective in increasing the efficacy of probiotics for juvenile sea cucumbers. So, further studies will be necessary to find out the proper methods to improve the efficacy of *B. subtilis* T13 in the juvenile sea cucumbers aquaculture, and to determine the optimal content of *B. subtilis* T13 in diet.

Modulation of immune system is one of the most commonly benefits of the probiotics [15]. The present study also demonstrated that *B. subtilis* T13 could significantly stimulate the immune response of sea cucumbers. Phagocytosis, respiratory bursts and T-NOS activity in coelomocytes were significantly increased by dietary *B. subtilis* T13 ($10^9$ CFU/g) (Fig. 2). Coelomocytes are the key components of immune system in sea cucumber. They recognize, engulf and/or encapsulate invaded microorganisms or foreign particles, and release humoral factors [37–39]. TCC was not significantly influenced by the dietary administration of *Bacillus subtilis* T13 in the present study. It was in agreement with the results in previous study on effects of dietary commercial probiotic product *B. subtilis* on the sea cucumber TCC [29]. In addition, the present study suggested that dietary *B. subtilis* T13 increased the immunity of sea cucumber not by increasing the total numbers of coelomocytes. Phagocytosis of coelomocytes is the primary line of immune defense in sea cucumbers [37–39]. Therefore, the increased phagocytosis would be more powerful against microbial infections. This observation in sea cucumbers coelomocytes was in agreement with those in fish leucocytes [40] and shrimp

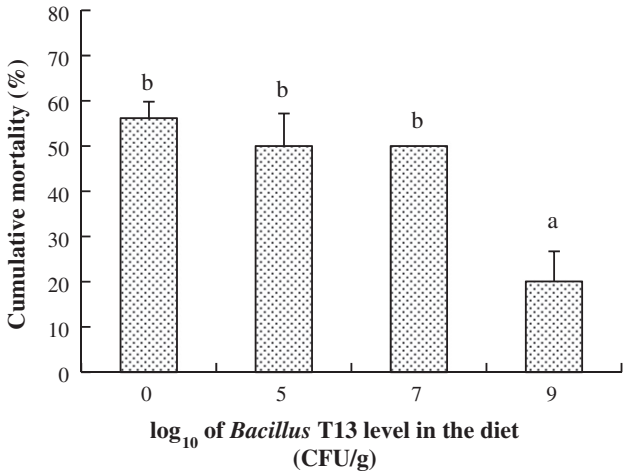


**Fig. 3.** Cumulative mortality during a 7-day *V. splendidus* challenge of juvenile sea cucumbers fed diets supplemented with graded levels of dietary *Bacillus* T13. Symbols represent means and standard errors of three replicate groups (means ± SE; $n = 3$). Different letters indicate significant difference in mean cumulative mortality after a 7-day *V. splendidus* challenge ($P < 0.05$).

hemocytes [10,20]. In shrimp, Rengpipat et al. [10] pointed out that a *Bacillus* surface wall peptidoglycan possibly elicited an immune function by acting on granulocytes for higher phagocytic activity. However, the precise mechanism by which *Bacillus* sp. increase phagocytic activity in aquatic animals is still not clear. Respiratory bursts are produced by phagocytes in order to attack invasive pathogens during phagocytosis, and have been widely used to evaluate the defense ability against pathogens [41]. Significant increase with this non-specific defense response in *Labeo rohita* and in grouper has been found following oral administration of *B. subtilis* and *Lactobacillus plantarum*, respectively [41,42]. Similar with this beneficial effect, such increased respiratory burst activity induced by the dietary *B. subtilis* T13 was also found in coelomocytes of sea cucumbers in the present study.

NOS is responsible for the production of NO, which is considered to be important mediators in the protective immune response system [43]. The role of NOS in the immune response of invertebrates has been reported by many studies [44]. For instance, the increased NOS activity has been demonstrated in the hemocytes of shrimp [45], crayfish [46] and snail [47] after exposure to LPS and in the shrimp *Fenneropenaeus chinensis* after administration of VP28 using *B. subtilis* as vehicles [48]. Besides, several immunostimulants such as β-glucan, mannan oligosaccharides and vitamin C were found to stimulate the NOS activity in coelomocytes of sea cucumber *in vitro* as well [49]. The stimulation of NOS activity by dietary *B. subtilis* T13 shown in the present study ratified the important role of NOS in immune response of sea cucumber coelomocytes *in vivo*.

The present study showed that the influences of dietary *B. subtilis* T13 on activities of SOD and ACP were not significant. SOD catalyses the dismutation of the extra bactericidal highly reactive $O^{2-}$ to $O_2$ and less reactive $H_2O_2$ [48], and is the important component of antioxidant defense system of the organism [50]. The effect of dietary administration of *Bacillus* sp. on SOD activity in shrimp and fish was studied extensively with inconsistent results. The significantly increased SOD activity was found in shrimp *L. vannamei* fed *B. licheniformis* at $10^5$ CFU/g [51]. But no significant difference in SOD activities was observed in shrimp *L. vannamei* fed *B. subtilis* E20 at $10^6$, $10^7$ or $10^8$ CFU/g [20] and in grouper *Epinephelus coioides* fed *Bacillus pumilus* or *Bacillus clausii* at $10^8$ CFU/g [16]. Similar with Tseng et al. [20] and Sun et al. [16], the present study also showed that dietary administration of *B. subtilis* T13 did not significantly influence SOD activity in coelomocytes of sea cucumbers. However, the variation of SOD activities was correlated well with the change of respiratory burst of sea cucumbers coelomocytes. ACP plays an important role in the immune system as a key compound of lysosomal enzymes to digest the invading organisms in invertebrate [52,53]. In the present study, it was showed that dietary levels of *B. subtilis* T13 from $10^5$ to $10^9$ CFU/g could not significantly influence the immunity of sea cucumbers through affecting ACP activity. This result was not consistent with Li et al. [18], who demonstrated that the dietary *Bacillus megeterium* at $10^{10}$ CFU/g had significantly increased the ACP activity in shrimp *L. vannamei*. As the precise mechanism of how the *Bacillus* to affect the ACP activity of aquatic invertebrate animals was not clear yet, therefore it was hard to explain this difference accurately. Superficially, the difference could be explained on the basis of differences in *Bacillus* strains, dietary level and animal species.

Many studies have reported that *Bacillus* strains supplementation in diet could increase disease resistance of fish [15,16,54,55] and shrimp [18,20,56] to pathogenic bacteria or virus, through the stimulation of cellular and humoral immune function. The present study showed that the oral administration of *B. subtilis* T13 significantly reduced cumulative mortality of juvenile sea cucumbers after being challenged with *Vibrio splendius*. In addition, this increased protection against *V. splendius* was only observed in the sea cucumbers fed with diet containing *B. subtilis* T13 at $10^9$ CFU/g, but not in those fed dietary *B. subtilis* T13 at dose of $10^5$ or $10^7$ CFU/g. Similar to the improved resistance, among all the doses of dietary *B. subtilis* T13 ($10^5$, $10^7$, $10^9$ CFU/g), only $10^9$ CFU/g dietary *B. subtilis* T13 resulted in significant growth increases and immunity stimulation of sea cucumbers through increased phagocytosis, respiratory bursts and T-NOS activity.

## 5. Conclusion

To conclude, potential probiotic *B. subtilis* T13 can improve growth performance, and up-regulate innate immunity together with increased resistance to skin ulceration disease caused by *V. splendidus* infection in juvenile sea cucumbers. Besides, this beneficial effect induced by *B. subtilis* T13 is only found at dosage of $10^9$ CFU/g diet, not at the lower dosage of $10^5$–$10^7$ CFU/g diet. The application of *B. subtilis* T13 may present a novel strategy for health management in sea cucumbers aquaculture.

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