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# Influences of immersion bathing in *Bacillus velezensis* DY-6 on growth performance, non-specific immune enzyme activities and gut microbiota of *Apostichopus japonicus**

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**Abstract** In this study, the influences of immersion bathing in different concentrations of *Bacillus velezensis* DY-6 on the body weight gain rate and non-specific immune enzyme activities of the coelom fluid of sea cucumber (*Apostichopus japonicus*) were determined in order to obtain the optimum bacterial concentration. The gut microbiota change in *A. japonicus* was then analyzed through high-throughput sequencing during the immersion bathing in *B. velezensis* DY-6 at the optimum concentration for 49 d. The results illustrate that the body weight growth rate of all bathing groups was higher than that of the control. The highest growth rate (25.3%) was achieved when the bacterial concentration was $1\times10^3$ CFU/mL. The activities of non-specific immune enzymes (ACP, AKP, SOD and LZM) of all bathing groups increased, and the activities of the enzymes of groups bathed with the bacterium at $1\times10^3$ and $1\times10^4$ CFU/mL reached the highest on day 21 and day 28. Taking the growth rate and economic cost into consideration, the optimum concentration of *B. velezensis* DY-6 was $1\times10^3$ CFU/mL. The influences of immersion bathing in *B. velezensis* DY-6 at $1\times10^3$ CFU/mL on the gut microbiota of *A. japonicus* were then evaluated through 16S rDNA sequencing analysis. Results showed that the gut microbiota changed with the addition of *B. velezensis* DY-6, and the richness and diversity of the gut microbiota peaked twice on day 14 and day 21, respectively. In association with the non-specific immune enzyme activities and if day 28 was selected as the dividing point, the community structure of the gut microbiota could be obviously divided into two types. The correlation analysis revealed that the non-specific immune enzyme activities were correlated significantly to some gut bacteria (in the phyla Firmicutes, Proteobacteria, and Bacteroidetes) after immersion bathing in *B. velezensis* DY-6. Our findings will provide the theoretical foundation for probiotic application in sea cucumber farming.

**Keyword**: *Apostichopus japonicus*; *Bacillus velezensis*; non-specific immune enzyme; gut microbiota; correlation

## 1 INTRODUCTION

Sea cucumber (*Apostichopus japonicus*) is consumed widely due to its high nutritional value and functions of promoting health and preventing cancers (Purcella et al., 2012), and its farming has been increasing year by year accordingly. The scale of sea cucumber farming reached 218 000 hectares in 2016, with a yield of 200 000 tons in China (Fisheries and Fisheries Administration Bureau of the Ministry of Agriculture, 2017). However, a skin ulceration syndrome (SUS) caused by *Vibrio splendidus* and other bacterial pathogens has been leading to massive losses of *A. japonicus* farming (Becker et al., 2004; Deng et al., 2009; Zhao et al., 2012). In addition, the abuses of broad-spectrum antibiotics and chemicals caused heavy pollution of the water and the environment nearby, which may also lead to drug

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residues in sea cucumber and rise drug-resistant bacteria (Moriarty, 1997; Van, 1997; Luzhetskyy et al., 2007; Keen and Montforts, 2011; Pflumm, 2011). Fortunately, probiotics as effective substitutes for antibiotics and chemicals promised to be applicable for the prevention of diseases and promotion of the growth of aquatic livestock. The mechanisms underlining the performance of probiotics include competition with pathogens for nutrition and attachment (Miranda and Zemelman, 2001; Zhou et al., 2007), secretion of various enzymes and antibacterial peptides and promotion to immune response (Shiri et al., 1997; Irianto and Austin, 2002; Tuohy et al., 2003; Aguilar-Macías et al., 2010; Hai, 2015).

Probiotics have been applied in aquaculture since 1986 (Kozasa, 1986), and experienced a development of more than 30 years. Their application has been increasing, and their study has evolved as one of the hot topics. Several *Bacillus* strains now are broadly used in aquaculture, especially in the farming of fish, shrimp, and shellfish. Probiotics now play a significant role in aquatic farming, water quality improvement, bacterial pathogen inhibition, immune promotion and gut microbiota optimization (Salinas et al., 2005; Ma et al., 2014; Fang et al., 2015; Li et al., 2016; Jiang et al., 2018). Gullian et al. (2004) showed that *Bacillus* sp. addition in *Fenneropenaeus chinensis* for 28 days reduced the death rate of shrimp infected by *Vibrio harveyi* for 24 h from 51.75% to 18.5%. Balcázar and Rojas-Luna (2007) demonstrated that the average weight of *Litopenaeus vannamei* with the addition of *Bacillus* sp. P64 was significantly higher than that of control. Zhao et al. (2012) found that *B. subtilis* could improve the non-specific immune enzyme activities of *A. japonicus*. Sun et al. (2008) fed *Epinephelus coioides with B. pumilus* and *B. clausii*, and found that the activity of superoxide dismutase (SOD) increased by 11.4% and 17.4%, respectively. Karim et al. (2016) proved that the death rate of *Crassostrea talienwhanensis* affected by *R. crassostreae* and *V. tubiashii* was significantly lower than that of control when *Phaeobacter* sp. S4 or *B. pumilus* RI06-95 was used. However, probiotics application in *A. japonicus* farming is blindly to some extension due to the insufficiency of research. Wang et al. (2000) illustrated that the unreasonable use of probiotics would threat the farmed animals. Therefore, it is very important to analyze the influence of probiotics on *A. japonicus*, and to decipher the mechanisms underlining such influence.

In our previous studies, a local probiotic strain has been screened out from the sediment of *A. japonicus* farming ponds and identified as *Bacillus velezensis* DY-6 (Wang et al., 2018). In this study, this strain was used to study the effect of different concentrations of *B. velezensis* DY-6 on the growth of sea cucumbers and the non-specific immune enzyme activities of its coelom fluid in order to optimize the application optimum concentration of *B. velezensis* DY-6. Simultaneously, the effect of *Bacillus velezensis* DY-6 at the optimum concentration on the gut microbiota of sea cucumber was also determined. Our findings would lay a foundation for the rational use of probiotics in *A. japonicus* farming.

## 2 MATERIAL AND METHOD

### 2.1 Experimental Materials and experimental grouping

*Bacillus velezensis* DY-6 used in the experiment was screened out from *A. japonicus* farming ponds ourselves. Previous studies have revealed that DY-6 inhibit obviously the growth of several pathogens such as *P. nigrifaciens*, *V. splendidus*, *V. parahaemolyticus* and *V. alginolyticus*. It also has wide temperature and salinity adaptability (Wang et al., 2018). Healthy sea cucumber juveniles from a culture corporation in Qingdao with an average weight of 10.3±2.1 g were randomly grouped and acclimatized in four plastic tanks (83 cm×64 cm×60 cm) contain filtered and ozone sterilized seawater for 3 days. The sea cucumber individuals were randomly divided into 21 tanks containing 35 L seawater, 50 individuals each.

The experimental groups were set to different bathing concentrations of *B. velezensis* DY-6 ($1\times10^2$, $1\times10^3$, $1\times10^4$, $1\times10^5$, $1\times10^6$ and $1\times10^7$ CFU/mL), three tanks each group. Simultaneously, three tanks (no *B. velezensis* DY-6) addition were used as control. The experiment lasted for 49 d, during which the temperature was maintained at 17±1.5°C, and 1/5 volume of seawater was replaced every day. The bacteria were added upon seawater was replaced. The sea cucumbers were fed a formula at 1% of the total weight of sea cucumber.

### 2.2 Determination of body weight gain rate of *A. japonicus*

Ahead and at the end of experiment, sea cucumber was counted, and the average weight, $W_0$ and $W_t$, were measured, respectively. The body weight gain rate (WGR) was calculated as follows:

$$\text{WGR}\ (\%)=(W_t-W_0)/W_0\times100\%.$$

### 2.3 Measurement of non-specific immune enzyme activities of *A. japonicus*

During the experiment, three individuals each tank were randomly every 7 days to collect coelom fluid and intestinal material. The coelom fluid was extracted with a disposable syringe and centrifuged at 4 000 r/min and 4°C for 10 min with the supernatant transferred into sterilized centrifuge tubes for determining the non-specific immune enzyme activities.

Acid phosphatase (ACP), alkaline phosphatase (AKP), lysozyme (LZM) and superoxide dismutase (SOD) were selected to represent the non-specific immune enzymes. Enzyme activities were determined using enzyme activity assay kits (Nanjing Jiancheng Bioengineering Institute) following the manufacturer's instructions.

### 2.4 Structure analysis of gut microbiota of *A. japonicus*

The optimum *B. velezensis* DY-6 concentration was selected based on the body weight gain rate of *A. japonicus* and non-specific immune enzyme activities. The microbiota community structure at this concentration was analyzed through 16S ribosomal RNA (rDNA) sequencing.

The sea cucumber was dissected with the intestine contents were removed and the intestine was washed three times with 1.5% NaCl. Each intestine was then transferred into a separate sterile centrifuge tube and stored at -80°C.

Microbial DNA was extracted using the E.Z.N.A. soil DNA Kit (Omega Biotek, Norcross, GA, U.S.) following the manufacturer's protocol. The V3–V4 region of the prokaryotic ribosomal RNA gene (16S rDNA) was amplified though PCR by denaturing at 95°C for 2 min which was followed by 27 cycles of denaturing at 98°C for 10 s, annealing at 62°C for 30 s, and extending at 68°C for 30 s and a final extension at 68°C for 10 min. The primers were 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′) in which an eight-base barcode was inserted, respectively. Amplicatio product was separated in 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) following the manufacturer's instructions, and quantified on QuantiFluor-ST (Promega, U.S.). Purified product was pooled in equimolar and paired-end sequenced (2×250) on an Illumina Hiseq2500 PE250 platform.

### 2.5 Bioinformatics analysis

Two short Illumina reads were assembled into a complete V3–V4 region, and analyzed with the standard methods. Sequences that were shorter than 55 bp, contained primer mismatches, ambiguous bases or uncorrectable barcodes, were removed. The sequences were clustered into operational taxonomic units (OTUs) at a similarity threshold of ≥97% using a UPARSE pipeline (v9.2.64). Chao1, Ace and Shannon indices were calculated using the QIIME (V1.9.1) and principal coordinate analysis (PCoA) was carried out with R (https://www.r-project.org/).

### 2.6 Correlation between OTU abundance and non-specific immune enzyme activities

The correlation coefficient between the abundance of OTU and non-specific immune enzyme activities was calculated with R (https://www.r-project.org/). OTUs with the Pearson correlation value >0.7 or<-0.7 and $P<0.05$ were selected out and annotated to the phyla based on Greengenes Database (https://www.arb-silva.de/), then the relationship between the OTU abundance and the non-specific immune enzyme activities was deduced.

### 2.7 Statistical analysis

Data were expressed as mean±standard deviations (SD). One-way analysis of variance (ANOVA) was performed to determine the significance of difference among groups. Duncan's multiple range test was used to compare the significance of difference among treatments. Statistical analyses were performed using SPSS version 17.0.

## 3 RESULT

### 3.1 Growth performance

The body weight gain rate of *A. japonicus* in all the experimental groups is listed in Table 1. The result showed that WGR of all experimental groups was higher than or equal to that of control. The WGR of $1\times10^2$ CFU/mL, $1\times10^3$ CFU/mL and $1\times10^4$ CFU/mL groups was significantly higher than that of other groups ($P<0.05$). The growth rate of the $1\times10^3$ CFU/mL group achieved the highest WGR (25.3%). The body weight gain rate of the $1\times10^5$ CFU/mL and $1\times10^6$ CFU/mL groups was not significantly different from that of control ($P>0.05$), which was 21.8% and 21.9%, respectively. From our results, it can be seen that *B. velezensis* DY-6 improved the growth of *A. japonicus* and that $1\times10^3$ CFU/mL was the optimum concentration.

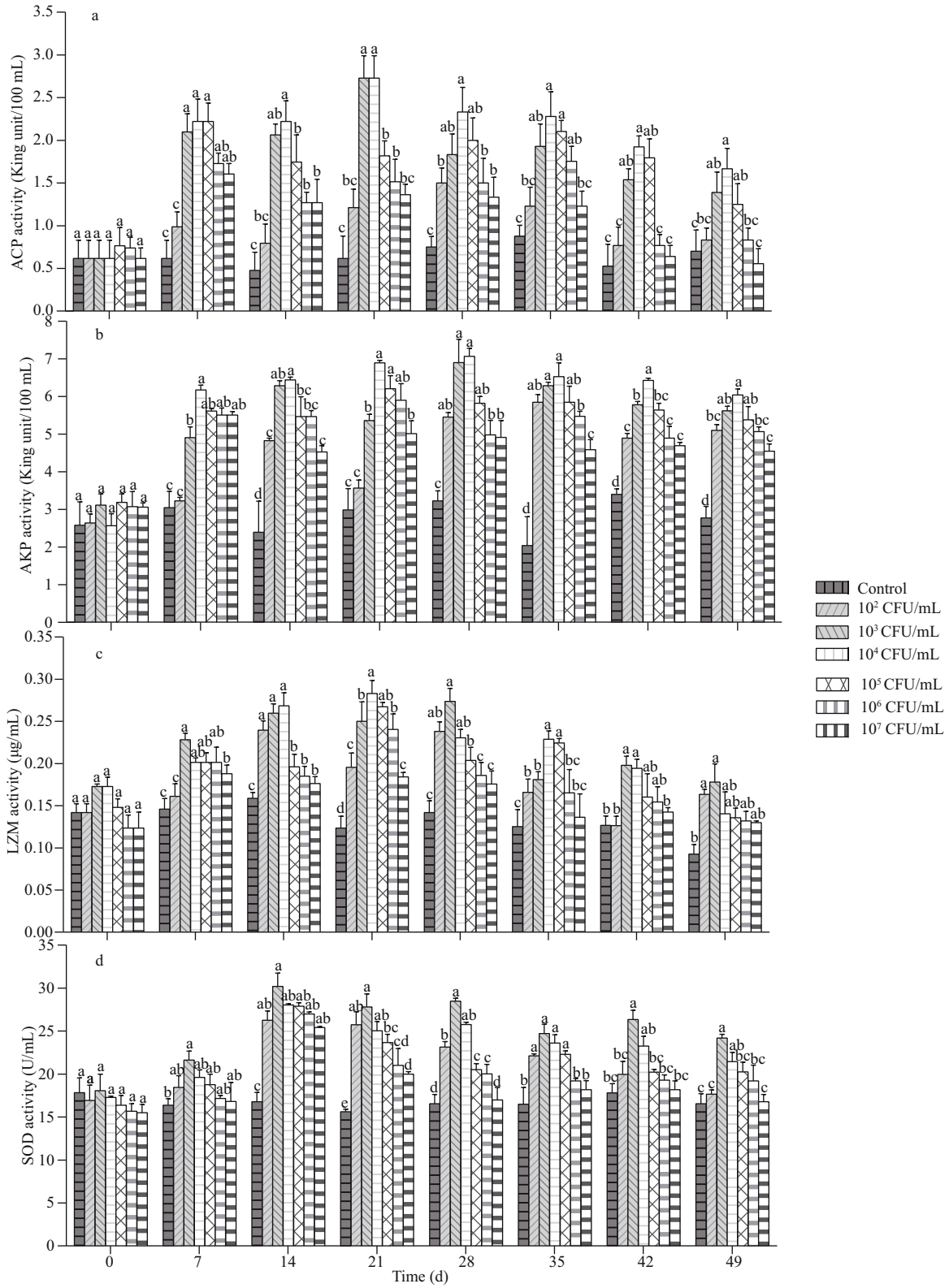


**Fig.1 The effect of different concentrations of *B. velezensis* on the activity of ACP (a), AKP (b), LZM (c) and SOD (d) of *A. japonicus* (mean±SD, *n*=3)**

### 3.2 Changes in non-specific immune enzyme activities

The effects of immersion bathing in different concentrations of *B. velezensis* DY-6 on the non-specific immune enzyme activities of *A. japonicus* are shown in Fig.1a–d. At the 0 d of the experiment, each

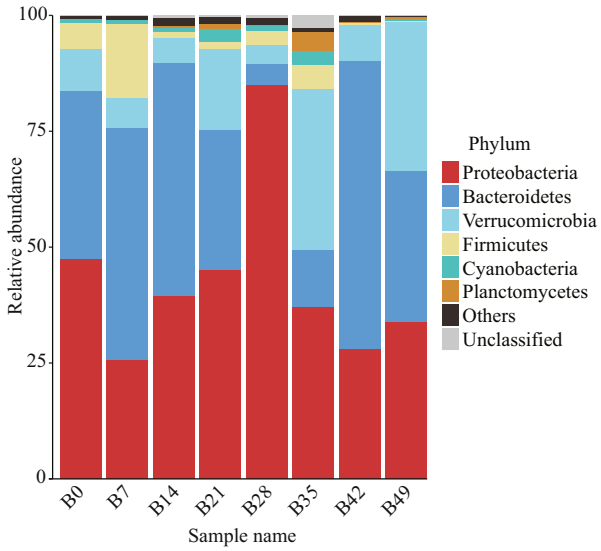


**Fig.2 Relative abundances of the gut microbiota at the phylum level in sea cucumber in 1×10³ CFU/mL group**

The stack column diagram indicates the bacterial phyla composition at different times, and different colors represent different kinds of bacterial phyla. The characters B0, B7, B14, B21, B28, B35, B42 and B49, respectively, represent 0 d, 7 d, 14 d, 21 d, 28 d, 35 d, 42 d, and 49 d ($n$=3).

non-specific immune enzyme activities had no significant difference among groups. Compared with the control, the non-specific immune enzyme activities fluctuated (increasing at early stages and decreasing at later stages) among experimental groups along observing dates. The ACP activity of 1×10³ and 1×10⁴ CFU/mL groups peaked on day 21, which was significantly higher than that of other two groups ($P$<0.05). The AKP activity of all experimental groups was significantly higher than that of control ($P$<0.05) after day 14, and reached the highest on day 28 in 1×10⁴ CFU/mL group. As to the activity of LZM, that of 1×10³ and 1×10⁴ CFU/mL groups was significantly higher than that of control ($P$<0.05) in the first 42 days, consistently peaked from day 14 to day 28, and then decreased to a value similar to that of control on day 49 in 1×10⁴ CFU/mL group ($P$>0.05). The SOD activity of *A. japonicus* was significantly higher than that of control ($P$<0.05) in 1×10³ CFU/mL group and reached its peak from day 14 to day 28. These findings indicated that 1×10³ and 1×10⁴ CFU/mL groups showed the best effect.

### 3.3 Diversity of the intestinal microflora of *A. japonicus* and intergroup difference analysis

According to the body weight gain rate and economic cost, the concentration of *B. velezensis* DY-6, 1×10³ CFU/mL, was accepted as the optimum. At 1×10³ CFU/mL, the influence of *B. velezensis* DY-6 on the gut microbiota of *A. japonicus* during the experimental period (from day 0 to day 49) was evaluated through 16S rDNA sequencing analysis.

The top 10 OTUs of each sample were selected, and the stack map of species distribution was constructed at phylum level using all selected OTUs (Fig.2). At the beginning (day 0), the relative abundances of the bacterial phyla Proteobacteria, Bacteroidetes, Verrucomicrobia and Firmicutes were relatively higher, which was 47.5%, 36.2%, 9.16% and 5.51%, respectively. On day 7, the species distribution did not change significantly. The relative abundance of Planctomycetes increased significantly on day 14 (0.045 4% vs. 0.513 2%, $P$<0.05). On day 21, the relative abundance of Planctomycetes increased significantly in comparison with that of day 0 (0.045 4% vs. 0.874 8%, $P$<0.01). On day 28, the relative abundance of Proteobacteria significantly increased (47.506 3% vs. 85.003 3%, $P$<0.01) and the relative abundance of Bacteroidetes decreased significantly (36.170 8% vs. 4.603 1%, $P$<0.05) compared with that on day 0. The species distribution

**Table 1 Body weight gain rate of sea cucumber at different concentrations of *B. velezensis* DY-6**

| Group (CFU/mL) | Body weight gain rate (%) | |
|---|---|---|
| | Mean | SD |
| Control | 21.7$^{c}$ | 0.054 |
| 1×10² | 24.9$^{a}$ | 0.248 |
| 1×10³ | 25.3$^{a}$ | 0.094 |
| 1×10⁴ | 25.2$^{a}$ | 0.088 |
| 1×10⁵ | 21.8$^{c}$ | 0.161 |
| 1×10⁶ | 21.9$^{c}$ | 0.564 |
| 1×10⁷ | 24.3$^{b}$ | 0.108 |

Superscript letters mean significant differences ($P$<0.05).

**Table 2 Alpha diversity index of the intestine samples collected at different time**

| Sample code ($n$=3) | Ace index | Chao1 index | Shannon index |
|---|---|---|---|
| B0 | 305.09±53.26 | 318.94±51.58 | 4.61±0.55 |
| B7 | 311.22±12.70 | 320.28±14.74 | 4.65±0.94 |
| B14 | 317.95±17.63 | 323.05±23.09 | 4.38±0.21 |
| B21 | 289.13±17.19 | 295.23±27.82 | 5.46±0.24 |
| B28 | 255.50±24.98 | 278.53±53.60 | 3.73±1.46 |
| B35 | 223.78±32.59 | 223.88±34.8 | 4.61±1.08 |
| B42 | 255.30±51.00 | 257.41±48.38 | 3.57±0.77 |
| B49 | 242.93±33.91 | 239.55±37.60 | 4.64±0.17 |

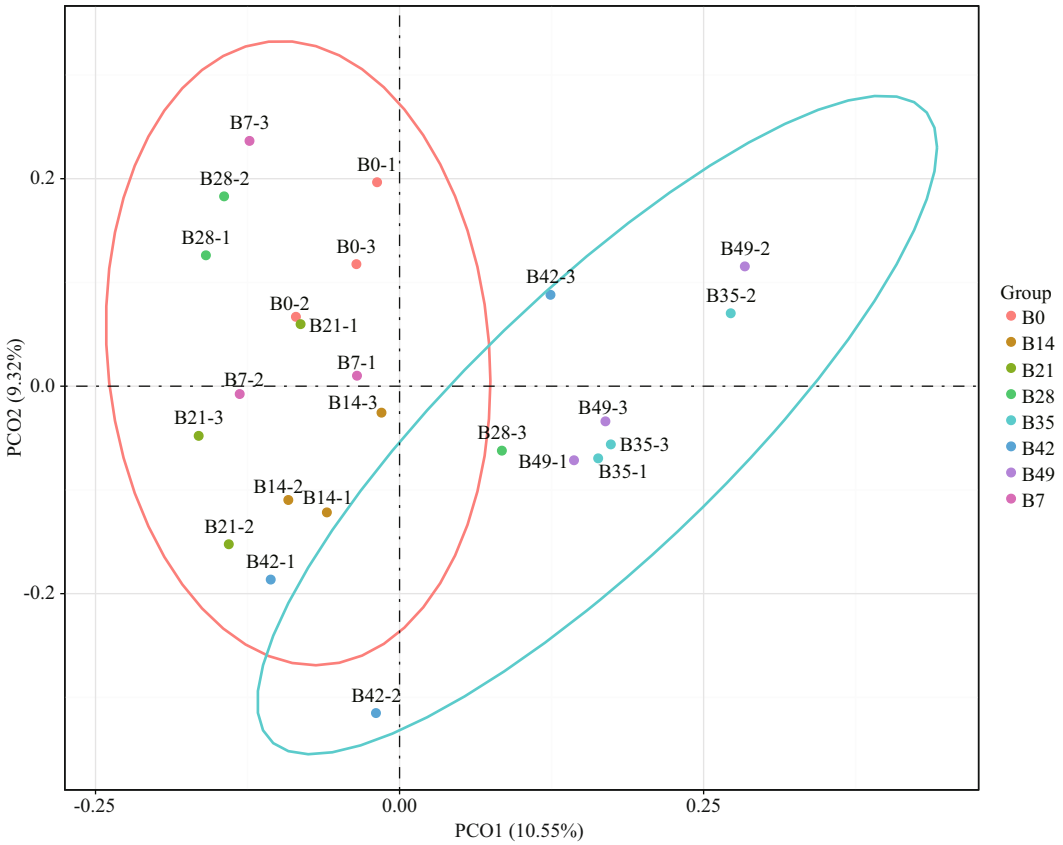


**Fig.3 Principal coordinates analysis (PCoA) of the gut microbiota in sea cucumber**

PCoA was plotted to summarize the microbial compositional differences between samples of different groups. Points that are closer each other represent microbial communities that are more similar. The characters B0, B7, B14, B21, B28, B35, B42 and B49, represent 0 d, 7 d, 14 d, 21 d, 28 d, 35 d, 42 d and 49 d, respectively, $n$=3.

did not change significantly on day 35 compared with that on day 0, but there was a significant difference from that on day 28. On day 42, the relative abundance of Cyanobacteria was significantly decreased (0.947 6% vs. 0.095 4%, $P$<0.05). On day 49, the relative abundances of Planctomycetes and Verrucomicrobia were significantly increased (0.045 4% vs. 0.233 7%, $P$<0.05; 9.162% vs. 32.149 2%, $P$<0.01, respectively), and the relative abundance of Firmicutes decreased significantly (5.507 7% vs. 0.244%, $P$<0.05). The addition of *B. velezensis* DY-6 induced gut microbiota of sea cucumbers into a dynamic process, the relative abundance of Planctomycetes, Proteobacteria, Bacteroidetes, Cyanobacteria, Verrucomicrobia and Firmicutes changed significantly during the experiment period.

The Ace and Chao1 index reached their highest on day 14, which indicated that the intestine of *A. japonicus* on day 14 had the highest bacterial richness. The Shannon index reached the highest on day 21, which indicated immersion bathing in *B. velezensis* DY-6, the biodiversity of the intestine reached the peak on day 21.

Principal coordinates analysis (PCoA) shows the similarity between samples in difference distance. This analysis divided the samples into two clusters based on a 95% confidence interval, B0, B7, B14, B21, and B28 as cluster one and B35, B42 and B49 as cluster 2, indicating that a dramatic change of intestinal microbiota community structure has taken place on day 28.

### 3.4 Correlation analysis between enzyme activities and the abundance of OTUs

The correlation efficiency between the abundance of OTUs and the changing trend of non-specific immune enzyme activities of coelom fluid is listed in Table 3 and illustrated in Fig.4a–d. The OTU which had significant correlationship with each enzyme activities and the highest Pearson correlation absolute value was screened out and annotated. Totally, four OTUs (OTU164, OTU304, OTU534, and OTU122)

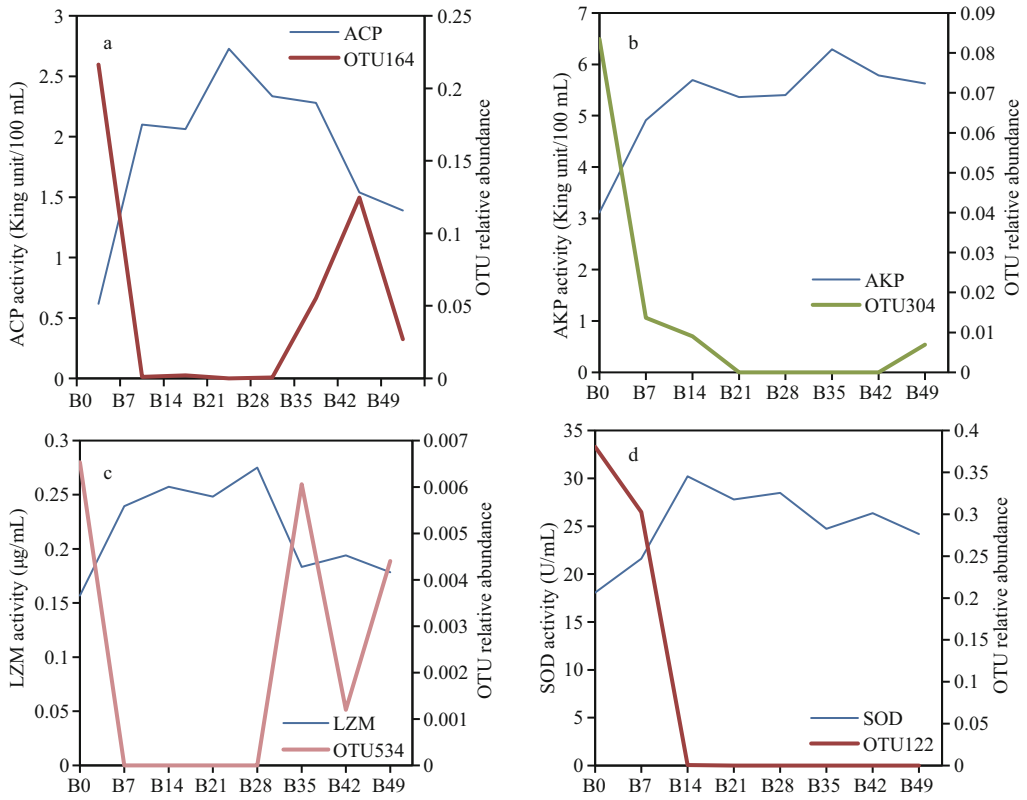


**Fig.4 The correlation analysis between the selected OTUs and the change trend of non-specific immune enzyme activities**

**Table 3 Non-specific immune enzyme activities and its associated OTU screening and annotation results**

| Name of enzyme activities | OUT-id | Pearson correlation | *P*-value | Taxonomy | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Phylum | Class | Order | Family | Genus |
| ACP | OTU164 | -0.83 | 0.010 1 | Firmicutes | Clostridia | Clostridiales | Clostridiaceae | *Clostridium* |
| AKP | OTU304 | -0.94 | 0.000 6 | Bacteroidetes | Gammaproteobacteria | Alteromonadales | Alteromonadaceae | *Agarivorans* |
| LZM | OTU534 | -0.89 | 0.003 0 | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Nautella* |
| SOD | OTU122 | -0.86 | 0.006 3 | Firmicutes | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Paracoccus* |

were selected. There is a positive correlation between OTUs and non-specific immune enzyme activity if the Pearson correlation is positive, while a negative value indicates a negative correlation. OTU164 was negatively correlated with ACP activity ($P$<0.05) with a Pearson correlation coefficient value of -0.83. OTU304 was negatively correlated with AKP activity ($P$<0.01) and the Pearson correlation coefficient value was -0.94. OTU534 was negatively correlated with LZM activity ($P$<0.01) and the Pearson correlation coefficient value was -0.89. OTU122 was negatively correlated with SOD activity ($P$<0.01) and Pearson's correlation coefficient value was -0.86. OTU164, OTU304, OTU534, and OTU122 were annotated as Firmicutes, Bacteroidetes, Proteobacteria, and Firmicutes, respectively.

## 4 DISCUSSION

Probiotics can stimulate the appetite of cultured organisms, promote nutrient absorption and optimize energy intake through the secretion of extracellular enzymes. They can also regulate the community structure of intestinal microorganisms, accelerate the digestion and absorption of food, and increase the growth rate of cultured organisms (Gómez and Balcázar, 2008; Ringø et al., 2010). Ghosh et al. (2003) revealed that adding *Bacillus* sp. to the feed of *Labeo rohita* could significantly increase the growth of *Labeo rohita*, reduce feed conversion rate, and improve protein efficiency. Ziaei-Nejad et al. (2006) added *Bacillus* sp. to *Fenneropenaeus indicus* and discovered that its viability increased by 11%–17%

and body weight increased by 8%–22% compared with control. In this study, we found that the *B*. *velezensis* DY-6 can improve the WGR of a sea cucumber to some extent, and the added bacteria at concentrations of $(1\times10^2)$–$(1\times10^4)$ CFU/mL had significantly larger effects than at other concentrations. Liu et al. (2009) showed that adding different concentrations of *Bacillus subtilis* into the culture water of *Oreochromis niloticus* could notably increase the body weight by 666.8% and improve water quality when the concentration was $1\times10^4$ CFU /mL.

Probiotics can increase the body's humoral immunity and cellular immunity by stimulating the transformation of lymphocytes in the *lamina propria* of the intestinal mucosa. Regulating the immune system of both humans and animals is one of the most common benefits of probiotics (Panigrahi et al., 2017). Studies have shown that *Bacillus* sp. could enhance the non-specific immune enzyme activities of aquatic animals (Salinas et al., 2005; Balcázar and Rojas-Luna, 2007). The mechanism involves the bacteria themselves or components of the cell wall stimulating the non-specific immune system of the host, thereby enhancing animal immunity (Rengpipat et al., 2000). This study found that non-specific immune enzyme activities (ACP, AKP, SOD, and LZM) in the coelom fluid of *A*. *japonicus* increased at different concentrations of *B*. *velezensis* DY-6, which demonstrated that the addition of *B*. *velezensis* DY-6 can improve the immunity of *A*. *japonicus*. Nair et al. (2011) found that non-specific immune enzyme activities of *Litopenaeus vannamei* significantly increased after inoculation with *Bacillus cereus*, and Suzer et al. (2008) reported that *Lactobacillus* sp. could improve the growth performance and digestive enzyme activities of *Sparus aurata* larvae. In this study, we found also that the enzyme activities in the coelom fluid of *A*. *japonicus* in $1\times10^3$ CFU/mL and $1\times10^4$ CFU/mL groups were higher than that of control and reached their peaks either on day 21 or day 28. Liu et al. (2011) added *Bacillus subtilis* to the feed of hybrid sturgeon (*Cipenser baerii* ♂×*Acipenser schrenckii* ♀) juveniles and found that the LZM and SOD activities first increased and then decreased. Sui (2003) reported that adding different combinations of probiotic preparations to the aquaculture water of *Litopenaeus vannamei* could significantly increase activities of different kinds of non-specific immune enzymes and decrease after they maximized at different times. After using probiotics, the trend of enzyme activities was consistent with that of this study, but the peak times of non-specific immune enzyme activities were different, which may be caused by varietal differences in probiotics and cultured organisms.

Gut microbiota plays important roles in the host's nutrition, metabolism, growth, and differentiation of epithelial cells, regulation of immune functions, and protection from outside attacks (Böttcher et al., 2000; Forchielli and Walker, 2005; Vael et al., 2008). This study used the 16S rDNA sequence to analyze the gut microbiota of *A*. *japonicus* at the concentration of $1\times10^3$ CFU/mL. The result indicated that the intervention of *B*. *velezensis* DY-6 induced the structure of the gut microbiota of *A*. *japonicus* into a dynamic process. The same phenomena were also investigated in farmed fish and mice: Huys et al. (2001) found that the use of probiotics significantly altered gut microbiota of farmed fish. Zhao et al. (2017) found that using *Bacillus subtilis* can significantly change the gut microbiota of mice, which is consistent with the results of this study. Zhao et al. (2017) also deduced that using *Bacillus subtilis* could regulate intestinal flora structure possibly via interacting with intestinal microbial but not via colonizing. As for this study, the mechanism of *B*. *velezensis* DY-6 on *A*. *japonicus* should be investigated in future.

Many studies have found that changes in the abundance of certain bacteria in the gut can affect immune processes of the host. Garrett et al. (2010) and Carvalho et al. (2009) found that Proteobacteria contain plenty of gut pathogens and may cause intestinal inflammation. Smith et al. (2013) and Furusawa et al. (2013) found that decreasing the abundance of Firmicutes can reduce the production of short chain fatty acids, whereas short chain fatty acids can increase T-cell tolerance in the intestinal mucosa. Carding et al. (2015) found that gut microbiota could help the host to gain access to essential nutrients and energy to prevent intrusion from outside germs, thus maintaining the integrity of the intestinal barrier and promoting the development of the host's immune system. This study correlated the abundance of OTUs at different time points and the non-specific immune enzyme activities in the body fluid of *A*. *japonicus*. The results showed that after an immersion bathing with *B*. *velezensis* DY-6, there was a significant correlation between the changes in non-specific immune enzyme activities of *A*. *japonicus* and some bacteria in the sea cucumbers gut. However, the specific mechanism of action between the changes in

the gut microbiota and non-specific immune enzyme activities after adding probiotics should be studied further.

Through analyzing the influence of different concentrations of *B. velezensis* DY-6 on non-specific immune enzyme activities in coelom fluid and the body weight gain rate of *A. japonicus*, this study determined that the best concentrations of *B. velezensis* DY-6 are $1\times10^3$ CFU/mL or $1\times10^4$ CFU/mL. Taking the cost into account, $1\times10^3$ CFU/mL is the optimum for practical use. The community structure of gut microbiota of *A. japonicus* was dynamic, and the change inflection point was on day 28 when *B. velezensis* DY-6 was used at the optimum concentration. Non-specific immune enzyme activities of the coelom fluid of *A. japonicus* reached their peaks on day 21 or day 28. This finding can provide a theoretical basis for using probiotics rationally in *A. japonicus* farming.

## 5 CONCLUSION

In summary, the addition of *B. velezensis* DY-6 could significantly improve the growth of and non-specific immune enzyme activities in sea cucumber, and the optimum concentration is $1\times10^3$ CFU/mL. The gut microbiota changed with the artificial involvement of *B. velezensis* DY-6 and could be obviously divided into two clusters on day 28. The activities changes for ACP, AKP, LZM, and SOD have significant correlations with the abundance of Firmicutes, Bacteroidetes, Proteobacteria, and Firmicutes, respectively.

## 6 DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are available from the corresponding author.

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**Introduction to the new book**

# Studies of the Biogeochemistry of Typical Estuaries and Bays in China

Springer has recently published the book entitled "Studies of the Biogeochemistry of Typical Estuaries and Bays in China". Professor Shen Zhiliang from the Institute of Oceanology, Chinese Academy of Sciences, China, authored and complied this book. The new book collects, compiles, and summarizes the studies on the biogeochemical cycling of the biogenic elements in mainly two typical regions in China, i.e., the Changjiang (Yangtze) River estuary and Jiaozhou Bay, and other regions are touched in the aspects of nutrients, suspended particulate matter, phytoplankton, and heavy metals, etc., in biogeochemistry, ecology, environmental science, oceanography, and biology etc.

Changjiang (Yangtze) River basins is the first focus of the book. The author treated the region as an integrated ecosystem from uppersteam to downstream, and to the estuarine, and from the atmosphere, continent, to the ocean. The authors systematically studied the concentration distributions, variations, and removals as well as the molar ratios of various forms of nitrogen, phosphorus, and silicate in the Changjiang River mainstream and tributaries in dry and flood seasons, and quantitatively estimated the budget and distribution mechanisms of nitrogen and phosphorus in the river basins for the first time. In addition, the author explored the behavior and removal of various forms of phosphorus and silicon during estuarine mixing and made the first measurement of sedimentation fluxes of suspended particulate matter, phosphorus, and silicon, and calculation of the mass balance of phosphorus and silicon in the turbidity maximum zone of the Changjiang River estuary. A novel method was established to estimate the ratio of resuspension of sediment. The long-term changes in nutrients and their structure, and ecological responses of phytoplankton community were studied in the Changjiang River estuary and Jiaozhou Bay, and the mechanism that triggers the occurrence of red tide was explored.

The Jiaozhou Bay is the second focus of the book, on which carbon, nitrogen, phosphorus, and silicon compositions (in seawater?), and their mole ratios of various size fractions of phytoplankton were reported; and the silicon limition on ophytoplankton growth in Jiaozhou Bay was discovered and determined. Based on these works, author proposed a new concept of nutrient structure formation in seawater and phytoplankton, and the balance between them. For the first time, author successfully isolated large diatom *Coscinodiscus asteromphalus* from natural seawater, determined its carbon, nitrogen, phosphorus, silicon, and chlorophyll *a* contents, and further estimated its contribution to the phytoplankton biomass in Jiaozhou Bay.

We warmly congratulate on the publication of this book as well as to the author on his 50$^{th}$ anniversary of scientific research. The book provides an important reference to the scientists and politicians who interested in the similar fields for the environmental protection and management in China and other regions of the world.

The book can be accessed via the following link:

https://link.springer.com/book/10.1007%2F978-3-662-58169-8.