Fish and Shellfish Immunology 94 (2019) 617–627

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of the probiotic *Bacillus amyloliquefaciens* on the growth, immunity, and disease resistance of *Haliotis discus hannai*

Gao Xiaolong[a,b], Ke Caihuan[a], Zhang Mo[b], Li Xian[b], Wu Fucun[b], Liu Ying[c,*]

[a] State Key Laboratory of Marine Environmental Science, College of Ocean and Earth Sciences, Xiamen University, Xiamen, 361102, China
[b] Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China
[c] Dalian Ocean University, Dalian, 116023, China

ARTICLE INFO

Keywords:
Bacillus amyloliquefaciens
Haliotis discus hannai
Growth
Immunity
Disease resistance

ABSTRACT

The effects of a diet containing the probiotic *Bacillus amyloliquefaciens* on the survival and growth of *Haliotis discus hannai* were evaluated by measuring growth and hematological parameters and the expression levels of nonspecific immune genes. In addition, the abalone's response to *Vibrio parahaemolyticus* infection was assessed. *H. discus hannai* (shell length: 29.35 ± 1.81 mm, body weight: 4.28 ± 0.23 g) were exposed to an 8-week culture experiment in indoor aquariums and a 2-week *V. parahaemolyticus* artificial infection experiment. In each experiment, the control group (C) was fed daily with the basal feed; the experimental groups were fed daily with the experimental feed, prepared by spraying *B. amyloliquefaciens* onto the basal feed at final concentrations of 10^3 (group A1), 10^5 (A2), and 10^7 (A3) cfu/g. The survival rate, body weight specific growth rate, and food conversion efficiency in A2 and A3 were significantly higher than those in A1 and C ($P < 0.05$). The total number of blood lymphocytes, the O_2^- and NO levels produced from respiratory burst, the activities of acid phosphatase, superoxide dismutase, and catalase, and the expression levels of catalase and thiol peroxidase in A2 were not significantly different from those in A3, but these factors were significantly higher in A2 compared to A1 and C ($P < 0.05$). The total antioxidant capacity and expression levels of glutathione S-transferase in A1, A2 and A3 were significantly higher than those in C ($P < 0.05$). At day 9 after infection with *V. parahaemolyticus*, all abalone in C were dead; at the end of the experiment, the cumulative mortality of abalone in A2 was significantly lower than that in any other group ($P < 0.05$). Thus, the experimental feed containing 10^5 cfu/g *B. amyloliquefaciens* not only facilitated the food intake and growth of abalone, but also effectively enhanced their non-specific immunity and resistance to *V. parahaemolyticus* infection. In this regard, *B. amyloliquefaciens* may be a useful probiotic strain for abalone aquaculture.

1. Introduction

Probiotics is a general term for beneficial microbes, which can improve the microbial environment of the intestinal tract, facilitate growth of aquatic animals, improve digestive enzyme activity and non-specific immunity of aquatic animals, regulate immune-related genes, and enhance resistance to pathogens [1–3]. Thus, adding exogenous probiotics to the feed of aquaculture species has a positive effect on the microbial flora in their digestive tracts. Exogenous probiotics not only directly influence the host through the presence of probiotic strains, but more importantly, they influence the host indirectly by affecting the microflora in the intestinal tract [4,5].

Bacillus spp. are Gram-positive aerobic bacteria that are present widely in the natural environment. Certain species of *Bacillus* can secrete a variety of antibacterial substances that have a strong inhibitory effect on the growth of various pathogenic fungi, pathogenic *Vibrio*, and other bacteria. Das et al. found that the activities of peroxidase and lysozyme in *Catla* that were fed with *Bacillus amyloliquefaciens* were significantly enhanced during the skin mucosal immune response [6]. Park et al. suggested that *B. amyloliquefaciens* KC16-2 could be used as a probiotic bacterium to control the mortality of abalone caused by opportunistic pathogenic vibrios. The mortality of the abalone fed with the probiotic diet was reduced to half that of the control group in a challenge test with *Vibrio tubiashii* [7]. Liu et al. reported that adding *Bacillus subtilis* E20 to food enhanced the phagocytic activity of *Epinephelus coioides* immune cells as well as the immunity and disease resistance of the host [8]. In another study, the addition of *Bacillus licheniformis* to the food of *Megalobrama terminalis* increased the activity of blood phenoloxidase and alkaline phosphatase as well as the content of total serum protein and globulin [9]. Chen et al. examined the effect

* Corresponding author. College of Marine Technology and Environment, Dalian Ocean University, 116023, Dalian, Liaoning Province, China.
E-mail address: yingliu@dlou.edu.cn (L. Ying).

https://doi.org/10.1016/j.fsi.2019.08.067
Received 8 April 2019; Received in revised form 23 August 2019; Accepted 26 August 2019
Available online 26 August 2019

of *B. licheniformis* XY-52 on the expression of *Cyprinus carpio* immune-related genes and found that the expression levels of *IL-1β*, *TNF-α*, and *HSP70* significantly increased after feeding [10]. Addition of *B. subtilis* to the food of *Sparus aurata* resulted in significantly increased expression levels of the inflammation-associated gene *IL-8* and the cytoskeleton-associated genes β-actin and atresia [11]. Kumar et al. reported that after feeding *Labeo rohita* with food containing various concentrations of *B. subtilis* for 2 weeks, the respiratory bursting of blood cells and the serum killing of *Aeromonas hydrophila* were significantly enhanced [12]. Feeding *C. catla* with food containing *Bacillus circulans* PB7 for 60 days resulted in significantly enhanced immunity and killing of *A. hydrophila* [13]. Tseng et al. fed *Litopenaeus vannamei* with food containing *B. subtilis* E20 for 98 days and found that the phenoloxidase and phagocytic activity and the clearance rate of bacteria were significantly improved; additionally, the survival rate of *L. vannamei* after challenge with *Vibrio alginolyticus* was significantly improved [14]. However, little is known about the effects of food containing *B. amyloliquefaciens* on the growth, survival, and disease resistance of *Haliotis discus hannai*.

Haliotis discus hannai is one of the most economically important marine shellfish species in China. The aquaculture production of abalone reached 148,500 tons in 2017, which was more than 90% of the total world output in that year [15]. Despite the rapid development of the abalone aquaculture industry, a lack of knowledge regarding water quality management and disease prevention and control has resulted in outbreaks and the wide prevalence of diseases. For example, atrophy-affected *Haliotis diversicolor supertexta* were found to contain large numbers of *Vibrio parahaemolyticus*, and *V. parahaemolyticus* isolated from the hemolymph of disease-affected individuals led to the death of juvenile abalone (LD_{50} was 8.36–8.41 × 10^4 cfu/g, and the morality rate was 50–60%) [16,17]. In another study, when the concentration of *V. alginolyticus* in water reached 10^5–10^6 cfu/mL, the morality rate of *Haliotis rufescens* significantly increased after 24 h [18].

The goal of this study was to examine the effects of a diet containing different concentrations of *B. amyloliquefaciens* on the survival, growth, and immune response of *H. discus hannai*. We used molecular biology techniques and a *V. parahaemolyticus* challenge experiment to further analyze the effects of the probiotic. The results provide a theoretical basis for the rational use of this potential probiotic in abalone aquaculture and for future studies of its probiotic mechanism.

2. Materials and methods

2.1. Source and acclimation of abalone

This experiment was carried out from March 6 to May 6, 2018 at the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, Shandong, China. Juvenile abalone were purchased from Changqing Ocean Science & Technology Co., Ltd. (Weihai, Shandong, China), and all experimental abalone were sourced from the same batch after artificial hatching. After purchasing the abalone, they were acclimated in four culture containers (length 1.2 m × width 1 m × height 1 m, water volume: 1200 L) for 15 d; water temperature was kept at 17 °C, salinity at 29 ± 1, pH at 7.9, dissolved oxygen concentration at > 6 mg/L, and the light cycle was set as the natural light cycle. Aquaculture water was obtained from a natural sea area and used after sedimentation and sand filtration. Two-thirds of the water was replaced with fresh seawater each day at 09:00 to ensure good water quality. During the period of acclimation, basal feed (crude protein: 38.42% ± 0.27%; fat: 5.38% ± 0.19%; ash: 14.32% ± 0.66%; humidity: 6.08% ± 0.14%; soluble carbohydrate: 23.81% ± 0.57%) purchased from Geosung Feed Co., Ltd. (Qingdao, Shandong, China) was added to the culture containers once a day at 17:00. The feeding quantity was equivalent to 3% of the wet body weight of the abalone.

2.2. Experimental strains

Bacillus amyloliquefaciens YL-10 was supplied by Qingdao BEPRO and was isolated and screened from the intestinal tracts of healthy individuals of *H. discus hannai*. During the course of the experiment, fresh viable bacteria (1 × 10^9 cfu/mL) were evenly sprayed onto the basal feed.

2.3. Experimental diets

Bacillus amyloliquefaciens was inoculated into 2216E liquid medium, shaken overnight (140 × *g*/min, 28 °C), then centrifuged at 9000 × *g*/min for 10 min. After the cells were collected, they were washed three times in sterile seawater. The cell concentration was measured with a hemocytometer, then the bacterial solution was diluted and evenly sprayed on the surface of the basal feed at a set concentration. The feed was then placed in a clean plastic bag and preserved at 4 °C. The amount of *B. amyloliquefaciens* in the feed was measured by the viable counting method every 3 days. The bacterial count of the experimental feed after it had been held for 7 days was the same as the initial count on Day 0. New food was prepared once a week.

2.4. Experimental design

This experiment was carried out in 60 L indoor aquariums (50 cm × 30 cm × 40 cm). The additive concentrations of *B. amyloliquefaciens* in treatments A1, A1, and A3 were 10^3, $10^{5,}$ and 10^7 cfu/g, respectively. Abalone fed the basal feed constituted the control group (C). Each treatment had four replicates, each of which was randomly stocked with 20 abalone (shell length: 29.35 ± 1.81 mm, body weight: 4.28 ± 0.23 g).

Throughout the experiment, the indoor temperature was kept at 17 °C by an air conditioner, and the salinity and pH were the same as those used during acclimation. Abalone were fed once each day at 18:00, and the feeding amount was 5% of the wet weight. The next morning, residual food and feces were removed separately from the bottom of the aquarium by siphon. Basal feed was placed in an additional three aquariums to measure the possible loss of food due to decay in the water. The next morning, the basal feed was collected, weighed, and dried, and the amount of residual food was calculated, representing food intake.

During the experiment, four-fifths of the water in each aquarium was exchanged each day, and each aquarium was washed once every 3 days. Each aquarium was provided with continuous aeration to maintain the concentration of dissolved oxygen > 6 mg/L. Aeration was stopped 20 min before the collection of residual food. The ammonia nitrogen concentration throughout the experiment was no higher than 0.2 mg/L.

2.5. Sample collection and determination

At the end of the culture experiment, the abalone were starved for 24 h. Twenty abalone were randomly selected from each group to measure the shell length and body weight, and the survival rate of abalone in each group was determined.

Eight additional abalone were randomly collected from each group. The body surface moisture was removed, the foot muscle surface was disinfected with alcohol, the foot muscle was removed with a disinfected scalpel, and the hemolymph was extracted to measure various hematological indexes. Each abalone was then shelled and their hepatopancreas was removed with scissors and forceps. The tissue was immediately placed in a 1.5 mL centrifuge tube, and the tube was quickly placed in liquid nitrogen. The samples were stored at −80 °C until analysis of the antioxidant index and the expression of various genes.

2.5.1. Determination of the total number of blood lymphocytes

For this analysis, 50 μL of hemolymph liquid was fixed with the equivalent amount of 10% neutral formalin. After mixing the solution evenly, the number of blood cells was counted using a counting plate at 400X under an optical microscope (Qiujing Inc., Shanghai, China).

2.5.2. Determination of phagocytic activity in blood lymphocytes

This analysis was conducted following the method proposed by Zhang et al. [19]. First, 100 μL of sterilized anticoagulant was added to 100 μL hemolymph. After evenly mixing the solution, a 50 μL cell suspension was coated on a glass slide, and 10–15 min later the cells were adhered to the slide. Following attachment, 50 μL of a yeast suspension (Baker's yeast, Type II, Sigma, St. Louis, MO, USA) was added to the cell monolayer. They were twice washed carefully with sterilized phosphate buffered saline (PBS), fixed with methanol after the serum was removed, stained with Giemsa, dried and restained with 1% methylene, and observed and counted under the oil immersion lens to calculate the phagocytic rate as follows:

Phagocytic rate = (Number of phagocytes/Total number of cells observed) × 100.

2.5.3. Determination of O_2^- levels produced by the respiratory burst of blood lymphocytes

Three hundred microliters of hemolymph from each abalone was used for this analysis. The sample was injected into three ELISA plate wells (100 μL hemolymph per well). The samples were incubated at room temperature for 20 min, and the hemolymph liquid was discarded. The remaining sample was washed with 100 μL of PBS once, and 100 μL of 0.3% nitroblue tetrazolium (NBT) solution was added. The mixture was allowed to react at room temperature for 30 min, followed by centrifugation at 800 × *g* at 4 °C for 10 min. The supernatant was discarded, and the remaining sample was fixed with 200 μL of anhydrous methanol, twice washed with 70% ethanol, and air dried. The precipitates were dissolved with 120 μL of 2 M potassium hydroxide and 140 μL of dimethyl sulfoxide. After even mixing, the optical density (OD) was measured using an ELISA reader (Bio-Rad Laboratories, Inc., Irvine, CA, USA) [20]. The respiratory burst levels of lymphocytes were expressed as the reduction amount of NBT produced per 100 μL of hemolymph (expressed in OD_{630}).

2.5.4. Determination of nitric oxide (NO) levels produced by the respiratory burst of blood lymphocytes

The NO levels in the lymphocytes were measured using a NO Detection Kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) following the method proposed by Villamil et al. [21]. First, 750 μL of hemolymph was centrifuged at 1500 × *g* at 4 °C for 10 min. The supernatant was discarded, the blood cells were resuspended in 150 μL of PBS (concentrated 5-fold), and 0.1% Triton-X100 liquid was added to break the blood lymphocytes. The solution was centrifuged at 10,000 × *g* at 4 °C for 15 min, and the supernatant was used to measure NO concentration as follows: 150 μL of the sample was placed in a clean centrifuge tube, evenly mixed with 60 μL of nitrate reducing agent, reacted at 27 °C for 60 min, fully and evenly mixed with Griess reagent, rested at room temperature for 10 min, and centrifuged at 5000 × *g* for 10 min. Finally, 120 μL of the supernatant was placed in a well of an ELISA plate, 90 μL of color developer was added, the solution was mixed and then rested at room temperature for 10 min, and OD_{550} was determined using an ELISA reader.

2.5.5. Determination of lysozyme (LZM) and acid phosphatase (ACP) activities in blood lymphocytes

LZM and ACP activities were measured using kits purchased from Nanjing Jiancheng Bioengineering Institute. LZM activity was measured using the turbidimetric method [22]: the reaction substrate was 0.2 mg/mL of a *Micrococcus* (Sigma) suspension prepared in 0.05 mol/L, pH 6.1 phosphate buffer, and the absorbance was measured at 0.5 min and 4.5 min at 530 nm using a spectrophotometer (721G-100, INESA CC, Shanghai, China). The activity of LZM was defined as the absorbance of the bacterial solution reduced by 0.001 per min (namely an active unit).

The activity of ACP was measured following the method described by Góth [23]: 500 μL of hemolymph was centrifuged at 1500 × *g* at 4 °C for 10 min, the supernatant was discarded, and 100 μL of sterilized PBS was added to resuspend the blood cells (concentrated 5-fold). The sample was then centrifuged at 15,000 × *g* at 4 °C for 5 min, and the supernatant was removed for testing. The activity of ACP was defined as the hemocyte dissolved matter in 100 mL of hemolymph reacted with the matrix solution (200 μL 4-aminoantipyrine, 400 μL potassium ferricyanide) at 27 °C for 15 min to produce 1 mg phenol (namely an active unit).

2.5.6. Determination of total antioxidant capacity (T-AOC) and superoxide dismutase (SOD) and catalase (CAT) activities of the hepatopancreas

T-AOC and SOD and CAT activities were measured using kits purchased from Nanjing Jiancheng Bioengineering Institute. First, 0.2–0.4 g of hepatopancreas tissue was mixed with 1.8 mL of 0.86% saline, fully ground in an ice-water bath, then centrifuged at 3500 g/min for 10 min to prepare the 10% tissue homogenate for the measurement of antioxidant enzyme activities.

T-AOC was measured using the ferric reducing ability of plasma method [24]. The antioxidant substances in an organism can reduce Fe^{3+} to Fe^{2+}, which can form a stable complex with phenanthroline-like substances. The level of T-AOC of a sample was measured using the colorimetric method. At 37 °C, a unit (U/mg) of T-AOC was defined as an increase of 0.01 of OD of all chemical reaction substances by 1 mg of tissue protein per minute.

The activity of SOD was measured using NBT photochemical reduction [25]. In the presence of methionine and riboflavin, NBT under illumination will generate blue methyl hydrazine through photochemical reduction. The absorbance of blue methyl hydrazine was measured at 560 nm using a spectrophotometer, and the enzyme activity was calculated based on the intensity of SOD-inhibited NBT photochemical reduction. SOD activity (i.e., an active unit) was defined as the amount of SOD present when the inhibition rate of SOD in 1 mL of the reaction liquid (10 mmol/L hydroxylamine hydrochloride, 7.5 mmol/L xanthine, 23.4 mU/mL xanthine oxidase, 3.3 g/L sulfanilic acid) reached 50%.

CAT activity was measured following Lygren et al. [26] as follows: 200 μL of homogenate were mixed with 3 mL of 0.05 mol/L phosphate buffer, then 200 μL of 0.3% H_2O_2 was added. The solution was mixed evenly, and 1 min later the absorbance was measured at 240 nm using a spectrophotometer; absorbance was recorded once every min for 5 min, and a 0.01 decrease per min was defined as an active unit.

The protein content in the homogenate was measured using Coomassie blue staining, as described by Bradford (1976) [27], and bovine serum albumin was used as the protein marker.

2.5.7. Expression of immune-related genes

The hepatopancreas tissues that were frozen and stored at −80 °C were ground in a mortar and pestle to which liquid nitrogen was added. Next, 0.05 mg of the obtained sample powder was rapidly and evenly mixed with 1 mL of TRIzol (Invitrogen, San Diego, CA, USA) to extract total RNA. Total RNA was extracted by removing the residual DNA from the sample using RQI RNase-Free DNase (TaKaRa, Kusatsu, Japan), and then RNA was reversely transcribed to cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time quantitative PCR was conducted using the SYBR® Premix Ex Taq™ II kit (Tli RNaseH Plus) (TaKaRa) and the TaKaRa Thermal Cycler Dice™ Real Time System TP800 instrument. The specific primers were designed based on cDNA complete sequences submitted to GenBank, and *Mn-SOD*, *CAT*, thiol peroxidase (TP_x), glutathione S-transferase (GST_s), heat shock protein 70 (*HSP70*), *HSP90*, and the reference gene β-actin were analyzed.

Table 1
Abalone antioxidant enzymes and heat shock proteins' genes analyzed in this study.

Gene	Basic function	Cellular localization	Reference
Mn-SOD	Detoxification of O_2^-	Mitochondria	Ekanayake et al. (2006) [68]
CAT	Detoxification of H_2O_2	Cytoplasm, nucleus mitochondria	Ekanayake et al. (2008) [69]
TP_x	Detoxification of H_2O_2	Cytoplasm, nucleus mitochondria	Pushpamali et al. (2008) [70]
GST_s	Detoxification of H_2O_2	Cytoplasm, nucleus mitochondria	Wan et al. (2008) [71]
HSP70	Chaperone, Antioxidant biomarker of stress	Cytoplasm, nucleus	Cheng et al. (2007) [72]
HSP90	Chaperone, Antioxidant biomarker of stress	Cytoplasm, nucleus	Zhang et al. (2011) [73]

Mn-SOD: Mn-superoxide dismutase; CAT: catalase; TP_x: thioredoxin peroxidase; GST_s: Sigma-glutathione-s-transferase; HSP70: heat shock protein 70; HSP90: heat shock protein 90.

Table 2
Real-time quantitative PCR primers for antioxidant enzymes and heat shock proteins' genes of *Haliotis discus hannai*.

Gene	Sequence (5′-3′)	Efficiency (%)	Reference
Mn-SOD	F: ACCTAGCTTTACCGAGTTA R: GCCACCTTACGGTAAATGGACC	99.17	Designed by author
CAT	F: ACTACCTGCAACTTCCCGTCAACT R: AGGTTGTTGATCAGATGGTCCCGT	100.31	Ekanayake et al. (2008) [69]
TP_x	F: TCAGACTACAGAGGGAAATA R: CATCCAAGGACCTCACAG	98.65	Pushpamali et al. (2008) [70]
GST_s	F: GCGGAGATTGTGACGGAAA R: GAGCAGCAAACAGGTCATCAAA	101.82	Designed by author
HSP70	F: ATGCCAATGGTATCCTC R: GTAATTCTCAGCCTCGTT	103.57	Cheng et al. (2007) [72]
HSP90	F: CACTGTGGACCAGAAATGC R: ACAGCAAAGCACGGAACT	98.36	Designed by author
β-Actin	F: GCTGCGTTGGTTATCG R: GGTACTTGAGGGTGAGGA	102.44	Designed by author

F: Forward primer; R: Reverse primer.

Tables 1 and 2 show genetic information and primer sequences.

Each sample was evenly mixed in a PCR tube and then placed in a PCR plate (Roche Diagnostics, Indianapolis, IN, USA). PCR amplification was conducted after transient centrifugation, and the reaction conditions were as follows: initial denaturation at 94 °C for 30 s; the cycling conditions were 94 °C for 5 s, 60 °C for 30 s, 40 cycles in total; the solubility curve was generated at the end of the experiment. Three replicates were run for each RNA sample and gene. mRNA levels of the target genes were calibrated using the Real-time PCR Ct ($2^{-\Delta\Delta Ct}$) relative quantitative method, with β-actin as the quantitative standard.

2.6. Vibrio parahemolyticus challenge experiment

At the end of the culture experiment, 24 abalone were randomly selected from each treatment group and the control group and transferred to 12 new aquariums, such that each group was replicated three times. The pathogen used to infect the experimental abalone was *V. parahemolyticus*, and it was supplied by the Aquaculture Engineering R& D Team of the Institute of Oceanology (Qingdao, China). Prior to use, *V. parahemolyticus* was cultured and activated by tryptic soy broth culture medium (3% NaCl) at 30 °C for 24 h. Subsequently, the pathogen was injected into *H. discus hannai*, and 24 h later hemolymph liquid from the infected abalones was collected, placed on thiosulfate citrate bile salts sucrose agar culture medium, and cultured at 30 °C for 24 h. Well-cultured bacterial colonies were selected to generate the bacterial suspension with sterilized PBS, and the concentration of the bacterial suspension was set at 1.0×10^8 cell/mL. The suspension was preserved at 4 °C.

Prior to the infection experiment, the 7-day median lethal concentration of *V. parahaemolyticus* to abalone was determined to be 1.0×10^7 cfu/mL. Using a sterilized syringe, 50 μL of the bacterial suspension at the median lethal concentration were injected into the foot muscle of each abalone. In the control group, each abalone was injected with 50 μL of sterilized PBS. Each treatment group was continuously fed, and the daily deaths of abalone were recorded. After 14 d, the infection experiment was ended and the cumulative mortality rate was calculated (as a percentage) as $D_t/D_0 \times 100\%$, where D_0 and D_t are the initial number of abalone and the number of cumulative deaths during the challenge experiment, respectively.

2.7. Data calculation

At the end of the culture experiment, the survival rate (S, %), body weight specific growth rate (SGR, % day^{-1}), food intake (FI, % body weight day^{-1}), and food conversion efficiency (FCE, %) of the abalone were calculated as follows:

$$S\ (\%) = [(N_1 - N_2)/N_1] \times 100$$

$$SGR\ (\%\ day^{-1}) = (\ln W_2 - \ln W_1)/T \times 100$$

$$FI\ (\%\ body\ weight\ day^{-1}) = F/[T \times (W_2 + W_1)/2] \times 100$$

$$FCE\ (\%) = (W_2 - W_1)/F \times 100$$

where N_1 and N_2 are the number of abalone at the beginning and end of the experiment, respectively; W_1 and W_2 are the body weight at the beginning and end, respectively; T is the number of days of the experiment; and F is the weight of food intake during the experiment (dry weight, g).

2.8. Statistical analysis

The statistical analysis was performed using SPSS, version 18.0 (Armonk, NY, USA). Data expressed in percentage format were arcsine transformed to satisfy the homogeneity test of variances. When one-way analysis of variance indicated statistical significance ($P < 0.05$), multiple comparisons were made using the Tukey test. Results are shown as the mean ± standard error.

3. Results

3.1. Survival, growth, and food intake

Addition of *B. amyloliquefaciens* to the basal feed significantly affected the survival rate of abalone. No significant difference in the survival rate was detected between A2 and A3; both groups had significantly higher survival rates than C and A1 ($P < 0.05$, Table 3). No significant difference in the body weight SGR of abalone was detected between C and A1; both groups had significantly lower body weight SGR than A2 and A3 ($P < 0.05$). The FI of abalone in A3 was highest; it was significantly higher than that in A1 ($P < 0.05$), but it did not differ significantly from that in A2 and C. The FCE of abalone in A2 was highest; it was significantly higher than that in C and A1, but it did not differ significantly from that in A3 ($P > 0.05$, Table 3).

Table 3
Effects of dietary supplemented graded levels of the *Bacillus amyloliquefaciens* on the survival rate, growth, food intake, and feed conversion efficiency of juvenile *Haliotis discus hannai* for 8 weeks.

Index	Treatment				ANOVA *P*
	C	A1	A2	A3	
Survival rate (%)	88.52 ± 3.69^{b}	86.03 ± 7.33^{b}	94.15 ± 5.97^{a}	92.31 ± 5.51^{a}	0.003
Specific growth rate (%/day)	0.17 ± 0.01^{b}	0.19 ± 0.02^{b}	0.28 ± 0.01^{a}	0.26 ± 0.01^{a}	0.001
Food intake (BW/day)	1.20 ± 0.05^{ab}	1.16 ± 0.14^{b}	1.24 ± 0.07^{ab}	1.33 ± 0.10^{a}	0.016
Feed conversion efficiency	0.79 ± 0.03^{b}	0.82 ± 0.05^{b}	0.99 ± 0.06^{a}	0.94 ± 0.18^{a}	0.001

Values represent means and standard errors of four replicates (means ± SE; n = 4). Values in the same row that had different superscripts are significantly different at $P < 0.05$ based on Tukey's test. ANOVA: One-way analysis of variance. C: control group; A1: *B. amyloliquefaciens* (10^3 cfu/g); A2: *B. amyloliquefaciens* (10^5 cfu/g); A3: *B. amyloliquefaciens* (10^7 cfu/g).

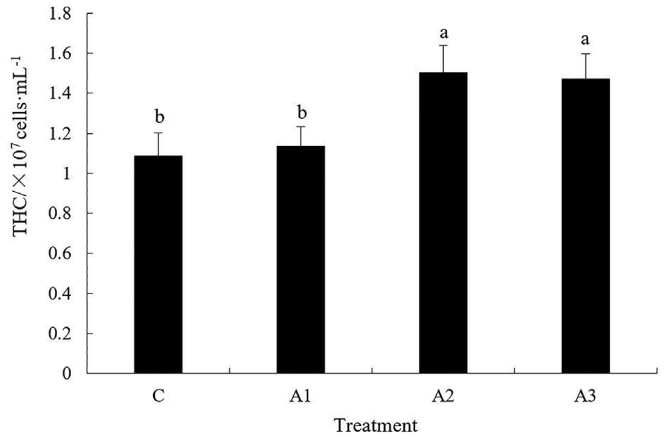

Fig. 1. Total hemocyte counts (THC) of juvenile *Haliotis discus hannai* fed with graded doses of dietary *Bacillus amyloliquefaciens* for 8 weeks. Values are means and standard errors of four replicates (means ± SE; n = 4). Treatments with different letters are significantly different ($P < 0.05$). C: control group; A1: *B. amyloliquefaciens* (10^3 cfu/g); A2: *B. amyloliquefaciens* (10^5 cfu/g); A3: *B. amyloliquefaciens* (10^7 cfu/g).

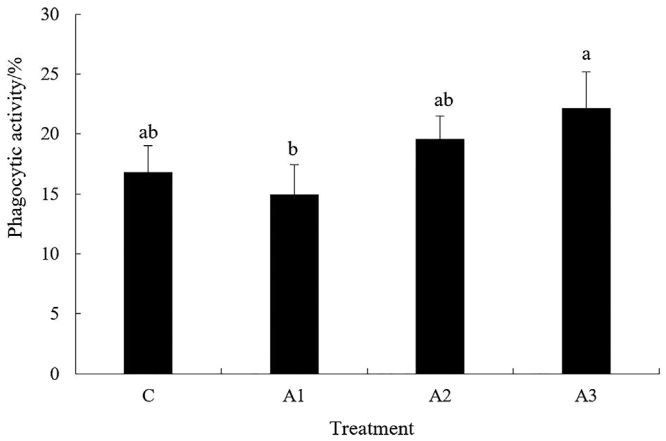

Fig. 2. Phagocytic activity of blood lymphocytes in juvenile *Haliotis discus hannai* fed with graded doses of dietary *Bacillus amyloliquefaciens* for 8 weeks. Values are means and standard errors of four replicates (means ± SE; n = 4). Treatments with different letters are significantly different ($P < 0.05$). C: control group; A1: *B. amyloliquefaciens* (10^3 cfu/g); A2: *B. amyloliquefaciens* (10^5 cfu/g); A3: *B. amyloliquefaciens* (10^7 cfu/g).

3.2. Total number of blood lymphocytes

Addition of *B. amyloliquefaciens* to the basal feed also significantly affected the total number of blood lymphocytes in abalone. No significant difference in the total number of blood lymphocytes was detected between A2 and A3; however, both groups had significantly higher numbers than A1 and C ($P < 0.05$, Fig. 1).

3.3. Phagocytic activity of blood lymphocytes

The phagocytic activity of blood lymphocytes in abalone was highest in A3, although the value did not differ significantly from that in A2 ($P > 0.05$, Fig. 2). The phagocytic activity of blood lymphocytes in abalone was lowest in A1, but it did not differ significantly from that in C and A2. However, phagocytic activity in A1 was significantly lower than that in A3 ($P < 0.05$).

3.4. O_2^- levels produced by respiratory burst of blood lymphocytes

Addition of *B. amyloliquefaciens* to the diet of abalone increased the O_2^- levels produced by respiratory burst of blood lymphocytes. The O_2^- levels were significantly lower in C than that in any other group ($P < 0.05$, Fig. 3). The O_2^- levels in A2 and A3 did not differ significantly, but the level in A2 was significantly higher than that in A1 ($P < 0.05$).

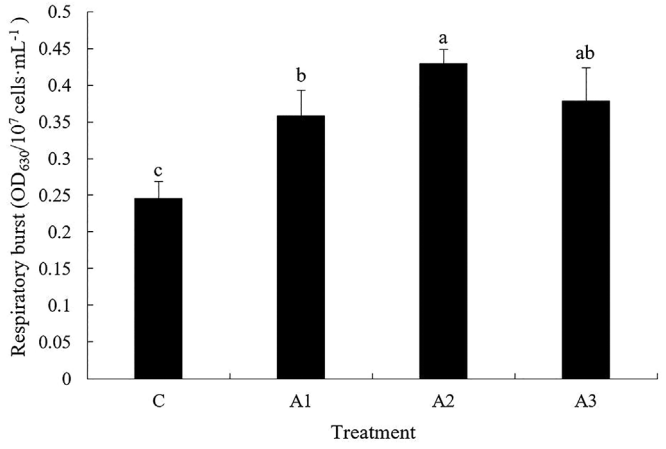

Fig. 3. Respiratory burst activity of blood lymphocytes in juvenile *Haliotis discus hannai* fed with graded doses of dietary *Bacillus amyloliquefaciens* for 8 weeks. Values are means and standard errors of four replicates (means ± SE; n = 4). Treatments with different letters are significantly different ($P < 0.05$). C: control group; A1: *B. amyloliquefaciens* (10^3 cfu/g); A2: *B. amyloliquefaciens* (10^5 cfu/g); A3: *B. amyloliquefaciens* (10^7 cfu/g).

3.5. NO levels produced by respiratory burst of blood lymphocytes

The NO levels produced by respiratory burst of blood lymphocytes was significantly higher in A1, A2 and A3 compared with the control group. No significant difference in the NO levels was detected between

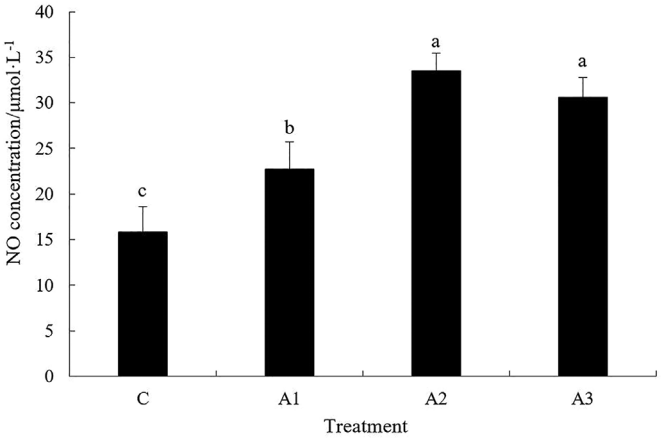

Fig. 4. NO concentration of juvenile *Haliotis discus hannai* fed with graded doses of dietary *Bacillus amyloliquefaciens* for 8 weeks. Values are means and standard errors of four replicates (means ± SE; n = 4). Treatments with different letters are significantly different ($P < 0.05$). C: control group; A1: *B. amyloliquefaciens* (10^3 cfu/g); A2: *B. amyloliquefaciens* (10^5 cfu/g); A3: *B. amyloliquefaciens* (10^7 cfu/g).

A2 and A3; both groups had significantly higher NO levels than A1 ($P < 0.05$, Fig. 4).

3.6. LZM and ACP activities of blood lymphocytes

No significant difference in LZM activity was identified among A1, A2, and A3, but LZM activity in A2 and A3 was significantly higher than that in C ($P < 0.05$, Table 4). ACP activity was highest in A2; there was no significant difference in ACP activity between A2 and A3. ACP activity in A2 was significantly higher than that in A1 and C ($P < 0.05$).

3.7. T-AOC and SOD and CAT activities of the hepatopancreas

Addition of *B. amyloliquefaciens* to the diet of abalone significantly improved T-AOC activity relative to the controls. The SOD activity of abalone in A1, A2, and A3 was significantly higher than that in C ($P < 0.05$, Table 4). The SOD activity of abalone was highest in A2; A2 had significantly higher SOD activity than that in A1 and A3 ($P < 0.05$). No significant difference in CAT activity was detected between A2 and A3; A2 had significantly higher CAT activity than that in A1 and C ($P < 0.05$).

3.8. Expression of immune-related genes

The presence of *B. amyloliquefaciens* in the abalone diet significantly affected the expression levels of *Mn-SOD*. The expression level of *Mn-SOD* in A2 was significantly higher than that of any other group ($P < 0.05$, Fig. 5A). Additionally, the expression level of *Mn-SOD* in A3 was significantly higher than that in A1 and C. No significant difference in expression levels of *CAT* was identified between A2 and A3, but both groups had significantly higher *CAT* expression than C or A1 ($P < 0.05$, Fig. 5B). The expression level of TP_x in C was significantly lower than that in A1, A2, and A3, and the expression level in A1 was significantly lower than that in A2 or A3 ($P < 0.05$, Fig. 5C). The expression levels of GST_s in A1 and A3 were significantly lower than in A2; A1 and A3 both had significantly higher GST_s levels than C ($P < 0.05$, Fig. 5D). The addition of *B. amyloliquefaciens* also significantly affected the expression of *HSP70*. No significant difference in expression level was detected among A1, A2, and A3; *HSP70* expression in A2 was significantly higher than that in C ($P < 0.05$, Fig. 5E). However, addition of *B. amyloliquefaciens* to the abalone diet had no significant effect on the expression levels of *HSP90* ($P > 0.05$, Fig. 5F).

3.9. Cumulative mortality

At the end of the *V. parahaemolyticus* infection experiment, a significant difference in the cumulative mortality of abalone was detected among the groups. At Day 2 after the infection experiment began, the cumulative mortality of abalone in C was significantly higher than that in A1, A2, and A3 ($P < 0.05$, Fig. 6). At Day 4, the cumulative mortality of abalone in C and A1 was significantly higher than that in A2 or A3 ($P < 0.05$). At Day 9, none of the abalone in C were alive. At the end of the experiment, the cumulative mortality of abalone in A1 was 93.33%, which was significantly higher than that in A2 or A3. The cumulative mortality of abalone in A2 was lowest and significantly lower than that in A3 ($P < 0.05$).

4. Discussion

Bacillus is a type of aerobic or facultative anaerobic bacterium. It usually is present in the intestinal microbial community in the form of endospores, which can produce proteases, amylases, and lipases, promote the decomposition and absorption of nutrients in the digestive tract, and improve food utilization and digestibility [28,29]. Addition of *B. amyloliquefaciens* to the diet of abalone in this study had positive effects on multiple parameters of *H. discus hannai*. At the end of the culture experiment, the survival rate, body weight SGR, and FCE of abalone in A2 and A3 were significantly higher than those in C or A1. The FI of abalone in A2 was lower than that in A3, but the growth increase of abalone in A2 was more obvious because the FCE of abalone in A2 was higher than that in A3.

Due to the high protein content of *Bacillus*, it is rich in amino acids and trace elements, which can be directly absorbed by the body as nutrients. Certain probiotics secrete nutrients such as organic acids, amino acids, and vitamins, which not only promote nutrient absorption by the host but also maintain the intestinal ecological equilibrium and stimulate development of the intestinal tract [30,31]. Sun et al.

Table 4
Lysozyme (LZM) activity, acid phosphatase (ACP) activity, total antioxidant capacity (T-AOC), superoxide dismutase (SOD) activity and catalase (CAT) activity of juvenile *Haliotis discus hannai* fed with graded doses of dietary *Bacillus amyloliquefaciens* for 8 weeks.

Index	Treatment				ANOVA *P*
	C	A1	A2	A3	
LZM (μg mL^{-1})	30.55 ± 3.19^{b}	34.25 ± 2.67^{ab}	38.93 ± 7.08^{a}	40.58 ± 5.94^{a}	0.005
ACP (U mL^{-1})	2.61 ± 0.35^{b}	3.01 ± 0.24^{b}	5.92 ± 0.41^{a}	5.76 ± 0.34^{a}	< 0.001
T-AOC (U mg^{-1} prot)	0.35 ± 0.02^{b}	0.62 ± 0.04^{a}	0.73 ± 0.04^{a}	0.70 ± 0.02^{a}	< 0.001
SOD (U mg^{-1} prot)	0.51 ± 0.11^{c}	0.89 ± 0.03^{b}	1.32 ± 0.15^{a}	0.93 ± 0.10^{b}	0.001
CAT (U mg^{-1} prot)	0.39 ± 0.02^{b}	0.34 ± 0.01^{b}	0.91 ± 0.04^{a}	0.67 ± 0.04^{ab}	0.001

Values represent means and standard errors of four replicates (means ± SE; n = 4). Values in the same row that had different superscripts are significantly different at $P < 0.05$ based on Tukey's test. ANOVA: One-way analysis of variance. C: control group; A1: *B. amyloliquefaciens* (10^3 cfu/g); A2: *B. amyloliquefaciens* (10^5 cfu/g); A3: *B. amyloliquefaciens* (10^7 cfu/g).

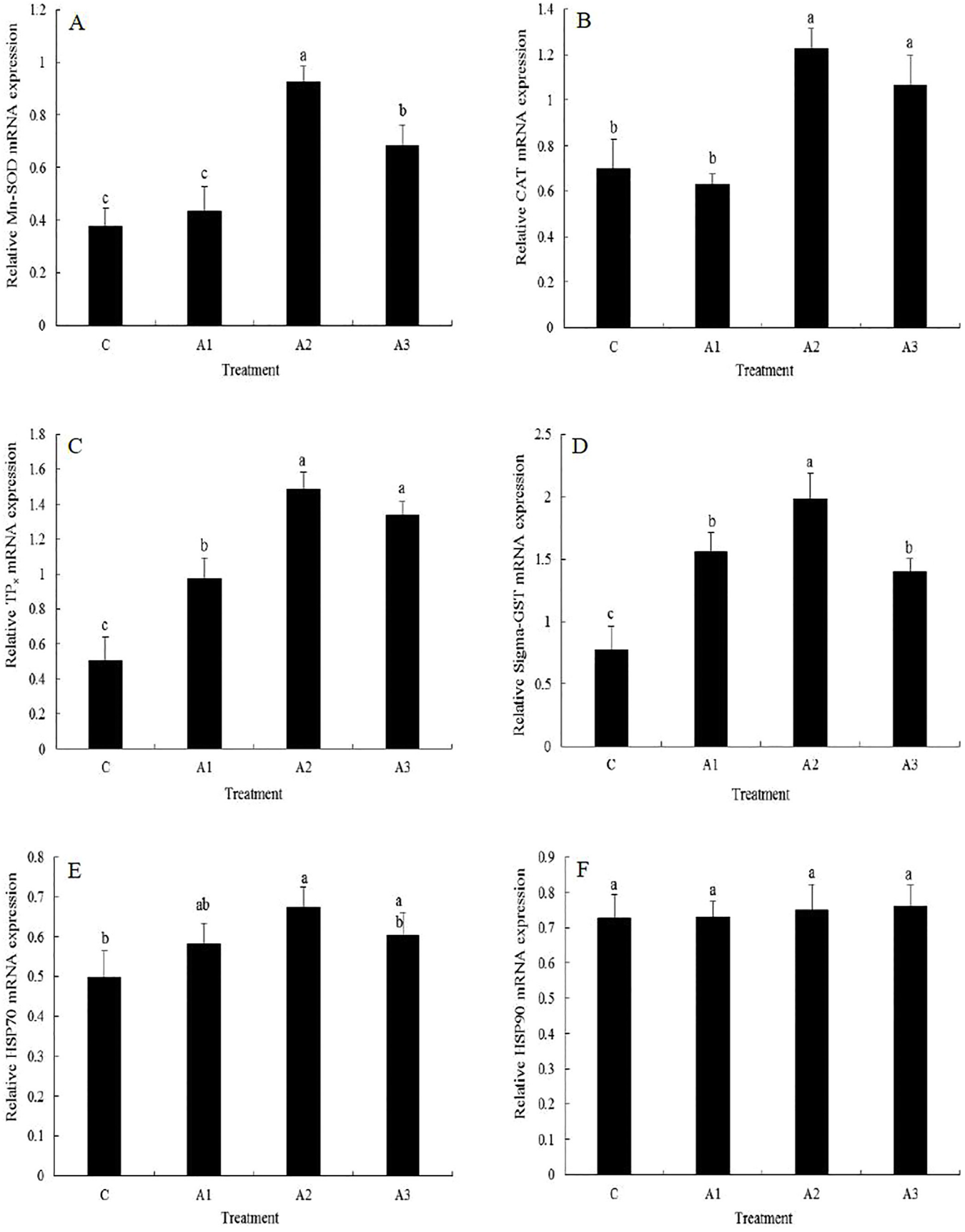

Fig. 5. Effects of dietary supplemented graded levels of the *Bacillus amyloliquefaciens* on the relative expression levels of *Mn-SOD* (A), *CAT* (B), TP_x (C), GST_s (D), *HSP70* (E) and *HSP90* (F) in hepatopancreas of juvenile *Haliotis discus hannai*. Values are means and standard errors of four replicates (means ± SE; n = 4). Treatments with different letters are significantly different ($P < 0.05$). C: control group; A1: *B. amyloliquefaciens* (10^3 cfu/g); A2: *B. amyloliquefaciens* (10^5 cfu/g); A3: *B. amyloliquefaciens* (10^7 cfu/g).

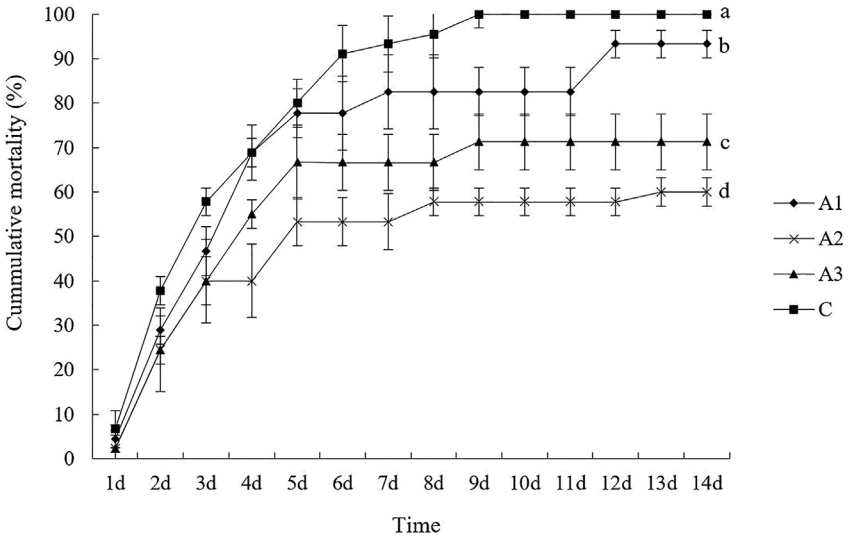

Fig. 6. Cumulative mortality during a 14-day *Vibrio parahaemolyticus* challenge of juvenile *Haliotis discus hannai* fed diets supplemented with graded levels of dietary *Bacillus amyloliquefaciens* for 8 weeks. Values are means and standard errors of three replicates (means ± SE; n = 3). Different letters indicate significant difference in mean cumulative mortality after a 14-day *V. parahaemolyticus* challenge ($P < 0.05$). C: control group; A1: *B. amyloliquefaciens* (10^3 cfu/g); A2: *B. amyloliquefaciens* (10^5 cfu/g); A3: *B. amyloliquefaciens* (10^7 cfu/g).

reported that *E. coioides* fed with *Enterococcus faecium* and *Lactococcus lactis* exhibited increased growth rate, food conversion rate, and protease activity in the digestive tract [32]. When Sun et al. fed *E. coioides* with *Psychrobacter* sp., which had no or weak ability to secrete digestive enzymes, they found that addition of the probiotic stimulated the digestive system and increased the output of digestive enzymes by the host, which in turn promoted body growth [33]. In our study, no significant difference in growth, FI, and FCE of abalone was detected between A1 and C, which likely was due to failure of bacteria to colonize after entry into the intestinal tract or due to less secretion of digestive enzymes after colonization because of the low concentration of *B. amyloliquefaciens* in A1. Therefore, the probiotic in this treatment group played a limited role in enhancing the conversion and absorption of nutrients by the abalone.

Doeschate and Coyne studied a strain of probiotic isolated from the intestinal tract of *Haliotis midae* called *Pseudoalteromonas* sp. C4, and they found that it could significantly increase the growth rate and food utilization rate of *H. midae* [34]. Iehata et al. examined the effects of different sources of lactic acid bacteria on the growth of *Haliotis gigantean*, and they found that applying a strain of *Pediococcus* sp. Ab1 significantly promoted its growth [35]. After feeding *Trachinotus ovatus* with food containing *B. subtilis* for 8 weeks, Zhang et al. found that the SGR and feeding efficiency was significantly higher than that in the control group [36]. El-Dakar et al. reported that food containing commercial probiotic preparations with *B. subtilis* would reduce the food coefficient and promote the growth of *Siganus rivulatus* [37]. Bagheri et al. added *Bacillus* spp. preparations (containing *B. subtilis* and *B. licheniformis*) to the basal feed of *Oncorhynchus mykiss*. After 63 days they found that the addition of 3.8×10^9 and 6.1×10^9 cfu/g of the *Bacillus* spp. preparations significantly increased the SGR [38]. In our study, the survival and growth of the abalone did not increase with increasing concentration of *B. amyloliquefaciens*. In fact, some of the growth and immune indexes in A3 tended to be lower relative to A2. These findings indicated that the addition of exogenous probiotics might lead to competition and exclusion among indigenous flora in the intestinal tract. Provided with limited adhesion sites on the mucous epithelium in the digestive tract, indigenous bacteria may inhibit the colonization and proliferation of excessive foreign bacteria, which could in turn maintain the equilibrium and homeostasis of the microbial community in the intestinal tract. Yan et al. reported that the animal digestive tract is an island-type microecosystem in which different microbial populations compete for niches, nutrients, and energy. This dynamic exchange is critical to the health of the host [39]. For this reason, adding only the appropriate concentration of probiotics will contribute to the equilibrium of the microecosystem in the intestinal tract; conversely, an excessive amount of probiotics may not achieve the expected result and could result in unintended waste.

Abalone are invertebrates, and cellular immunity is a critical component of their non-specific immune response. Thus, the number of blood cells and phagocytic activity play crucial roles in cellular immunity [40]. The total number of lymphocytes and phagocytic activity were higher in A2 and A3 compared to C, indicating that the addition of *B. amyloliquefaciens* to the feed enhanced the non-specific immunity of the abalone, thus enhancing the immune function of the host. The phagocytic activity of blood lymphocytes is a critical indicator of the defense function of blood lymphocytes [41,42]. Hooper et al. suggested that the temporary decrease in the number of blood lymphocytes of "withering"-affected *Haliotis cracherodii* can be attributed to apoptosis and aging of blood lymphocytes in the hemolymph [43]. Balcázar et al. isolated four probiotics (*V. alginolyticus* UTM 102, *B. subtilis* UTM 126, *Roseobacter gallaeciensis* SLV03, and *Pseudomonas aestumarina* SLV22) from the intestine of *L. vannamei*, then used them to conduct a feeding test. They reported that the cumulative mortality of individuals fed with the probiotics was significantly lower compared to the control group after *L. vannamei* was infected with *V. parahaemolyticus* for 14 days [44]. Yang et al. found that the phagocytic activity and respiratory burst levels of the coelomocytes of *Apostichopus japonicas* fed for 60 days with *Bacillus cereus* G19-added basal feed were significantly higher compared with the control group [25]. These findings indicate that *B. amyloliquefaciens* can act as an immune activator to enhance the phagocytic activity of cells and the innate cellular immune response of the host.

Respiratory burst activity refers to a large number of reactive oxygen species (ROS) being produced when blood cells phagocytose pathogens. ROS can quickly attack pathogens, which is closely associated with the bactericidal activity of blood cells. Superoxide anion (O_2^-) is the initial product of ROS, thus the concentration of O_2^- can accurately reflect the levels of ROS [45]. In our study, the O_2^- levels produced by respiratory burst of blood cells in A1, A2, and A3 were significantly higher than that in C. The O_2^- levels produced by respiratory burst of blood cells in A2 were nearly double that of C. Nikoskelainen et al. demonstrated that food containing 10^5 cfu/g of *Lactobacillus rhamnosus* induced the respiratory burst capacity of *O. mykiss* [46]. Brunt et al. and Kim and Austin also showed that *O. mykiss* fed with the probiotics *Aeromonas sobria* GC2 and *Carnobacterium divergens* B33 for 2 weeks exhibited increased levels of ROS produced by respiratory burst activity [47,48]. In our study, the addition of *B. amyloliquefaciens* to the diet of *H. discus hannai* increased the O_2^- levels produced by respiratory burst of blood cells, leading to enhanced immune defense in the host. This process might explain the higher

survival rate of abalone in A2 and A3.

LZM is an integral part of humoral immunity in invertebrates [49,50]. The LZM activity of abalone in A1, A2, and A3 was enhanced relative to that in C, and the LZM activity of abalone in A2 and A3 was significantly higher than that in C. Newaj-Fyzul et al. found that *O. mykiss* fed with 10^7 cells/g of *B. subtilis* AB1 for 14 d exhibited a significant improvement in respiratory burst and intestinal LZM activity and a significant decrease in mortality rate after being infected with *Aeromonas* [51]. Sharifuzzaman and Austin (2009) added 10^8 cells/g of *Kocuria* SM1 to the food of *O. mykiss* and found that it enhanced the phagocytic activity of macrophages and the LZM activity of serum; it also significantly reduced the mortality rate after challenge with *Vibrio anguillarum* [52]. These findings are similar to those of our study, and they suggest that the addition of probiotics can enhance the humoral immunity of abalone.

ACP is an integral component of lysosomes in the immune system and can effectively guard against the invasion of pathogens. The invasion of pathogens into the body of an animal stimulates the release of ACP by phagocytes, and ACP then acts by hydrolyzing the phosphate esters on the surface of pathogenic bacteria [53,54]. Compared with C, the ACP activity of abalone in A2 and A3 was significantly enhanced, and in the subsequent bacterial challenge test, the cumulative mortality of abalone in A2 and A3 was significantly lower than that in A1 and C. Similarly, Li et al. reported that a basal feed mixed with *Bacillus* OJ significantly enhanced the activity of ACP in the serum of *L. vannamei* [55]. These results suggest that the addition of *B. amyloliquefaciens* led to enhanced disease resistance of abalone and to a lower probability of disease occurrence. Moreover, it enhanced the recognition of foreign matter by blood cells as well as humoral immunity.

T-AOC is an integrated index for measuring the functional state of the antioxidant system of the body, and its levels reflect the compensatory capacity of the antioxidant enzyme system and the non-enzymatic system of the body and the state of free radical metabolism of the body [56]. In our study, addition of *B. amyloliquefaciens* to the abalone diet resulted in significantly higher T-AOC compared with the control. Moreover, in the subsequent bacterial infection test, the cumulative mortality of abalone in the treated groups was significantly lower than that of the controls. These results suggested that the variation of T-AOC could effectively reflect the antioxidant capacity of the host.

SOD has a strong antioxidant defense function, as it catalyzes O_2^- to generate H_2O_2. CAT and GST_s catalyze H_2O_2 to generate water and oxygen, thereby scavenging excess free radicals and reducing lipid peroxidation damage. Therefore, the activities of SOD, CAT, and GST_s reflect the functional variations of the antioxidant system of the body in response to environmental stress [57,58]. Selim and Reda fed 10^4–10^6 cfu/g of *B. amyloliquefaciens* to *Oreochromis niloticus* for 30 d and found that the relative expression levels of the immune-related genes interleukin-1 and tumor necrosis factor alpha in the experimental group were significantly higher than in the controls [59]. In our study, SOD activity and expression levels of *Mn-SOD* in A2 were significantly higher than those in any other group. No significant differences in CAT activity and expression levels of *CAT* were detected between A2 and A3, but A2 had significantly higher *CAT* expression compared to C and A1. These results suggested that *B. amyloliquefaciens* not only promoted the antioxidant activity of the body by enhancing the activity of SOD and CAT, but also resulted in effective scavenging of free radicals and prevention of lipid peroxidation by secreting antioxidant enzymes or promoting the secretion of antioxidant enzymes as activators.

HSPs are relatively conserved proteins that are found commonly in animals and plants. HSP70 and HSP90 play key roles in protein folding, degradation, redox equilibrium, and the innate immune response [60–62]. In our study, the expression levels of *HSP70* in A1, A2, and A3 were significantly higher than that in C, and the expression level of *HSP70* in A2 was significantly higher than that in C. These findings suggested that adding *B. amyloliquefaciens* to the abalone diet enhanced the antioxidant capacity of the body, which in turn facilitated HSP participation in the protection and repair processes of the body. No significant difference in the expression levels of *HSP90* was detected among the groups, but the expression levels of *HSP90* in A2 and A3 were higher than those in C and A1. Basu et al. showed that host cells bound to HSP during the immunization process recognized tumor cell-associated polypeptides, repaired phagocyte damage, and prevented autolysis and apoptosis as a result of the intracellular defense system [63]. In summary, the presence of *B. amyloliquefaciens* in the abalone feed increased the expression levels of HSP genes, which helped maintain the normal physiological function of the cells and their resistance to environmental stress factors.

Cumulative mortality is a critical index for assessing the health of cultured organisms and the efficacy of immune enhancers. At the end of the bacterial infection experiment, the cumulative mortality of abalone in A1, A2, and A3 was significantly lower than that in C, indicating that the immunity and disease resistance of the abalone were significantly enhanced after *B. amyloliquefaciens* was added to the feed. Macey and Coyne added probiotics (*Vibrio midae* SY9, *Cryptococcus* sp. SS1, *Debaryomyces hansenii* AY1) isolated from the intestinal tracts of *H. midae* to feed and examined the growth and disease resistance of *H. midae*. They found that the addition of probiotics not only promoted abalone growth, but also significantly enhanced its disease resistance [64,65]. Rengpipat et al. fed *Penaeus monodon* with feed containing *Bacillus* S11, and 90 days later exposed the prawns to *Vibrio harveyi*. The survival rate of *P. monodon* in the probiotic group was 100%, whereas it was only 26% in the control group [66]. Gibson et al. found that when separately infected with the pathogenic bacteria *Vibrio tubiashii*, juvenile *Crassostrea gigas* died within 5 d; in the presence of *Aeromonas media* A199, however, none of the infected juveniles died [67]. These results showed that the addition of probiotics at an appropriate concentration can significantly enhance the resistance of a host to pathogenic infection and ultimately significantly increase survival rates.

In summary, after the basal feed was treated with 10^5 and 10^7 cfu/g of *B. amyloliquefaciens*, the survival rate, body weight SGR, and FCE of abalone were significantly higher compared to the control group. In the 10^5 cfu/g group (A2), the total number of blood lymphocytes, the O_2^- and NO levels produced from respiratory burst, and the T-AOC and activity of antioxidant enzymes were higher compared to the other groups. The expression levels of antioxidant genes *Mn-SOD*, *CAT*, GST_s, TP_x, and *HSP70* in A2 were also higher than in any other group, which might explain the significantly lower cumulative mortality of abalone in A2 at the end of the bacterial infection experiment. These findings illustrated the potential for *B. amyloliquefaciens* to be used as a dominant probiotic strain, as its presence facilitated abalone growth, enhanced the antioxidant capacity of the body, and reduced the mortality rate after bacterial infection. Subsequent experimental trials should focus on determining the optimum *B. amyloliquefaciens* feeding concentration, frequency, and mode. Use of this probiotic could help reduce aquaculture costs and improve breeding efficiency.

Ethics statement

All abalones in this study were handled in strict accordance with China legislation on scientific procedures on living animals. The protocol was approved by the ethics committee at University of Chinese Academy of Science (permit number: 399 20021109).

Conflicts of interest

Authors declare that he/she has no conflict of interest.

Acknowledgements

We would like to thank Hao Jingwei and Li Wenya for help with analyzing antioxidant capacity and enzyme activity of body tissues. This research was supported by the earmarked fund for the Modern

Agro-industry Technology Research System [CARS-49], the China Postdoctoral Science Foundation Grant [2019M650153], the National Natural Science Foundation of China of China [31672673], and the Outstanding Postdoctoral Scholarship from the State Key Laboratory of Marine Environmental Science at Xiamen University.

References

[1] N.V. Hai, Research findings from the use of probiotics in tilapia aquaculture: a review, Fish Shellfish Immunol. 45 (2015) 592–597.
[2] A. Newaj-Fyzul, A.H. Al-Harbi, B. Austin, Review: developments in the use of probiotics for disease control in aquaculture, Aquaculture 431 (2014) 1–11.
[3] S.K. Song, B.R. Beck, D. Kim, J. Park, J. Kim, H.D. Kim, et al., Prebiotics as immunostimulants in aquaculture: a review, Fish Shellfish Immunol. 40 (2014) 40–48.
[4] R. Harikrishnan, M.C. Kim, J.S. Kim, C. Balasundaram, M.S. Heo, Probiotics and herbal mixtures enhance the growth, blood constituents, and nonspecific immune response in *Paralichthys olivaceus* against *Streptococcus parauberis*, Fish Shellfish Immunol. 31 (2011) 310–317.
[5] M.B. Hovda, B.T. Lunestad, R. Fontanillas, J.T. Rosnes, Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.), Aquaculture 272 (2007) 581–588.
[6] A. Das, K. Nakhro, S. Chowdhury, D. Kamilya, Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*), Fish Shellfish Immunol. 35 (2013) 1547–1553.
[7] J.Y. Park, W.S. Kim, H.Y. Kim, E. Kim, Potential use of *Bacillus amyloliquefaciens* as a probiotic bacterium in abalone culture, J. Fish. Pathol. 29 (2016) 35–43.
[8] C.H. Liu, C.H. Chiu, S.W. Wang, W. Cheng, Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*, Fish Shellfish Immunol. 33 (2012) 699–706.
[9] C.N. Zhang, X.F. Li, W.N. Xu, G.Z. Jiang, K.L. Lu, L.N. Wang, W.B. Liu, Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on innate immunity, antioxidant capability and disease resistance of triangular bream (*Megalobrama terminalis*), Fish Shellfish Immunol. 35 (2013) 1380–1386.
[10] X.M. Chen, H.M. Lu, X.T. Niu, G.Q. Wang, D.M. Zhang, Enhancement of secondary metabolites from *Bacillus Licheniformis* XY-52 on immune response and expression of some immune-related genes in common carp, *Cyprinus carpio*, Fish Shellfish Immunol. 45 (2015) 124–131.
[11] R. Cerezuela, J. Meseguer, M.Á. Esteban, Effects of dietary inulin, *Bacillus subtilis* and microalgae on intestinal gene expression in gilthead seabream (*Sparus aurata* L.), Fish Shellfish Immunol. 34 (2013) 843–848.
[12] R. Kumar, S.C. Mukherjee, R. Ranjan, S.K. Nayak, Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*, Fish Shellfish Immunol. 24 (2008) 168–172.
[13] P. Bandyopadhyay, P.K.D. Mohapatra, Effect of a probiotic bacterium *Bacillus circulans* PB7 in the formulated diets: on growth, nutritional quality and immunity of *Catla catla* (Ham.), Fish Physiol. Biochem. 35 (2009) 467–478.
[14] D.Y. Tseng, P.L. Ho, S.Y. Huang, S.C. Cheng, Y.L. Shiu, C.S. Chiu, C.H. Liu, Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20, Fish Shellfish Immunol. 26 (2009) 339–344.
[15] China Bureau of Fisheries, China Fisheries Yearbook in 2017, Agricultural Press of China, Beijing, 2018, p. 29 (in Chinese).
[16] P.C. Liu, Y.C. Chen, C.Y. Huang, K.K. Lee, Virulence of *Vibrio parahaemolyticus* isolated from cultured small abalone, *Haliotis diversicolor* supertexta, with withering syndrome, Lett. Appl. Microbiol. 31 (2000) 433–437.
[17] K.K. Lee, P.C. Liu, C.Y. Huang, *Vibrio parahaemolyticus* infectious for both humans and edible mollusk abalone, Microb. Infect. 5 (2003) 481–485.
[18] C. Anguiano-Beltrán, R. Searcy-Bernal, M.L. Lizárraga-Partida, Pathogenic effects of *Vibrio alginolyticus* on larvae and postlarvae of the red abalone *Haliotis rufescens*, Dis. Aquat. Org. 33 (1998) 119–122.
[19] Q. Zhang, H.M. Ma, K.S. Mai, W.B. Zhang, Z.G. Liufu, W. Xu, Interaction of dietary *Bacillus subtilis* and fructooligosaccharide on the growth performance, nonspecific immunity of sea cucumber, *Apostichopus japonicas*, Fish Shellfish Immunol. 29 (2010) 204–211.
[20] C.J. Secombes, Isolation of salmonid macrophages and analysis of their killing activity, Tech. Fish Immunol. 1 (1990) 137–154.
[21] L. Villamil, A. Figueras, R. Aranguren, B. Novoa, Non-specific immune response of turbot, *Scophthalmus maximus* (L.), experimentally infected with a pathogenic *Vibrio Pelagius*, J. Fish Dis. 26 (2003) 321–329.
[22] A.E. Ellis, Lysozyme assay, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Robertsen, W.B. Van Muiswinkel (Eds.), Techniques in Fish Immunology, SOS Publication, Fair Haven, NJ, 1990, pp. 101–103.
[23] L. Góth, A simple method for determination of serum catalase activity and revision of reference range, Clin. Chim. Acta 196 (1991) 143–151.
[24] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[25] G. Yang, X.L. Tian, S.L. Dong, M. Peng, D.D. Wang, Effects of dietary *Bacillus cereus* G19, *B. cereus* BC-01, and *Paracoccus marcusii* DB11 supplementation on the growth, immune response, and expression of immune-related genes in coelomocytes and intestine of the sea cucumber (*Apostichopus japonicus* Selenka), Fish Shellfish Immunol. 45 (2015) 800–807.
[26] B. Lygren, K. Hamre, R. Waagbø, Effects of dietary pro- and antioxidants and some health parameters in Atlantic salmon (*Salmo salar*), J. Aquat. Anim. Health 11 (1999) 211–221.
[27] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[28] N.T.N. Tinh, N.N. Phuoc, K. Dierckens, P. Sorgeloos, P. Bossier, Gnotobiotically grown rotifer *Brachionus plicatilis* sensu strictu as a tool for evaluation of microbial functions and nutritional value of different food types, Aquaculture 253 (2006) 421–432.
[29] V.M. Son, C.C. Chang, M.C. Wu, Y.K. Guu, C.H. Chiu, W. Cheng, Dietary administration of the probiotic, *Lactobacillus plantarum*, enhanced the growth, innate immune responses, and disease resistance of the grouper *Epinephelus coioides*, Fish Shellfish Immunol. 26 (2009) 691–698.
[30] J.H. Wang, L.Q. Zhao, J.F. Liu, H. Wang, S. Xiao, Effect of potential probiotic *Rhodotorula benthica* D30 on the growth performance, digestive enzyme activity and immunity in juvenile sea cucumber *Apostichopus japonicus*, Fish Shellfish Immunol. 43 (2015) 330–336.
[31] X.L. Gao, M. Zhang, X. Li, Y. Han, F.C. Wu, Y. Liu, Effects of a probiotic (*Bacillus licheniformis*) on the growth, immunity, and disease resistance of *Haliotis discus hannai* Ino, Fish Shellfish Immunol. 76 (2018) 143–152.
[32] Y.Z. Sun, H.L. Yang, R.L. Ma, K. Song, J.S. Li, Effect of *Lactococcus lactis* and *Enterococcus faecium* on growth performance, digestive enzymes and immune response of grouper *Epinephelus coioides*, Aquacult. Nutr. 18 (2012) 281–289.
[33] Y.Z. Sun, H.L. Yang, R.L. Ma, C.X. Zhang, W.Y. Lin, Effect of dietary administration of *Psychrobacter* sp. on the growth, feed utilization, digestive enzymes and immune responses of grouper *Epinephelus coioides*, Aquacult. Nutr. 17 (2011) 733–740.
[34] K.I. ten Doeschate, V.E. Coyne, Improved growth rate in farmed *Haliotis midae* through probiotic treatment, Aquaculture 284 (2008) 174–179.
[35] S. Iehata, T. Inagaki, S. Okunishi, M. Nakano, R. Tanaka, H. Maeda, Improved gut environment of abalone *Haliotis gigantea* through *Pediococcus* sp. Ab1 treatment, Aquaculture 305 (2010) 59–65.
[36] Q. Zhang, H.R. Yu, T. Tong, W.P. Tong, L.F. Dong, M.Z. Xu, Z.C. Wang, Dietary supplementation of *Bacillus subtilis* and fructooligosaccharide enhance the growth, non-specific immunity of juvenile ovate pompano, *Trachinotus ovatus* and its disease resistance against *Vibrio vulnificus*, Fish Shellfish Immunol. 38 (2014) 7–14.
[37] A.Y. El-Dakar, S.M. Shalaby, I.P. Saoud, Assessing the use of a dietary probiotic/prebiotic as an enhancer of spinefoot rabbitfish *Siganus rivulatus* survival and growth, Aquacult. Nutr. 13 (2007) 407–412.
[38] T. Bagheri, S.A. Hedayati, V. Yavari, M. Alizade, A. Farzanfar, Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding, Turk. J. Fish. Aquat. Sci. 8 (2008) 43–48.
[39] Q. Yan, C.J. van der Gast, Y. Yu, Bacterial community assembly and turnover within the intestines of developing zebrafish, PLoS One 7 (2012) e30603.
[40] I.M. Soares-da-Silva, J. Ribeiro, C. Valongo, R. Pinto, M. Vilanova, R. Bleher, et al., Cytometric, morphologic and enzymatic characterisation of haemocytes in *Anodonta cygnea*, Comp. Biochem. Physiol. A 132 (2002) 541–553.
[41] X. Geng, X.H. Dong, B.P. Tan, Q.H. Yang, S.Y. Chi, H.Y. Liu, et al., Effects of dietary chitosan and *Bacillus subtilis* on the growth performance, non-specific immunity and disease resistance of cobia, *Rachycentron canadum*, Fish Shellfish Immunol. 31 (2011) 400–406.
[42] J.H. Cha, S. Rahimnejad, S.Y. Yang, K.W. Kim, K.J. Lee, Evaluations of *Bacillus* spp. as dietary additives on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*) against *Streptococcus iniae* and as water additives, Aquaculture 402–403 (2013) 50–57.
[43] C. Hooper, R. Day, R. Slocombe, J. Handlinger, K. Benkendorff, Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models, Fish Shellfish Immunol. 22 (2007) 363–379.
[44] J.L. Balcázar, T. Rojas-Luna, D.P. Cunningham, Effect of the addition of four potential probiotic strains on the survival of pacific white shrimp (*Litopenaeus vannamei*) following immersion challenge with *Vibrio parahaemolyticus*, J. Invertebr. Pathol. 96 (2007) 147–150.
[45] M. Maeda, A. Shibata, G. Biswas, H. Korenaga, T. Kono, T. Itami, et al., Isolation of lactic acid bacteria from kuruma shrimp (*Marsupenaeus japonicus*) intestine and assessment of immunomodulatory role of a selected strain as probiotic, Mar. Biotechnol. 16 (2014) 181–192.
[46] S. Nikoskelainen, A.C. Ouwehand, G. Bylund, S. Salminen, E.M. Lilius, Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*), Fish Shellfish Immunol. 15 (2003) 443–452.
[47] J. Brunt, A. Newaj-Fyzul, B. Austin, The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum), J. Fish Dis. 30 (2007) 573–579.
[48] D.H. Kim, B. Austin, Cytokine expression in leucocytes and gut cells of rainbow trout, *Oncorhynchus mykiss* Walbaum, induced by probiotics, Vet. Immunol. Immunopathol. 114 (2006) 297–304.
[49] R.S. Anderson, L.L. Brubacher, L.R. Calvo, M.A. Unger, E.M. Burreson, Effects of tributyltin and hypoxia on the progression of *Perkinsus marinus* infections and host defence mechanisms in oyster, *Crassostrea virginica* (Gmelin), J. Fish Dis. 21 (1998) 371–380.
[50] M.C. Ordás, A. Ordás, C. Beloso, A. Figueras, Immune parameters in carpet shell clams naturally infected with *Perkinsus atlanticus*, Fish Shellfish Immunol. 10 (2000) 597–609.
[51] A. Newaj-Fyzul, A.A. Adesiyun, A. Mutani, A. Ramsubhag, J. Brunt, B. Austin, *Bacillus subtilis* AB1 controls Aeromonas infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum), J. Appl. Microbiol. 103 (2007) 1699–1706.

[52] S.M. Sharifuzzaman, B. Austin, Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout, Fish Shellfish Immunol. 27 (2009) 440–445.
[53] C. Yang, J. Kong, Q. Wang, Q. Liu, Y. Tian, K. Luo, Heterosis of haemolymph analytes of two geographic populations in Chinese shrimp *Fenneropenaeus chinensis*, Fish Shellfish Immunol. 23 (2007) 62–70.
[54] N.W. Ross, K.J. Firth, A. Wang, J.F. Burka, S.C. Johnson, Changes in hydrolytic enzyme activities of naïve Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation, Dis. Aquat. Org. 41 (2000) 43–51.
[55] J.Q. Li, B.P. Tan, K.S. Mai, Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*), Aquaculture 291 (2009) 35–40.
[56] A. Vosloo, A. Laas, D. Vosloo, Differential responses of juvenile and adult South African abalone (*Haliotis midae* Linnaeus) to low and high oxygen levels, Comp. Biochem. Physiol. A 164 (2013) 192–199.
[57] S. Rengpipat, A. Tunyanun, A.W. Fast, S. Piyatiratitivorakul, P. Menasveta, Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic, Dis. Aquat. Org. 55 (2003) 169–173.
[58] R.M. Martínez-Álvarez, A.E. Morales, A. Sanz, Antioxidant defenses in fish: biotic and abiotic factors, Rev. Fish Biol. Fish. 15 (2005) 75–88.
[59] K.M. Selim, R.M. Reda, Improvement of immunity and disease resistance in the *Nile tilapia, Oreochromis niloticus*, by dietary supplementation with *Bacillus amyloliquefaciens*, Fish Shellfish Immunol. 44 (2015) 496–503.
[60] Q. Gao, J.M. Zhao, L.S. Song, L.M. Qiu, Y.D. Yu, H. Zhang, D.J. Ni, Molecular cloning, characterization and expression of heat shock protein 90 gene in the haemocytes of bay scallop *Argopecten irradians*, Fish Shellfish Immunol. 24 (2008) 379–385.
[61] D. Picard, Heat-shock protein 90, a chaperone for folding and regulation, Cell. Mol. Life Sci. 59 (2002) 1640–1648.
[62] J. Robert, Evolution of heat shock protein and immunity, Dev. Comp. Immunol. 27 (2003) 449–464.
[63] N. Basu, A.E. Todgham, P.A. Ackerman, M.R. Bibeau, K. Nakano, P.M. Schulte, G.K. Iwama, Heat shock protein genes and their functional significance in fish, Gene 295 (2002) 173–183.
[64] B.M. Macey, V.E. Coyne, Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment, Aquaculture 245 (2005) 249–261.
[65] B.M. Macey, V.E. Coyne, Colonization of the gastrointestinal tract of the farmed South African abalone *Haliotis midae* by the probionts *Vibrio midae* SY9, *Cryptococcus* sp. SS1, and *Debaryomyces hansenii* AY1, Mar. Biotechnol. 8 (2006) 246–259.
[66] S. Rengpipat, A. Tunyanun, A.W. Fast, S. Piyatiratitivorakul, P. Menasveta, Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic, Dis. Aquat. Org. 55 (2003) 169–173.
[67] L.F. Gibson, J. Woodworth, A.M. George, Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*, Aquaculture 169 (1998) 111–120.
[68] P.M. Ekanayake, H.S. Kang, M. De Zoysa, Y. Jee, Y.H. Lee, J. Lee, Molecular cloning and characterization of Mn-superoxide dismutase from disk abalone (*Haliotis discus discus*), Comp. Biochem. Physiol. B 145 (2006) 318–324.
[69] P.M. Ekanayake, M. De Zoysa, H.S. Kang, W. Qiang, Y. Jee, Y.H. Lee, et al., Cloning, characterization and tissue expression of disk abalone (*Haliotis discus discus*) catalase, Fish Shellfish Immunol. 24 (2008) 267–278.
[70] W.A. Pushpamali, M. De Zoysa, H.S. Kang, C. Oh, I. Whang, S.J. Kim, et al., Comparative study of two thioredoxin peroxidases from disk abalone (*Haliotis discus discus*): cloning, recombinant protein purification, characterization of antioxidant activities and expression analysis, Fish Shellfish Immunol. 24 (2008) 294–307.
[71] Q. Wan, I. Whang, J. Lee, Molecular characterization of mu class glutathione-S-transferase from disk abalone (*Haliotis discus discus*), a potential biomarker of endocrine-disrupting chemicals, Comp. Biochem. Physiol. B 150 (2008) 187–199.
[72] P.Z. Cheng, X. Liu, G.F. Zhang, J.G. He, Cloning and expression analysis of a HSP70 gene from Pacific abalone (*Haliotis discus hannai*), Fish Shellfish Immunol. 22 (2007) 77–87.
[73] W.B. Zhang, C.L. Wu, K.S. Mai, Q.Y. Chen, W. Xu, Molecular cloning, characterization and expression analysis of heat shock protein 90 from Pacific abalone, *Haliotis discus hannai* Ino in response to dietary selenium, Fish Shellfish Immunol. 30 (2011) 280–286.