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Characterization of *Bacillus subtilis* from gastrointestinal tract of hybrid Hulong grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) and its effects as probiotic additives

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ABSTRACT

Probiotics are widely used for the improvement of animals' growth and health. However, few marine aquatic probiotics are applied and licensed in China. In this study, a *Bacillus* spp. strain was isolated from the Hulong grouper gastrointestinal tract, which was identified as a new strain of *Bacillus subtilis* and was named as 7k. *B. subtilis* 7k showed desirable capability of sporulation and resistance to heat, simulated gastric juice and simulated duodenum juice, indicating its potential as probiotics. Seven antimicrobial chemicals were found in the secretion of the *B. subtilis* 7k. *B. subtilis* 7k addition in diet promoted the growth rate of Hulong groupers. Moreover, *B. subtilis* 7k can inhibit infection by iridovirus, making *B. subtilis* 7k a suitable kind of probiotic for maintaining fishes' health. Our results also revealed that *B. subtilis* 7k induced non-specific immune response in Hulong grouper under virus infection. Hulong grouper fed by diets containing *B. subtilis* 7k at 10^8 and 10^{10} cfu g^{-1} for 4–8 weeks were significantly strengthened in serum lysozyme activity, serum alternative complement activity (ACH50), serum bactericidal activity, respiratory burst, superoxide dismutase activity (SOD), and phagocytic activity of head kidney leucocytes when compared with those fed by control diets. In conclusion, *B. subtilis* 7k was isolated and characterized to be a kind of process enduring, growth stimulating, immunity enhancing and health promoting probiotic using in grouper culture.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO) report, global fishers produced 167 million tonnes of fish in 2014, leading to a global trade worth almost US$ 148 billion. People have never consumed so much fish as they do today [1]. However, the widespread use of antibiotics leads to antibiotic resistance that is detrimental to both aquatic and human health [2]. It is reported that at least 2,000,000 people suffered from serious infections with antibiotic-resistant bacteria, and at least 23,000 people died each year as a direct result of these antibiotic-resistant infections in the United States [3]. The horrible influence on people's health urged authorities like Food and Drug Administration to limit the usage of antibiotic as animal growth promoters [4].

At this scenario, the application of probiotics could be alternative strategy to prevent pathogenic infections. Probiotics affect animals' health in many positive ways, including: (i) competitive exclusion of pathogens, (ii) production of nutrients and enzymatic contribution to digestion, (iii) secretion of inhibitory substance, (iv) enhancement of immune response and (v) improvement of water quality [5–7]. *B. subtilis* was found to produce bacteriocins and subtilin, which could be used in food preservation and control of food poisoning [8]. It was reported that the administration of *B. subtilis* strains in rearing water could enhance water quality and improve resistance to *Vibrio harveyi*

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infection in juvenile white shrimps, bringing about better growth performance [7]. What's more, the spore formation often promises high resistance in extreme environment, such as gastric juice, bile and heat treatment, which might be advantageous in feeding [9,10].

Hulong grouper is a novel hybrid grouper (*Epinephelus fuscoguttatus* (♀) × *Epinephelus lanceolatus* (♂)) of high economic potential, which luckily inherited the merits of parents [11]. In recent years, many studies have examined its excellent performance in growth rate, however, diseases had resulted in high mortality in this species [12,13]. Viral pathogens were frequently reported to infect groupers, among which iridovirus such as Singapore grouper iridovirus (SGIV) have caused heavy economic losses especially in juvenile groupers [14].

In this paper, a *B. subtilis* strain was isolated from Hulong grouper gastrointestinal tract (GIT). We examined its antagonistic ability against several pathogens and identified its possibility of surviving in simulated digestive system and resistance to heat treatment. Then its probiotic properties in growth, non-specific immune gene expression and innate immunity were characterized. The data also provided new insight into the function of probiotics during virus infection.

2. Materials and methods

2.1. Isolation and identification of bacteria

Samples of fresh intestinal contents were collected from the gastrointestinal (GI) tract of Hulong groupers referring to the method of Guo X' s study [15]. In order to exclude non-spore producing bacteria, 1 g samples were diluted 1:100 in sterile PBS buffer, and heat for 15 min at 80 °C. After centrifuged at 4000 g for 10 min at 4 °C, the supernatant was added into LB culture medium and incubated for 24 h at 37 °C. Then the medium was serially diluted and plated for monoclones. The typical colonies showing a dark yellow plaque (2–4 mm in diameter) with rough edge were selected. Cell morphology of strains was observed via a light Microscope. After an overnight incubation at 37 °C, the purified strain were picked up and re-incubate in LB culture medium for 12 h at 37 °C. The strains were stored at 4°Cfor further biochemical and molecular characterization.

To identify isolated bacterial species, 16s rDNA sequences were amplified by using two primers K1F and K2R (Supplementary file Table S1). Bacteria DNA were extracted using the DNA Isolation Kit (Takara) according to the manufacture's instructions. A 25 μl PCR reaction mixture contains bacteria DNA 1 μl, *Taq* 0.2 μl, dNTP 1 μl, 10 × buffer 2.5 μl, K1F 1 μl, K2R 1 μl and ddH_2O. The RCR amplification consisted of a pre-denaturation for 3 min at 95 °C, 1 cycle , followed by 35 cycles of 95 °C for 1 min, 55 °C annealing for 45s and 72 °C extension for 45s, and a final extension at 72 °C for 5min. The PCR products were sequenced and analyzed through https://www.ncbi.nlm.nih.gov/BLAST by comparing them with bacterial 16s rDNA sequences in Genbank to determine the genus. The 16s rDNA sequences of other selected strains were down loaded and aligned with the isolated probiotic strain using MEGA5.0 software. The phylogenetic tree was constructed with a neighbour joining DNA distance algorithm to determine the evolutionary distance between strains. Conventional physiological and biochemical identifications of the isolates were carried out as described in the instructions of Bergey's Manual® of Systematic Bacteriology [16].

2.2. Preparation of spores and vegetative cells

For the preparation of the vegetative cells, colonies of *B. subtilis* 7k were picked up from LB agar plates and cultured in a sterilized 250 ml shake flask with LB medium at 37 °C. The vegetative cells were taken from logarithmic phase cells that contain little spores (Fig. 2). For the preparation of spores, the pure *B. subtilis* colonies were transferred into 5L auto fermentor (Zhen Jiang Dong Fang, China) with the medium as follows: soybean meal 10 gl^{-1}, yeast extract 2.5 gl^{-1}, glucose 10 gl^{-1}, $(NH_4)_2SO_4$ 2.5 gl^{-1}, K_2HPO_4 0.69 gl^{-1}, KH_2PO_4 0.25 gl^{-1}, $MgSO_47H_2O$ 0.2 gl^{-1}, $MnSO_44H_2O$ 0.1 gl^{-1}. Following a 48h cultivation at 37 °C, the medium were centrifuged at 8000 × *g* for 15 min at 4 °C, after which the supernatant was removed. In order to remove residual vegetative cells, the spores were transferred into a lysozyme solution (4 mg/ml) for 15 min at room temperature and then washed twice with PBS buffer. The washed spores were heat-treated in water bath at 68 °C for 1 h and diluted in water to approximately 10^9 cfu ml^{-1} [14]. The spores were stored at −20 °C prior to use. The number of vegetative cells was evaluated by plating out on LB medium. Before cultured on LB agar plate, each sample was heated at 65 °C for 35 min and then cooled on ice for 10 min [17].

2.3. Determination of tolerance to heat and simulated GIT conditions

The heat treatment was carried out in boiling water bath at 100 °C. *Escherichia coli*, spores and vegetative cells of *B. subtilis* 7k were sampled at suitable interval (10–30 s) during the heat treatment, and then cultured on LB agar plates for 24 h at 37 °C and selected the plates with 30–300 colonies for cell counting. Three parallel plates were carried out for each sample, and survival curves were constructed by plotting the log_{10} number of survivors against time, and data were expressed as means ± SD from three independent trials with duplicated plating in each trial.

A simulated gastric fluid was prepared as described by Clavel, T et al. [18]. The solution consisting of 4.8 g l^{-1} NaCl, 1.56 g l^{-1} $NaHCO_3$, 2.2 g l^{-1} KCl and 0.22gl^{-1} $CaCl_2$ was adjusted to pH 1.5 by using sterile 1 M HCl or 1 M NaOH and sterilized by autoclaving at 121 °C for 20min. Immediately prior to testing, a filter (0.22 μm) sterile water pepsin solution was added (final concentration at 500U l^{-1}).

A Simulated duodenum juice (SDJ) was prepared as described by Ziarno, M et al. [19]. The solution consisting of 5 g NaCl, 0.6 g KCl, 0.25 g $CaCl_2$ and 8.5 g bovine bile dissolved in 1 L of 1 M solution of $NaHCO_3$ and the pH was adjusted to 7.0 by using sterile 1 M HCl or 1 M NaOH. Then the solution was sterilized by autoclaving at 121 °C for 20 min. After sterilization, the solution was supplemented with 20,000 U l^{-1} of lipase solution, 16, 000 Ul^{-1} of amylase solution and 1200 Ul^{-1} of protease solution. Each enzyme was dissolved in water and sterile filtered (0.22 μm).

E. coli, spores and vegetative cells of *B. subtilis* 7k were used to test the tolerance to simulated GIT conditions. Samples were collected at suitable interval (5–20 min) during the treatment. Counting method was as described in heat-resistance experiment.

2.4. Extracellular antimicrobial assay

B. subtilis 7k was incubated in LB medium for 24 h at 37 °C and diluted (1:100) with the culture medium (soybean meal 10 g l^{-1}, yeast extract 2.5 g l^{-1}, glucose 10 g l^{-1}, $(NH_4)_2SO_4$ 2.5 g l^{-1}, K_2HPO_4 0.69 g l^{-1}, KH_2PO_4 0.25 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.2 g l^{-1} and $MnSO_4{\cdot}4H_2O$ 0.1 g l^{-1}). Then it was cultured in a 5L auto fermentor (Zhen Jiang Dong Fang, China) and incubated for 48 h at 37 °C. The culture medium was centrifuged at 4000 × *g* for 10 min at 4 °C, after which the supernatant was collected. The supernatant was sterilized through 0.22 μm pore-size filters and stored at −20 °C for use.

The extracellular antimicrobial activity of the *B. subtilis* 7k was determined by measuring the zone of inhibition on LB agar plate. *Staphylococcus aureus*, *Aeromonas hydrophila, Micrococcus lysodeikticus*, *Vibrio harveyi*, *Vibrio vulnificus* and *Vibrio alginolyticus* were used as pathogens (Lab collection). 150 μL supernatant were added to the Oxford cup in the LB agar incubated with 10^5 CFU ml^{-1} of pathogen. All assays were conducted in triplicate. In order to investigate the chemical properties of antimicrobial compounds in the supernatant, high performance liquid chromatography coupled to a high-resolution mass spectrometer (LC-HRMS, Bruke maXis) was used to analyze the chemical composions in the supernatant.

2.5. Effects of probiotic supplemented feed on the growth of groupers

Healthy Hulong groupers were purchased from a grouper culture farm in Hainan province, China. The grouper acclimating in tanks with laboratory recirculating aerated seawater for 2 weeks were fed with steriled diet (Purchase from Guangdong Haid Group Co., Ltd, China) before the start of the experiment. In order to avoid cannibalization, groupers weighing between 7.5 g and 10.0 g were chosen for the trial and randomly distributed into four diet groups [0 (control), 10^6, 10^8, 10^{10} cfug^{-1} of *B. subtilis* 7k-supplemented diets], each of which comprised of 40 fish. In the experiment, the groupers were fed with the diet twice daily (9:00 a.m., 5:00 p.m.), and 70% water was renewed daily.

Feed was prepared by mixing the *B. subtilis* 7k with the commercial feedstuff (Guangdong Haid Group Co., Ltd, China). Except for the *B. subtilis* 7k, the basic ingredients were sterilized at 121 °C for 20 min before mixing. After the process of mixing, granulation and drying (4 h at 60 °C), the concentration of *B. subtilis* 7k in each diet was measured by counting the colonies on the LB plate both at the start and the end of the trial. After feeding with *B. subtilis* 7k-supplemented diets for 4 weeks, the final weight, weight gain, average daily gain (ADG), survival rate, percent weight gain (PWG, PWG = [100 × (final body weight – initial body weight)(initial body weight)$^{-1}$]) and feed efficiency (FE, FE = [(final body weight – initial body weight)(feed intake)$^{-1}$]) of different groups were measured for evaluation of growth effect.

2.6. Challenge test

Singapore grouper iridovirus (SGIV), which can propagated in grouper spleen cells [14], was detected by PCR amplification of MCP gene to ensure that the groupers are uninfected by SGIV before the assay. After feeding with *B. subtilis* 7k-supplemented diets at concentrations of 0, 10^6, 10^8 and 10^{10} cfu g^{-1} for 5 weeks, the experimental viral infection was conducted by immersion of the SGIV in the seawater containing SGIV at 10^3 pfu l^{-1} for 3 days [14]. The test consisted of 5 groups, each of which was comprised of 40 fish. Among them, four challenged groups fed with 0, 10^6, 10^8 and 10^{10} cfug^{-1} *B. subtilis* 7k-supplemented diet respectively and one group served as the unchallenged and negative control. Each group composed of four 20L-tanks containing 10 fish each, among which three tanks were used for calculating survival rate and the fourth tank serving as the gene expression samples. After a 48h of infection, the spleen of 4 groupers were collected from each group for determining the immune-related genes expression levels. In order to minimize the bias, all the samples were collected at 4:00 p.m. before feed and water renewal. The tissue samples were collected from the groupers and then immediately immersed in Sample Protector for RNA/DNA (Takara), followed by storage at −80 °C until analysis. The mortality was monitored daily for a whole trial of 10 days. PCR detection of the MCP gene of SGIV [14] was performed for each dead fish (spleen) to confirm the infection of the SGIV in the challenged fish. During the period, groupers were fed with their respective diet twice daily (9:00 a.m., 5:00 p.m.) with 70% water renewal daily.

2.7. Immune-related gene expression of grouper

The spleen of the challenged fish were collected to detect the expression of target genes, including interferon-induced Mx genes (*MXI*, *MXII*), interferon-stimulated gene (*ISG15*), interleukin (*IL-1β*, *IL-8*), and tumor necrosis factor alpha (*TNF-α*). Total RNA was extracted from spleen by using the SV Total RNA Isolation Kit (Promega) and the genes expression were measured by quantitative real-time PCR (qPCR) in a Roche 480 Real Time Detection System (Roche, German). SYBR Green I real-time PCR Kit (TOYOBO) was used for qPCR. The assay conditions were as follows: 94 °C for 5 min, followed by 45 cycles of 5 s at 94 °C, 10 s at 60 °C and 15 s at 72 °C. All the experiments were carried out with biological repetition in triplicate. The relative mRNAs expression levels were analyzed as target genes expression to β-actin using typical Ct method ($2^{-\triangle\triangle ct}$ method) [20]. The results were expressed as mean ± SD, means were separated by least significant difference ($p < 0.05$)(SPSS). All the primers sequences are shown in Supplementary files (Table S1).

2.8. Immunological assay

Hulong groupers with weight between 45 g and 50 g were chosen and fed with *B. subtilis* 7k-supplemented diets at concentrations of 0, 10^6, 10^8 and 10^{10} cfu g^{-1} for 2 months. At the 0, 4 and 8 weeks, three groupers from each group were sampled to determine the non-specific immune parameters. Blood was individually withdrawn, serum was collected, head kidneys of the groupers were excised, and the leucocytes were harvested following the method of Yeh et al. [21]. Harvested cells were adjusted to 10^6 cells ml^{-1} for the assay.

2.8.1. Lysozyme activity

The serum lysozyme activity analysis was determined as described by Obach et al. [22] based on the lysis of the lysozyme sensitive Gram positive bacterium, *Micrococcus lysodeikticus*. Three groupers from each group were sampled to determine the lysozyme activity.

2.8.2. Respiratory burst assay

Respiratory burst activity produced by leucocytes of the head kidneys was detected according to the detailed procedures of Cook et al. [23]. 2 × 10^5 head kidney leucocytes of each sample were used for respiratory burst assay.

2.8.3. Alternative complement activity (ACH_{50}) assay

ACH_{50} was determined according to the method described by Sunyer and Tort [24]. The volume of serum complement producing 50% hemolysis (ACH_{50}) was determined, and the number of ACH_{50} unit ml^{-1} was calculated for each experimental grouper.

2.8.4. SOD assay

For SOD assay, 2 × 10^5 head kidney leucocytes of each sample were used. Leucocytes were homogenized in phosphate-buffered saline buffer (PBS) on ice and centrifuged at 5 × 10^3 g for 5 min at 4 °C. The supernatant was immediately used for the SOD analysis. SOD activity was measured by its ability to inhibit superoxide radical-dependent reactions using a Superoxide Dismutase (SOD) assay kit (Nan Jing Jian Cheng, China) according to the detailed procedures of the kit. The specific activity was expressed as units (mg protein)$^{-1}$. The concentration of protein in the leucocyte suspension was determined by the Bradford method [23] using a Bradford protein assay kit (Nan Jing Jian Cheng, China).

2.8.5. Bactericidal assay

Three groupers from each group were sampled to determine the bactericidal activity. Grouper blood was withdrawn at 0, 4 and 8 weeks after feeding with *B. subtilis*7k-supplemented diet and 100 μL of serum was mixed with 100 μL of *V. alginolyticus* (1 × 10^6 CFU ml^{-1}) and kept at 25 °C for 1 h prior to enumeration of the survival *V. alginolyticus* by plate counting on TCBS agar. The bactericidal activity of fish serum was expressed as 1- percentage of survival of *V. alginolyticus* after exposure to fish serum.

2.8.6. Phagocytic activity assays

The phagocytic activity analysis was described previously [21]. Three hundred phagocytes were counted under a light microscope using oil immersion. The percentage of macrophages that engulfed one or more fluorescent latex beads, defined as the phagocytic rate (PR) was expressed as: PR = [100 × (phagocytic leucocytes) (total leucocytes)$^{-1}$].

2.9. Statistic alanalysis

One-way analysis of variance (ANOVA) was used to analyze the data. When ANOVA identied differences among groups, Tukey's multiple-comparisons' Test was conducted to examine significant differences among treatments using the SAS computer software (SAS Institute, Cary, NC, USA). Before the analysis, the data (from the growth effect, susceptibility, bactericidal activity and phagocytic activity assays) were normalized by arcsine-transformation. Statistically significant differences required $p < 0.05$.

3. Results

3.1. Identification of the bacteria isolated from the GIT of groupers

According to the comparison of the 16s rDNA sequence of isolated strain 7k to those deposited in the Genebank of NCBI database, the phylogenetic analysis by MEGA5 indicated the most related species was *B. subtilis* (> 99% homology) (Fig.S1). As shown in Table S2 (Supplementary file), biochemical identification and physiological results suggested that both the standard strain of *B. subtilis* ATCC6051 and the isolated bacteria were Gram positive and aerobic. Furthermore, both of them were not only capable of producing catlase, amylase, protease and acid, but utilizing glucose, arabinose, xylose, mannitol, sucrose, nitrate acid and nitrate. The phylogenetic analysis, physiological and biochemical characters indicated that the isolated strain 7k belonged to *B. subtilis*.

3.2. Bacteria growth curve

The bacterial growth of *B. subtilis* 7k was presented in Fig. 1. After 30h of growth in auto fermenter, the culture entered the stationary phase and the sporulation percentages of *B. subtilis* 7k reached its peak at 48h (more than 95%). In the fermentation of shake flask, the culture entered the stationary phase at 18h, while the count of vegetative cell started to rapidly fall at 36h. The sporulation percentages of *B. subtilis* 7k reached its highest point at 33h (nearly 20%).

3.3. Determination the tolerance to heat and simulated git conditions

As shown in Fig. 2a, the resistance to heat of *B. subtilis* 7k was characterized. The survival curves were obtained by treating the strain in the boiling water bath. After heated at 100 °C for 2 min, the spore of *B. subtilis* 7k, with a slight decrease of survival rate, showed high resistance to heat, while the vegetative cell of *B. subtilis* 7k showed a sharp decrease of survival rate after heat treatment for 30 s. *E. coli* was also susceptible to heat, since no cell survived after heat treatment at 100 °C for 60 s.

As shown in Fig. 2b, decrease in the spore count of *B. subtilis* 7k was less than 5% in the first hour under simulated gastric juice condition. The same experiment was carried out with *E.coli* and vegetative cell of *B. subtilis* 7k, which caused sharp reduction in bacterial population. But as Fig. 2b shown, the vegetative cell of *B. subtilis* 7k showed a better tolerance to simulated gastric juice condition than *E. coli* ($p < 0.05$) .

When incubated in the simulated duodenum juice for 3h, spores of *B. subtilis* 7k showed higher tolerance, with survival rate almost 100%. Nevertheless, the vegetative cells of *B. subtilis* 7k decreased from 6.43 log CFU ml^{-1} to 1.56 log CFU ml^{-1}, showing a high susceptibility to simulated duodenum juice condition (Fig. 2c). Surprisingly, the number of *E. coli* was stable in simulated duodenum juice indicating that *E. coli* was tolerant to the simulated duodenum juice.

3.4. Extracellular antimicrobial assay

The results of extracellular antimicrobial activity assays of *B. subtilis* 7k are listed in Table 1. The supernatant of fermentative broth of *B. subtilis* 7k could form inhibiting zones and displayed extracellular antimicrobial activity. Among the 6 selected pathogens, the supernatant had antimicrobial effect on *S. aureus*, *V. alginolyticus*, *M. lysodeikticus* and *V. harveyi*, but less antimicrobial effect on *A. hydrophila* and *V. vulnificus*. According to the LC-HRMS analysis (Fig. 3), there were 7 main substances (S1-S7) in the supernatant that might contribute to the antimicrobial effect. All those substances were suggested to be Iturins compounds, a family of lipopeptides extracted from the culture media of various strains of *B. subtilis*, on the base of the HRMS data and Antibase database search. The molecular formulas of 7 Iturins were deduced to be $C_{47}H_{72}N_{12}O_{14}$ (S1, maybe Iturin A1), $C_{48}H_{74}N_{12}O_{14}$ (S2, maybe Iturins A2 or AL-1), $C_{48}H_{74}N_{12}O_{14}$ (S3, maybe Iturins A2 or AL-1), $C_{49}H_{76}N_{12}O_{14}$ (S4, maybe Iturins A3, A4 orA5),$C_{49}H_{76}N_{12}O_{14}$ (S5, maybe Iturin A3, A4or A5), $C_{49}H_{76}N_{12}O_{14}$ (S6, maybe Iturins A3, A4 or A5), and $C_{50}H_{78}N_{12}O_{14}$ (S7, maybe Iturins A6 or A7), according to the accurate molecular weight of those substances showed in Fig. 3.

3.5. B. subtilis 7k promoted the growth of grouper

The results of animal experiments were shown in Table 2. At the beginning of the trial, no significant difference ($p > 0.05$) was observed in weight between the groupers fed with the probiotic-supplemented diet and control diet. However, there were significant differences ($p < 0.05$) in the final weight, weight gain, ADG, PWG, and FE between the fish fed with 10^6, 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k-supplemented diet and the control diet after 28 days. No significant difference was observed between the fish fed with 10^6, 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k-supplemented diet in the final weight, weight gain, PWG, and FE. There was no significant difference ($p > 0.05$) in survival rate between the fish fed with probiotic-supplemented diet and control diet.

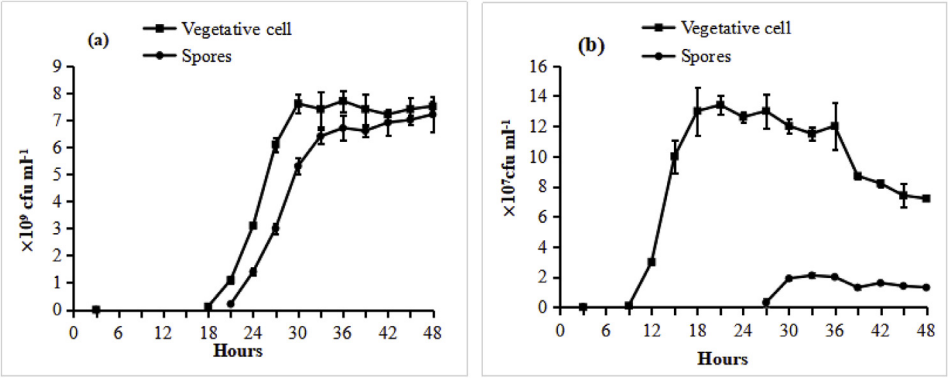
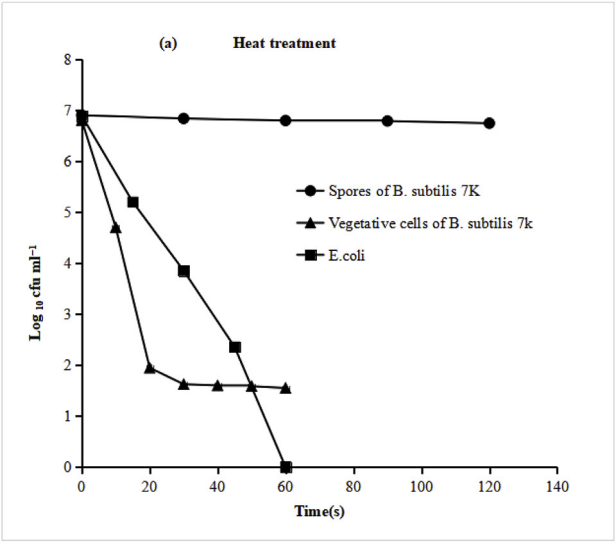
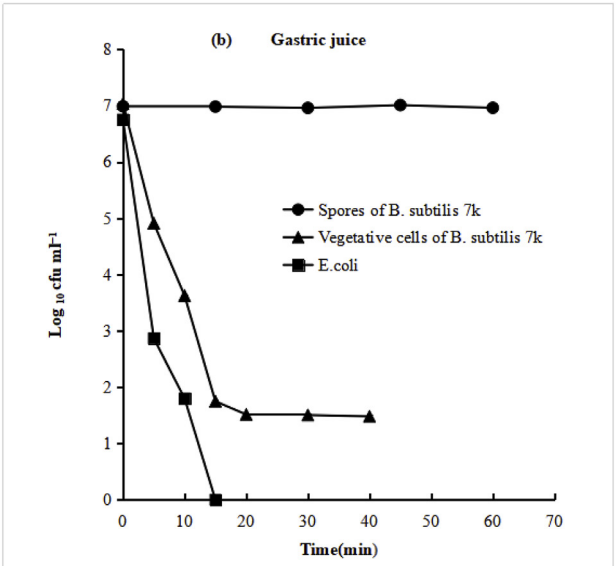


Fig. 1. The bacterial growth curves of *B. subtilis* 7k. Spore and vegetative cell of *B. subtilis* 7k were Counted in the auto fermentor (a) and shake flask (b).





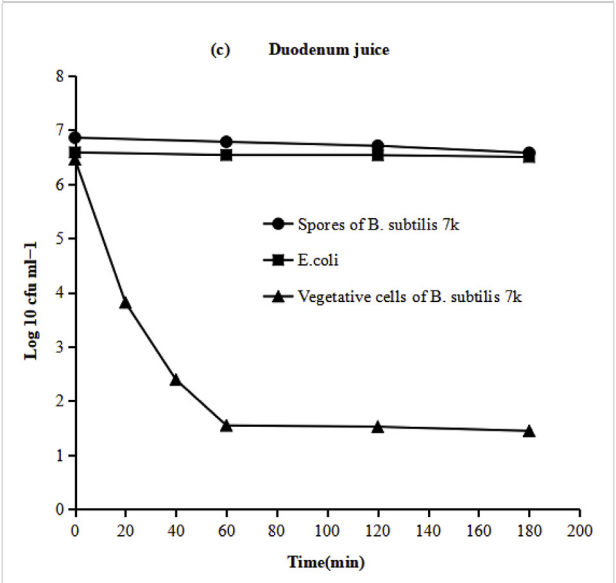


Fig. 2. The resistances of *B.subtilis*7kto heat andsimulated git conditions.Survival curves of the vegetative cell and spores of the *B. subtilis* 7k, and *E. coli* at 100 °C (a) under simulated gastric juice condition (b) and simulated duodenum juice (c). The data were expressed as means ± SD from three independent trials.

Table 1
Antimicrobial activities of the probiotic *B. subtilis* 7k isolated from grouper against fish pathogens.

Name of the fish pathogens	Zone of inhibition
A.hydrophila	+ +
V. vulnificus	+
V. harveyi	+ + +
S. aureus	+ + +
M. lysodeikticus	+ + +
V. alginolyticus	+ + +

+ zone of inhibition between 1 mm and 5 mm, + + zone of inhibition between 5 mm and 15 mm, + + + zone of inhibition between 15 mm and 20 mm.

3.6. Challenge test with SGIV infection

After challenged with SGIV by immersion infection, no dead fish was observed in the fish fed with the 10^8 and 10^{10} cfu g^{-1}*B. subtilis* 7k-supplemented diet until the 3rd day. As shown in Fig. 4, after 10 days of challenge there were significant differences in cumulative mortalities in the fish fed with the 10^8 (46.67 ± 11.55%) and 10^{10} cfu g^{-1} (30 ± 0%) probiotic-supplemented diet, compared to the control diet (73.33 ± 5.77%). Besides, the mortality of fish fed with the 10^{10} cfu g^{-1} probiotic-supplemented diet was significantly lower than those fed with 10^8 and 10^6 cfu g^{-1} diet (60.00 ± 10.00%) after 10 days of challenge. However, no significant difference in mortality was detected between the fish fed with 10^6 cfu g^{-1} diet and the control diet. The protective effect of *B. subtilis* 7k-supplemented diet seemed to be in a dose-dependent manner.

3.7. Immune-related genes expression of groupers

No dead fish was observed in the fish fed on *B. subtilis* 7k-supplemented diet at 0, 10^6, 10^8 and 10^{10} cfu (g diet)$^{-1}$ after challenged with SGIV for 48h. For the expression of the three interferon related gene, including *MXI* (Fig. 5 a), *MXII* (Fig. 5 b) and *ISG15* (Fig. 5 c), the expression level of fish fed 10^8 diet were significantly higher than those fed with 10^6 and 0 cfu g^{-1} diet. The expression level of fish fed with 10^8 diet were significantly lower than 10^{10} cfu g^{-1} fed fish in *MXI* and *ISG15* expression. Only in the expression of *ISG15*, we observed no significant difference between 10^6 and 0 cfu g^{-1} diet fish.

For the expression of the three pro-inflammatory cytokines genes, including *IL-1β* (Fig. 5d), *IL-8* (Fig. 5e) and *TNFα* (Fig. 5f), the fish fed with 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k-supplemented diet were significantly higher than that of fish fed with 10^6 and 0 cfu g^{-1} diet. Moreover, *IL-8* expression in fish fed 10^{10} cfu g^{-1} diet was significantly up-regulated than 0 cfu g^{-1} diet fish after 48h virus infection. It deserves attention that groupers fed 10^{10} cfu g^{-1} diet had significantly higher expression in *IL-1β*, however, significantly lower expression in *TNFα*, as compared to the fish fed with 10^8 cfu g^{-1} diet.

3.8. Non-specific immune parameters of grouper after feeding with B. subtilis 7k-supplemented diet

Lysozyme activities of groupers fed the 10^6, 10^8, and 10^{10} cfu g^{-1} *B. subtilis* 7k diets were significantly higher than those fed the control diet, and had respectively increased by 1.2-, 1.3- and 1.3-fold compared to the control diet after 4 weeks of feeding, and had increased by 1.4-, 1.9-, and 2.2-fold after 8 weeks of feeding (Fig. 6a). The lysozyme

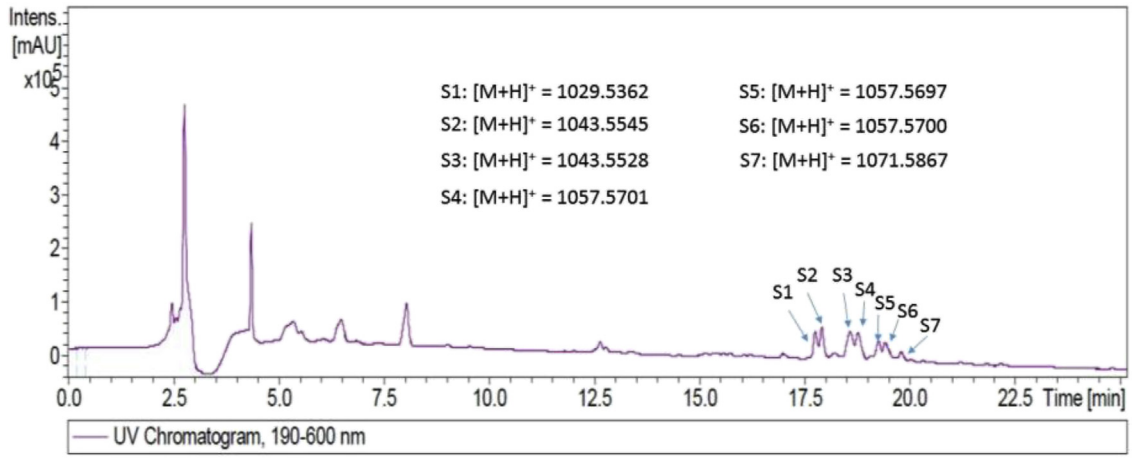


Fig. 3. LC-HRMS analysis of the supernatants of fermentative broth of *B.subtilis*7k.

activities exhibited to have been dose-dependent.

Four weeks after feeding assays, respiratory burst was not significantly different ($p > 0.05$) between the *B. subtilis* 7k-supplemented and the control groups. However, at 8 weeks the groupers fed the 10^6, 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k containing diet showed significantly ($p < 0.05$) higher respiratory burst than the control group, with 1.3-, 1.5-, and 1.7-fold increase, respectively (Fig. 6b).

ACH50 levels of groupers fed the diet containing *B. subtilis* 7k at 10^8 and10^{10} cfu g^{-1} for 4 weeks were significantly higher than those fed the 10^6 cfu g^{-1} *B. subtilis* 7k diet and control diet, and had respectively increased by 1.2- and 1.3-fold compared to the control group. After 8 weeks of feeding, the ACH50 levels of fish fed the 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k supplemented diets were significantly higher than those fed the control diet, and had respectively increased by 1.3-, and 1.5-fold compared to the control group (Fig. 6c).

SOD activity of groupers fed a diet containing *B. subtilis* 7k at 10^8 and 10^{10} cfu g^{-1} was significantly higher than those fed the 10^6 cfu g^{-1} *B. subtilis* 7k-supplemented diet and the control diet after 4 weeks of feeding. Eight weeks later, SOD activity of groupers fed the 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k diets was significantly higher than the control group, increasing by 1.5-, and 1.9-fold, respectively (Fig. 6d). No significant difference in SOD activity was observed between groupers fed the 10^6 cfu g^{-1} *B. subtilis* 7k diets and the control diet after 8 weeks of feeding.

In the bactericidal assay, grouper serum' ability to kill the pathogenic *V. alginolyticus* showed no difference between the *B. subtilis* 7k-supplemented diet groups and the control at the 4th week. However, after 8 weeks, the bactericidal activity of grouper fed the 10^6, 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k diets was significantly higher than the control group, increasing by 1.4-, 2.1-, and 2.1-fold (Fig. 6e). No significant difference in bactericidal activity was observed between groupers fed the 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k diets after 8 weeks of feeding.

Phagocytic activities (PA) of head kidney leucocytes were slightly increased ($p > 0.05$) in *B. subtilis* 7k-supplemented diet groups

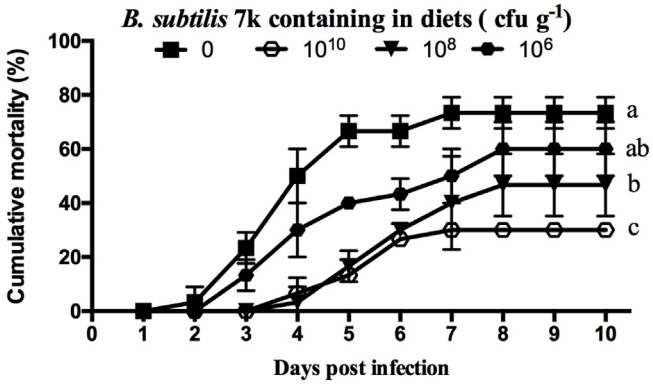


Fig. 4. The cumulative mortality of Hulong grouper fed on *B. subtilis*7k-supplemented diet after challenged with SGIV for 10 days. Results are expressed as mean value ± SDs (n = 3). Curves with different letters denote statistical significance ($p < 0.05$) in cumulative mortality among groups at the 10th day.

comparing with the control after 4 weeks of assay. However, after 8 weeks, the groupers fed 10^6, 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k-supplemented diets showed significantly higher PAs than the control groupers. The PAs of groupers fed the 10^6, 10^8 and 10^{10} cfu g^{-1} *B. subtilis* 7k-supplemented diets for 8 weeks increased by 1.4-, 1.5-, and 1.6-fold (Fig. 6f).

4. Discussions

Since Kolloath, in 1953, firstly suggested the term “probiotics” as an essential role in the development of animal health, probiotics have been used in aquaculture practices for such a long time [25]. Apart from probiotics' growth effect on host and the benefit for water quality, it is also regarded as an alternative to antibiotics due to the competitive exclusion of potential pathogen germs [5–7]. Besides, several probiotics

Table 2
The effect of *B.subtilis*7k-supplemented diet on the growth of grouper.

	control	10^6 cfu g^{-1}	10^8 cfu g^{-1}	10^{10} cfu g^{-1}
Initial weight (g)	7.66 ± 0.45[a]	7.83 ± 0.33[a]	7.47 ± 0.45[a]	7.78 ± 0.34[a]
Final weight(g)	14.84 ± 0.27[a]	17.08 ± 1.15[b]	16.93 ± 0.64[b]	16.88 ± 0.32[b]
Weight gain (g)	7.19 ± 0.33[a]	9.25 ± 1.08[b]	9.46 ± 0.34[b]	9.10 ± 0.30[b]
ADG (g)	0.26 ± 0.01[a]	0.33 ± 0.04[b]	0.34 ± 0.01[b]	0.33 ± 0.01[b]
PWG (%)	93.92 ± 3.24[a]	118.31 ± 14.13[b]	126.90 ± 7.705[b]	117.15 ± 8.21[b]
FE	0.88 ± 0.04[a]	1.14 ± 0.13[b]	1.16 ± 0.04[b]	1.12 ± 0.04[b]
Survival rate (%)	100[a]	100[a]	100[a]	100[a]

Data (mean ± SD) in the same row with different letters show significant differences ($p < 0.05$).

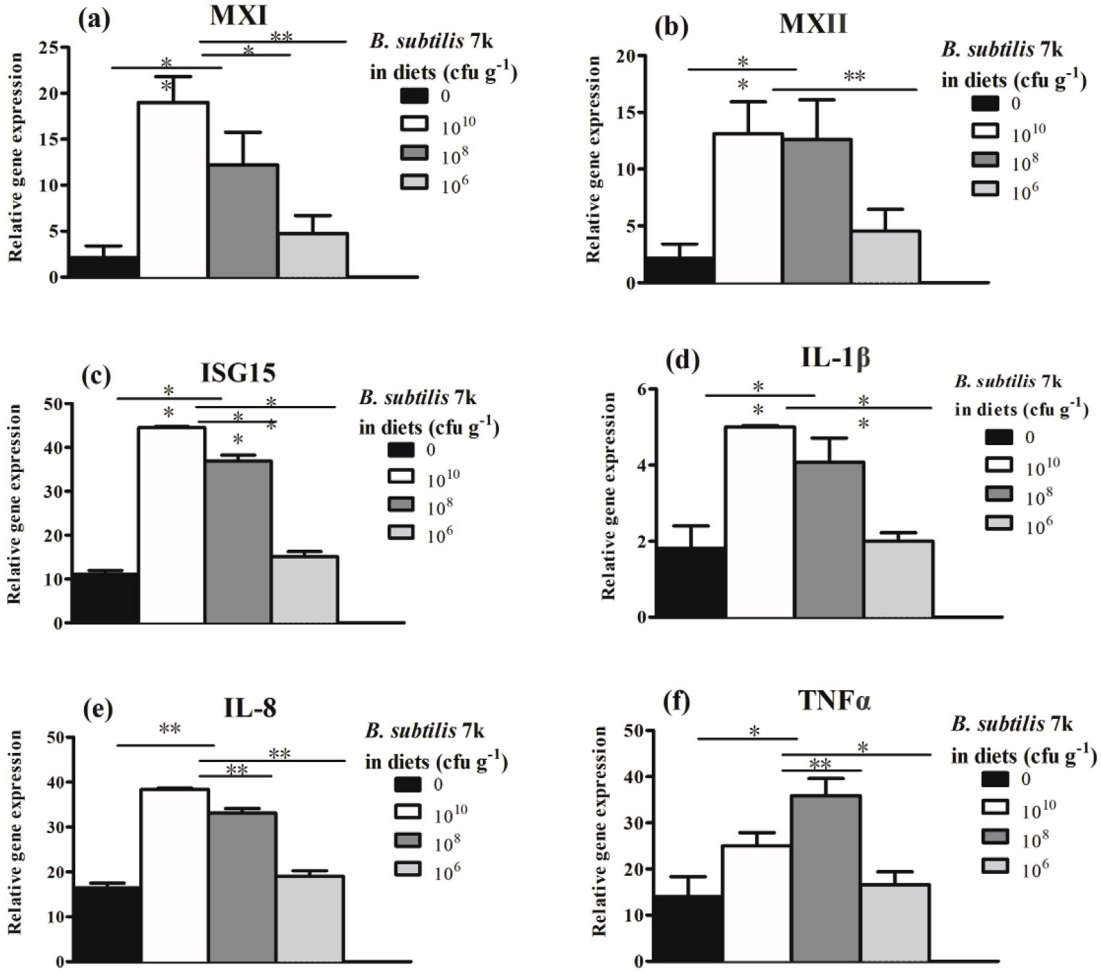


Fig. 5. The immune-related gene expression of grouper fed on *B. subtilis*7k-supplemented diet after challenged with SGIV for 48h. Only show the results of comparisons between fish fed 10^{10} cfu g^{-1} of *B. subtilis* 7k diet and other groups. $*p < 0.05$; $**p < 0.01$.

were demonstrated that they could improve hosts' resistance to certain diseases caused by viruses [26–28].

In this study, the bacteria isolated from groupers intestine was tested to be used as potential aquatic probiotics for Hulong groupers. The vegetative cells and spores of *B. subtilis* 7k were tested in the heat resistance and simulated GIT tolerance experiment. In this study, the *B. subtilis* 7k spores have shown high resistance to high temperature. There was little reduction in the number of the spores of *B. subtilis* 7k under the simulated GIT condition, which was coordinated with the recent reports [17–19].

Several studies have demonstrated the growth-promotion effect on the use of probiotics as dietary supplementation for grouper [26–29]. After feeding for 4 weeks, Hulong grouper fed diet containing *B. subtilis* 7k at 10^6-10^{10} showed significantly better performance in the final weight, weight gain, ADG, PWG and FE than the control group. The improved growth rate could benefit from some secretion of digestive enzymes, such as amylase, lipases and proteases, which could help the host break down the large units in the digestion process. Besides, probiotic itself can secrete some important nutrients, such as vitamins K, B_{12} and amino acids, when colonized in the GIT [30,31].

In the extracellular antimicrobial assay, clear inhibitory zones appeared in kinds of pathogens culture broth, which meant the secretion from *B. subtilis* 7k could inhibit different potential pathogens. Similar results, for example, were also observed in other strains of *Bacillus* spp., in which the supernatant of culture broth showed strong inhibitory effect on *S. typhimurium*, *L. monocytogenes*, *S. Aureus*, *V. vulnificus*, *V.harveyi*, *E. coli*, and *S. aureus* [32]. Bacillus can produce various antimicrobial metabolites, such as bacteriocin, surfactins, iturin, fengycin, bacilysin, subtilin, and/or sublancin, and some had been widely used in the food industry [8,33]. According to HPLC-HRMS results, 7 lipopeptides substances, iturins, secreted by *B. subtilis* 7k may contribute to its inhibitory effect. Further work on purification and identification of the metabolite of *B. subtilis* 7k will help to understand its antimicrobial mechanism.

The ubiquitous outbreak of viral diseases seriously affects the aquatic culture. In this study, the potential antiviral effect of *B. subtilis* 7k was tested. In the challenge test, significant differences in mortality between Hulong groupers fed with different doses of *B. subtilis* 7k-supplemented diet were observed. Previous studies have demonstrated that Germ-free mice tend to be more susceptible to certain virus, such as influenza virus, Coxsackie virus and Friend leukemia virus, which might indicate microbiota's essential role in virus infection [34]. Turner R B et al. showed that probiotics could reduce nasal lavage virus titer and the proportion of subject shedding viral in nasal secretion among probiotic treated volunteers [35]. In teleost, several studies also showed that the addition of *Lactobacillus plantarum, Saccharomyces cerevisiae* or *B. subtilis* in diet contributed to the antiviral activity in *E. coioides* [26–28]. However, the antiviral mechanisms modulated by probiotics are still not totally understood. In order to elucidate the effect of *B. subtilis* 7k on the virus infection, the expression of three *IFN* related genes and three pro-inflammatory cytokines were investigated. In response to the virus infection, *ISG15*-liked gene could conjugate to target

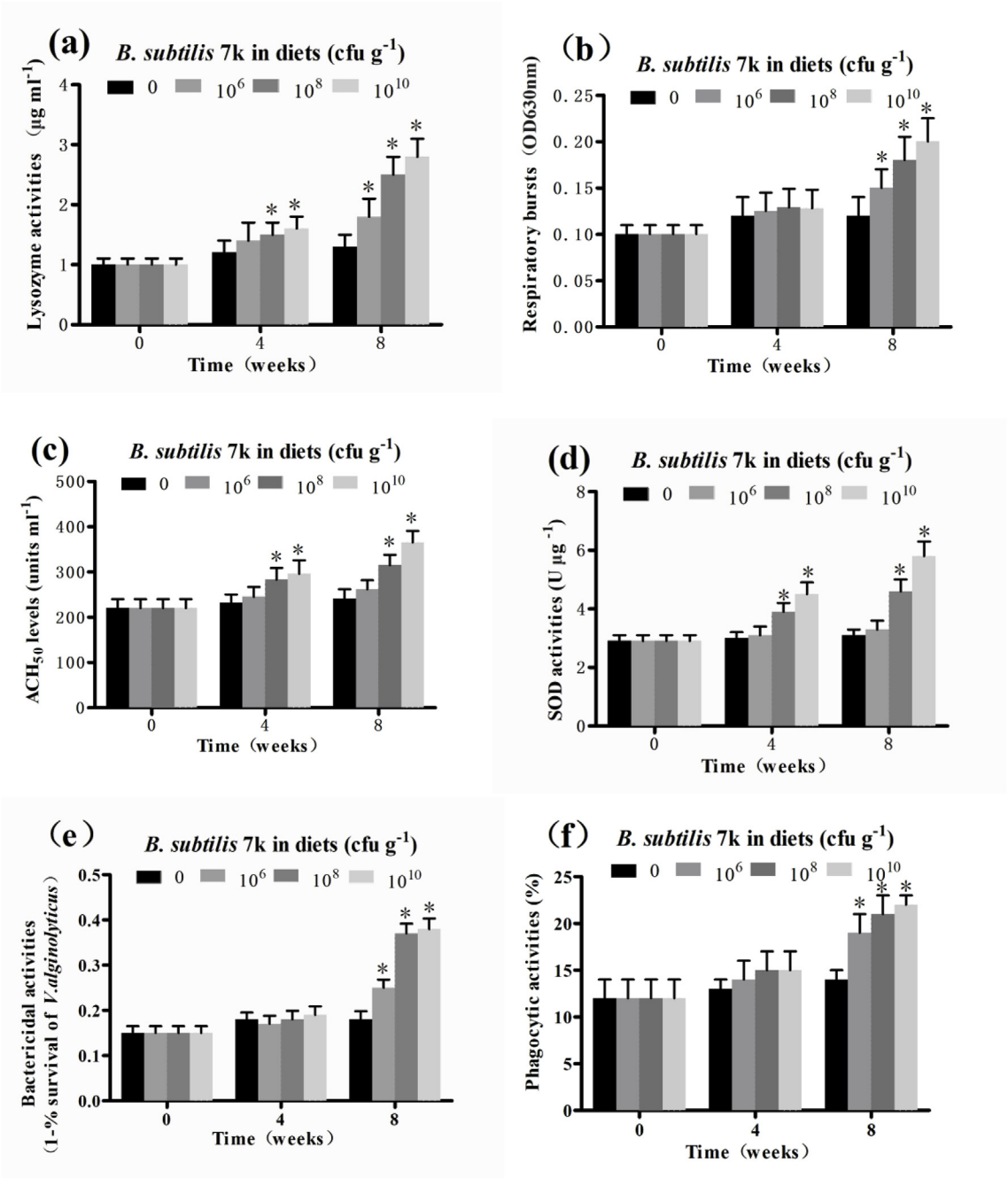


Fig. 6. Non-specific immune parameters of Hulong grouper after administration of *B. subtilis*7k-supplemented diet. Lysozyme activity (a), respiratory burst (b), alternative complement activity (ACH50) (c), superoxide dismutase activity (d), bactericidal activity (e) and phagocytic activity (f) of Hulong grouper (*E. fuscoguttatus* (♀) × *E. lanceolatus* (♂)) fed *B. subtilis*7k-supplemented diets at 0, 10^6, 10^8 and 10^{10} (cfu) (g diet)$^{-1}$. Each bar represents the mean value from three determinations with the standard error. *$p < 0.05$.

proteins via a pathway named "Isgylation", which could inhibit the replication of virus [36]. The effect of fish *MX*-gene, which has already been proved to play a fundamental role in mammal antiviral activity, is still scarcely understood and varies in different species [37–39]. In this study, *ISG15* was up-regulated in the *B. subtilis* 7k addition groups and showed a correlation with doses. The expression of *MX* genes was significant higher in the fish fed 10^8 and 10^{10} cfu g^{-1} diet. Since high *MX* genes expression is often related to good antiviral performance in grouper and correlated with other antiviral genes, such as *ISG15*, Viperin and *IRF3/7*, these findings indicate *MX* gene's antiviral effect may be induced by *B. subtilis* 7k addition in the diet of groupers [40–42]. Besides, the probiotics up-regulate the three pro-inflammatory cytokines, which may benefit inflammatory migration and lead to adaptive immunity. In present study, fish fed 10^8 and 10^{10} cfu g^{-1} diet did have higher expression of pro-inflammatory cytokines than other groups, but the 10^{10} cfu g^{-1} diet fish was lower than the 10^8 cfu g^{-1} diet fish in *TNFα* expression. Similar result was also observed in inactivated SGIV vaccines in orange-spotted grouper [40].

Lysozymes are one of the defensive factors protecting against invasive microorganisms in vertebrates [43]. They lyse gram-positive bacteria, and kill gram-negative bacteria after a complement and other enzymes have disrupted the outer cell walls [44,45]. Lysozymes promote phagocytosis as opsonin, or by directly activating polymorpho nuclear leukocytes and macrophages [46,47]. An alternative pathway

of complement activity is a nonspecific defense mechanism for protecting host against a wide range of potentially invasive organisms, such as bacteria, fungi, viruses, and parasites [48]. The research results showed that Hulong groupers fed *B. subtilis* 7k supplemented diets at 10^6 to 10^{10} cfu g^{-1} after 4 weeks showed significant dose-dependent increase in both serum lysozyme and alternative complement pathway activities (ACH_{50}). The similar result was observed in *E. coioides* fed 10^6 to10^{10} cfu kg^{-1} *L. plantarum* supplemented diets for 4 weeks [49], and fed 10^5 to 10^7 cfu kg^{-1} *S. cerevisiae* supplement diets for 4 weeks [28]. It is suggested that resistance to bacterial and viral pathogens is correlated with increases in lysozyme activities and ACH_{50} of grouper fed diets containing probiotic.

The present study indicated that dietary containing *B. subtilis* 7k administration at 10^6, 10^8 and 10^{10} cfu g^{-1} for 8 weeks significantly increased the spiratory burst activity in Hulong grouper. This was similar to studies on *E. coioides* fed *L. plantarum*- [49] and *S. cerevisiae*-supplemented diets [28], and also agreed with studies on respiratory burst activity in *Oreochromis niloticus* fed diets containing *B. subtilis* and *Lactobacillus acidophilus* for 1–2 months [50]. The results suggested that head kidneys SOD activities of Hulong grouper fed *B. subtilis* 7k-supplemented diets at 10^8 and 10^{10} cfu g^{-1} significantly increased after 1–2 months of administration, which agreed with studies on *E. coioides* fed *S. cerevisiae*-supplemented diets [28]. Former studies also demonstrated that the SOD activities of *O. niloticus* significantly increased after administration with *B. subtilis* and *Bacillus coagulans* as water additives for 40 days [51]. However, serum SOD concentrations showed no significant difference between *E. coioides* fed *Bacillus pumilus*- or *Bacillus clausii*-supplemented diets and the control diet for 1 month [29].

There were many studies about probiotics regulating immunity, including the ability to increase serum bactericidal activity in aquatic animals [52,53]. In the present study, bactericidal activity of serum has been found to have increased in Hulong groupers fed *B. subtilis*7k-supplemented diets. The serum bactericidal activity against *V. alginolyticus* of probiotic-fed grouper was significantly higher than the control fish after 8 weeks of feeding administration. Similar findings indicated that the serum bactericidal activity of *Labeo rohita* after administration of *B. subtilis* (10^7 cfu g^{-1}) supplemented diets was more effective than the control group against *A. hydrophila* [53]. Macrophages play an important role in regulation of innate immune system. This study indicated that dietary containing *B. subtilis* 7k administration at 10^6, 10^8, and 10^{10} cfu g^{-1} for 8 weeks significantly increased the phagocytic activities in Hulong grouper. It agrees with the studies in *Sparus aurata*, fed diets containing *B. subtilis* on the phagocytic activity [54]. Similar, the phagocytic activity were significantly improved in *E. coioides* fed *B. pumilus*- or *B. clausii* supplemented diets at 10^8 cfu g^{-1} for 2 months [29], and *Catla catla* fed *B. circulans*-supplemented diets at 10^6 cfu kg^{-1} for 2 months [55].

In summary, our study showed that the *B. subtilis* 7k isolated from grouper GIT has desirable resistance and sporulation as potential probiotics. Several antimicrobial substances were found in the secretion of the *B. subtilis* 7k. The prominent growth promotion effect as well as its effect on inhibition of the infection by pathogenic microorganism make *B. subtilis* 7k to be a promising probiotics. The results also revealed that *B. subtilis* 7k may contribute to better performance in non-specific immune response of grouper and increase the immune-related genes expression under virus infection. In all, *B. subtilis* 7k was well-founded to be an effective probiotic using in aquatic culture.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.058.

The accurate molecular weights of 7 main substances (S1-S7) were provided by HRMS. The molecular formulas of 7 Iturins were deduced as follows: $C_{47}H_{72}N_{12}O_{14}$ (S1), $C_{48}H_{74}N_{12}O_{14}$ (S2), $C_{48}H_{74}N_{12}O_{14}$ (S3), $C_{49}H_{76}N_{12}O_{14}$ (S4), $C_{49}H_{76}N_{12}O_{14}$ (S5), $C_{49}H_{76}N_{12}O_{14}$ (S6), and $C_{50}H_{78}N_{12}O_{14}$ (S7).

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