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Effects of potential probiotic *Bacillus velezensis* K2 on growth, immunity and resistance to *Vibrio harveyi* infection of hybrid grouper (*Epinephelus lanceolatus*♂ × *E. fuscoguttatus*♀)

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ABSTRACT

Nowadays, there is no suitable treatment for vibriosis in groupers. So an eco-efficient and environmentally friendly treatment is necessary for the grouper industry. Probiotic-feeding has been a promising strategy to control the bacterial pathogens in aquaculture. A new *Bacillus velezensis* strain named K2 was isolated from the intestinal tract of healthy grouper, and exhibited wide antimicrobial spectrum of against fish pathogens, including *Vibrio harveyi*, *Vibrio alginolyticus*, *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas caviae*, *Enterococcus casseliflavus* and *Lactococcus garvieae*. Moreover, results of the safety of *B. velezensis* K2 showed that intraperitoneal injection of K2 in healthy grouper did not cause any pathological abnormality or death, indicating this bacteria could be considered as a candidate probiotic in aquaculture. Groupers were fed with the diets containing 1×10^7 cfu/g of *B. velezensis* K2 for 4 weeks. Various immune parameters were examined at 1, 2, 3, and 4 weeks of post-feeding. Results showed that diets supplemented with K2 significantly increased serum acid phosphatase (ACP) activity ($P < 0.05$). Results of the mRNA expression of immune-related genes in the head kidney of hybrid grouper showed that the expression of lysozyme gene was significantly upregulated after 1 and 2 weeks of feeding ($P < 0.05$). A significant up-regulation of the expression of piscidin, IgM and MyD88 were detected at day 21, whereas the TLR3 and TLR5 showed lower expression compared to the controls during 21 days, and a significant decrease of TLR3 gene was found at day 28 ($P < 0.05$). After challenge with *V. harveyi*, the survival rate of fish administrated with the strain K2 for 28 days was signifiacantly higher than the controls without this strain ($P < 0.05$). These results collectively suggest that *B. velezensis* K2 is a potential probiotic species to improve health status and disease resistance and can be developed as a probiotic agent in grouper industry.

1. Introduction

The outbreaks of bacterial diseases lead to significant economic losses in aquaculture, and have become a major threat to fish farming [1]. In the past few decades, chemical drugs such as antibiotics and disinfectants were used to control fish bacterial diseases. However, application of these chemicals has caused a series of serious threats to human health, for example, the development of resistant bacteria, and residual antibiotics ingested by humans through the food chain [2–5]. Of late, probiotic feeding is considered to be a promising strategy for controlling fish diseases, because it may promote host health mainly through enzymatic contribution to digestion, inhibition of pathogenic microorganisms, growth promoting factors, and host immune enhancement [6]. Some studies showed that probiotic bacteria isolated from fish intestine have the ability to inhibit the growth of pathogens [7,8]. Moreover, the isolate has the property of surviving and colonizing in the gastrointestinal tract of fish. Therefore, gastrointestinal tract of fish is a good source of potential probiotic for controlling infectious diseases in fish [9]. Ramesh et al. reported that two novel probiotic strains *Bacillus licheniformis* KADR5 and *Bacillus pumilus* KADR6 isolated from intestinal tract of *Labeo rohita* effectively inhibited the growth of pathogens such as *A. hydrophila* [9]. Peng et al. found that probiotic *Bacillus* PC465 isolated from the gut of *Fenneropenaeus chinensis* improved the resistance of *Litopenaeus vannamei* against white

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spot syndrome virus [10].

Hybrid grouper (*Epinephelus lanceolatus*♂ × *E. fuscoguttatus*♀) as a novel species of grouper was first produced by University Malaysia Sabah in 2007 [11]. The female parent of the hybrid grouper, *E. fuscoguttaus*, commonly known as brown marbled grouper or tiger grouper, is a slow-growing but long-lived species with high disease resistance [12]. The male parent of this hybrid grouper, *E. lanceolatus* or giant grouper with a common name of queensland grouper, is popular breeding species for its rapid growth, reaching up to 3 kg in the first year [12]. Hybrid grouper inherits good traits from parents, and has rapid growth and high disease resistance [12], which has turn into a new driving force for the development of the grouper industry. Nevertheless, because of intensive aquaculture and polluted aquatic environment, the groupers farming also suffered increasing difficulties, especially with a whole host of contagious diseases including different kinds of viral, bacterial, and parasitic pathogens [13]. The grouper larvae are highly susceptible to bacterial infection, which often results in economic losses [14]. Species within the *Vibrio* genus, including *V. alginolyticus*, *V. angullarum*, *V. parahaemolyticus* and *V. harveyi* [15], are the most common marine fish pathogens that cause bacterial diseases in groupers, such as orange-spotted grouper (*E. coioides*) [16] and giant grouper (*E. lanceolatus*) [14]. After infection with *Vibrio*, fish may have skin coloration loss, external hemorrhage, a depressed abdomen, hemorrhagic liver and lethality [17]. To date, there is no suitable treatment for vibriosis in groupers. Therefore, an eco-efficient and environmentally friendly treatment is necessary for the grouper industry.

In this study, a potential probiotic strain K2 was isolated from the intestinal tract of healthy grouper, and identified as *B. velezensis* by *gyrB* gene sequence comparison and phylogenetic analysis. The in vitro antimicrobial assay showed that this strain K2 has significant antibacterial activity against several fish pathogenic bacteria. In addition, this work was also conducted in vivo to evaluate the potential of *B. velezensis* K2 as dietary probiotics on growth, immunity and disease resistance against *V. harveyi* in hybrid grouper.

2. Materials and methods

2.1. Bacterial isolation and identification

The bacteria strain K2 used in this study was isolated from the mid gut of healthy grouper. The tissue was collected aseptically and rinsed with sterile saline (0.85%), and then homogenized in 1 mL of sterile saline (0.85%). The suspension was diluted 100 folds in sterile saline (0.85%). Afterwards, 0.1 mL of the suspension was aseptically plated onto Luria-Bertani (LB) agar plates and incubated at 37 °C for 24 h. The distinctively single colonies were randomly selected and streaked on LB agar plates. The sample process was repeated in order to get pure cultures. The purified strains were stored in 50% (w/v) glycerol at −80 °C for further study. The identificantion of this bacterial strain was based on cluster analysis on the sequence of *gyrB*. The primer for this gene was listed in Table S1, and the method of PCR amplification and sequence analysis was performed according to Yi et al. [18].

2.2. Antimicrobial assay

Eight fish pathogenic bacteria (*Vibrio harveyi, Vibrio alginolyticus, Aeromonas hydrophila, Aeromonas veronii, Aeromonas caviae, Streptococcus agalactiae, Enterococcus casseliflavus and Lactococcus garvieae*), as well as the gut bacteria strain were grown distinctively in LB liquid medium at 37 °C, 160 rpm for 24 h. Afterwards, 0.1 mL of pathogen-diluted culture (1×10^7 cells/mL, counted with hemocytometer) was plated on LB agar plates. A sterile filter paper tray (6 mm diameter) containing 5 μL of the putative probiotic bacterial culture was placed in the center of the plate and incubated at 37 °C for 24 h to detect anti-pathogenic activity by transparent zone around the inoculated paper.

2.3. Safety evaluation

2.3.1. In vitro hemolysis test

The hemolysis test uses goat blood agar plates (Qingdao Haibo Biotechnology Co., Ltd.) to detect the production of hemolysin to rule out the potential pathogenicity. The culture solution containing 5 μL of activated strain K2 (1×10^7 cells/mL) was inoculated on a blood agar plate and observed after incubation at 37 °C for 24 h. Determine whether hemolysis is based on the following types of hemolysis: the production of α-hemolysin destroys the decomposed red blood cells and forms a green circle around the colonies. The production of β-hemolysin causes a transparent circle around the colonies. No transparent circles without hemolysin.

2.3.2. Grouper pathogenicity assay

The grouper was divided into seven groups (N = 10), and each group was separately intraperitoneally injected with 0.1 mL of strain K2 suspension at a concentration of 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} cells/mL and sterile saline (0.85%) to observe the survival rate within 7 days.

2.4. Fish and experimental set-up

Groupers (~5 g) were obtained from a grouper farm in Guangzhou Maoyu Biotechnology Co., Ltd (Guangdong, China). The healthy state of fish is confirmed by normal physiology and behavior (movement, appetite) and without disease symptoms or injuries (fins, gills, eyes, skin). The basal diet was fed for 2 weeks before the experiment started.

The selected groupers were randomly divided into 2 groups of 150 fish of similar size and each group contains three replicates (50 fish per tank). The control group (basic diet) and the experimental group (diet supplemented with 1×10^7 cfu/g of K2 strain) were divided into three tanks. The amount of feed is 1% of the grouper weight twice a day (9:00 and 17:00). During the 28 days, the temperature was controlled at the optimum temperature for growth, pH 7.8, dissolved oxygen 5.4 mg/L, ammonia nitrogen 0.130 mg/L, nitrite 0.014 ± 0.005 mg/L. Replace one-third of the water in the water tank every afternoon. The water should be aerated one day in advance and the water temperature should be adjusted to the suitable temperature.

2.5. Sampling and assessment of growth performance

To macroscopically reflect the effect of K2 (1×10^7 cfu/g) on the growth of groupers, ten fish were randomly selected from each replicate per 7 days to measure the body length, weights and carcass weight.

2.6. Sample collection

Ten fish from each replicate were randomly collected per seven day. Approximately 0.5 mL of blood sample was collected from the caudal vein of each fish with 1 mL heparinized syringe and placed in plastic Eppendorf tube. The tubes were kept at 4 °C overnight and centrifuged at 3500 × *g* for 10 min at 4 °C to obtain serum and store at −80 °C. In addition, head kidney tissue was collected and immersed in 0.2 mL Trizol reagent (TaKaRa, Japan) for subsequent RNA isolation to analyze expression of immune-related genes.

2.7. Non-specific immunological measurement

Serum samples were collected per 7 days for determination of the Acid phosphatase (ACP) activity, Alkaline phosphatase (AKP) activity and Complement component-3 (C3) activity. The measurement was performed with commercial kits from Nanjing Jiancheng Institute (Nanjing, China) according to the manufacturer's instructions.

Table 1
Primer sequences for qRT-PCR.

Genes	Primers (5′–3′)	Species
β-actin	F: TGTGTCATCTTCTCCCTGT R: GAGAGGTATCCTGACTCTGAAGTA	*Epinephelus bruneus*
Piscidin	F: ATGAGGTGCATCATCCTCTTTC R: TCAGGCAAAAGCTTTCTCTCGTTC	*Epinephelus coioides*
Lysozyme	F: GACATTCAGTCCAGCCTAGAGTCC R: CCCGACTCCCACTGGCTC	*Epinephelus coioides*
IL-8	F: CATGATGGAAGCCATGAG R: GTCAGCGTGTCAGGAAT	*Oplegnathus fasciatus*
CC chemokine 1	F: ATCTCAGTCTCAGGTTTCATTG R: TCTCCATCATCACATCCACT	*Epinephelus coioides*
MyD 88	F: AGCTGGAGCAGACGGAGTG R: GAGGCTGAGAGCAAACTTGGTC	*Epinephelus coioides*
Ig M	F: ACCGTGACCCTGACTTGCTATG R: CCCGATGGACCTGACAATAGC	*Epinephelus coioides*
TLR 3	F: TCTCCATTCCGTCACCTTCC R: TCATCCAGCCCGTTACTATCC	*Epinephelus lanceolatus*
TLR 5	F: CTGACCCTGATGCTTTTCG R: GCTACTTTACTGCTGTGTG	*Epinephelus coioides*

2.8. RNA isolation and real-time quantitative PCR

Total RNA was isolated from the head kidney with TRIzol reagent (TaKaRa, Japan). The tissue was homogenized in 1 mL TRIzol reagent. After centrifugation at 12 × *g* for 5 min at 4 °C, the supernatant was transferred to a new 1.5 mL tube and mixed with 0.5 mL of chloroform. The mixture was centrifuged and the aqueous phase was transferred to a new tube. RNA was precipitated by adding 0.5 mL isopropanol. Following centrifugation, the RNA pellet was washed twice with 75% ethanol and dissolved in RNase free water. One microgram of total RNA was treated with DNase-I (TaKaRa, Japan) to remove genomic DNA, and cDNA was synthesized using a PrimeScript™ RT reagent Kit (TaKaRa, Japan). Real-time quantitative PCR (RT-qPCR) was performed on a CFX96™ Real-time PCR system (Bio-rad, USA). Primer pairs are listed in Table 1 and β-actin gene was included as a reference gene. For each reaction, the total 20 μL volume contains 10 μL of AceQ® qPCR master mix (Vazyme, China), 0.8 μL of each primer (10 μM), 1.4 μL of cDNA, and 7 μL of nuclease-free water. The PCR cycling conditions were as follows: one cycle at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. After the PCR reaction, melting curve analysis was performed to exclude the formation of nonspecific products. The Ct value of each gene was recorded and used to quantify gene expression. At least three biological replicates were performed for each sample, and the fold-changes in the relative quantities of the amplified targets were calculated by the 2-△△Ct method.

2.9. Challenge test

The preliminary test was conducted before the challenge test. *V. harveyi* was grown in LB broth and incubated at 37 °C for 24 h. The *V. harveyi* culture was diluted to obtain a concentration of 1×10^5, 1×10^6, 1×10^7, 1×10^8 and 1×10^9 cells/mL bacterial suspension (counted with a hemocytometer). Hybrid groupers were intraperitoneally injected with 0.1 mL V. *harveyi* suspension with different concentrations mentioned above, and the results showed that the LD_{50} of *V. harveyi* for 7 days was 10^7 cells/mL. Therefore, each fish in the control group and the experimental group (N = 20) was intraperitoneally injected with 0.1 mL of *V. harveyi* bacterial suspension at a concentration of 1×10^7 cells/mL after 28 days of feeding. The protective effect of strain K2 against *V. harveyi* was evaluated by survival rate (number of survivors in the immunized group/total number of experimental fish) × 100. Observe pathological changes daily and regularly remove dead fish from the tanks.

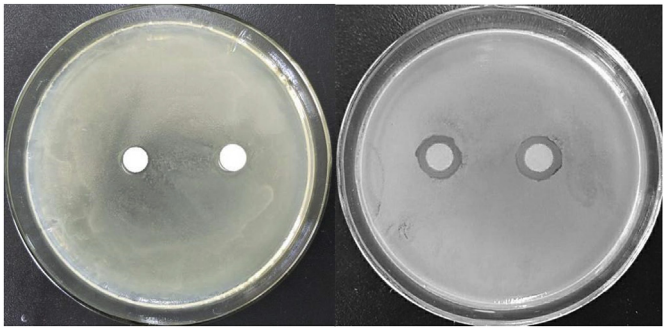

Fig. 1. Antibacterial activity of *B. veleszensis* K2. Sterile saline (0.85%)(A) and *B. veleszensis* K2 (B) were performed on LB agar plate. *Vibrio harveyi* as a pathogen indicator.

2.10. Statistical analysis

All data were statistically analyzed using IBM SPSS Statistics 24.0 software and presented as the mean ± SE of mean (SEM). Comparisons were made using Independent-Samples T test or Mann-Whitney *U* test for normally distributed data and for non-normally distributed data, respectively. $P < 0.05$ and $P < 0.01$ were considered statistically significant and extremely significant, respectively.

3. Results

3.1. Bacterial isolation, characterization and identification

A total of 107 strains of bacteria were isolated and purified from the intestines of healthy grouper ($N = 10$) for next in vitro antagonistic test. Seven strains showed antagonistic effect against *V. harveyi* (Table S2), and one of these strains, designed as K2, exhibited the strongest antibacterial activity (Fig. 1). This bacterial strain formed white and wet colonies of 3–5 mm in diameter on LB medium and possessed typical characteristics of *Bacillus* species which was rod-shaped, Gram-positive, motile and capable of producing endospores and forming biofilms. The partial 16S rRNA sequence analysis of this strain showed 99% sequence homology with several *Bacillus* species including *B. velezensis* A2 (MG727659.1), *B. subtilis* S64 (FJ763648.1) and *B. amyloliquefaciens* B10 (KY685067.1). The identity of this strain was further determined by the sequence of the *gyrB* (DNA gyrase B) gene which showed 100% similarity to *B. velezensis* GQJK49 (CP021495.1). The gyrB sequence of K2 was submitted to GenBank and the accession number was assigned as MN365038. Furthermore, ML (maximum likelihood) phylogenetic tree reconstructed based on *gyrB* gene sequences showed that strain K2 firmly clustered with *B. velezensis* (Fig. 2).

3.2. Antimicrobial activity

The agar diffusion test was used to determine the antimicrobial effect of *B. velezensis* K2. As shown in Table 2, strain K2 has excellent antimicrobial activity against Gram-negative fish pathogens, including *V. harveyi*, *V. alginolyticus*, *A. hydrophila*, *A. veronii* and *A. caviae*. Antagonistic activity against Gram-positive two fish pathogens (*E. casseliflavus* and *L. garvieae*) was also observed. But *B. velezensis* K2 has no antagonistic activity against the pathogen *S. agalactiae*.

3.3. Safety evaluation

B. velezensis K2 has no hemolytic activity after incubation on the goat blood agar plates at 37 °C for 24 h, while *A. hydrophila,* as a negative control, had a distinct hemolytic ring (Fig. 3). In addition, all fish intraperitoneally injected with 0.1 mL of *B. velezensis* K2 (10^5, 10^6, 10^7,

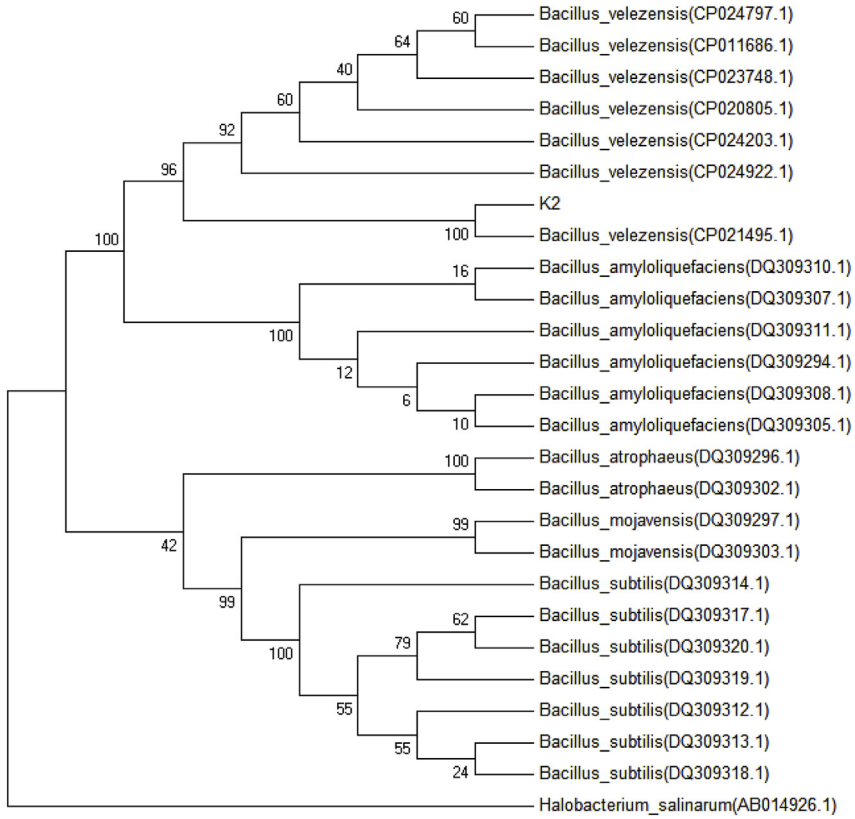

Fig. 2. Phylogenetic tree based on *gyrB* gene sequences of *B. veleszensis* K2. The *gyrB* gene sequences were aligned using ClustalW tool in MEGA 7.0.26, and phylogenetic analysis was performed using the Maximum likelihood method.

Table 2
The inhibition zone size of Strain K2 for common aquatic pathogens.

Indicator bacteria	Bacteriostatic circle(mm)
Vibrio harveyi	12.46
Vibrio alginolyticus	9.14
Aeromonas hydrophila	9.14
Aeromonas veronii	14.20
Aeromonas caviae	12.04
Enterococcus casseliflavus	12.84
Lactococcus garvieae	13.50
Streptococcus agalactiae	No bacteriostatic circle

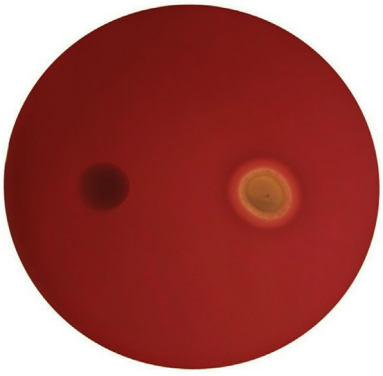

Fig. 3. Hemolysis test results of *B. veleszensis* K2 (left) and positive control *A. hydrophila* strain (right) were performed on goat blood plate.

10^8, 10^9 and 10^{10} cells/mL) didn't show mortality or any pathological symptoms for 7 days (data not shown).

3.4. Growth performance

The effects of dietary administration of *B. velezensis* K2 on growth performance of groupers were displayed in Fig. 4. There was no significant difference in body length, body weight and carcass weight between the experimental and the control groups during 28 days of feeding.

3.5. Acid phosphatase (ACP), alkaline phosphatase (AKP) and component-3 (C3) activities of serum

Fish administrated with the strain K2 showed significantly higher ACP activity than the controls after first and second weeks of feeding ($P < 0.05$ and 0.01, respectively, Fig. 5A). However, no significant differences in AKP (Fig. 5B) and C3 (Fig. 5C) activity were observed between the treatment and control groups after 7, 14, 21 and 28 days of feeding, respectively.

3.6. Expression of immune-related genes after dietary supplementation with *B. velezensis K2*

The relative mRNA expression of immune-related genes including interleukin-8 (IL-8), Toll-like receptor 5 (TLR5), CC chemokine 1, lysozyme, piscidin, Immunoglobulin M (IgM), Myeloid differentiation primary response gene 88 (MyD88) and Toll-like receptor 3 (TLR3) in the kidney of grouper fed with the diet containg strain K2 is shown in Fig. 6.

The results showed that a significant upregulation of IL-8 was observed after the first week of feeding ($P < 0.01$, Fig. 6A), while the mRNA expression of this gene was remarkablely down-regulated at day

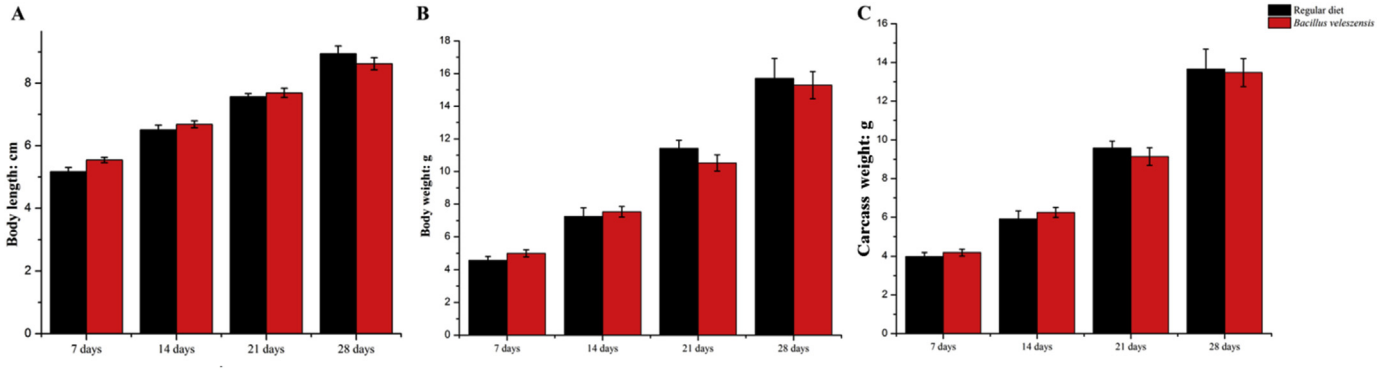

Fig. 4. Effect of dietary supplementation of *B. veleszensis* K2 on the growth of groupers. Groupers were fed daily with regular diet containing 10^7 cfu/g of *B. veleszensis* for 28 days. Body length (A), weights (B) and carcass weight (C) of fish were measured every 7 days. Data represent mean ± SEM (n = 10).

28 ($P < 0.05$, Fig. 6A). In addition, an extreme down-regulation of the expression of CC chemokine 1 gene was detected at day 14 ($P < 0.01$, Fig. 6C), but the expressions of both IL-8 and CC chemokine 1 genes were down-regulated at day 14 and 28, especially a significant difference at day 28 and 14, respectively ($P < 0.05$ and 0.01, respectively, Fig. 6A, C). However, there were no significant differences in the mRNA expression of TLR5 (Fig. 6B) between the fish administrated with *B. velezensis* K2 and controls during 28 days of feeding. In contrast, the expression of the lysozyme gene was up-regulated from first to third week of feeding, and the significant difference was observed after 1 and 2 weeks of feeding ($P < 0.05$, Fig. 6D). Similarly, the mRNA expressions of piscidin, IgM and MyD88 were also up-regulated during 3 weeks of feeding, especially a significant upregulation of MyD88 and piscidin at day 7 and 21, respectively, while at day 28, the expressions of these three genes were simultaneously down-regulated, including a significant change of the IgM and MyD88 gene ($P < 0.05$, Fig. 6E, F, G). On the contrary, the mRNA expression of TLR3 in probiotic-treated fish was down-regulated during 28 day of feeding compared to the controls, and a significant decrease was found at day 28 ($P < 0.05$, Fig. 6H).

3.7. Disease resistance against V. harveyi

Fish mortality was recorded daily for 1 week after challenge with *V. harveyi* in order to evaluate the protective effect of *B. velezensis* K2 on grouper against bacterial infection, and the survival curve was shown in Fig. 7. Grouper fed with a regular diet or a diet containing *B. velezensis* K2 showed 25% and 55% survival, respectively, and statistics analysis demonstrated strain K2 significantly increased fish survival ($P < 0.05$, Fig. 7).

4. Disucussion

Some researchers reviewed advantages of using probiotics in aquaculture [19,20]. For example, probiotics can increase the non-specific immune response of fish and then enhance the resistance against pathogens [19]. Live bacteria in probiotics, as a substitute for antibiotics and chemicals, can act as an alarm molecule to activate the immune system. Until now, a lot of the common Gram-positive probiotic organisms, such as *Bacillus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Streptococcus*, *Enterococcus*, *Carnobacterium*, and *Weissella* species, have been used in aquaculture industry [21,22]. Among probiotics, *Bacillus* spp. are increasingly and are diffusely applied in aquaculture. Compared with other non-spore-forming bacteria, *Bacillus* spp. has more obviously innate superiorities [23]. The formation of endospores allows them to live in extreme stresses, and provides biological solutions for formulation and preservation problems at industrial scale production. Most of *Bacillus* are both aerobic and facultatively anaerobic, which implies they can grow in various niches to compete against potential pathogens. A large number of studies have shown that feeding with *Bacillus* have an effect of antagonizing intestinal pathogenic bacteria and improving disease prevention ability [24]. Furthermore, *Bacillus* spp. can be introduced into the culture environment to compete with pathogenic bacteria as well as to promote the growth of the cultured organisms. In addition, *Bacillus* spp. are non-pathogenic and non-toxic microorganisms, which has no side-effects when used to aquatic organisms [25]. For example, *B. subtilis* strains significantly enhance the weight gain and digestive enzyme activity in juvenile white shrimp (*Litopenaeus vannamei*) [26], *B. amyloliquefaciens* and *B. pumilus* stimulated growth performance, innate immunity, and stress tolerance of striped catfish [27].

B. velezensis, as a surfactant producing bacterium, was firstly

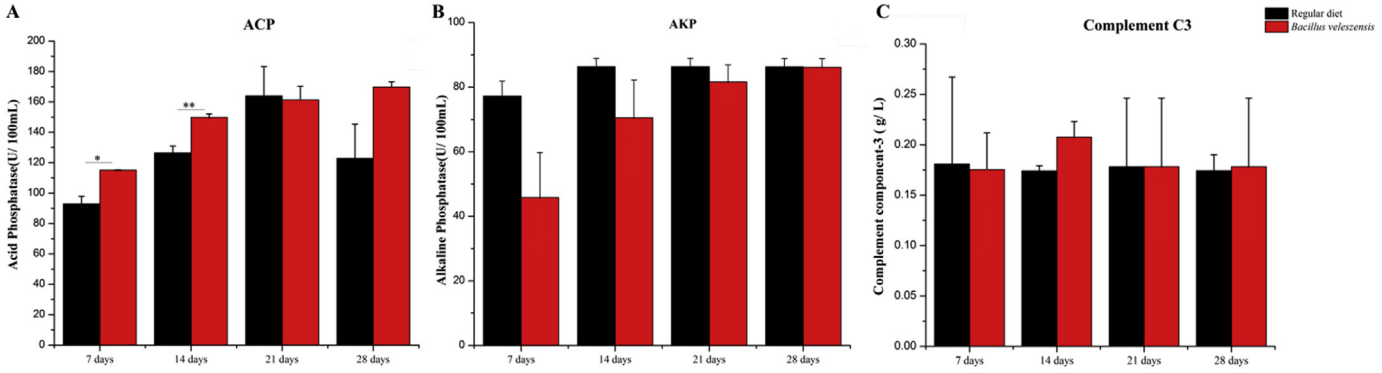

Fig. 5. Serological immune index after *B. veleszensis* K2 supplementation. Groupers were fed daily with regular diet and 10^7 cfu/g of *B. veleszensis*-containing diets for 28 days. Acid phosphatase (ACP) activity (A), Alkaline phosphatase (AKP) activity (B) and Complement component-3 activity (C) were measured. Each value represents mean ± SEM (n = 10). Asterisks indicate statistical significance compared with control ($P < 0.05$).

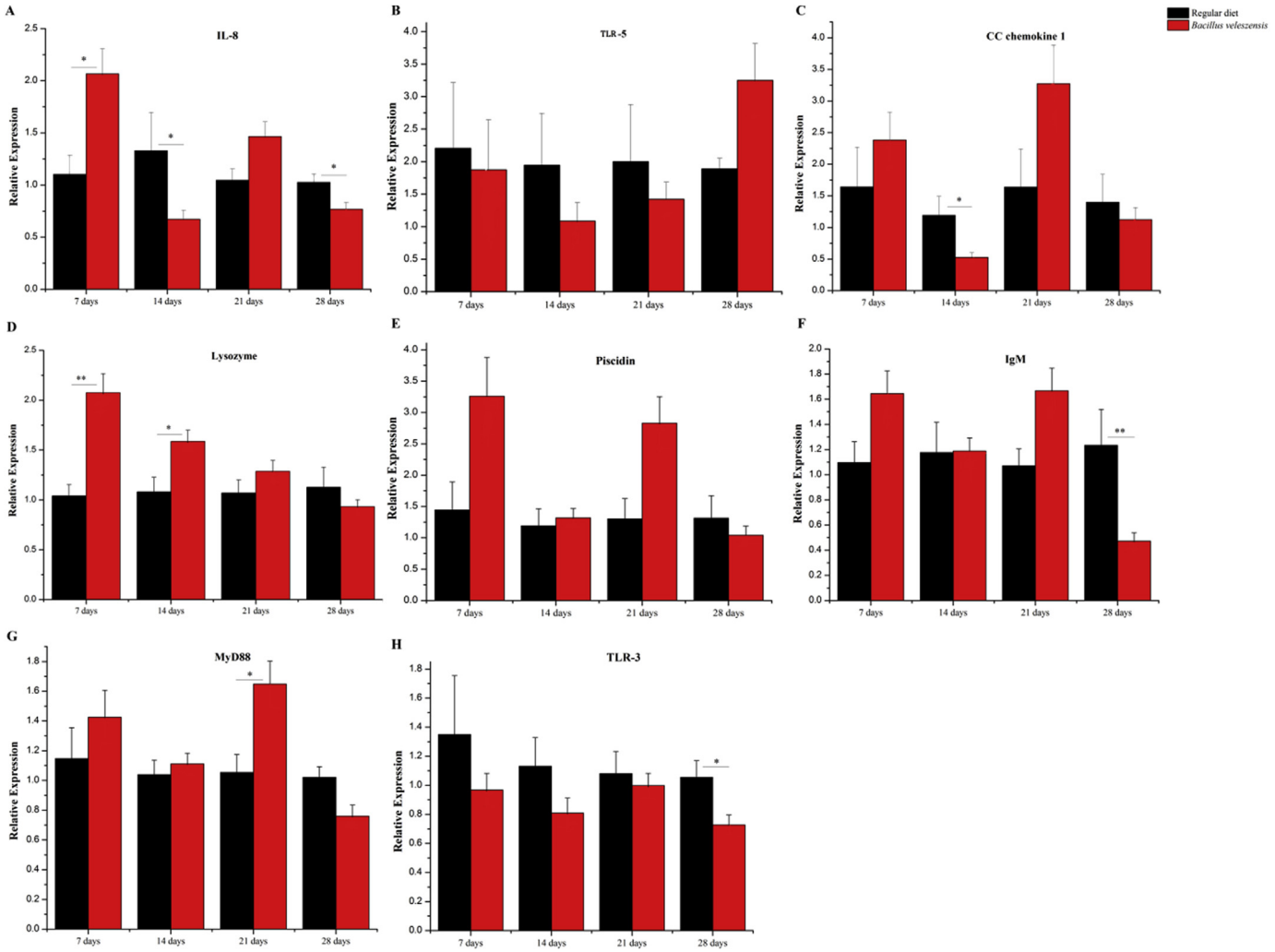

Fig. 6. Expression of immune defense-associated genes after *B. veleszensis* K2 supplementation. Fish liver RNA was isolated after feeding with different diets for the indicated number of days. Relative expression of IL-8 (A), TLR-5(B), CC chemokine 1(C), lysozyme (D), piscidin (E), IgM(F), MyD88(G) and TLR-3(H) were assessed using quantitative real-time PCR. Each value represents mean ± SEM (n = 10). Asterisks indicate statistical significance compared with control (* and ** indicated $P < 0.05$ and 0.01, respectively).

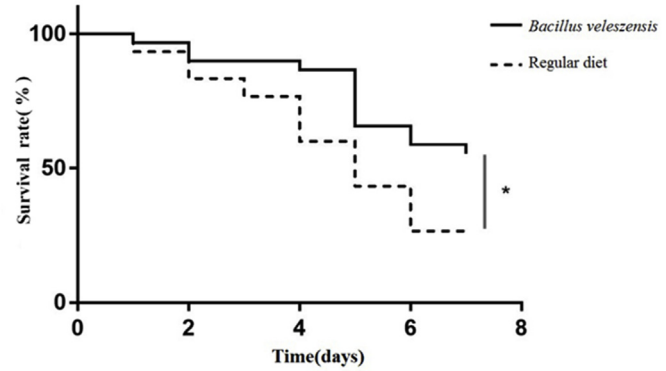

Fig. 7. Supplementation of *B. veleszensis* K2 effects on survival rate of fish upon challenge with *V. harveyi*. Grouper were fed with regular diet and *B. veleszensis*-containing diets for 28 days, followed by challenge with *V. harveyi*.. Fish survival was monitored for 8 days (n = 10). Asterisks indicate statistical significance compared with control ($P < 0.05$).

isolated from the river Velez in Malaga by Ruiz-Garcia et al. [28]. Recently, Wang et al. provided strong evidence that *B. velezensis* is a later heterotypic synonym of *B. amyloliquefaciens* [29]. A comparative analysis of the 16S ribosomal RNA sequence is the most commonly used genotypic method for bacterial identification [30]. Strains that show more than 97% sequence similarity in the 16S rRNA are generally considered to be the same species [31]. However, phylogenetic analysis of the 16S rRNA gene sequence has insufficient power to discriminate among *Bacillus* species because of the highly conserved nature of this gene [32]. In contrast, the *gyrB* gene encodes the subunit B protein of DNA gyrase, a type II DNA topoisomerase, which has a crucial role in DNA replication and is distributed universally among bacterial species [33,34]. Sequence of the *gyrB* was generally considered to be a higher discrimination ability than the 16S rRNA gene [35], and thus this phylogenetic target has been suggested as an excellent candidate molecular marker for the identification of bacterial species [36,37]. This gene was first applied to the identification of *Pseudomonas* relatives by Yamamoto et al., in 1995 [38]. In this study, phylogenetic tree based on gryB gene sequence showed that strain K2 firmly allocated within *B. velezensis* clade (Fig. 2).

In previous studies, selection criteria for probiotic bacteria should evaluate the capability of a bacterial strain to restrict or control the growth of pathogenic bacteria [39]. Most studies carried out to select microorganisms as candidate probiotics have focused on in vitro antagonism tests, in which pathogens are exposed to the candidate probiotics or their extracellular products [40]. The present in vitro study

showed that *B. velezensis* exhibited antagonistic activity against the pathogenic indicator bacteria *V. harveyi*, *V. alginolyticus*, *A. hydrophila*, *A. Veronii*, *A. caviae*, *E. casseliflavus* and *L. garvieae*. The present finding is similar to Yi et al. [18]. According to some researchers, the inhibitory property of *B. amyloliquefaciens* may be attributed to production of secondary metabolites with antimicrobial activity [41]. Similarly, this result indicates that K2 is likely to produce antibacterial compounds to inhibit the growth of pathogenic bacteria. Another most important criterion to select probiotics is that the probiotics should not be pathogenic to the hosts and it is to be confirmed through testing in vivo before acceptance [42]. The probiotics used in this study were no pathogenicity in grouper because no hemolysis occurred in the blood plate and no death/morbidity was observed within 7 days after the challenge. A similar result was seen in Austin et al. [43] and Aly et al. [44] who showed the safety of probiotics via intramuscular and intraperitoneal injection in Atlantic salmon and tilapia, respectively.

The blood parameters can reflect fish health condition and nutritional metabolism, which are useful for determining the health status of the fish in response to dietary supplements [45,46]. AKP is an extracellular enzyme that hydrolyzes phosphate conjugates in various organic compounds such as proteins, lipids and carbohydrates [47]. In rainbow trout, the plasma AKP activity was significantly increased when fish were fed with diets containing 10^9 cfu/g *LactoBacillus rhamnosus* for 20 and 30 days [48]. In this study, dietary supplement *B. velezensis* K2 had no significant effect on AKP activity of hybrid groupers; however, this strain significantly increased plasma ACP activity in the first 14 days. It is well known that ACP as a key compound of lysosomal enzymes, plays a crucial role in the immune system, and has been used as a marker of macrophage activation in animal models [49]. Here, our results indicated that administration with *B. velezensis* K2 could stimulate macrophage of the groupers, but Furthermore, the alternative complement pathway functions as a powerful component of nonspecific defense mechanism and plays a role to eliminate a wide range of potentially invasive organisms, such as bacteria, fungi, viruses, and parasites [50]. Liu et al. reported that the addition of 10^4-10^8 cfu/g *B. subtilis* E20 strain in diet significantly improved the serum replacement complement activity (ACH50) of the grouper, and the amount of added probiotic was dose-dependent [51]. Cerezuela et al. found that adding a mixture of 10^7 cfu/g heat-killed *B. subtilis* and 10 g/kg inulin to the bait for feeding the golden head mites 2w and 4w significantly improved the hemolytic complement level of the fish serum [52]. In contrast, in this study, there is no significant difference in the Complement component-3 (C3) activity between the experimental and control groups throughout the experiment. These results suggest that *B. velezensis* K2 may have little influence on the alternative pathway of complement activity of grouper.

Cytokines secreted by immune cells or others initiate inflammation at the site of infection, and induce the arrival of phagocytic cells to eliminate the invading pathogen [53]. Probiotics regulate host immunity by altering the expression of pro-inflammatory cytokines. Toll-like receptors (TLRs), a group of type I transmembrane receptors, is involved in innate immunity by recognizing microbial conserved structures [54]. In humans and mice, the expression of TLR3 was up-regulated in the presence of double-stranded RNA viruses in vitro [55]. The bacterial flagellin recognition and activation of host inflammatory responses are mediated by Toll-like receptor 5 [56]. However, in our study, TLR3 and TLR5 in the head kidney of hybrid grouper showed down-regulation or no significant difference compared to the controls during 28 days of feeding with K2, and therefore we hypothesized that K2 might not increase the ability to resist viruses, and it might enhance resistance to the bacterial pathogens through other immune pathways. IL-8 is an essential pro-inflammatory cytokines and major neutrophil chemoattractant, which can recruit and activate macrophages and neutrophils to kill the microorganisms [57]. The present study found that IL-8 was significantly up-regulated only in the first week, indicating K2 might increase IL-8 levels in the short term to promote the migration of neutrophils transfer to inflammatory sites and subsequently adhesion to endothelial cells; however, the down-regulation of IL-8 gene might occur in the absence of invasive pathogens.

Likewise, Piscidin, one of the most common AMPs in fish, is a family of peptides with a highly conserved, histidine-rich, phenylalanine-rich N-terminus and a more variable C-terminus [58], and highly active against bacteria, viruses, fungi, and parasites [58,59]. Moreover, MyD88 is a common adaptor that is essential for proinflammatory cytokine production [60]. Absence of MyD88 in mice leads to susceptibility in extensive pathogens in experimental settings of infection [61]. Similarly, IgM was regarded as the major component for humoral adaptive immunity in teleost fish and played a key role in acquired immunity [62]. Expression of IgM would be altered immediately after immunogenic stimulation. The present result shown that the mRNA expressions of piscidin, IgM and MyD88 were up-regulated after 21 days of feeding, it is possible that the up-regulation of these genes may be related to the enhancement of the resistance to *V. harveyi* in hybrid grouper.

As a barrier for microbial invasion, lysozyme is an important defense molecule of fish innate immune system [63]. Lysozyme kills the sensitive bacteria is known to be the degradation of the peptidoglycan in the bacterial cell wall leading to rapid lyse gram-positive bacteria, and kill gram-negative bacteria after a complement and other enzymes have disrupted the outer cell walls [64,65]. In this study, the expression of the lysozyme gene was up-regulated from first to third week of feeding, and the significant difference was observed after 1 and 2 weeks of feeding, this result suggested that the resistance of grouper to pathogens may be related to the upregulation of lysozyme.

In conclusion, the present study determined the efficacy of *B. velezensis* as a potential probiotic for application in aquaculture. We demonstrated that feeding with a diet that contains 1×10^7 cfu/g *B. velezensis* K2 for 28 days can enhance the resistance of the grouper to *V. harveyi*, but no improvement on growth performance was shown. K2 can induce the up-regulation of certain innate cellular and humoral immune responses, thereby enhancing the resistance to *V. harveyi*. Therefore, the addition of appropriate fresh probiotic preparations in the diet can maintain the sustained activation of immune cells. However, more studies are required to clarify other ways of action of this probiont to further characterize its efficacy.

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Appendix A. Supplementary data

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