Fish and Shellfish Immunology 93 (2019) 711–719

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Evaluation of the potential probiotic *Bacillus subtilis* isolated from two ancient sturgeons on growth performance, serum immunity and disease resistance of *Acipenser dabryanus*

Jun Di[a,b], Zhipeng Chu[b], Shuhuan Zhang[b], Jun Huang[b], Hao Du[b], Qiwei Wei[a,b,*]

[a] Key Laboratory of Freshwater Fish Reproduction and Development, Ministry of Education of China, Southwest University, Chongqing, 400715, China
[b] Key Laboratory of Freshwater Biodiversity Conservation, Ministry of Agriculture of China, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, 430223, China

ARTICLE INFO

Keywords:
Bacillus subtilis, probiotic
Serum immunity
Disease resistance
Dabry's sturgeon
Chinese sturgeon

ABSTRACT

In the present study, we aimed to screen the potential probiotic *Bacillus subtilis* isolated from the gut of healthy fish using *in vitro* assays and to evaluate its effect on Dabry's sturgeon (*Acipenser dabryanus*) using *in vivo* feeding experiments. Among the isolates, *B. subtilis* BSth-5 and BSth-19 exhibited antimicrobial effect against four sturgeon-pathogenic bacteria, including *Aeromonas hydrophila*, *A. veronii*, *A. media*, and *Streptococcus iniae*. The cell number of *B. subtilis* BSth-5 and BSth-19 changed little after 2 h of exposure to pH 3.0 or fresh Dabry's sturgeon bile at 2.5% and 5.0%. Meanwhile, *B. subtilis* BSth-5 and BSth-19 produced extracellular protease, cellulose, and lipase. And it was proved that *B. subtilis* BSth-5 and BSth-19 were harmless after injection of Dabry's sturgeon. One group of Dabry's sturgeon was fed a control diet and two groups were fed experimental diets containing 2.0×10^8 CFU/g BSth-5 (T1 group) or BSth-19 (T2 group) for 8 weeks. No significant differences in final weight, weight gain rate, and special growth rate were observed in the T1 and T2 groups compared to the control group ($P > 0.05$), but a significant improvement in survival rate was detected after 4 and 8 weeks of feeding ($P < 0.05$). After 8 weeks, serum total antioxidant capacity, total superoxide dismutase activity, and IgM levels were significantly higher in the T1 and T2 groups compared to the control group ($P < 0.05$). Moreover, serum lysozyme activity was significantly higher in the T1 group relative to the control group during the whole experiment period ($P < 0.05$); however, the differences were not significant between the T2 and control groups ($P > 0.05$). Serum malondialdehyde levels in the T1 and T2 groups were significantly lower than those in the control group after 4 weeks ($P < 0.05$). Sturgeons in the T1 and T2 groups showed a higher survival rate after *Aeromonas hydrophila* infection. To summarize, dietary supplementation with BSth-5 and BSth-19 could enhance the survival rate, antioxidant activity, serum immunity, and disease resistance in *A. dabryanus*.

1. Introduction

Sturgeons are ancient fish species belonging to the order Acipenseriformes [1]. Three sturgeon species are mainly distributed in the Yangtze River system and the East China Sea: Chinese paddlefish (*Psephurus gladius*), Chinese sturgeon (*Acipenser sinensis*), and Dabry's sturgeon (*A. dabryanus*) [2]. The wild populations of these species have drastically reduced during the past decades due to the construction of hydroelectric dams, habitat destruction, and overfishing [2]. Chinese sturgeon and Dabry's sturgeon were listed as critically endangered species on the International Union for Conservation of Nature (IUCN) Red List in 2010 [3,4]. In response, the Chinese government implemented a series of strategies to restore both these endangered species, such as prohibiting commercial fishing, establishing nature reserves and artificial breeding [5]. With intensive farming, fish are subjected to stress conditions that weaken immune systems, leading to increased susceptibility to pathogens [6–8]. To date, the reported diseases of sturgeon in China were mainly caused by bacterial pathogens such as non-tuberculous *mycobacteria*, *A. hydrophila*, and *A. veronii* [9,10]. For decades, treatment with antimicrobial compounds has been the traditional strategy for controlling bacterial diseases in aquaculture. However, antibiotics overuse may result in public health hazards, including the evolution and spread of resistance genes and antimicrobial-resistant pathogens, as well as the retention of antibiotics in fish [11].

* Corresponding author. Key Laboratory of Freshwater Fish Reproduction and Development, Ministry of Education of China, Southwest University, Chongqing, 400715, China.
E-mail address: weiqw@yfi.ac.cn (Q. Wei).

https://doi.org/10.1016/j.fsi.2019.08.020
Received 5 May 2019; Received in revised form 5 August 2019; Accepted 8 August 2019
Available online 13 August 2019
1050-4648/ © 2019 Elsevier Ltd. All rights reserved.

To reduce the abuse of antibiotics in aquaculture, many alternative solutions have been suggested, including herb extracts, antibacterial peptide, prebiotics, and probiotics [12,13]. Among them, probiotics have received particular attention due to the high abundance, low cost and convenience in application [12]. Probiotics provide a friendly solution to bacterial diseases in fish and help overcome the adverse effects of antibiotics. Probiotics can exert beneficial effects on the host by inhibiting pathogenic microorganisms, activating the host immune defense, enhancing intestinal microbial balance, and promoting growth and survival rate [11,14,15]. The most commonly used probiotic candidates are *Lactobacillus* spp. and *Bacillus* spp [16]. *Bacillus* sp. have been proven to possess more obvious inherent advantages than other non-spore-forming probiotic bacteria [17]. The ability to form endospores makes them survive in extreme environments such as high temperatures, low pH, and high bile concentrations [14,17]. Besides, *Bacillus* sp. are capable of colonizing in the gastrointestinal tract, producing digestive enzymes, and secreting antimicrobial metabolites [14].

Several studies have revealed that dietary administration of *Bacillus subtilis* can improve growth, immune response, antioxidant function, and disease resistance in fish [18–20]. Although *B. subtilis* has been reported as a probiotic bacteria in sturgeons, most of them are non-fish sourced, which may be unreliable in aquaculture [13,21]. Therefore, it is essential to screen the potential probiotic *B. subtilis* to meet the requirements of sturgeon aquaculture. In the present study, four endogenous *B. subtilis* isolates were obtained from the gut of Chinese sturgeon and Dabry's sturgeon. The isolates were screened for probiotic properties based on their antimicrobial activity *in vitro*, extracellular enzyme production, tolerance to low pH and high concentration of bile, and safety. We also performed *in vivo* experiments to evaluate the effects of dietary administration of *B. subtilis* on the growth performance, serum immunity, digestive enzyme activity, and disease resistant to *Aeromonas hydrophila* in *A. dabryanus*.

2. Materials and methods

2.1. B. subtilis and probiotic properties

2.1.1. Ethics statement

All fish handling and experimental procedures were approved by the Animal Care and Use Committee of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences.

2.1.2. Isolation and identification of bacteria

Nine apparently healthy fish (Dabry's sturgeon, n = 8; Chinese sturgeon, n = 1) were obtained from the Taihu Hatchery, a breeding site in Jingzhou City of Hubei Province, China. They belonged to the second captive generation (F2) descended from wild individuals and were anesthetized by administering an overdose of tricaine methanesulfonate (MS-222, Sigma, USA). The gut from each animal was obtained under sterile conditions. Intestinal tissues were rinsed with sterile phosphate-buffered saline (PBS) to remove gut contents and then homogenized in PBS. The homogenate was heated at 80 °C for 20 min, diluted serially, spread on tryptone soy agar (TSA; BD-Difco, USA), and incubated at 37 °C for 48 h. *Bacillus*-like colonies were picked and subcultured five times, and subsequently identified according to Bergey's Manual [22] and the Manual for the Identification of Medical Bacteria [23].

Bacterial genomic DNA was extracted using a high salt extraction method [24]. The 16S rRNA gene was amplified using the universal primers 16S–F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-R (5′-TAC GGTTACCTTGTTACGACTT-3′) [25], and gyrB gene (encoding the DNA gyrase β-subunit) was amplified using gyrB-F (5′-ATCATACAGGAACG ACGACAC-3′) and gyrB-R (5′-CATTCTTGCTCTTGCCGCCA-3′) [17]. PCR products were detected using 1.5% agarose gel containing ethidium bromide, and the products were purified with a PCR purification kit (Omega, Georgia, USA). The purified products were sequenced at Sangon Biotech (Shanghai, China). Sequences of the 16S rRNA and gyrB genes from the isolates were aligned and compared with related sequences available in GenBank. Phylogenetic trees were constructed using the neighbour-joining method in MEGA v7.0. Genetic distances were calculated with the Kimura two-parameter model. A bootstrap analysis with 1000 replicates was used to estimate the reliability of each tree topology.

2.1.3. Antimicrobial assay in vitro

Antimicrobial activity was measured using the Oxford cup agar diffusion method [17,26]. Four sturgeon-pathogenic bacteria (*A. hydrophila*, *A. veronii*, *A. media* and *Streptococcus iniae*), as well as *B. subtilis* isolates were separately cultured in brain heart infusion (BHI, BD-Difco, USA) broth at 28 °C, 200 rpm for 20 h. The culture of pathogenic bacteria were adjusted to approximately 10^8 cells ml^{-1} with sterile PBS using spectrophotometry (OD600) and plate count methods, and 150 μL culture was spread on TSA agar plate. The plates spread with different pathogenic bacteria were dried for 15 min on vertical flow clean cabinet. Then a sterile Oxford cup (8 mm in diameter) containing 200 μL of *B. subtilis* isolates (10^8 cells ml^{-1}) was placed on the plates. The diameter of the clear zones around each Oxford cup was measured after incubation at 37 °C for 24 h. Each sample was tested twice in triplicate.

2.1.4. Extracellular enzyme assay

Extracellular cellulase, protease, and lipase production by *B. subtilis* isolates was studied using the agar diffusion method. Cellulase assay was conducted using the method described by Guo et al. [17], with slight modifications. Briefly, 2 μL of *B. subtilis* (1.0×10^8 CFU/ml) isolates was spotted onto carboxymethyl cellulose agar plates and incubated at 37 °C for 48 h. Congo red (1 mg/ml) was used for staining for 15 min; bleaching was then performed with NaCl (1 M) for 15 min until the background turned blue. Cellulase activity was identified by transparent zones around a colony. To determine extracellular protease production, 2 μL of *B. subtilis* (1.0×10^8 CFU/ml) isolates was spotted onto peptone gelatin-enriched nutrient agar (4% gelatin) plates, followed by incubation at 32 °C for 48 h. The plates were stained with $HgCl_2$ until the appearance of a clear zone around a colony. Protease activity was recorded as the diameter of the clear zones [27]. Similarly, 2 μL of *B. subtilis* (1.0×10^8 CFU/ml) isolates was spotted onto basal medium plates supplemented with Tween-80 (1%), and clear zones on the plates indicated lipase activity [28]. Each sample was tested twice in triplicate.

2.1.5. pH and bile tolerance

B. subtilis isolates were analyzed for their pH and bile tolerance abilities using the method described by Ramesh et al. [29], with some modifications. The isolates were cultured in tryptone soy broth (TSB, BD-Difco, USA) at 37 °C for overnight. The cultures were then inoculated in a spore preparation medium (broth: 8.0 g/l, NaCl 5.0 g/l, $GaCL_2$ 0.1 g/l, K_2HPO_4 2.0 g/l, $MgSO_4{\cdot}7H_2O$ 0.25 g/l, $MnSO_4$ 0.01 g/l) and incubated at 37 °C with shaking at 200 rpm for 96 h. *B. subtilis* spores were harvested and adjusted to approximately 1.0×10^8 CFU/ml with sterile PBS using the method of Guo et al. [17]. The spores were separately subjected to sterile PBS at pH 1.0, 2.0, and 3.0, followed by incubation at 37 °C for 2 h. Total viable bacteria were estimated by the plate count method on TSA. To assess bile tolerance, the spores were exposed to fresh Dabry's sturgeon bile at concentrations of 0%, 2.5%, 5.0%, 7.5%, and 10% (v/v). The cells in the bile were incubated at 37 °C for 2 h, and viable bacteria were estimated using the plate count methods on TSA.

2.1.6. Safety evaluation of probiotic isolates

A total of 150 healthy F2 Dabry's sturgeon (approximately 28 g) were obtained from the Taihu Hatchery. They were randomly divided into 5 groups (n = 30 per group) and placed in 5 tanks (1 tank per

group). Four experimental groups were intraperitoneally (IP) injected with the four *B. subtilis* isolates (BSth-1, BSth-4, BSth-5, and BSth-19) at the same dosage of 2.0×10^7 cells per fish, respectively. Whereas the control group was IP injected with 200 μL of sterile PBS. Fish were fed a commercial sturgeon diet (Tianma®, Fujian, China). Mortality was monitored daily for 2 weeks, after which a part of the survivors were sacrificed to evaluate for the presence of diseases.

2.2. Diet preparation

BSth-5 and BSth-19 were grown in TSB at 37 °C for overnight. The cell suspensions were inoculated in spore preparation medium and incubated at 37 °C with shaking at 200 rpm for 96 h. *B. subtilis* spores were harvested and adjusted to 2.0×10^9 CFU/ml with sterile PBS, as described earlier. The spore suspensions were kept at 4 °C until required. A commercial sturgeon feed (crude protein 43.21%, crude lipid 12.12%, and ash 11.48%; Tianma®, Fujian, China) was used as the basal diet. Two experimental diets were formulated: one with BSth-5 added to the basal diet (T1) and the other with BSth-19 added to the basal diet (T2). Briefly, the bacterial suspensions containing *B. subtilis* isolates were added to commercial feed to obtain 2.0×10^8 cells per gram diet, by mixing tap water with automatic mixer. The control diet was made by mixing the basal diet powder with the same volume of tap water (without *B. subtilis*). The pellets were dried overnight at 45 °C and stored at −20 °C until use. Viable *B. subtilis* cells in the feed were assessed every week as described by Zaineldin et al. [18]. As the number of cells decreased by approximately 10% after 2 weeks of storage, new pellets were prepared every 2 weeks to ensure BSth-5 and BSth-19 vitality in the experimental diets.

2.3. Feeding experiments

The feeding trial was performed at the Taihu Hatchery. The third captive generation (F3) of Dabry's sturgeon juveniles was obtained from the above hatchery and acclimatized for 2 weeks to experimental conditions. A total of 360 acclimatized sturgeons were randomly divided into 9 plastic tanks (2000-L water) at a density of 40 fish per bank. Each group consisted of three replicates. One group served as the control group (C) and was fed the control diet. The other two groups (T1 and T2) were fed one of the two experimental diets: T1 diet or T2 diet. Feeding was performed twice daily (9 a.m. and 6 p.m.) for 8 weeks up to apparent satiation. Water quality parameters were monitored during the experiment: water temperature 20.5–22.4 °C; dissolved oxygen 6.4–7.7 mg/l; pH 7.1–7.7 and ammonia < 0.05 mg/l.

2.4. Growth parameters

Fish were weighed in each tank at 0, 4, and 8 week stages of the feeding trial to assess growth performance, as per the method of Liu et al. [19] and Thy et al. [30]. Weight gain rate (WGR) = 100 × (final weight − initial weight)/initial weight; Special growth rate (SGR) = 100 × [ln (final mean body weight) − ln (initial mean body weight)]/days; Survival rate (SR) = 100 × (final numbers/initial numbers).

2.5. Sample collection

Three fish were randomly selected from each tank (n = 9/treatment) after 4 and 8 weeks of feeding and sampled for analyses. Fish were anesthetized by administering an overdose of MS-222. Blood samples were obtained from the caudal vein using a 10 ml non-heparinized vacuum blood collector. The extracted blood from the three fish in each tank was pooled into an Eppendorf tube. After clotting at 4 °C for overnight, the samples were centrifuged at 4 °C and 3000 rpm for 15 min, and the serum was collected and stored at −80 °C until use [31]. In addition, the intestines were separated, rinsed with PBS, and stored at −80 °C for digestive enzyme analysis.

2.6. Serum immune responses

Total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD) activity, malondialdehyde (MDA) content, and lysozyme (LZM) activity in the serum were measured following the manufacturer's instructions manual from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China) [19].

IgM levels were measured using a FISH IgM ELISA Kit (Cusabio, Wuhan, Hubei, China), according to the manufacturer's protocol. Briefly, 50 μL of serum samples were added to 96-well microplates (precoated with fish IgM antibody). Samples were incubated with 50 μL of horseradish peroxidase-conjugated IgM at 37 °C for 1 h. Each well was aspirated and washed three times with a wash buffer. Then, 50 μL each of substrate A and substrate B was added to each well, followed by incubation at 37 °C for 15 min. The reaction was stopped by adding 50 μL of stop solution to each well. OD of each well was measured at 450 nm using a microplate reader. IgM levels have been expressed as μg/ml.

2.7. Digestive enzyme activities

The intestine samples were homogenized in 0.85% saline water at a ratio of 1:9 (w/v) using a hand-held tissue grinder at 4 °C. The homogenate was centrifuged at 4 °C and 2500 rpm for 10 min. The supernatant was kept at −80 °C to analyze different enzyme activities within 24 h. Total protein content of the supernatant was measured using bovine serum albumin as the standard, according to previously described methods [32].

Protease activity was measured using casein as the substrate, which reacted with the Folin phenol reagent [33]. One unit of protease activity was defined as the number of micromoles of tyrosine released per minute per milligram of tissue protein at 37 °C. Amylase activity was analyzed using the method of iodine starch colorimetry using an assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). One unit of amylase activity was defined as 10 mg starch hydrolyzed per milligram of tissue protein at 37 °C for 30 min. Lipase activity was also evaluated using the assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). One unit of lipase activity was defined as the number of micromoles of fatty acids released per min per milligram of tissue protein at 37 °C.

2.8. A. hydrophila infection

As Dabry's sturgeon are listed as a first-class protected animal in China and a critically endangered species on the IUCN Red List, we limited the number of fish used for bacterial challenge experiments. At the end of the 56-day feeding trial, 15 randomly selected Dabry's sturgeon (n = 5 per tank) from each experimental group were IP injected with 200 μL sturgeon-pathogenic *A. hydrophila* (1.0×10^8 CFU/ml). Fish were fed the commercial diet during the experiment. Clinical signs and mortality were monitored daily for 10 days. *A. hydrophila* was re-isolated from the liver and spleen of morbid fish to confirm infection establishment. The isolated bacteria were identified using 16S rDNA sequencing.

2.9. Statistical analyses

Data are presented as mean ± standard deviation (SD) and analyzed using one-way ANOVA. A multiple comparison (Duncan's) test was used to determine differences among treatment groups. Differences were regarded as significant when P was < 0.05 [17,30]. Statistical analyses were performed using SPSS 23 software.

Table 1
Phenotypic characteristics of the four isolates.

Test items	Strains			
	BSth-1	BSth-4	BSth-5	BSth-19
Gram stain	+	+	+	+
Growth at 2% NaCl	+	+	+	+
Growth at 5% NaCl	+	+	+	+
Growth at 7% NaCl	+	+	+	+
Amylolysis	+	+	+	+
Catalase test	+	+	+	+
V–P test	+	+	+	+
Glucose oxidation	+	+	+	+
Gelatin liquefaction	+	+	+	+
Citrate utilization	+	+	+	+
Production of indole	–	–	–	–

Symbol '+' denotes a positive result, '–' denotes a negative result.

3. Results

3.1. B. subtilis isolation and identification

Three isolates (BSth-1, BSth-4, and BSth-5) were obtained from Dabry's sturgeon and one (BSth-19) from Chinese sturgeon. These Gram-positive bacteria could produce amylase, catalase, and acid from glucose, and grow in 2%–7% NaCl (Table 1). They could utilize gelatin and citrate but did not produce indole. These bacterial isolates were positive for Voges–Proskauer (V–P) test (Table 1). Based on Bergey's manual and the Manual for the Identification of Medical Bacteria, the phenotypic characteristics of these isolates resembled those of *B. subtilis*. Moreover, BLAST analysis of the 16S rRNA and gyrB gene sequences from the four isolates revealed high homology (98%–100%) with *B. subtilis*. Phylogenetic trees constructed based on the sequences of 16S rRNA and gyrB genes showed that the four isolates grouped along with the reference strains of *B. subtilis* (Fig. 1 **and** Fig. 2). Based on the phenotypic characteristics and phylogenetic analysis, these isolates were thus identified as *B. subtilis*.

3.2. Antimicrobial assay in vitro

Antimicrobial activities of the four *B. subtilis* isolates against four sturgeon-pathogenic bacteria are presented in Table 2. BSth-5 and BSth-19 were found to inhibit all the tested pathogens: *A. hydrophila*, *A. veronii*, *A. media*, and *S. iniae*. However, BSth-1 and BSth-4 did not inhibit *A. veronii*. Among the four isolates, BSth-5 had the highest antagonistic effect against the four pathogens *in vitro*, followed by BSth-19.

3.3. Extracellular enzyme production

Extracellular enzyme activities of four *B. subtilis* isolates are shown in Table 3. Protease activities of BSth-4, BSth-5, and BSth-19 were significantly higher than those of BSth-1 ($P < 0.05$), while there were no significant differences between BSth-4, BSth-5, and BSth-19 ($P > 0.05$). Likewise, lipase activities of BSth-4, BSth-5, and BSth-19 were significantly higher than those of BSth-1 ($P < 0.05$), and BSth-5 showed the highest lipase activity. There were no significant differences in cellulase activities among the four isolates ($P > 0.05$).

3.4. pH and bile tolerance

All four *B. subtilis* isolates were able to survive after 2 h of exposure to pH 3.0 to 1.0 and showed no significant differences after exposure to pH 3.0 compared to the control group ($P > 0.05$) (pH 7.0 served as the control) (Table 4). However, the viable cell counts for all the isolates significantly decreased after 2 h of exposure to pH 2.0 to 1.0 ($P < 0.05$) (Table 4). Likewise, the viable cell counts of all isolates decreased after 2 h of exposure to bile concentrations ranging from 2.5% to 10.0% (Table 5). However, all isolates showed relatively high resistance to fresh Dabry's sturgeon bile as the survivabilities showed no significant differences upon exposure to bile concentrations ranging from 2.5% to 5.0% ($P > 0.05$) (Table 5).

3.5. Safety evaluation of probiotic isolates

All four isolates of *B. subtilis* were found to be harmless to Dabry's

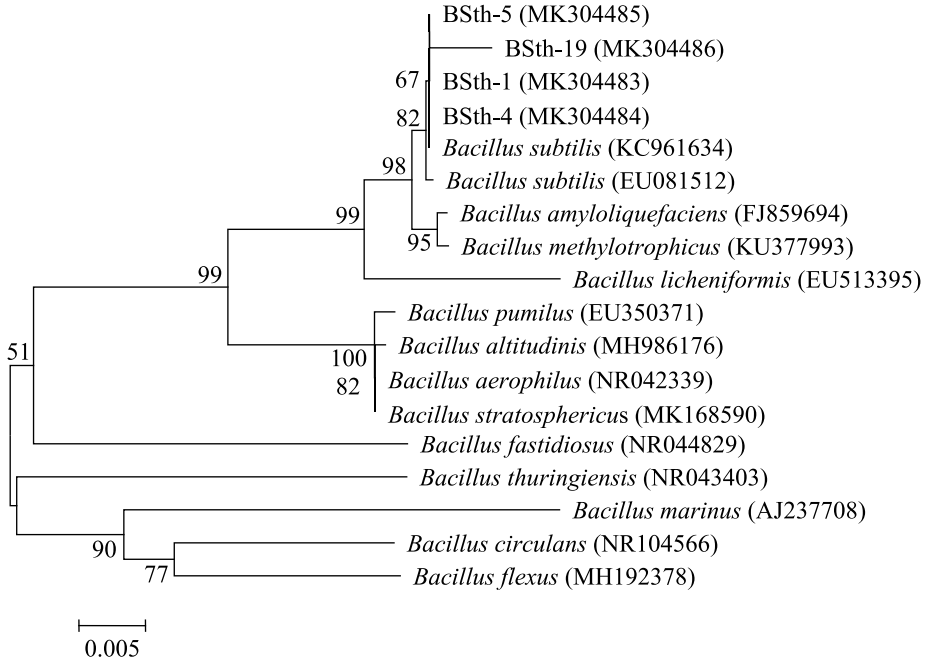

Fig. 1. Phylogenetic tree of the four isolates (BSth-1, BSth-4, BSth-5, and BSth-19) based on the 16S rRNA gene sequences using the neighbour-joining method. The scale bar represents 0.005 per nucleotide site. The gene accession numbers are shown in the parentheses after each species.

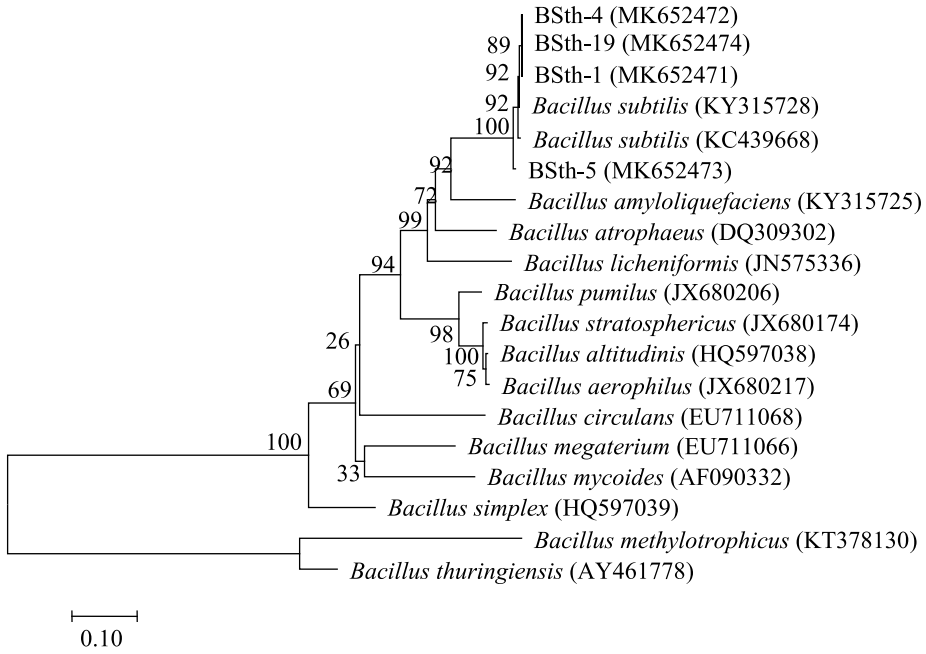

Fig. 2. Phylogenetic tree of the four isolates (BSth-1, BSth-4, BSth-5, and BSth-19) based on the gyrB gene sequences using the neighbour-joining method. The scale bar represents 0.10 per nucleotide site. The gene accession numbers are shown in the parentheses after each species.

Table 2
Antimicrobial activities of the four isolates *in vitro*.

Sturgeon pathogens	Strains			
	BSth-1	BSth-4	BSth-5	BSth-19
Aeromonas hydrophila	+	+	+ +	+
Aeromonas veronii	–	–	+ +	+
Aeromonas media	+	+	+	+
Streptococcus iniae	+ +	+ +	+ +	+

Symbol '+' denotes inhibition zone of 10 mm or more, '+ +' denotes inhibition zone of 15 mm or more, '–' denotes absence of inhibition zone.

Table 3
Extracellular enzyme activities of the four isolates.

Activity (cm)	Strains			
	BSth-1	BSth-4	BSth-5	BSth-19
Protease	1.33 ± 0.12^{a}	1.93 ± 0.26^{b}	2.04 ± 0.11^{b}	2.14 ± 0.03^{b}
Cellulase	1.26 ± 0.29	1.10 ± 0.30	1.07 ± 0.08	1.28 ± 0.07
Lipase	1.47 ± 0.23^{a}	2.12 ± 0.04^{bc}	2.30 ± 0.13^{c}	1.94 ± 0.08^{b}

Values (mean ± SD, n = 3) with different superscripts in each row are significantly different (P < 0.05).

Table 4
The viable cell counts of the four isolates at different pH.

Strains	Viability of bacteria in CFU/ml (× 10^{7})			
	pH			
	7.0	3.0	2.0	1.0
BSth-1	16.33 ± 1.53^{a}	14.73 ± 0.21^{a}	9.43 ± 0.95^{b}	3.47 ± 0.15^{c}
BSth-4	23.00 ± 2.65^{a}	22.77 ± 2.91^{a}	7.13 ± 1.84^{b}	1.70 ± 1.13^{c}
BSth-5	34.67 ± 8.08^{a}	28.07 ± 3.04^{a}	16.83 ± 3.36^{b}	3.03 ± 0.93^{c}
BSth-19	16.33 ± 7.77^{a}	15.80 ± 6.97^{a}	13.37 ± 0.81^{a}	3.03 ± 0.12^{b}

Values (mean ± SD, n = 3) with different superscripts in each row are significantly different ($P < 0.05$).

sturgeon as no mortality was observed upon experimental infection. At the end of the experiment, no abnormal clinical signs were observed in the infected fish, including swimming behavior, feeding activity, and internal organs (data not shown). This suggested that the four isolates were not pathogenic to Dabry's sturgeon.

3.6. Growth performance

Growth performance and SR of Dabry's sturgeon fed experimental diets are presented in Table 6. There were no significant differences in final weight (FW), WGR, and SGR between fish fed diet T1 and diet T2 and fish fed the control diet after 4 and 8 weeks of feeding ($P > 0.05$). However, FW, WGR, and SGR of fish fed diet T1 and diet T2 slightly increased compared with those of fish fed the control diet. SR of fish fed diet T1 and diet T2 was significantly higher than SR of fish fed the control diet after 4 and 8 weeks ($P < 0.05$).

3.7. Serum immune responses

Serum T-AOC in the T1 and T2 groups was higher than that in the control group after 4 weeks of feeding, but the differences were not significant ($P > 0.05$) (Fig. 3). After 8 weeks, T-AOC in the T1 and T2 groups was significantly higher than that in the control group ($P < 0.05$), but the differences were not significant between the T1 and T2 groups ($P > 0.05$) (Fig. 3).

Serum T-SOD activity in the T1 and T2 groups was significantly higher than that in the control group after 4 and 8 weeks of feeding ($P < 0.05$), but the differences were not significant between the T1 and T2 groups ($P > 0.05$) (Fig. 4).

Serum MDA levels in the T1 and T2 groups were significantly lower than those in the control group after 4 weeks of feeding ($P < 0.05$), but the differences were not significant between the T1 and T2 groups ($P > 0.05$) (Fig. 5). After 8 weeks, MDA levels in the T1 group were significantly lower than those in the control group ($P < 0.05$), but no significant differences were observed between the T2 and control groups ($P > 0.05$) (Fig. 5).

Serum LZM activity in the T1 group was significantly higher than that in the control group after 4 and 8 weeks ($P < 0.05$) (Fig. 6). LZM activity in the T2 group was higher than that in the control group after

Table 5
The viable cell counts of the four isolates at different concentration of bile.

Strains	Viability of bacteria in CFU/ml ($\times 10^7$)				
	Bile %				
	0	2.5	5.0	7.5	10.0
BSth-1	16.33 ± 1.53^{a}	15.30 ± 0.69^{a}	14.60 ± 0.73^{a}	11.30 ± 1.39^{b}	10.13 ± 1.64^{b}
BSth-4	23.00 ± 2.65^{a}	22.03 ± 1.37^{ab}	19.47 ± 1.50^{ab}	18.47 ± 1.96^{bc}	16.33 ± 1.81^{c}
BSth-5	34.67 ± 8.08^{a}	32.00 ± 5.72^{a}	29.50 ± 3.82^{a}	19.70 ± 1.25^{b}	18.80 ± 1.51^{b}
BSth-19	16.33 ± 7.77^{a}	14.70 ± 1.91^{a}	13.40 ± 1.47^{ab}	9.30 ± 0.89^{ab}	7.03 ± 0.65^{b}

Values (mean ± SD, n = 3) with different superscripts in each row are significantly different ($P < 0.05$).

Table 6
Growth performance of Dabry's sturgeon fed the control diet (C), 2.0×10^8 CFU/g BSth-5 (T1) and 2.0×10^8 CFU/g BSth-19 (T2) for 4 and 8 weeks.

Time	Parameters	C	T1	T2
4 weeks	IW (g)	28.41 ± 0.64	28.54 ± 0.44	28.31 ± 0.42
	FW (g)	80.80 ± 0.92	82.79 ± 1.24	80.95 ± 1.23
	WGR (%)	184.42 ± 3.25	190.04 ± 4.33	185.93 ± 4.36
	SGR (%)	3.73 ± 0.04	3.80 ± 0.05	3.75 ± 0.05
	SR (%)	96.67 ± 1.44^{a}	100^{b}	99.17 ± 1.44^{b}
8 weeks	IW (g)	28.41 ± 0.64	28.54 ± 0.44	28.31 ± 0.42
	FW (g)	154.97 ± 6.58	163.99 ± 3.11	160.58 ± 4.24
	WGR (%)	445.53 ± 23.15	474.50 ± 10.90	462.40 ± 19.75
	SGR (%)	44.53 ± 9.56	54.68 ± 5.27	46.3 ± 8.43
	SR (%)	90.00 ± 2.5^{a}	95.83 ± 1.44^{b}	95.00 ± 2.5^{b}

Values (mean ± SD, n = 3) with different superscripts in each row are significantly different ($P < 0.05$).
IW = Initial weigh; FW = Final weight; WGR = Weight gain rate; SGR = Special growth rate; SR = Survival rate.

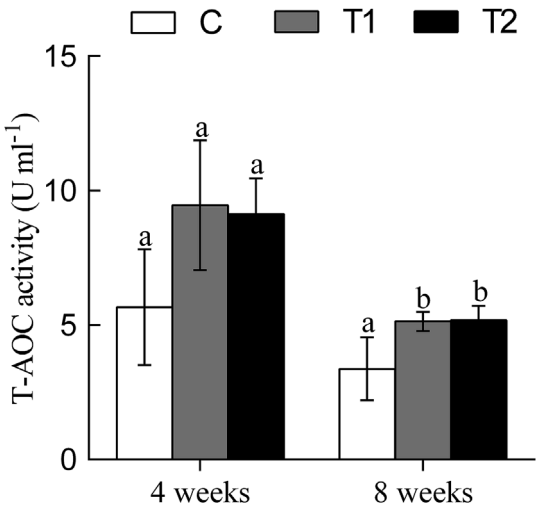

Fig. 3. Total antioxidant capacity (T-AOC) of Dabry's sturgeon fed the control diet (C), 2.0×10^8 CFU/g BSth-5 (T1) and 2.0×10^8 CFU/g BSth-19 (T2) for 4 and 8 weeks. Values (mean ± SD,n = 3) with different letters are significantly different ($P < 0.05$) between the different groups at the same sampling week.

4 and 8 weeks, but the differences were not significant ($P > 0.05$) (Fig. 6).

Serum IgM levels showed no significant differences between the T1 and T2 groups and the control group after 4 weeks of feeding ($P > 0.05$) (Fig. 7). After 8 weeks, IgM levels in the T1 and T2 groups were significantly higher than those in the control group ($P < 0.05$) (Fig. 7).

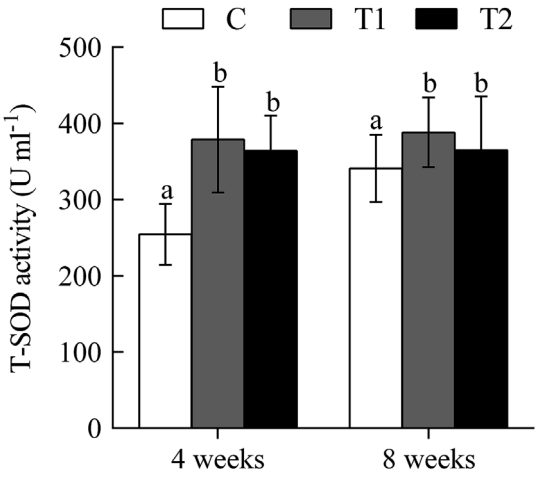

Fig. 4. Total superoxide dismutase (T-SOD) activity of Dabry's sturgeon fed the control diet (C), 2.0×10^8 CFU/g BSth-5 (T1) and 2.0×10^8 CFU/g BSth-19 (T2) for 4 and 8 weeks. Values (mean ± SD,n = 3) with different letters are significantly different ($P < 0.05$) between the different groups at the same sampling week.

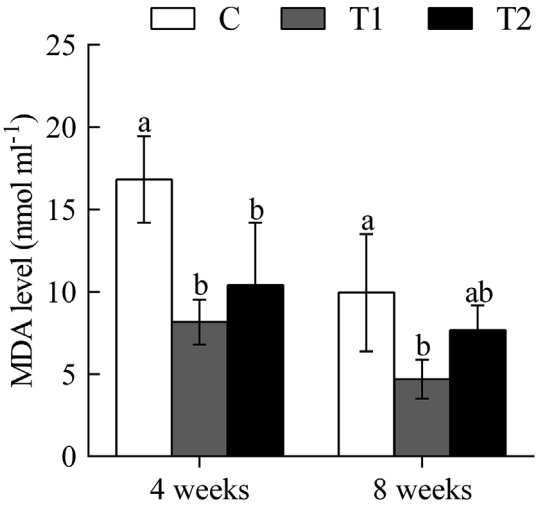

Fig. 5. Malondialdehyde (MDA) levels of Dabry's sturgeon fed the control diet (C), 2.0×10^8 CFU/g BSth-5 (T1) and 2.0×10^8 CFU/g BSth-19 (T2) for 4 and 8 weeks. Values (mean ± SD,n = 3) with different letters are significantly different ($P < 0.05$) between the different groups at the same sampling week.

3.8. Digestive enzyme activity

After 8 weeks of feeding, protease, amylase, and lipase activities of fish fed diet T1 and diet T2 were higher than those of fish fed the control diet, but the differences were not significant ($P > 0.05$) (Table 7).

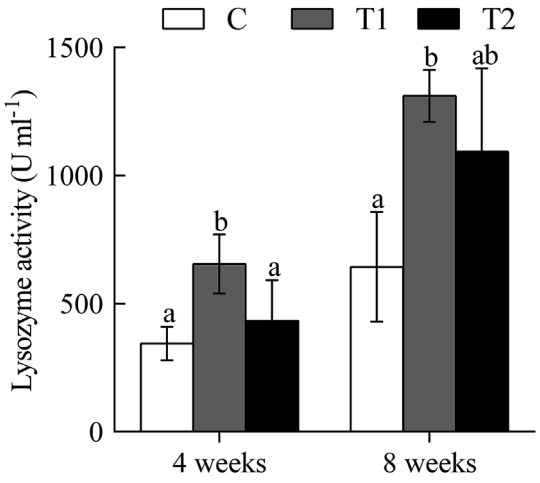

Fig. 6. Lysozyme (LZM) activity of Dabry's sturgeon fed the control diet (C), 2.0×10^8 CFU/g BSth-5 (T1) and 2.0×10^8 CFU/g BSth-19 (T2) for 4 and 8 weeks. Values (mean ± SD,n = 3) with different letters are significantly different ($P < 0.05$) between the different groups at the same sampling week.

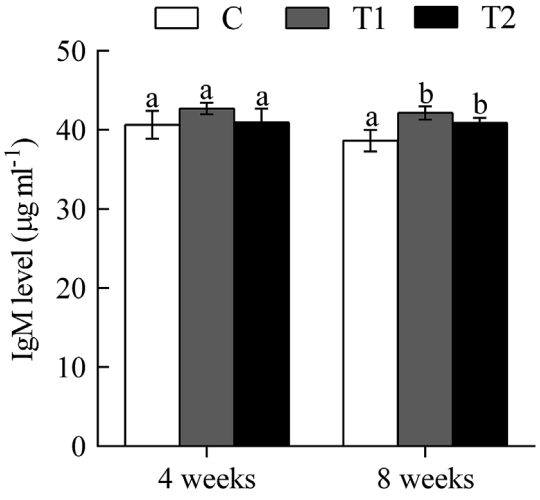

Fig. 7. Immunoglobulin M (IgM) levels of Dabry's sturgeon fed the control diet (C), 2.0×10^8 CFU/g BSth-5 (T1) and 2.0×10^8 CFU/g BSth-19 (T2) for 4 and 8 weeks. Values (mean ± SD,n = 3) with different letters are significantly different ($P < 0.05$) between the different groups at the same sampling week.

Table 7
Intestinal digestive enzyme (amylase, lipase, and pepsin) activities of Dabry's sturgeon fed the control diet (C), 2.0×10^8 CFU/g BSth-5 (T1) and 2.0×10^8 CFU/g BSth-19 (T2) for 8 weeks.

Parameters	C	T1	T2
Amylase (U/g prot)	266.02 ± 36.63	304 ± 62.70	270 ± 52.77
Lipase (U/g prot)	25.11 ± 7.45	35.13 ± 22.10	27.31 ± 8.86
Protease (U/mg prot)	0.64 ± 0.09	1.16 ± 0.41	0.82 ± 0.16

Values (mean ± SD, n = 6) with different superscripts in each row are significantly different ($P < 0.05$).

3.9. A. hydrophila infection

The challenge test showed that Dabry's sturgeon fed diet T1 and diet T2 enhanced protection against *A. hydrophila* infection (Fig. 8). SR was highest in the T1 group (66.67%), followed by the T2 group (53.33%) and then the control group (33.33%) (Fig. 8).

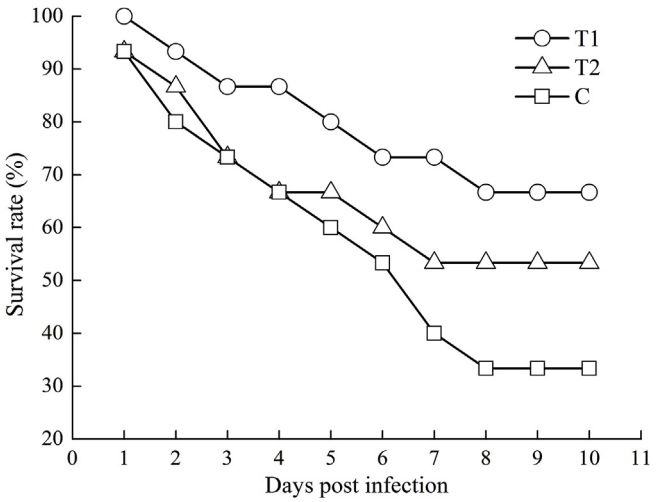

Fig. 8. Survival rate (%) of Dabry's sturgeon after challenge with *Aeromonas hydrophila* for 10 days. T1: fish fed 2.0×10^8 CFU/g BSth-5, T2: 2.0×10^8 CFU/g BSth-19, C: fish fed the control diet.

4. Discussion

There is increasing evidence that *B. subtilis* is effective in inhibiting the growth of fish pathogens such as *Aeromonas* spp., *Vibrio* spp., *Edwardsiella* spp., and *Streptococcus* spp [34,35]. Herein BSth-5 and BSth-19 could effectively inhibit the four sturgeon-pathogenic bacteria, namely *A. hydrophila*, *A. veronii*, *A. media*, and *S. iniae*. The antimicrobial activity of *B. subtilis* may be attributed to the production of antibacterial substances, alteration of pH in the growth medium, and competition of essential nutrients [15]. Tolerance to low pH and high bile concentration is a prerequisite for probiotics to survive and grow in the gastrointestinal tract of fish [19]. In this study, four *B. subtilis* isolates showed relatively high resistance to low pH and high concentration of bile. In case of all the isolates, the survivability was > 90% after 2 h of exposure to 2.5% Dabry's sturgeon bile and > 80% after 2 h exposure to pH 3.0. Moreover, it is noteworthy that the bile concentration in the gastrointestinal tract of Dabry's sturgeon is < 1.3% and that pH is estimated to be 5.0–7.5 (data not shown). Thus, the four isolates of *B. subtilis* can be added as supplements in Dabry's sturgeon diets.

It is widely accepted that the extracellular activity of *B. subtilis* is one of the main reasons for its ability to improve digestive activities of the host [15,19]. Cellulase can evidently increase protease and amylase activities and promote the growth of fish [17]. In this study, the four isolates of *B. subtilis* could produce cellulase, lipase, and protease, and this observation was in accordance with results of previous studies [17,19]. Furthermore, improvements in FW, WGR, and SGR were noted in fish fed diet T1 and diet T2 after 4 and 8 weeks. The growth-promoting effects of *B. subtilis* may be attributed to the production of extracellular enzymes, synthesis of vitamins, and degradation of harmful substances [15]. This sort of enhanced growth has also been reported in Nile tilapia (*Oreochromis niloticus*) fed *B. subtilis* at 10^8 CFU/g diet for 8 weeks [19] and sea cucumber (*Apostichopus japonicus*) fed *B. subtilis* at 10^9 CFU/g diet for 30 days [36]. Although *B. subtilis* can promote the growth of aquatic animals, an appropriate dose in feed is required [37]. This could be attributed to the differences in animals (species and sizes), feeding methods, and experimental conditions [36]. Therefore, further studies should aim to identify appropriate methods to improve the efficacy of *B. subtilis* in sturgeon aquaculture and to determine the optimal demand for *B. subtilis* in feed.

Probiotics can effectively improve host antioxidant activities and innate immunity [14,18]. T-AOC includes non-enzymatic and enzymatic antioxidant activities. The role of antioxidant enzymes is to protect the host cells and tissues from oxidative stress. SOD is one of the

most important antioxidant defense enzymes, catalyzing the dismutation of two superoxide anions into hydrogen peroxide and oxygen [38]. T-AOC and SOD activities, as reliable indicators of the antioxidant status of fish, are utilized to measure the capacity of all antioxidants [19]. In the present study, serum T-AOC and T-SOD activities in T1 and T2 groups were significantly higher than those in the control group after 8 weeks. Our results are in agreement with those of Liu et al. [19] who reported that T-AOC and SOD activities significantly increased after *O. niloticus* were fed *B. subtilis* for 8 weeks. Moreover, significantly higher T-AOC and SOD activities were observed upon feeding African catfish (*Clarias gariepinus*) with *B. subtilis*, *B. cereus*, and *B. amyloliquefaciens* for 30 days [14]. MDA is the end-product of lipid peroxidation; it is generated when reactive oxygen species (ROS) degrade polyunsaturated fatty acids in the cell membrane, and it can indirectly reflect the extent of cell damage [39,40]. Our results showed that Dabry's sturgeon fed diet T1 and diet T2 exhibited a significant decrease in MDA levels compared to when they were fed the control diet after 4 weeks. Meanwhile, MDA levels in fish fed diet T1 were significantly lower than those in fish fed the control diet after 8 weeks. Similarly, serum MDA levels hve been reported to be significantly reduced upon feeding *B. subtilis* to Yoshitomi tilapia [40] and broiler chickens [41]. These results indicated that BSth-5 and BSth-19 can enhance antioxidant activities in Dabry's sturgeon by effectively alleviating ROS stress.

LZM is one of the important antibacterial proteins in the non-specific immune system and an indispensable tool for fish to evade viral and bacterial infections [42]. LZM activity has been reported to significantly increase in fish fed *B. subtilis*-supplemented diets [14,18,43]. In line with these findings, Dabry's sturgeon fed diet T1 showed a significant increase in serum LZM activity than fish fed the control diet after 4 and 8 weeks. In contrast, serum LZM levels in rainbow trout were not significantly affected on feeding the fish with *B. subtilis*-supplemented diets [44]. No significant differences in LZM activity was also found between olive grouper (*Paralichthys olivaceus*) fed a diet containing *B. subtilis* and fish fed the control diet for 8 weeks [45]. In the present study, LZM activity in fish fed diet T2 was numerically higher than that in fish fed the control diet, but the differences were not significant. The differences in LZM activity between the T1 and T2 groups may be associated with different sources of *B. subtilis* in the feed [37,46]. Hence, further research is needed to investigate host specificity differences of BSth-5 and BSth-19 in Dabry's sturgeon.

Immunoglobulins are main components of the adaptive immune system, and IgM is a major immunoglobulin type in fish [47,48]. High serum levels of IgM act as a natural defense system, providing instant protection against bacterial pathogens and reflecting the immune system status of fish [38,47]. Probiotics can significantly enhance serum IgM activity in fish [14,49]. In the present study, IgM levels in fish fed T1 diet and diet T2 were significantly higher than those in fish fed the control diet after 8 weeks. In contrast, no significant differences in IgM levels were observed upon feeding *B. subtilis* to gilthead sea bream (*Sparus aurata*) [50] and grouper (*Epinephelus coioides*) [51]. The differences in IgM levels may be attributed to strain-specific effects [37,52]. Probiotics often exert host specific and strain specific differences in their modes of action [37]. Likewise the origin and source of probiotics, viability, dose and duration of supplementation can regulate their activities. Probiotics can stimulate piscine immune system but inappropriate dose or duration of probiotics supplementation can cause undesirable results [37]. Therefore, the type of probiotics, dose, and method of administration with respect to fish are critical factors that can regulate immune responses in fish [37].

A. hydrophila is associated with outbreaks of motile aeromonad septicemia in cultured sturgeons and other freshwater fish, causing significant economic losses in fish aquaculture [9]. In the present study, Dabry's sturgeon fed diet T1 and diet T2 showed higher SR than fish fed the control diet after *A. hydrophila* challenge. Meanwhile, we found that the natural SR by fish in the T1 and T2 groups was significantly higher than that by fish in the control group after 4 and 8 weeks of feeding. In line with our results, previous studies have reported that dietary administration of *Bacillus* spp. promoted immune responses, SR, and disease resistance in fish [15,53,54]. The effectiveness of probiotics in enhancing SR and disease resistance is usually attributed to increased immunity [15,33].

In conclusion, under *in vitro* experimental conditions, the *B. subtilis* isolates BSth-5 and BSth-19 demonstrated good probiotic properties considering their antimicrobial activity, extracellular enzyme production, and tolerance to low pH and high bile concentration. *In vivo* experiments showed that BSth-5 and BSth-19 enhanced the SR, antioxidant activity, serum immunity, and disease resistance in Dabry's sturgeon. To the best of our knowledge, this study is the first time to demonstrate *B. subtilis* isolated from Chinese sturgeon and Dabry's sturgeon can be used as potential probiotics for aquaculture.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 31772854), the Special Fund for Basic Scientific Research Business of Central public Research Institutes of China (2017HY-ZD0405), and the National Basic Research Program of China (973 Program, 2015CB150702).

References

[1] E.K. Pikitch, P. Doukakis, L. Lauck, P. Chakrabarty, D.L. Erickson, Status, trends and management of sturgeon and paddlefish fisheries, Fish Fish. 6 (2005) 233–265.
[2] Q.W. Wei, F.E. Ke, J.M. Zhang, P. Zhuang, J.D. Luo, R.Q. Zhou, et al., Biology, fisheries, and conservation of sturgeons and paddlefish in China, Environ. Biol. Fish. 48 (1997) 241–256.
[3] Q.W. Wei, *Acipenser Dabryanus*. The IUCN Red List of Threatened Species, (2010) e.T231A13041556.
[4] Q.W. Wei, *Acipenser Sinensis*. The IUCN Red List of Threatened Species, (2010) e.T236A13044272.
[5] J.H. Wang, Q.W. Wei, Y.C. Zou, Conservation strategies for the Chinese sturgeon, *Acipenser sinensis*: an overview on 30 years of practices and future needs, J. Appl. Ichthyol. 27 (2011) 176–180.
[6] K. Luo, J. Di, P.P. Han, S.H. Zhang, L.H. Xia, G.M. Tian, et al., Transcriptome analysis of the critically endangered Dabry's sturgeon (*Acipenser dabryanus*) head kidney response to *Aeromonas hydrophila*, Fish Shellfish Immunol. 83 (2018) 249–261.
[7] K. Luo, S.H. Zhang, D.D. Tang, L.H. Xia, W.H. Gao, G.M. Tian, et al., Analysis of the expression patterns of the cytokine receptor family B (CRFB) and interferon gamma receptor (IFNGR) in Dabry's sturgeon (*Acipenser dabryanus*), Dev. Comp. Immunol. 84 (2018) 1–7.
[8] Q.Q. Xu, K. Luo, S.H. Zhang, W.H. Gao, W.B. Zhang, Q.W. Wei, Sequence analysis and characterization of type I interferon and type II interferon from the critically endangered sturgeon species, *A. dabryanus* and *A. sinensis*, Fish Shellfish Immunol. 84 (2019) 390–403.
[9] J. Di, S.H. Zhang, J. Huang, H. Du, Y. Zhou, Q. Zhou, et al., Isolation and identification of pathogens causing hemorrhagic septicemia in cultured Chinese sturgeon (*Acipenser sinensis*), Aquacult. Res. 49 (2018) 3624–3633.
[10] S.H. Zhang, J. Huang, J. Di, H. Du, Q. Zhou, Q.Q. Xu, et al., The genome sequence of a new strain of *Mycobacterium ulcerans* ecovar Liflandii, emerging as a sturgeon pathogen, Aquaculture 489 (2018) 141–147.
[11] Z.Z. Qi, X.H. Zhang, N. Boon, P. Bossier, Probiotics in aquaculture of China—current state, problems and prospect, Aquaculture 290 (2009) 15–21.
[12] A.N. Wang, C. Ran, Y.B. Wang, Z. Zhang, Q.W. Ding, Y.L. Yang, et al., Use of probiotics in aquaculture of China—a review of the past decade, Fish Shellfish Immunol. 86 (2019) 734–755.
[13] S.H. Hoseinifar, E. Ringø, M.A. Shenavar, E.M. Angeles, Probiotic, prebiotic and synbiotic supplements in sturgeon aquaculture: a review, Rev. Aquac. 8 (2016) 89–102.
[14] R.M. Reda, M.A. El-Hady, K.M. Selim, H.M. El-Sayed, Comparative study of three predominant gut Bacillus strains and a commercial *B. amyloliquefaciens* as probiotics on the performance of *Clarias gariepinus*, Fish Shellfish Immunol. 80 (2018) 416–425.
[15] Y. Cai, W. Yuan, S.F. Wang, W.L. Guo, A. Li, Y. Wu, et al., In *vitro* screening of putative probiotics and their dual beneficial effects: to white shrimp (*Litopenaeus vannamei*) postlarvae and to the rearing water, Aquaculture 498 (2019) 61–71.
[16] Y.L. Yi, Z.H. Zhang, F. Zhao, H. Liu, L.J. Yu, J.W. Zha, et al., Probiotic potential of *Bacillus velezensis* JW: antimicrobial activity against fish pathogenic bacteria and immune enhancement effects on *Carassius auratus*, Fish Shellfish Immunol. 78 (2018) 322–330.
[17] X. Guo, D.D. Chen, K.S. Peng, Z.W. Cui, X.J. Zhang, S. Li, et al., Identification and characterization of *Bacillus subtilis* from grass carp (*Ctenopharynodon idellus*) for use as probiotic additives in aquatic feed, Fish Shellfish Immunol. 52 (2016) 74–84.

[18] A.I. Zaineldin, S. Hegazi, S. Koshio, M. Ishikawa, A. Bakr, A.M.S. El-Keredy, et al., *Bacillus subtilis* as probiotic candidate for red sea bream: growth performance, oxidative status, and immune response traits, Fish Shellfish Immunol. 79 (2018) 303–312.

[19] H.T. Liu, S.F. Wang, Y. Cai, X.H. Guo, Z.J. Cao, Y.Z. Zhang, et al., Dietary administration of *Bacillus subtilis* HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, *Oreochromis niloticus*, Fish Shellfish Immunol. 60 (2016) 326–333.

[20] A. Newajfyzul, A.H. Alharbi, B. Austin, Review: developments in the use of probiotics for disease control in aquaculture, Aquaculture 431 (2014) 1–11.

[21] X. Liu, Y. Zhang, Q.I. Qian, M. Zhao, L. Mai, Effects of *Bacillus subtilis* on growth, digestive enzyme activity, and non-specific immunity in hybrid sturgeon (*Acipenser baeri* ♂ × *Acipenser schrenkii* ♀) juveniles, J. Fish. Sci. China 18 (2011) 1315–1320.

[22] J.H. Brown, Bergey's Manual of Determinative Bacteriology, fifth ed., American Journal of Public Health and the Nations Health, 1939.

[23] S.T. Cowan, K.J. Steel, Manual for the identification of medical bacteria, Proceedings of the Royal Society of Medicine, 1966.

[24] S.M. Aljanabi, I. Martinez, Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques, Nucleic Acids Res. 25 (1997) 4692–4693.

[25] W.G. Weisburg, S.M. Barns, D.A. Pelletier, D.J. Lane, 16S ribosomal DNA amplification for phylogenetic study, J. Bacteriol. 173 (1991) 697–703.

[26] R. Chao, A. Carrias, M.A. Williams, N. Capps, B.C.T. Dan, J.C. Newton, et al., Identification of *Bacillus* strains for biological control of catfish pathogens, PLoS One 7 (2012) e45793.

[27] S. Saha, R.N. Roy, S.K. Sen, A.K. Ray, Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella* (Valenciennes), Aquacult. Res. 37 (2010) 380–388.

[28] K.Y. Leyva-Madrigal, A. Luna-González, C.M. Escobedo-Bonilla, J.A. Fierro-Coronado, I.E. Maldonado-Mendoza, Screening for potential probiotic bacteria to reduce prevalence of WSSV and IHHNV in whiteleg shrimp (*Litopenaeus vannamei*) under experimental conditions, Aquaculture 322 (2011) 16–22.

[29] D. Ramesh, A. Vinothkanna, A.K. Rai, V.S. Vignesh, Isolation of potential probiotic *Bacillus* spp. and assessment of their subcellular components to induce immune responses in Labeo rohita against *Aeromonas hydrophila*, Fish Shellfish Immunol. 45 (2015) 268–276.

[30] H.T.T. Thy, N.N. Tri, O.M. Quy, K. Kannika, S. Unajak, N. Areechon, Effects of the dietary supplementation of mixed probiotic spores of *Bacillus amyloliquefaciens* 54A, and *Bacillus pumilus* 47B on growth, innate immunity and stress responses of striped catfish (*Pangasianodon hypophthalmus*), Fish Shellfish Immunol. 60 (2016) 391–399.

[31] G. Zahra, S. Caroline, R. Eugene, D.M. Luc, C.M. Courtin, J.A. Delcour, et al., Effects of dietary arabinoxylan-oligosaccharides (AXOS) and endogenous probiotics on the growth performance, non-specific immunity and gut microbiota of juvenile Siberian sturgeon (*Acipenser baerii*), Fish Shellfish Immunol. 35 (2013) 766–775.

[32] M.M. Bradford, A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[33] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[34] A. Fečkaninová, J. Koščová, D. Mudroňová, P. Popelka, J. Toropilová, The use of probiotic bacteria against *Aeromonas* infections in salmonid aquaculture, Aquaculture 469 (2017) 1–8.

[35] A.O.D. Mahmoud, K. Shunsuke, M.A. Mohamed, V.D. Hien, Probiotic application for sustainable aquaculture, Rev. Aquac. 10 (2018) 1–18.

[36] Y. Zhao, W. Zhang, W. Xu, K. Mai, Y. Zhang, F.Z. Liu, Effects of potential probiotic *Bacillus subtilis* T13 on growth, immunity and disease resistance against *Vibrio splendidus* infection in juvenile sea cucumber *Apostichopus japonicus*, Fish Shellfish Immunol. 32 (2012) 750–755.

[37] S.K. Nayak, Probiotics and immunity: a fish perspective, Fish Shellfish Immunol. 29 (2010) 2–14.

[38] E.D. Abarike, J. Cai, Y.S. Lu, H. Yu, L. Chen, J. Jian, et al., Effects of a commercial probiotic BS containing *Bacillus subtilis* and *Bacillus licheniformis* on growth, immune response and disease resistance in Nile tilapia, *Oreochromis niloticus*, Fish Shellfish Immunol. 82 (2018) 229–238.

[39] Y. Duan, Y. Zhang, H. Dong, Y. Wang, J. Zhang, Effect of the dietary probiotic *Clostridium butyricum* on growth, intestine antioxidant capacity and resistance to high temperature stress in kuruma shrimp *Marsupenaeus japonicus*, J. Therm. Biol. 66 (2017) 93–100.

[40] L.N. Tang, K. Huang, J. Xie, D. Yu, L. Sun, Q. Huang, et al., 1-Deoxynojirimycin from *Bacillus subtilis* improves antioxidant and antibacterial activities of juvenile Yoshitomi tilapia, Electron. J. Biotechnol. 30 (2017) 39–47.

[41] L. Gao, B.K. Wang, X.Q. Mei, H. Xu, Y. Qin, W.F. Li, et al., Effects of three probiotic Bacillus on growth performance, digestive enzyme, antioxidative capacity, serum immunity, and biochemical parameters in broilers, Anim. Sci. J. 89 (2018) 1561–1571.

[42] C. Li, Y. Zhao, T. Liu, J. Huang, Q. Zhang, B. Liu, et al., The distribution and function characterization of the i type lysozyme from *Apostichopus japonicus*, Fish Shellfish Immunol. 74 (2018) 419–425.

[43] D. Ramesh, S. Souissi, Effects of potential probiotic *Bacillus subtilis* KADR1 and its subcellular components on immune responses and disease resistance in Labeo rohita, Aquacult. Res. 49 (2017) 1–11.

[44] D.L. Merrifield, A. Dimitroglou, G. Bradley, R.T.M. Baker, S.J. Davies, Probiotic applications for rainbow trout (*Oncorhynchus mykiss* Walbaum): I. Effects on growth performance, feed utilization, intestinal microbiota and related health criteria, Aquacult. Nutr. 16 (2010) 504–510.

[45] 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 (2013) 50–57.

[46] X.M. Li, R. Einar, H.H. Seyed, L.L. Helence, B. Harry, D.G. Yang, The adherence and colonization of microorganisms in fish gastrointestinal tract, Rev. Aquac. (2018), https://doi.org/10.1111/raq.12248 1–16.

[47] Y.Z. Sun, H.L. Yang, R.L. Ma, W.Y. Lin, Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*, Fish Shellfish Immunol. 29 (2010) 803–809.

[48] M. Choudhury, K.P. Prasad, Isolation and characterization of immunoglobulin M of Asian sea bass, *lates calcarifer* and its level in serum, Cent. Eur. J. Biol. 6 (2011) 1064–1064.

[49] R. Cerezuela, F.A. Guardiola, P. González, J. Meseguer, M.Á. Esteban, Effects of dietary *Bacillus subtilis, Tetraselmis chuii*, and *Phaeodactylum tricornutum*, singularly or in combination, on the immune response and disease resistance of sea bream (*Sparus aurata L.*), Fish Shellfish Immunol. 33 (2012) 342–349.

[50] I. Salinas, L. Abelli, F. Bertoni, S. Picchietti, A. Roque, D. Furones, et al., Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.), Fish Shellfish Immunol. 25 (2008) 114–123.

[51] J. Wang, H.L. Yang, H.Q. Xia, J.D. Ye, K.L. Lu, X. Hu, et al., Supplementation of heat-inactivated *Bacillus clausii* DE5 in diets for grouper, *Epinephelus coioides*, improves feed utilization, intestinal and systemic immune responses and not growth performance, Aquacult. Nutr. 24 (2018) 821–831.

[52] N. Ibnou-Zekri, S. Blum, J.S. Eduardo, T.V.D. Weid, Divergent patterns of colonization and immune response elicited from two intestinal *Lactobacillus* strains that display similar properties *in vitro*, Infect. Immun. 71 (2003) 428–436.

[53] S.M. Aly, A.G. Ahmed, A.A. Ghareeb, M.F. Mohamed, Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections, Fish Shellfish Immunol. 25 (2008) 128–136.

[54] H. Zokaeifar, J.L. Balcázar, C.R. Saad, M.S. Kamarudin, K. Sijam, A. Arshad, et al., Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp, *Litopenaeus vannamei*, Fish Shellfish Immunol. 33 (2012) 683–689.