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Dietary administration of probiotic *Paenibacillus ehimensis* NPUST1 with bacteriocin-like activity improves growth performance and immunity against *Aeromonas hydrophila* and *Streptococcus iniae* in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Bacteria-induced diseases are a major cause of mortality in aquaculture. Probiotics have commonly been used to replace antibiotics for prophylactic biocontrol in aquaculture. In the present study, *Paenibacillus ehimensis* NPUST1 was isolated from a tilapia culture pond. This probiotic has bacteriocin-like activities against *Aeromonas hydrophila* and was characterized by biochemical analysis and 16S rDNA sequencing. The physiochemical properties of a crude extract of the bacteriocin-like substance revealed low pH and high thermal tolerance. The substance exhibited broad-spectrum antimicrobial activity against diverse aquatic pathogens, food spoilage, clinical pathogens, and plant pathogens. The effect of dietary supplementation with *P. ehimensis* NPUST1 was evaluated in regard to the growth of Nile tilapia (*Oreochromis niloticus*) and immunity against pathogenic infection. The results showed significantly increased weight gain (WG), feed conversion ratio (FCR), and feed efficiency (FE) in Nile tilapia fed *P. ehimensis* NPUST1 for 2 months compared with fish fed a control diet. When challenged with *A. hydrophila* and *S. iniae,* the fish fed *P. ehimensis* NPUST1 also exhibited a higher survival rate than fish fed the control diet. The immune parameters revealed that the *P. ehimensis* NPUST1-fed fish had significantly higher phagocytic activity, respiratory burst, and superoxide dismutase (SOD) of the head kidney leukocytes, as well as higher serum lysozyme activity and expression of cytokines TNF-α and IL-1β than the fish fed the control diet. These results indicate that dietary supplementation with *P. ehimensis* NPUST1 improved the growth performance, immunity, and disease resistance in Nile tilapia.

1. Introduction

Global aquaculture has made significant contributions to the global economy, and there is increasing demand for seafood worldwide. Aquaculture now accounts for more than 50% of the world's food fish and has been responsible for impressive growth in the supply of fish for human consumption. Tilapia is the most popular aquaculture fish species and is farmed in over 100 countries because of its fast growth, suitability for aquaculture, and high marketability. However, intensive culture of tilapia has caused serious stress to culture environments and increased the incidence of infectious diseases, especially bacteria-induced diseases. This has resulted in high mortality of culture fishes and severe economic loss. For example, tilapia infected with *Aeromonas hydrophilia* and *Streptococcus iniae* have clinical symptoms of hemorrhagic septicemia, such as loss of appetite, red discoloration at the anus and base of the fins, and hemorrhagic eyes, gills, internal organs, and muscle [1,2]. There is widespread use of antibiotics, antimicrobials, and disinfectant agents for prophylactic and therapeutic purposes in fish farming, which has led to the emergence of antibiotic-resistant pathogens, residual antibiotics in aquatic products, and alteration of the microbiota in aquaculture environments. These issues have led to an increasing number of investigators making efforts to develop alternative strategies for disease control.

Probiotics potentially offer an alternative approach to control

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disease in aquaculture [3]. Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amount. Gatesoupe reported the first use of probiotics in aquaculture in 1999, and studies on the applications of probiotics in aquaculture have increased rapidly [4]. A variety of Gram-positive and Gram-negative probiotics have been applied in aquaculture to contribute multiple benefits to the host, including providing digestive enzymes to increase feed efficiency, metabolism, and growth. They also improve the beneficial microflora in the GI tract and enhance the host's innate immunity against infections [5]. However, probiotics improve intestinal microbiota and booster up defense mechanism in diverse ways and the effects are thought to be species specific or even strain-specific. Thus, development of different probiotics are required for diverse aquatic animals in aquaculture.

Species of *Paenibacillus* were originally included in the genus *Bacillus* due to their common morphological and physiological characteristics with the type species *Bacillus subtilis.* In 1993, *Paenibacillus* was reassigned as a new genus based on phylogenetic analysis according to 16S rRNA gene sequences. The genus *Paenibacillus* has been isolated from a variety of environments that are relevant to humans, animals, and plants. Many species of *Paenbacillus* produce antimicrobial peptides or proteins called bacteriocins, which are useful in medicine or as pesticides in biocontrol [6]. Bacteriocins are ribosomally synthesized bioactive antimicrobial peptides or proteins that are produced by a variety of microorganisms. They have been suggested to replace broad-spectrum classical antibiotics due to their advantageous properties including low toxicity, immunomodulation of host immunity, the possibility of *in situ* production by probiotics, and the fact that these peptides can be bioengineered [7]. Recently, the bacteriocin-producing *Paenbacillus* has come into use as microbial agent in plant industry. Bosmans et al. reported that the application of the bacteriocin-producing *Paenbacillus* clade significantly reduced hairy root disease (HRD) in the hydroponic cultivation of tomato [8]. Treatment with bacteriocin-producing *P. polymyxa* promoted watermelon growth and induced host-defense responses against Fusarium wilt in cucumber [9,10]. These studies show that bacteriocins-producing *Paenbacillus* have the potential to be used as effective probiotics against plant pathogenic pathogens for disease control. However, there are few investigations on the application of bacteriocin-producing *Paenbacillus* species against aquatic pathogens in aquaculture.

In addition to bacteriocin, many species also yield hydrolytic enzymes that could potentially be applied in agriculture. The cell walls of fungi comprise large fractions of β-1, 3-glucan, chitin, cellulose, and protein. Several *Paenbacillus* species have been found to produce glucanase, chitinase, cellulase, and protease with the ability to destroy fungal cell walls. For example, chitinase, amylase, and protease from a strain of *P. polymyxa* inhibited mycelial growth and reduced anthracnose caused by *Colletotrichum gloeosporioides* and *C. acutatum* [11,12]. Hydrolytic enzymes produced by probiotics are also widely used as additives to enhance nutritional digestion and increase feed efficiency in aquaculture. For example, wild shrimp (*Penaeus monodon*) were fed a diet containing extracellular enzymes (amylase, protease, lipase, and phytase) produced by *B. cereus*, which effectively improved feed efficiency and enhanced growth performance [13]. In South African abalone (*Haliotis midae*), protease-producing *Vibrio midae* SY elevated the levels of digestive enzymes and thus enhanced feed digestion [14]. Reports have shown that *Paenbacillus geneus* is capable of producing diverse extracellular enzymes, but there are few investigations on the effects of extracellular enzyme-producing *Paenbacillus* on growth and feed utilization [15].

In the present study, *Paenibacillus ehimensis* NPUST1 was isolated-from tilapia culture pond as a potential probiotic with bacteriocin-like activity and the ability to produce amylase, cellulase, and xylanase. The physiochemical properties and antagonistic ability of the bacteriocin-like substance against a variety of pathogens were determined. The effects of dietary supplementation with *P. ehimensis* NPUST1 on the growth performance, feed efficiency, immune immunity, and survival rate were evaluated in Nile tilapia after challenge with *A. hydrophila* and *S. iniae*. Furthermore, the effect of *P. ehimensis* NPUST1 on the immune status of tilapia was evaluated by determining immune parameters including the respiratory burst activity, phagocytic activity, and superoxide dismutase (SOD) activity of head kidney leukocytes, as well as serum lysozyme activity and expression of cytokines TNF-α and IL-1β. These assessments were used to evaluate the usefulness of *P. ehimensis* NPUST1 as a probiotic in aquaculture.

2. Materials and methods

2.1. Fish, bacteria, and culture system

Nile tilapia (*Oreochromis niloticus*) were obtained from a domestic fish farm and transferred to circulating aerated water at 28 °C in the Aquatic Laboratory Animal Facility, which is certificated by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All fish were handled in compliance with local welfare regulations. The pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) was kindly provided by Dr. Jyh-Yih Chen [16], and *Streptococcus iniae* was kindly provided by Dr. Hong-Yi Gong [17]. *Burkholderia gladioli* BCRC 13899 was purchased from the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Taiwan. Other pathogens were obtained as described in previous reports [18,19]. All bacteria were cultured in tryptic soy broth (TSB) medium at individual optimal temperatures overnight and then stored in 20% glycerol at 20 °C until use.

2.2. Screening of potential probiotics producing bacteriocin-like substances

Water samples from local tilapia culture pools were obtained and serially diluted with sterile water. One milliliter of each diluted suspension was sampled and spread onto the surface of De Man, Rogosa, and Sharpe (MRS) agar media. The plates were then incubated under aerobic conditions at 28 °C for 48 h. After incubation, each colony was spotted on TSB agar, which had previously been inoculated with indicator strain *Aeromonas hydrophila* at a level of approximately 1.0×10^6 CFU/ml. The appearance of clear zones after incubation overnight at 28 °C indicated that the isolate produced antimicrobial substances. Based on the antimicrobial efficacy performance, one of these isolates, NPUST1, was selected to conduct the subsequent antagonistic experiments with other pathogens. The NPUST1 isolate was identified as *Paenibacillus ehimensis* based on biochemical analysis and 16S rDNA gene sequencing performed by the BCRC.

2.3. Antimicrobial spectrum and physiochemical properties of bacteriocin-like substance

A 0.5-mL aliquot of *P. ehimensis* NPUST1 from the glycerol stock was cultured in 50 ml of TSB (Difco, USA) in an incubation shaker at 28 °C and 175 rpm for 16 h as the seed culture. Next, 1 ml of seed culture was inoculated into a 500-mL flask containing 100 mL of TBS medium and cultured at 28 °C and 175 rpm. A 1-ml sample of culture medium was obtained to measure bacterial growth by determining the optical density (OD) at 600 nm. The culture medium was centrifuged at 13,600 × *g* at 4 °C for 15 min, and then the cell-free supernatant was used to determine the protein concentration and antimicrobial activity to evaluate the production profile of the antimicrobial substance during the growth of *P. ehimensis* NPUST1. The protein concentration was measured using Bradford's method [20]. The antimicrobial activity was determined by a modified resazurin assay using sterile 96-well plates. Briefly, 100 μl of reaction mixture per well composed of 10 μl of *E. coli* (10^7 CFU/ml), 67 μl of TSB medium, 13 μl of resazurin solution, and 10 μl of various dilutions of cell-free supernatant was cultured at 28 °C for 12 h. To quantify the antimicrobial activity, the arbitrary unit (AU) of antimicrobial activity was defined as the lowest amount of cell-free supernatant that prevented a color change in the test well [21].

The bactericidal activity of the antimicrobial substance against diverse pathogens was determined by agar diffusion assay. The cell-free supernatant of the TBS medium was cultured at 28 °C and 175 rpm for 96 h and then separated by centrifuging at 1700 × *g* for 15 min. The antimicrobial substance in the cell-free supernatant was precipitated by adding ammonium sulfate to a final saturation of 60%, and the mixture was kept at 4 °C for 16 h. After centrifugation at 13,600 × *g* for 15 min, the pellet was suspended in 1 ml of phosphate buffered saline (PBS; pH 7.0) and dialyzed against PBS buffer overnight at 4 °C. The dialyzed solution was considered as a purified bacteriocin-like substance and was sterilized by filtration through a 0.22-μm syringe filter (Millipore, Bedford, MA, USA). Plates were prepared with TSB agar seeded with an overnight culture of the indicator strain *A. hydrophila* at a level of approximately 1.0×10^6 CFU/ml. Next, 8-mm filter paper discs (Tokyo Roshi Kaisha, Ltd., Japan) were placed on the surface of the TSB agar plate, and 20 μl of purified bacteriocin-like substance was loaded onto the paper discs. The diameter of the inhibitory zone around the filter paper was measured after incubation for 24 h. A filter paper filled with 20 μl of sterile water was used as control. To determine the thermal stability of the purified bacteriocin-like substance, it was exposed to 30, 40, 50, 60, 70, 80, 90, or 100 °C for 1 h or to 121 °C for 15 min in an autoclave, and then the residual antimicrobial activity was measured. The antimicrobial activity of the purified bacteriocin-like substance stored at 4 °C was used as a control. To determine the pH sensitivity of the substance, it was suspended in different pH buffers (pH 2–11). After 4 h of incubation at 28 °C, the residual antimicrobial activity was determined. The antimicrobial activity of the purified bacteriocin-like substance suspended in PBS buffer (pH 7.0) was used as a control. To determine the stability of the substance during storage, samples were stored at −20, 4, 25, and 40 °C for 70 days. Samples were taken from the stored material at 2-week intervals to determine the antimicrobial activity.

2.4. Biosafety evaluation of isolated P. ehimensis NPUST1

Juvenile Nile tilapia (*O. niloticus*) with an average weight of 5.23 ± 0.8 g were acclimatized in circulating aerated water at 28 °C for 7 days. A total of 90 fish were divided into three groups with three replicates for each group. *P. ehimensis* NPUST1 was grown in TSB broth for 24 h at 28 °C and centrifuged at 1700 × *g* and 4 °C for 15 min. The cell pellets were then suspended in an appropriate volume of sterile water. Experimental fish (10 fish per tank) were injected intraperitoneally with 50 μl of the bacteria diluted at concentrations of 1×10^6, 1×10^7, and 1×10^8 CFU per fish and kept in 60-L aquaria. The biosafety of *P. ehimensis* NPUST1 was evaluated by observing the health status for 14 days and recording mortality every day.

2.5. Diet preparation and experimental design

Juvenile Nile tilapia (*O. niloticus*) with an average weight of 5.53 ± 0.45 g were randomly divided into three groups (C, G1, and G2). Each group contained 20 fish, and experiments were performed in triplicate. The control group (C) was fed a basal diet with the following formulation (w/w): 10% fish meal, 47% soybean meal, 18% gluten, 5.4% corn starch, 5.7% canola oil, 1% carboxymethyl cellulose (CMC), 10% α-starch, 1.9% mineral mixture, and 1% vitamin mixture. The basal diet was approximately 39% crude protein and 8% crude lipid. The probiotic *P. ehimensis* NPUST1 was added to the basal diet at levels of 1×10^6 CFU/g (G2) and 1×10^7 CFU/g (G3). The experimental diet was prepared as described previously [22] and stored at 4 °C. The fish were fed twice daily at 5% of the body weight. During the feeding trial, the fish were weighed once every two weeks, and the amount of diet provided was adjusted accordingly. The tanks were cleaned by siphoning the water daily, and uneaten food was collected 1 h after each feeding to determine feed intake. After 2 months of cultivation, the growth-performance parameters and non-specific immune parameters of the fish were determined.

2.6. Growth performance analysis

The fish were weighed every two weeks to assess growth performance. The weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), feed efficiency (FE), and survival rate (SR) were calculated using the following equations: WG = final body weight - initial body weight, FE = (final body weight-initial body weight) x $(\text{feed intake})^{-1}$, FCR = (total food intake) x $(\text{weight gain})^{-1}$, SR = 100 x (final number of tested fish)/(initial number of tested fish).

2.7. Non-specific immune analysis

Six fish were sampled from the control, G1, and G2 groups after 2 months of feeding. Blood samples were collected from the caudal vasculature using a 5-mL syringe, and serum was then collected by centrifugation at 1500 × *g* and 4 °C for 10 min for a lysozyme activity assay. The head kidneys of the fish were excised, and the leucocytes were harvested. The harvested cells were adjusted to 10^5 cells/ml for assays of the phagocytic activity, respiratory burst, and SOD activity. The detailed procedures for collecting leucocytes from the head kidney and conducting these assays were described previously [22].

2.8. Pathogen challenge test

Pathogens *A. hydrophila* and *S. iniae* were cultured individually in TSB and incubated at 28 °C for 24 h. The cells were harvested by centrifugation at 6100 × *g* for 15 min at 4 °C and then resuspended in an appropriate volume of distilled water. The 7 days lethal dose of 50 (LD_{50}) was determined by intraperitoneal (i.p.) injection of grade doses of *A. hydrophila* and *S. iniae* respectively (10^5, 10^6, 10^7, 10^8 CFU/fish) into 10 fish. The bacterial challenge test was conducted in triplicate by intraperitoneally injecting 20 μL of diluted *A. hydrophila* and *S. iniae* into the fish at LD_{50} concentrations of 1.0×10^6 CFU and 1.0×10^5 per fish, respectively. Experimental fish (10 fish per tank) were kept in a 60-L tank containing 40 L of fresh water at 28 ± 1 °C. Fish fed the control diet and injected with saline were used as a negative control. Each group was tested in triplicate. The challenged fish were observed daily, and all mortalities were recorded for 7 days.

2.9. RNA extraction and real-time quantitative PCR

Total RNA was extracted from the head kidneys of tilapia at the end of the probiotic administration using TriPure isolation reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. Approximately 1 μg of total RNA from each of the tissue samples was reverse transcribed to produce cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) according to the manufacturer's instructions. The expression levels of tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and β-actin were determined using quantitative PCR. The expression of β-actin was used as internal control. The specific primers used for detecting each gene are listed in Table 1. The quantitative PCR was performed by Applied Biosystem StepOnePlus Real-Time PCR system with a KAPA SYBR FAST qPCR Kit (KAPA KR0389, MA, USA). The cycling profile was as follows: 60 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s, annealing, and primer extension at 60 °C for 1 min. Equal quantities of the total RNA from six fish in different group were examined in triplicate. The relative expression levels of each group were normalized to β-actin and are expressed as the mean ± standard error (SE).

Table 1
Primer sequences and names of genes used in this study.

Gene name	Primer sequence (5′-3′)	PCR size (bp)	Accession No.
TNF-α	F:CCAGAAGCACTAAAGGCGAAGA R:CCTTGGCTTTGCTGCTGATC	82	AY428948.1
IL-1β	F:TGGTGACTCTCCTGGTCTGA R:GCACAACTTTATCGGCTTCCA	86	XM_005457887.1
β-actin	F:TGACCTCAGACTACCTCATG R:TGATGTCACGCACGATTTCC	89	KJI26772.1

2.10. Statistical analyses

A one-way ANVOA and Tukey's multiple comparison tests were used to determine the significant differences ($P < 0.05$) in the relative gene expression level and immune parameters between the control and experimental groups. For the results from the challenged test, the cumulative survival were analyzed by Kaplan-Meier method. The data were analyzed using SAS software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Preliminary screening of putative probiotics with antimicrobial activity against Aermonas hydrophila

Five water samples from distinct culture pools of tilapia were obtained, and then six bacterial clones were randomly selected from 10^{-2}-10^{-5} dilutions of each sample. A total of 30 bacterial isolates were cultured and then screened for antagonistic activity against *A. hydrophila*. Only one isolate had strong antimicrobial activity with a diameter of the inhibition zone greater than 10 mm. The isolate was genotypically identified using the 16S rDNA gene sequence. A partial 16S rDNA sequence of 1431 bp was obtained and analyzed by BLAST against known bacterial 16S rDNA sequences from the NCBI GenBank database. The results revealed that the isolate had a similarity of 99% to *Paenibacillus ehimensis* IFO 15659. Consequently, the isolate was named as *Paenibacillus ehimensis* NPUST1. The partial 16S rDNA sequence of *P. ehimensis* NPUST1 was registered at GenBank with accession number MF692769. The biochemical characteristics of Gram-positive endospore-forming *P. ehimensis* NPUST1 are shown in Table 2.

3.2. The antimicrobial activity and physiochemical properties of bacteriocin-like substance from P. ehimensis NPUST1

The growth curve of *P. ehimensis* NPUST1 in a flask revealed significant increases in the protein concentration accompanied by antimicrobial activity in TSB medium after 40 h of cultivation (Fig. 1A). This result suggested that the bacteriocin-like substance produced by *P. ehimensis* NPUST1 occurred in the post-stationary phase when bacterial cells are no longer growing. The bacteriocin-like substance from the cell-free supernatant was partially purified by ammonium sulfate precipitation, after which its bactericidal potency against *A. hydrophila* was significantly increased (Supplementary Fig. 1). The physiochemical properties of partially purified bacteriocin-like substance were determined by diffusion assay against *A. hydrophila,* including pH susceptibility, thermal susceptibility, and thermal stability. Although the antimicrobial activity of the substance was slightly reduced at pH 2 (reduced 12%), it maintained stable antimicrobial activity in the range of pH 3 to 10 (Fig. 1C). The substance also revealed a high thermal tolerance, and the antimicrobial activity did not decrease even at temperatures of 100–121 °C (Fig. 1D). The thermal stability of the bacteriocin-like substance at different temperatures decreased gradually with the storage time. The antimicrobial activity remained 71.8% and 78.6% when the substance was maintained at −20 °C and 4 °C for 70 days, respectively. The antimicrobial activity of the substance remained at 53.2% at room temperature (25 °C) for 70 days. The antimicrobial activity dramatically decreased to 30% when the substance was maintained at 40 °C for two weeks, and the antimicrobial activity was mostly lost after 70 days of storage (Fig. 1B). The partially purified bacteriocin-like substance revealed antimicrobial activity against aquatic pathogens *A. hydrophilia*, *S. agalactiae*, *V. vulnificus*, *D. hansenii* and food spoilage bacteria *S. aureus*, *S. typhimurium* and *L. monocytogenes*. It also showed activity against clinical pathogen *P. aeruginosa* and plant pathogen *B. gladioli*. Furthermore, the substance also revealed antimicrobial potency against antibiotic-resistant pathogens *V. alginolyticus*, *V. parahaemolyticus,* and MRSA (Table 3).

Table 2
Morphology and biochemical characteristics of *P. ehimensis* NPUST.

Test item	Result	Test item	Result
Gram staining	Positive	Anaerobic growth	–
Cell morphology	Rod	Nitrate reductase	+
Spore-formation	+	Starch hydrolysis	+
Motility	+	Casein hydrolysis	–
Catalase	+	Cellulose hydrolysis	+
Oxidase	+	Xylan hydrolysis	+

3.3. Dietary supplementation of P. ehimensis NPUST1 enhanced growth performance

Before evaluating the effect of *P. ehimensis* NPUST1 on tilapia growth, different dosages of *P. ehimensis NPUST1* were intraperitoneally injected into tilapia to confirm their biosafety. Even at high injected concentrations of 10^8 CFU per fish, no pathological symptoms, disease, or mortalities occurred during a period of 14 days post-injection, indicating *P. ehimensis NPUST1* is harmless for the fish (Supplementary Fig. 2). To determine whether *P. ehimensis* NPUST1 can effectively enhance nutrient utilization and growth performance, the parameters WG, FCR, and FE were evaluated after 2 months of dietary supplementation with *P. ehimensis* NPUST1, as shown in Table 4. The growth performance showed WG of 7.5 ± 1.85 g and 50.0 ± 0.48 g for the tilapia fed the basal diets containing 10^6 and 10^7 CFU/g of *P. ehimensis* NPUST1, respectively, which were significantly higher than that of fish fed the basal diet (29.6 ± 0.46 g). FCR and FE of tilapia supplemented with *P. ehimensis* NPUST1 were markedly higher to those of the control fish. There were no significant differences in WG, FCR, and FE between fish fed with 10^6 and 10^7 CFU/g of *P. ehimensis* NPUST1. These results suggest that dietary supplementation with this probiotic could improve nutrition utilization and enhance growth performance.

3.4. Dietary supplementation with P. ehimensis NPUST1 increased the survival rate of tilapia challenged with A. hydrophilia and S. iniae

The effect of the probiotic on the disease resistance against bacterial infection was determined by measuring the survival rate of tilapia fed the basal diet containing 10^6 and 10^7 CFU/g of *P. ehimensis* NPUST1 after challenging with *A. hydrophilia* and *S. iniae*. As shown in Fig. 4, the survival rate at 7 days post-infection was 100% for the group injected with PBS buffer. However, the survival rate in the control group injected with *S. iniae* was dramatically reduced during the first 2 days post-infection and then maintained at 17.8 ± 7.7% until 7 days post-infection. Notably, at the end of the 7-day experiment, the survival rates were 51 ± 8.6% and 50 ± 8.6% in tilapia fed 10^6 and 10^7 CFU/g of *P. ehimensis* NPUST1, respectively. These survival rates were significantly higher than that of fish fed the basal diet. This suggests that dietary supplementation with *P. ehimensis* NPUST1 enhances the resistance against *S. iniae* infection in tilapia (Fig. 2). The survival rates were not significantly different between fish given 10^6 and 10^7 CFU/g, suggesting that the dosage of 10^6 CFU/g is sufficient to obtain the function of disease resistance.

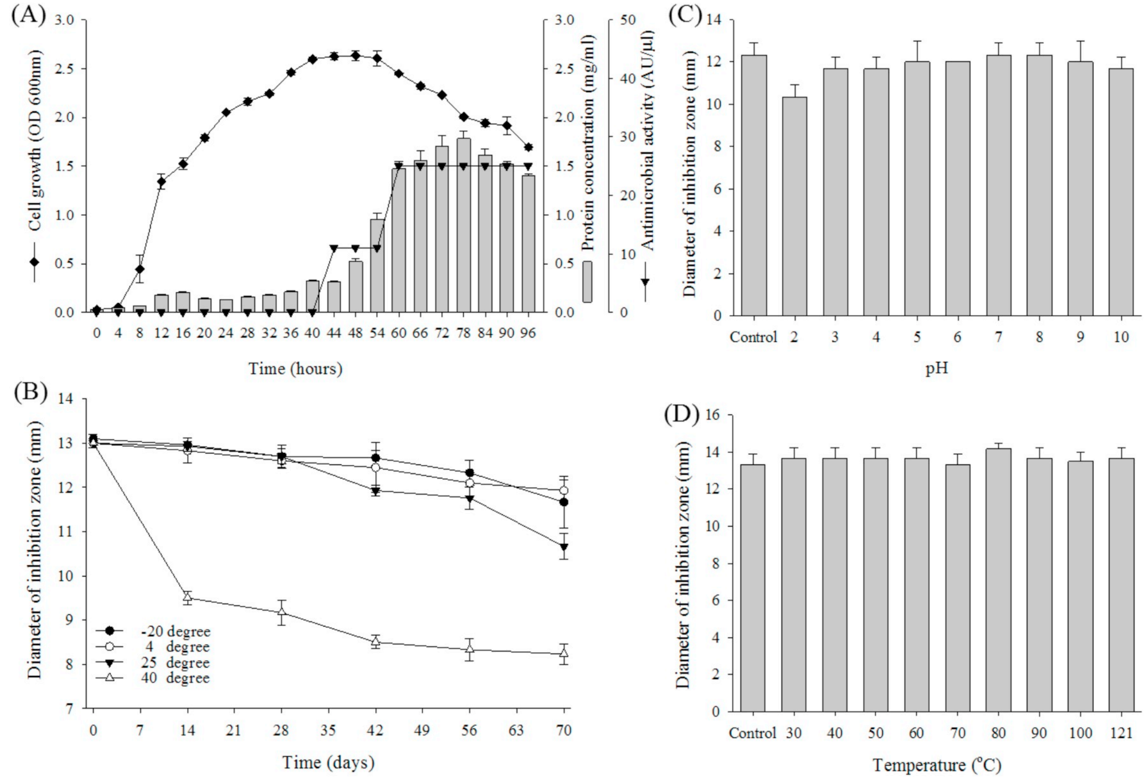

Fig. 1. Physiochemical properties of bacteriocin-like substance produced from *P. ehimensis* NPUST1. (A) Bacterial growth, protein concentration, and antimicrobial activity were monitored during growth in TSB at 28 °C. (B) The antimicrobial activity of bacteriocin-like substance at pH 2–10 for 4 h. (C) Preservation of the residual antimicrobial activity of bacteriocin-like substance at −20, 4, 25, and 40 °C for 70 days. (D) Antimicrobial activity of partially purified antimicrobial substance at different temperatures for 1 h. Each of the data represents the mean of triplicate experiments.

3.5. Effects of *P. ehimensis* NPUST1 supplementation on immune parameters

The significantly increased survival rates led us to investigate the effects of *P. ehimensis* NPUST1 on fish immunity further. The phagocytic activity (PA) of head kidney leukocytes in fish supplemented with *P. ehimensis* NPUST1 was significantly increased compared with that of fish fed the basal diet. However, no significant differences in PA were observed between that tilapia supplemented with 10^6 and 10^7 CFU/g of *P. ehimensis* NPUST1. Compared with fish fed the basal diet, the relative PAs of fish supplemented with 10^6 and 10^7 CFU/g of *P. ehimensis* NPUST1 for 2 months increased by 1.9-fold and 1.8-fold, respectively (Fig. 3A). The respiratory burst activity in fish fed 10^6 and 10^7 CFU/g of *P. ehimensis* NPUST1 was also significantly higher (5-fold and 9.7-fold at 2 months, respectively) than that of fish fed the basal diet (Fig. 3B). Similarly, the serum lysozyme activity of fish fed with *P. ehimensis*

Table 4
Growth performance of tilapia fed with basal diet only and basal diet containing 10^6 or 10^7 CFU/g of probiotic *P. ehimensis* NPUST1 for 2 months.

Parameters	Treatments		
	Control	10^6 CFU/g (G1)	10^7 CFU/g (G2)
Initial weight (g)	5.5 ± 0.45[a]	5.4 ± 0.47[a]	5.6 ± 0.69[a]
Final weight (g)	37.3 ± 3.59[a]	52.9 ± 2.32[b]	55.6 ± 0.22[b]
Weight gain (WG) (g)	31.93 ± 3.53[a]	47.46 ± 1.85[b]	55.6 ± 0.22[b]
Survival rate (%)	88.9 ± 3.85[a]	95.5 ± 3.85[a]	97.7 ± 3.85[a]
Feed conversion ratio (FCR)	1.38 ± 0.09[a]	1.10 ± 0.04[b]	1.08 ± 0.01[b]
Feed efficiency (FE)	0.73 ± 0.05[a]	0.91 ± 0.04[b]	0.92 ± 0.01[b]

Data are presented as the mean ± aS.E. from triplicates. Different superscripts in the same rows represent significant differences ($p < 0.05$).

Table 3
Antimicrobial potency of purified bacteriocin-like substance from *P. ehimensis* NPUST1 against various pathogens.

Pathogens	Antimicrobial activity[a]	Pathogens	Antimicrobial activity[a]
Aeromonas hydrophila	+ + +	*Escherichia coli*	+ + +
Streptococcus agalactise	+ + +	*Staphylococcus aureus*	+ +
Streptococcus iniae	+ +	*Methicillin-resistant S. aureus*[b]	+ +
Vibrio vulnificus[b]	+ + +	*Salmonella typhimurium*	+ +
Vibrio parahaemolyticus[b]	+ +	*Listeria monocytogenes*	+ +
Vibrio alginolyticus	+ +	*Pseudomonas aeruginosa*	+ +
Debaryomyces hansenii	+ + +	*Burkholderia gladioli*	+ + +

[a] Antimicrobial activity was determined by well-diffusion agar assay. + + + diameter of inhibition zone > 1.3 cm; + + diameter of inhibition zone between 1.0 and 1.3 cm. All tests were done in triplicate.
[b] Antibiotic-resistant pathogens.

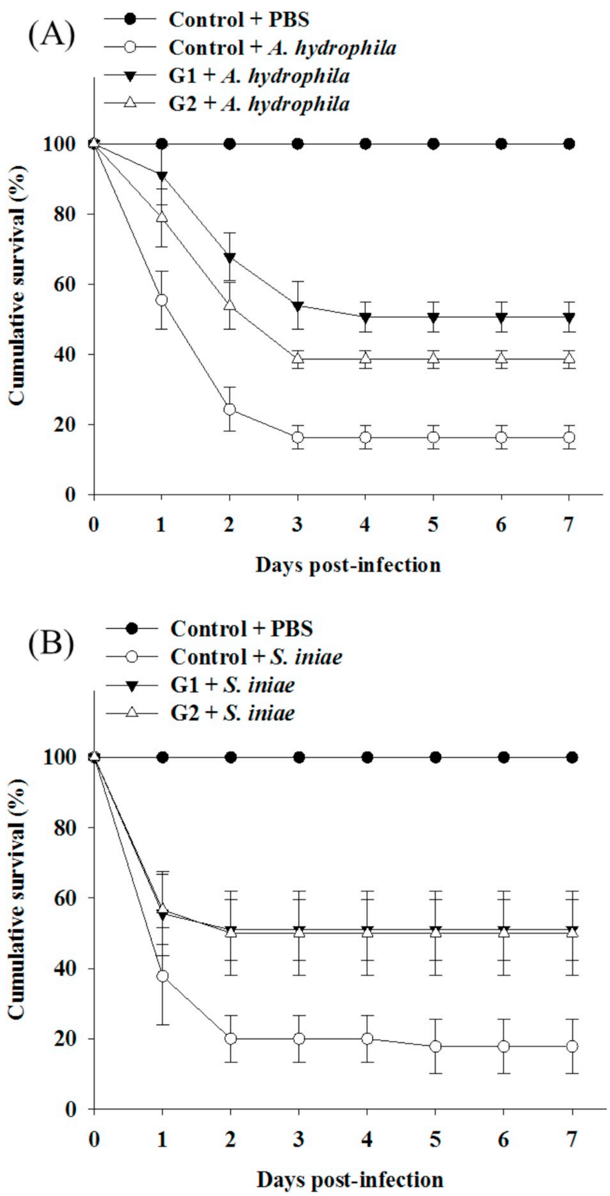

Fig. 2. Cumulative survival of tilapia challenged with (A) *A. hydrophila* and (B) *S. iniae* after feeding basal diet only (control) and basal diet containing 10^6 CFU/g (G1) and 10^7 CFU/g (G2) of *P. ehimensis* NPUST1 for 2 months. The survival rate in the probiotic groups was significantly higher ($p < 0.05$) than that in the control group according to the Kaplan-Meier method.

NPUST1 was significantly higher than that of fish fed the basal diet (Fig. 3C). There was no significant difference in the PA, respiratory, and lysozyme activity between tilapia supplemented with 10^6 and 10^7 CFU/g of *P. ehimensis* NPUST1. The SOD activity of fish fed 10^7 CFU/g of *P. ehimensis* NPUST1 was obviously higher than that of fish fed the basal diet and 10^6 CFU/g of *P. ehimensis* NPUST1. The SOD activity between fish fed 10^6 CFU/g and fish fed the basal diet was not significantly different (Fig. 3D). The expression of cytokine genes *TNF-α* and *IL-β* in the head kidneys and spleen were also determined by real-time quantitative PCR to evaluate the immune status. The expression of *TNF-α* and *IL-β* in the head kidneys and spleen were significantly increased in Nile tilapia fed the diet containing *P. ehimensis* NPUST1 compared with the fish fed the control diet (Fig. 4). This enhanced immune parameters could enhance fish immunity.

4. Discussion

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins from bacteria. They have become one of the effective weapons against pathogens due to the specific characteristics of broad-spectrum inhibition, natural occurrence, and heat stability, and tolerance of pH variation. Moreover, bacteriocins play important roles in the recognition and modulation of the immune system for host protection from infections [23]. The administration of bacteriocin-producing probiotics has potential to serve as an effective solution for issues resulting from the antibiotics used in aquaculture, such as the spread of antibiotic-resistant pathogens and the risk of antibiotic residue in foods [24]. Once the administered probiotics establish a microbial community in the host, they can block the colonization of pathogenic bacteria in the intestinal epithelium through the production of bacteriocin, which improves the innate immunity, nutrition metabolism, and growth of the host through the production of diverse digestive enzymes. The present study isolated an antimicrobial substance-producing probiotic *P. ehimensis* NPUST1 from the water of a tilapia culture pool. As shown in Supplementary Fig. 1, the antimicrobial substance can be partially purified by ammonium sulfate precipitation, indicating that it is a proteinaceous substance. The purified antimicrobial substance maintains stable antimicrobial activity at temperatures more than 100 °C and at pH 3 to 10, suggesting that the substance is heat stable and pH tolerant. Thus, the antimicrobial substance was assumed to be a bacteriocin-like substance, although it has not been confirmed in the present study. High temperature and pH variation are important conditions during the production procedure of food, medicine, and feed. In the present study, the bacteriocin-like substance from *P. ehimensis* NPUST1 revealed heat stability, pH tolerance, and wide-spectrum bactericidal activity against aquatic pathogens, food-spoilage bacteria, clinical pathogens, a plant pathogen, and multiple antibiotic-resistant pathogens. This suggests a potential for its application in preserving food, disease prevention in plant production, and as an alternative to antibiotics for disease control in medicine, animals, and aquaculture feed. The storage study of the bacteriocin-like substance showed stable bactericidal activity at −20 °C and 4 °C, suggesting cold temperature conditions are appropriate for the storage of products derived from this substance.

Starch and plant-derived protein such as soybean meal, rice gluten meal, and wheat middlings are widely used in tilapia feed to reduce the amount of fish meal. Amylase can catalyze the hydrolysis of starch into disaccharides or trisaccharides to increase the absorption efficiency of feed. However, the non-starch polysaccharide (NSP) components of these plants are recognized as anti-nutritional factors that increase feed viscosity and limit feed utilization and growth performance. Cellulose and xylan are the most abundant NSPs in plant feedstuffs. Cellulose is the basic structural component of plant cell walls and constitutes about 33% of all vegetable materials. Xylan is the most common hemicellulose and represents the major non-cellulosic cell wall polysaccharide in plants. Unfortunately, endogenous cellulase and xylanase are lacking or scarce in the gastrointestinal (GI) tract of fish. Thus, supplementing with probiotics that produce these enzymes is a potential way to reduce feed viscosity and improve feed efficiency. Reports showed that dietary supplementation with cellulase, amylase or xylanase-producing probiotics could significantly increase intestinal hydrolytic enzymes and enhance growth performance in mori (*Cirrhinus mrigala*) and tilapia (*O. niloticus*) [22,25]. These studies demonstrated that supplementation with hydrolytic enzyme-producing probiotics can increase digestive enzymes and contribute to the growth performance in fish by improving nutritional utilization. The present study showed that *P. ehimensis* NPUST1 possess ability to secret amylase, cellulase. The growth parameters WG and FE significantly improved in fish fed *P. ehimensis* NPUST1 for 2 months compared to fish fed a control diet. This

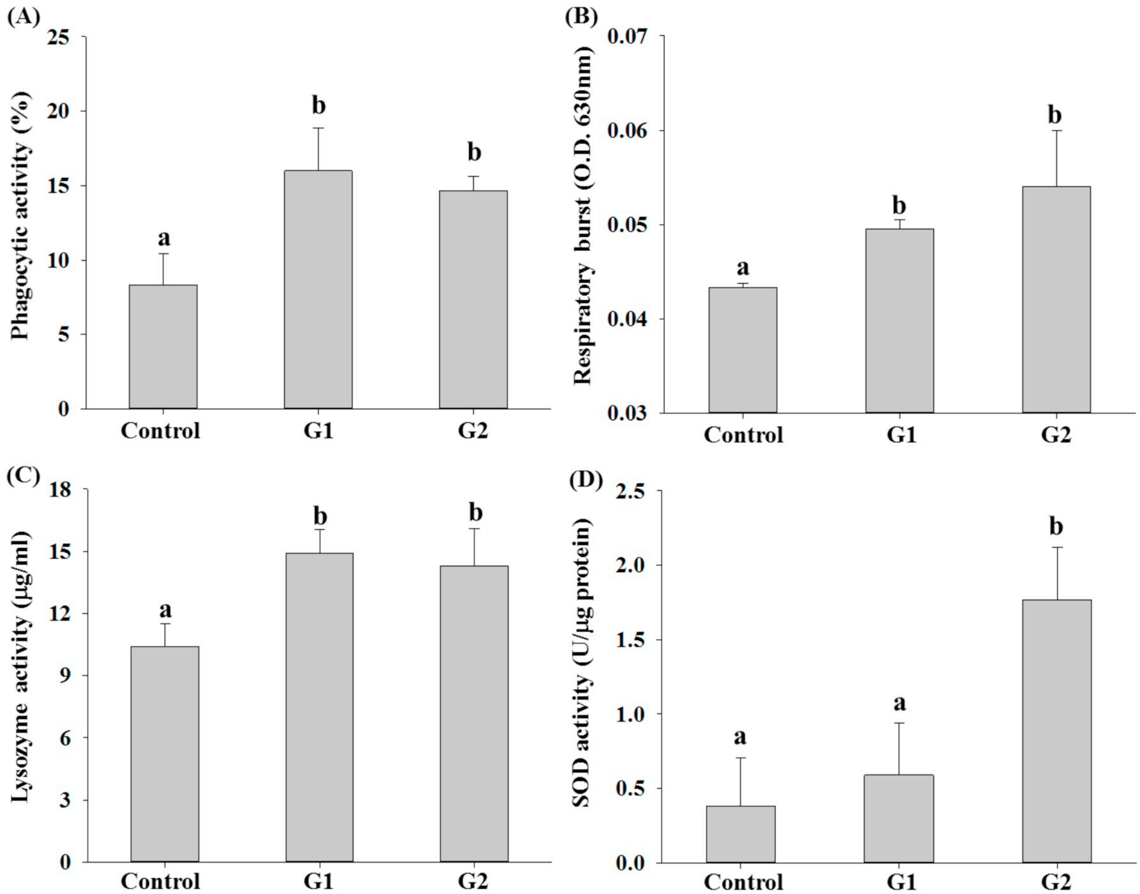

Fig. 3. Non-specific immune response of Nile tilapia that were fed 10^6 (G1) or 10^7 CFU/g (G2) of *P. ehimensis* NPUST1. (A) Phagocytic activity. (B) Respiratory burst activity. (C) Lysozyme activity. (D) SOD activity. Bars with different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

suggests that the growth-promoting benefit may be attributed to better dietary nutrient utilization due to the colonization of *P. ehimensis* NPUST1 and its exogenous production of amylase, cellulase, and xylanase.

Probiotics regulate the host immune response to disease infection, which is one of the major beneficial effects in aquaculture. In the past few decades, a wide range of bacteria have been used as probiotics to enhance immunity and resistance to diseases. Nevertheless, there have not been many studies on the genus *Paenibacillus* as probiotics [26]. So far, only three studies have reported on the use of *Paenibacillus* as probiotics to enhance the health status of the host in aquaculture. The first report showed that *Paenibacillus* spp. isolated from marine sediment exhibited antagonistic activity against the pathogenic *Vibrio* bacteria and has potential for use as a probiotic in the culture of tiger shrimp larva (*Penaeus monodon*) [27]. The second report showed that dietary supplementation of *P. polymoxa* enhances innate immunity and increases the survival rate of common carp (*Cyprinus carpio*) challenged with *Aeromonas hydrophila* [28]. The third report showed the effects of the dietary supplementation of single or multiple probiotics including *P. polymoxa* on the non-specific immune responses and disease resistance in starry flounder (*Platichthys stellatus*). The results suggest that the single and multiple probiotics had equal beneficial effects [29]. The present study is the first report demonstrating *P. ehimensis* as a probiotic to contribute beneficial effects in fish. Our results showed that the survival rate of tilapia challenged with *A. hydrophila* and *S. iniae* increased in fish given *P. ehimensis* NPUST1, suggesting that dietary supplementation with this probiotic can enhance the innate immunity against pathogenic infection. The innate immune parameters such as PA, respiratory burst, and lysozyme activity were evaluated to demonstrate the immunomodulatory function of *P. ehimensis* NPUST1.

In teleosts, the head kidney is extremely sensitive to the change of the external environment and plays a critical role in the regulation of immunity in response to pathogenic invasion. The PA of head kidney leukocytes such as neutrophils, monocytes, and macrophages in fish plays an antibacterial role through the activation of the early inflammatory response. The phagocytic cells exhibit an ability to recognize pathogens directly or indirectly and they engulf and kill invading pathogens via phagocytosis. During phagocytosis, phagocytes produce highly microbicidal reactive oxygen species (ROS) to kill invasive pathogens, such as superoxide anions ($O_2{}^-$), hydroxyl free radicals (OH^-), and hydrogen peroxide (H_2O_2), which is called respiratory burst activity. In the present study, the tilapia fed with the diet containing *P. ehimensis* NPUST1 for 2 months showed significantly higher PA and respiratory burst compared with the activity in the tilapia fed the control diet. This suggests that *P. ehimensis* NPUST1 exhibits immunostimulatory functions in tilapia. Our results are consistent with a report in which respiratory burst was found to be significantly increased in common carp (*Cyprinus carpio*) after 80 days of feeding with *P. polymoxa* [28].

The antioxidant enzyme SOD provides cellular defense against ROS by catalyzing the dismutation of $O2^-$ to O_2 and H_2O_2. The reaction reduces destructive oxidative stress and plays an important role in protecting cells against the potential oxidative damage induced by pathogen infection. The effects of probiotics from the *Paenibacillus* genus on the SOD activity of fish have not been studied much, so the knowledge on the *Bacillus* genus is discussed. Although *bacillus* spp. have been commonly used as probiotics in aquaculture, the effects of probiotic *Bacillus* spp. on SOD activity are not consistent. Reports show that serum SOD activity increased in tilapia after feeding them with *B. licheniformis* and *B. subtilis* HAINUP40 for 4 and 8 weeks, respectively

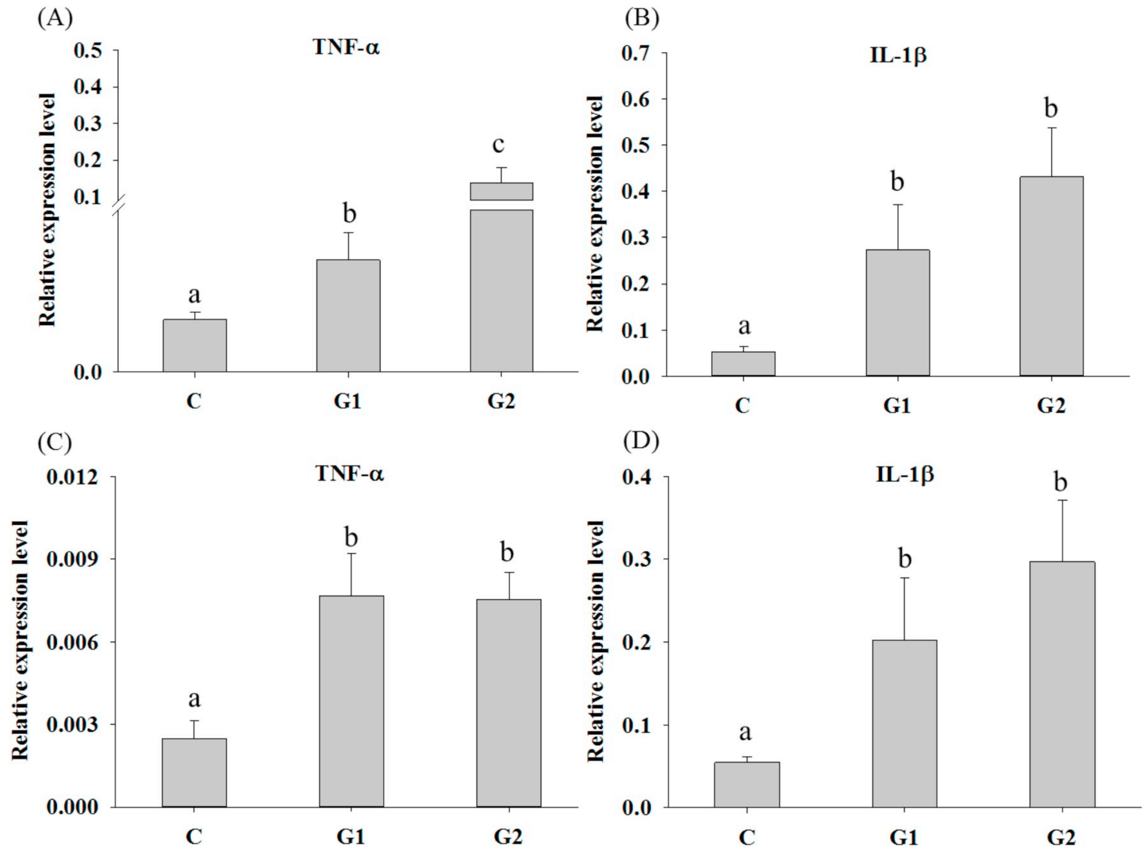

Fig. 4. Quantitative PCR analysis of cytokine gene expression in Nile tilapia fed 10^6 (G1) or 10^7 CFU/g (G2) of *P. ehimensis* NPUST1 for 2 months. Expression of *TNF-α* and *TGF-β* in (A & C) head kidney and (C and D) spleen. Bars with different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

[30–32]. However, SOD activity in the head kidney leukocytes and serum was not affected in Nile tilapia fed a diet containing the probiotic *B. amyloliquefaciens* for 8 weeks and *B. licheniformis* for 10 weeks [22,33]. Moreover, another report found lower serum SOD activity in *Epinephelus coioides* fed a diet containing *B. pumilus* and *B. clausii* for 1 week [34]. Son et al. found that dietary administration of *Lactobacillus plantarum* for 4 weeks significantly decreased the SOD activity. They hypothesized that the decreased SOD may occur in order to retain the levels of ROS such as $O2^-$ or OH^- to enhance the microbial-killing capacity of phagocytes [35]. Recently, Liu et al. reported that SOD activity in *E. coioides* fed diets containing *B. subtilis* at different concentrations (10^4, 10^6, and 10^8 CFU/g) was obviously reduced after feeding for 1 week. However, the SOD activity increased after feeding for 4 weeks, suggesting that the difference in SOD level may be associated with probiotic strain, dosage, and administrative duration [36]. In the present study, the SOD activity was significantly increased in the head kidneys of tilapia fed *P. ehimensis* NPUST1 at a concentration of 10^7 CFU/g compared with that of tilapia fed the control diet. This suggests that *P. ehimensis* NPUST1 stimulates a better immune response and protective efficiency from oxidative damage.

Lysozyme is an indispensable enzyme that functions as a humoral component of the non-specific defense system. It exhibits bactericidal activity and prevents the growth of infectious pathogens by hydrolyzing the glycosidic linkages between N-acetyl-D-glucosamine (NAG) and N-acetylmuramic acid (NAM) in the peptidoglycan of bacterial cell walls. In addition to bactericidal activity, recent evidence shows that lysozyme activity has a function of modulating innate immune responses. When invading pathogens are engulfed by phagocytic cells via phagocytosis, neutrophils and macrophages kill the pathogenic bacteria by releasing lysozyme. The lysozyme-mediated bacterial lysis can activate pattern-recognition receptors and the complement system as an opsonin to modulate the host immune response against pathogen infection [37]. Gupta et al. found that *P. polymyxa* supplemented in feed of common carp (*Cyprinus carpio*) enhanced lysozyme activity against *A. hydrophila* infection [28]. The results of the present study showed that tilapia fed a diet containing *P. ehimensis* NPUST1 for 2 months exhibited significantly increased serum lysozyme activity suggesting *P. ehimensis* NPUST1 supplemented with feed has beneficial effects on the lysozyme activity of the nonspecific immune system.

The head kidney and spleen are thought to be major lymphoid tissues in teleost fishes. Cytokine genes are important mediators secreted from macrophages or monocytes that regulate the immune system and defense mechanisms against infection. Proinflammatory cytokines including TNF-α and IL-1β are biomarkers of immuno-modulators, which are expressed at the early stage of infection in fish and have a key role in inflammatory regulation. TNF-α and IL-1β can activate the PA of leucocytes, marcophages, and natural killer cells in fish, as well as perform bactericidal activity by increasing respiratory and ROS production during phagocytosis. Munoz-Atienza et al. reported that in vitro and vivo administration of probiotics significantly increased the expression of *TNF-α* and *IL-1β* in the head kidneys and spleen of turbot (*Scophthalmus maximus* L.) against *Tenacibaculum maritimum* and *Vibrio splendidus* infection [38]. The present study showed 10^6 CFU/g of *P. ehimensis* NPUST1-adminstrated tilapia produced 2.13- and 3.10-fold, and 3.10- and 3.69-fold significantly higher expression level of *TNF-α* and *IL-1β* in head kidney and spleen compared with control diet-treated tilapia, respectively. The significantly higher expression of *TNF- α* and *IL-1β* in tilapia fed *P. ehimensis* NPUST1 suggested that *P. ehimensis* NPUST1 can enhance the cytokine expression (TNF-α and IL-1β) of innate immune system and provide defense against pathogen infection.

In conclusion, the present study isolated *P. ehimensis* NPUST1, a potent probiotic with bacteriocin-like activity and extracellular hydrolytic enzyme activity (amylase, cellulase, and xylanase). Dietary supplementation of *P. ehimensis* NPUST1 not only enhanced growth performance and feed efficiency but also modulated innate immunity, including PA and respiratory burst activity of the head kidney leukocytes, serum lysozyme activity, and the cytokine genes expression of *TNF-α* and *IL-1β*. The increased survival rate in Nile tilapia after challenge with *A. hydrophila* and *S. iniae* was correlated with increases in the innate immune parameters in the group fed *P. ehimensis* NPUST1. The resent study is the first to examine the effects of *P. ehimensis* as a probiotic, and the results suggest that it can be used in aquaculture to improve growth performance, feed utilization, and immunity against diseases.

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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.2018.10.059.

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