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A potential probiotic *Chromobacterium aquaticum* with bacteriocin-like activity enhances the expression of indicator genes associated with nutrient metabolism, growth performance and innate immunity against pathogen infections in zebrafish (*Danio rerio*)

Che-Chun Yi[a,1], Chun-Hung Liu[b,d,1], Kuo-Pin Chuang[c,d], Yi-Ting Chang[a], Shao-Yang Hu[a,d,*]

[a] Department of Biological Science and Technology, National Pingtung University of Science and Technology, Pingtung, 912, Taiwan
[b] Department of Aquaculture, National Pingtung University of Science and Technology, Pingtung, 912, Taiwan
[c] Graduate Institute of Animal Vaccine Technology, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, 912, Taiwan
[d] Research Center for Animal Biologics, National Pingtung University of Science and Technology, Pingtung, 912, Taiwan

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ABSTRACT

The use of probiotics as alternatives to antibiotics for disease control is a relatively eco-friendly approach in aquaculture; hence, studies isolating and assessing the benefit of potential probiotics to fish farming are common. The zebrafish is an excellent model system for validating beneficial functions of potential probiotics before their practical application in aquaculture. Here, a potentially probiotic *Chromobacterium aquaticum* was isolated from lake water samples and characterized by biochemical analysis and 16S rDNA sequencing. The probiotic produced extracellular enzymes (protease and xylanase) and a bacteriocin-like substance, which exhibited tolerance to extreme pH and high-temperature conditions and broad-spectrum bactericidal activity against diverse pathogens, including aquatic, foodborne, clinical and plant pathogens. The effects of *C. aquaticum* on zebrafish nutrient metabolism, growth performance and innate immunity were evaluated by measuring the expression of indicator genes after *C. aquaticum* feeding for 8 weeks. Fish administered the probiotic exhibited significantly increased hepatic mRNA expression of carbohydrate metabolism-related genes, including glucokinase (GK), hexokinase (HK), glucose-6-phosphatase (G6Pase), and pyruvate kinase (PK-L), and growth-related genes, including the growth hormone receptor (GHR) and insulin-like growth factor-1 (IGF-1). Innate immune-related genes (IL-1β, IL-6, TNF-α, IL-10, IL-21, NF-κb, lysozyme and complement C3b) were induced in fish with probiotic supplementation. Probiotic-treated fish exhibited a higher survival rate than control fish after challenge with *Aeromonas hydrophila* and *Streptococcus iniae*. Together, these data suggest that *C. aquaticum*, as a probiotic feed supplement, could enhance nutrient metabolism and growth performance and could modulate innate immunity against *A. hydrophila* and *S. iniae* in zebrafish.

1. Introduction

With capture fishery production at its upper limit, worldwide aquaculture is responsible for fish production to supply the demand for food of human consumption. According to FAO 2018, aquaculture accounts for 53% of the total fishery production, for a production value of USD 232 billion [1]. However, the increasing incidence of diseases from pathogen infection due to intensive aquaculture and water contamination has caused a severe economic loss in aquaculture. Among pathogen infections, bacterial diseases are a major contributor to blocking the sustainable development of aquaculture. For instance, hemorrhagic septicemia and vibriosis caused by pathogenic *Aeromonas* or *Streptococcus* and *Vibrio*, respectively, usually result in mass mortality in both fresh and marine fish [2–4]. Thus, antibiotics or chemicals are commonly used in fish farming sectors for preventing disease outbreaks or for therapeutic purposes. However, the misuse of antibiotics has caused issues such as the rapid spread of antibiotic-resistant pathogens, reduced therapeutic efficacy of antibiotics and increased risk of food

* Corresponding author. Department of Biological Science and Technology, National Pingtung University of Science and Technology, No. 1 Hseufu Road, Neipu, Pingtung, 912, Taiwan.
E-mail address: syhu@mail.npust.edu.tw (S.-Y. Hu).
[1] Che-Chun Yi and Chun-Hung Liu contributed equally to this work.

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safety by residual antibiotic contamination. Thus, identifying an alternative to antibiotics for disease prevention is quite important and urgent.

Probiotics have been considered a relatively eco-friendly alternative to antibiotics for biocontrol in aquaculture. Numerous studies have shown that probiotics are beneficial to growth performance, nutrient metabolism, immune responses, disease prevention, the gut microbiota and water quality [5]. A review of these studies indicates that most probiotics used in aquaculture are lactic acid or *Bacillus* spp. because of their production of hydrolytic enzymes (e.g., phytase, cellulase, protease and xylanase) that increase nutrient utilization, and their long-term safety for mammalian species. In particular, *Bacillus* spp. are commonly used as probiotics due to their ability to produce endospores that are resistant to the low pH of the gastrointestinal (GI) tract [6,7]. However, the interaction between probiotics and hosts is considered species-specific or strain-specific; hence, diverse probiotics need to be developed for treating a variety of fish species [8]. Recently, criteria for screening probiotics used in fish farming, such as nonpathogenicity, a lack of antibiotic resistance, extracellular enzyme production and antagonistic activity, have been reviewed [9]. Extracellular enzyme-producing probiotics have been demonstrated to exhibit positive effects on enhancing nutrient utilization and growth performance in fish. For instance, dietary supplementation with extracellular enzyme (protease, amylase, lipase, phytase or xylanase)-producing probiotic bacteria of the genus *Bacillus* can improve nutrient metabolism and growth in zebrafish, tilapia and tiger shrimp [10–12]. Antagonistic activity against diverse pathogens is a very important property for probiotics. Probiotics can produce bacteriocin to inhibit or kill pathogen competitors and can then prevent harmful bacteria from colonizing the gut epithelial surface. Bacteriocins are ribosomal proteins or peptides synthesized by bacteria and exhibit bacteriostatic or bactericidal activity against competitors to maintain the environmental predominance in microbial communities. In general, bacteriocins have the advantages of thermal stability, low toxicity, immunity to host cells and the ability to be produced by probiotics or by recombinant engineering techniques. Unlike most antibiotics, bacteriocins do not induce antimicrobial resistance in bacteria, because their structures and antimicrobial mechanisms are different from those of antibiotics [13–15]. Thus, bacteriocins have been considered a potential alternative to antibiotics. In aquaculture, although several reports have shown that probiotics confer positive effects on fish, studies of the effects of bacteriocin-producing probiotics on fish are relatively rare.

Chromobacterium is a genus of gram-negative bacteria with rod-shaped morphology. To date, no study has reported the use of the genus *Chromobacterium* as a probiotic in aquaculture. *Chromobacterium aquaticum* CC-SEYA-1 was first isolated from spring water samples and identified as a new species of this genus [16]. Next-generation sequencing of the *C. aquaticum* CC-SEYA-1 genome was recently performed [17]. In the present study, a protease- and xylanase-producing *C. aquaticum* bacterium with bacteriocin-like activity was isolated from a water sample collected from Jing-Si Lake at National Pingtung University of Science and Technology (NPUST). Proteases can break down complex proteins into oligopeptides or amino acids. Exogenous protease supplementation in feed can effectively increase protein digestibility, resulting in increased nutrient utilization and feed efficiency. Xylanase is a typical enzyme that hydrolyzes linear polysaccharides and hemicellulose, which is a component of plant cell walls. Because of this activity, dietary supplementation with xylanase-producing probiotics has attracted attention as an emerging strategy for improving feed efficiency and degrading nonstarch polysaccharides (NSPs), which is an antinutritional factor in fish feed ingredients of plant origin [11]. Thus, the present study attempted to evaluate the effects of *C. aquaticum* on fish health status and confirm the feasibility of *C. aquaticum* as a potential probiotic in aquaculture.

The discovery of probiotics that exhibit multiple beneficial effects on aquaculture stimulated many studies on the isolation of potential probiotics, and practical applications of probiotics in a variety of fish have been rapidly developed. However, directly evaluating the efficacy of potential probiotics on food fish species usually requires a large area for culture and is expensive. Zebrafish is an excellent animal model widely used in aquaculture research due to its advantages, including the previous exploration of its genome sequence, its copious production of offspring, its easy manipulation and its relatively low culture area and cost requirements. Moreover, the important genes, including immune-, growth-, and stress-related genes, are highly conserved among vertebrates and thus provide an effective tool to validate various immunostimulants, including probiotics [10,18]. Investigations have reported that the probiotic *B. amyloliquefaciens* R8 exerts similar beneficial effects on zebrafish and Nile tilapia (*O. niloticus*), suggesting that the zebrafish model system could allow effective assessment of probiotic function [10,11]. The present study evaluated a protease- and xylanase-producing *C. aquaticum* bacterium with bacteriocin-like activity as a potential probiotic in zebrafish. The biochemical characteristics and antagonistic ability of the bacteriocin-like substance against a variety of pathogens were evaluated by a disk diffusion assay. In addition, the effects of dietary supplementation with *C. aquaticum* on nutrient metabolism and growth were evaluated by determining the expression levels of indicators such as hepatic glucokinase (GK); hexokinase 1 (HK1); glucose-6-phosphatase (G6Pase); pyruvate kinase, liver isoform (PK-L); the growth hormone receptor (GHR); and insulin-like growth factor-1 (IGF-1). Moreover, the effect of *C. aquaticum* on immunity to pathogen infection was evaluated by assessing immune parameters, including the expression of cytokine genes and the survival rate after challenge with *A. hydrophila* and *S. iniae*. The results could be considered a basis for the practical implementation of *C. aquaticum* as a probiotic in aquaculture.

2. Materials and methods

2.1. Fish and bacterial strains

Adult 2.5-month-old AB strain zebrafish (*Danio rerio*) were purchased from the Taiwan Zebrafish Core Facility at Academia Sinica (Taipei, Taiwan). Fish were reared in a 90-L aquarium at 28 °C to acclimate to the environment of the aquatic laboratory animal facility for 7 days. Fish were fed twice daily with a commercial diet (MeM Prime, BERNAQUA, Olen, Belgium). All fish were treated in accordance with local animal welfare regulations. The pathogen culture conditions and maintenance of bacterial species culture stocks were as described in a previous report [19].

2.2. Screening and identification of potential probiotics with bacteriocin-like activity

The protocol for screening potential probiotics with bacteriocin-like activity was modified in accordance with a previous report [19]. Briefly, water samples were collected from Jing-Si Lake at NPUST and serially diluted ten times with sterile water. A 100-μL aliquot of each diluted suspension was spread onto the surface of tryptic soy broth (TSB) agar medium and incubated at 28 °C for 24 h. After incubation, each colony was spotted on TSB agar containing pathogenic *A. hydrophila* at a concentration of approximately 1.0×10^6 CFU/mL and incubated at 28 °C for 4 days. Formation of an inhibition zone around an isolate colony indicated that the isolate produced antimicrobial substances. Based on the antimicrobial potency, the colony with the strongest antagonistic activity against *A. hydrophila* was isolated. The isolate identification was performed by the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) using 16S rDNA sequencing and biochemical analysis. The 16S rDNA fragment was amplified by PCR using universal primers 27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′). The PCR program cycles were set as follows: denaturation at 95 °C for 4 min; 32 cycles at 94 °C

for 1 min, 58 °C for 1 min, and 72 °C for 95 s; and final extension at 72 °C for 5 min. The amplified products were separated by 1.5% agarose gel and purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). The amplified 16S rDNA fragments (1500 bp) were sequenced with an ABI 3730xl DNA Analyzer (Foster City, CA, USA). The sequences were compared with known sequences in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/blast). Biochemical analysis for isolate identification was performed by classic microbiology tests including Gram-staining; catalase and oxidase tests; motility, indole production, and enzyme activity (arginine dihydrolase, urease, β-glucosidase, protease and β-galactosidase) tests and carbohydrate fermentation (glucose, arabinose, mannose, mannitol, maltose and N-acetyl-glucosamine) tests.

2.3. Biochemical properties and antimicrobial spectrum of the bacteriocin-like substance

The seed culture and growth curve of *C. aquaticum* grown on TSB medium was determined according to a protocol described in a previous report [19]. A 1-mL aliquot of culture medium was sampled at different cultivation times to monitor growth by measuring the optical density (OD) at 600 nm using a spectrophotometer. Cell-free supernatants obtained from the culture medium by centrifugation at 13,600 × *g* and 4 °C for 15 min were used to determine the protein concentration and antimicrobial activity in order to evaluate the production profile of the bacteriocin-like substance during the growth of *C. aquaticum*. The Bradford assay was used to determine the protein concentration [20]. The protocol for the resazurin assay to assess the antimicrobial activity of the cell-free supernatants and the definition of the arbitrary unit (AU) for antimicrobial activity were described in a previous report [19]. The antagonistic activity of the bacteriocin-like substance against a variety of pathogens was determined by a disk diffusion assay. Cell-free supernatant from the TSB culture medium of *C. aquaticum* at 96 h was obtained by centrifugation at 1700 × *g* for 15 min. An 8-mm filter disk (Tokyo Roshi Kaisha, Ltd., Japan) was placed on the surface of a TSB agar plate inoculated with a variety of indicator pathogens at a concentration of approximately 1.0×10^6 CFU/mL. A 20-μl aliquot of cell-free supernatant was loaded onto the filter disks and incubated at 28 °C for 24 h. The diameter of the inhibition zone surrounding the filter disks was measured to evaluate the antimicrobial potency of the bacteriocin-like substance. To confirm that the bacteriocin-like substance is a proteinaceous compound, 180 μL of the cell-free supernatant was coincubated with 20 μL of trypsin (Sigma-Aldrich Corporation, USA) at a final concentration of 1 mg/mL at 37 °C for 1 h. The antimicrobial activity of the trypsin-treated substance was determined by a disk diffusion assay as described above. To determine the effect of temperature on the bacteriocin-like substance, a 1.5-mL centrifuge tube containing 1 mL of cell-free supernatant was incubated at 30, 40, 50, 60, 70, 80, 90 and 100 °C for 1 h. The residual antimicrobial activity against *A. hydrophila* was determined by a disk diffusion assay. The antimicrobial activity of the cell-free supernatant at 4 °C was used as the control. To determine the effect of pH on the bacteriocin-like substance, 1 mL of cell-free supernatant was lyophilized to a powder and redissolved in 1 mL of buffers with different pH values, including glycine-HCl buffer for pH 2, citrate-phosphate buffer for pH 3–6, tris(hydroxymethyl) aminomethane buffer for pH 8–9 and glycine-NaOH buffer for pH 10–11, for 4 h. The antimicrobial activity of cell-free supernatant redissolved in PBS buffer (pH 7.0) was used as the control. All experiments were performed in triplicate.

2.4. Experimental design and fish husbandry

Zebrafish (*D. rerio*) (approximately 4.1 cm in length and weighing 0.46 g) were randomly divided into the control, G1, and G2 groups. Twenty fish per group were cultured in a 10-L tank at 28 °C, and each experiment was conducted in triplicate. The formulation of the basal diet is described in a previous report [10]. The approximate composition of the basal diet was 39.2% crude protein and 8% crude lipid as determined according to AOAC analysis methods (AOAC 1997). The control group was fed a basal diet. For the G1 and G2 groups, the probiotic *C. aquaticum* was added to the basal diet at levels of 1×10^6 CFU/g (G1) and 1×10^7 CFU/g (G2). The experimental diets were prepared according to a previously described protocol [11]. The fish in the control, G1 and G2 groups were fed twice daily at 2% of body weight. After 8 weeks of feeding, the hepatic expression levels of indicator genes related to nutrient metabolism and growth and the systemic expression levels of indicator genes related to the innate immune response were determined in the zebrafish.

2.5. Toxicity assessment of C. aquaticum

Adult zebrafish with an average weight of 4.62 ± 0.45 g were acclimatized in circulating aerated water at 28 °C for 7 days. A total of 120 fish were divided into four groups to assess the toxicity of *C. aquaticum* at 1×10^6 CFU, 1×10^7 CFU and 1×10^8 CFU per fish. Fish injected with PBS buffer were used as the control group. Each group was analyzed in triplicate. *C. aquaticum* was cultured 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. Fish in each tank (n = 10) were injected intraperitoneally with 20 μL of the diluted bacterial solution and cultured in 60-L aquaria. The toxicity of *C. aquaticum* was evaluated by recording the mortality of the injected fish daily for 7 days.

2.6. In vitro and in vivo determination of xylanase activity

For the in vitro assay, 0.5 g of the basal diet or the basal diet containing *C. aquaticum* was placed into a 15-mL plastic centrifuge tube containing 6 mL of sterile water and incubated at 28 °C for 7 days. After cultivation, the mixture was centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatant was used for the determination of xylanase activity. For the in vivo assay, three fish from each group were sacrificed and dissected on ice at the end of the feeding trial. Fish intestine samples were harvested in 1.5-mL microcentrifuge tubes containing 1 mL of sterile water. After vigorous vortexing and centrifugation at 12,000 × *g* for 15 min at 4 °C, the supernatant was used for the measurement of xylanase activity. Xylanase activity was evaluated by measuring the amounts of reducing sugars released from xylan according to the dinitrosalicylic acid (DNS) method described in a previous report [10].

2.7. Detection of indicator gene expression by quantitative polymerase chain reaction (PCR)

Six zebrafish from each group were sampled for extraction of total RNA. Total RNA was extracted separately from the liver and whole body of zebrafish using TriPure isolation reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. The expression levels of indicator genes involved in nutrient metabolism, growth and innate immune responses were assessed by real-time PCR (Applied Biosystems StepOnePlus, Foster City, CA, USA) using SYBR Green PCR reagents. The specific primers used for amplifying each gene are described in previous reports [10,21,22] and are listed in Table 1. Expression of the EF-1α gene was used as the internal control. Real-time PCR was carried out according to previously reported conditions [10]. The relative expression level of each gene was normalized to that of EF-1α and expressed as the mean ± standard error (SE).

2.8. Challenge experiment

A. hydrophila and *S. iniae* were grown in TSB and incubated at 28 °C for 24 h. Bacterial cells were collected after centrifugation at 6,100 × *g*

Table 1
Primer sequences used in this study.

Gene name	Primer sequence (5′→ 3′)	Accession number
Nutrient metabolism and growth-related genes		
Glucokinase (GK)	GCTGTGAAGTCGGCATGATA CTTCAACCAGCTCCACCTTAC	BC122359.1[a]
Hexokinase 1 (HK1)	ACTTTGGGTGCAATCCTGAC AGACGACGCACTGTTTTGTG	BC067330.1[a]
Glucose-6-phosphatase (G6Pase)	TCACAGCGTTGCTTTCAATC AACCCAGAAACATCCACAGC	BC164161.1[a]
Pyruvate kinase, liver isoform (PK-L)	TCCTGGAGCATCTGTGTCTG GTCTGGCGATGTTCATTCCT	BC152219.1[a]
Growth hormone receptor (GHR)	TGAGTCGTTCAGGGTTGCACTT CGCTGTCGCTGAATTCACCAAA	NM_001083578
Insulin-like growth factor-1 (IGF-1)	AGTGTACCATGCGCTGTCTC AAAAGCCCCTGTCTCCACAC	NM_131825
Immune-related genes		
Interleukin-1β (IL-1β)	TGGACTTCGCAGCACAAAATG CACTTCACGCTCTTGGATGA	AY340959
Interleukin-6 (IL-6)	TCAACTTCTCCAGCGTGATG TCTTTCCCTCTTTTCCTCCTG	JN698962
Interleukin-10 (IL-10)	TCACGTCATGAACGAGATCC CCTCTTGCATTTCACCATATCC	BC163031
Interleukin-21 (IL-21)	AATCATTCATCGTGGACAGTGTGT AACGTTCGGCTGTTGACCAT	NM_001128574
Tumor necrosis factor-α (TNF-α)	AAGGAGAGTTGCCTTTACCG ATTGCCCTGGGTCTTATGC	BC165066
Nuclear factor-κB (NF-κB)	AAGAGGACCAAAATAAGCACAG AAG TCCAAGGTACATCGCCATGA	AY163838
Lysozyme	CGTGGATGTCCTCGTGTGAAG CCAATGGAGAATCCCTCAAA	NM_139180
Complement component C3b	CGTCTCCGTACACCATCCATT GGCGTCTCATCAGGATTTGTTAC	NM_131243
Elongation factor-1α (EF-1α)	AACAGCTGATCGTTGGAGTCAA TTGATGTATGCGCTGACTTCCT	AY422992

for 15 min at 4 °C and resuspended in different volumes of distilled water to adjust the cell concentration. The 7-day median lethal dose (LD_{50}) was determined by intraperitoneal (i.p.) injection of serial doses of *A. hydrophila* and *S. iniae* separately (10^5, 10^6, 10^7, and 10^8 CFU/fish) into 10 fish. At the end of the feeding trial, 15 fish from each group were administered an i.p. injection of 20 μL of *A. hydrophila* or *S. iniae* solution diluted to the LD_{50} doses of 1.0×10^6 CFU and 1.0×10^5 CFU per fish, respectively. Fish fed the control diet and injected with PBS were used as the negative control group. Each group was analyzed in triplicate. Infected fish with pathogenic signs were observed daily, and dead fish were removed from the tanks. The cumulative survival in each group was recorded for 7 days post infection.

2.9. Statistical analysis

Significant differences in xylanase activity and relative gene expression levels between each group were analyzed statistically using one-way ANOVA and Tukey's multiple comparison tests. A probability value of less than 0.05 ($p < 0.05$) was considered significant. Cumulative survival in the challenge experiment was analyzed by the Kaplan-Meier method. Data analysis was performed using SAS software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Screening of potential probiotics with antagonistic activity against A. hydrophila

Four water samples from Jing-Si Lake at NPUST were serially diluted and individually cultured on TSB agar plates. Based on morphological characteristics, 10 different colonies were selected from each water sample, and a total of 40 bacterial isolates were cultured on selective agar plates for screening antagonistic activity against *A. hydrophila*. Although three isolates with antimicrobial activity were selected, only one exhibited potent antimicrobial activity. This isolate was identified by 16S rDNA sequencing and biochemical analysis and was found to share 99.11% similarity with *Chromobacterium aquaticum*. Physicochemical analysis revealed that the isolate was a gram-negative, rod-shaped, motile facultative anaerobic bacteria with catalase, oxidase and nitrate reductase activity. Analysis of digestive enzyme activities revealed that the isolate exhibited protease and xylanase activity (Supplementary Fig. 1). Consequently, this protease- and xylanase-producing isolate with antimicrobial activity was identified as *Chromobacterium aquaticum*.

3.2. Antibacterial spectrum and biochemical properties of the bacteriocin-like substance produced by C. aquaticum

The production profile of the antimicrobial substance produced by *C. aquaticum* was monitored by measuring the growth curve, protein concentration and antimicrobial activity. The antimicrobial activity clearly increased with increasing protein concentration after 44 h of cultivation in TSB medium, suggesting that the production of this bacteriocin-like substance by *C. aquaticum* occurred in mid-stationary phase (Fig. 1A). The antimicrobial activity of the cell-free supernatant against *A. hydrophila* at 30 h, 48 h and 96 h was evaluated by a disk diffusion assay. An inhibition zone was formed around the cell-free supernatant samples at 48 h and 96 h (Fig. 1B), consistent with the antimicrobial profile indicated by the *C. aquaticum* growth curve. In addition, the antimicrobial activity of the cell-free supernatant was evaluated in the presence of trypsin. The antagonistic activity was lost after trypsin treatment (Fig. 1C). Moreover, the effects of pH and temperature on the antimicrobial activity of the bacteriocin-like substance was determined by a disk diffusion assay, which showed that the bacteriocin-like substance retained stable antimicrobial activity over the range of pH 2–10. However, the antimicrobial activity of the bacteriocin-like substance was slightly reduced, by 12%, in an extremely alkaline environment (pH 11) (Fig. 2A). The substance also exhibited a high thermal tolerance and retained stable antimicrobial activity at temperatures of 40 °C–90 °C. Moreover, the substance maintained 80% of its baseline antimicrobial activity even at the high temperature of 100 °C (Fig. 2B). The antimicrobial spectrum of the bacteriocin-like substance against diverse pathogens was determined by a disk diffusion assay. As shown in Table 2, the substance exhibited antimicrobial activity against the aquatic pathogens *A. hydrophila*, *S. agalactiae*, *V. parahaemolyticus*, *V. alginolyticus* and *D. hansenii*; the foodborne pathogens *S. aureus*, *S. typhimurium* and *L. monocytogenes*; the clinical pathogens methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa*; and the plant pathogen *B. gladioli*. Notably, the substance exhibited antagonistic potency against the antibiotic-resistant pathogens *V. alginolyticus*, *V. parahaemolyticus,* and MRSA (Table 2).

3.3. Fish fed the C. aquaticum-supplemented diet exhibited enhanced expression of indicator genes involved in nutrient metabolism and growth

Nonpathogenicity to fish is the basic requirement for potential probiotics. The toxicity of *C. aquaticum* was evaluated in zebrafish by i.p. injection of different doses of *C. aquaticum*. Neither mortality nor signs or symptoms of infection occurred in the fish, even at high doses of 1×10^7 CFU/per fish, suggesting that *C. aquaticum* is not toxic to fish (Supplementary Fig. 2). The level of xylooligosaccharides (XOSs) released from xylanase degradation in *C. aquaticum* was measured before assessing the effect of *C. aquaticum* on indicators of nutrient metabolism in zebrafish. The in vitro and in vivo results revealed that the XOS level was significantly increased in the feed containing *C. aquaticum* and in the intestine of zebrafish fed the *C. aquaticum*-supplemented diet (Fig. 3). The expression of genes involved in nutrient metabolism and growth were evaluated in fish from the G1 and G2 groups after 8 weeks of feeding. The hepatic expression levels of the GK and HK1 genes in

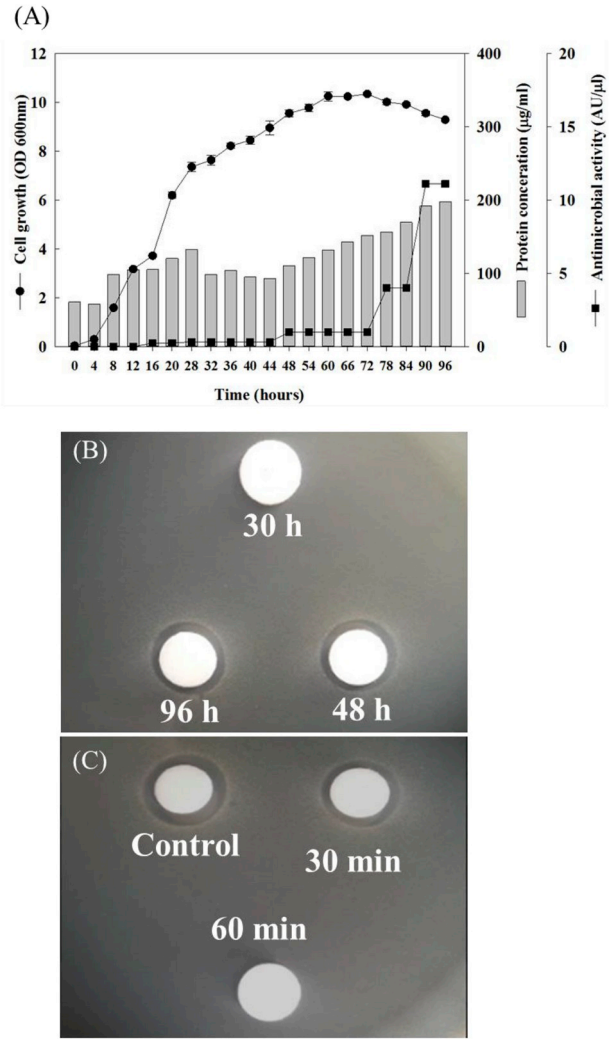

Fig. 1. Characterization of the bacterial physiology of *Chromobacterium aquaticum*. (A) The protein concentration and antimicrobial activity were monitored with respect to the growth curve. (B) The antimicrobial activity of the cell-free supernatant of *C. aquaticum* culture broth against *A. hydrophila* at 30 h, 48 h and 96 h was determined by a disk diffusion assay. A 20-μL aliquot of culture broth was added to the disk. (C) The antimicrobial activity of the cell-free supernatant of *C. aquaticum* culture broth at 96 h was measured in the presence of trypsin for 30 min and 60 min "Control" indicates cell-free supernatant at 96 h without trypsin treatment.

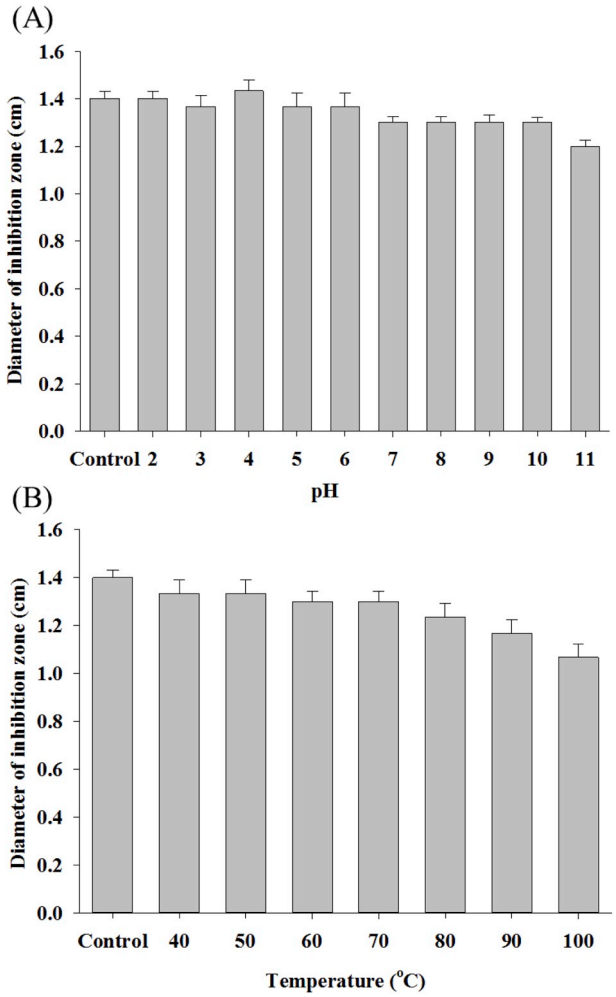

Fig. 2. Biochemical characteristics of the bacteriocin-like substance produced by *Chromobacterium aquaticum*. (A) pH tolerance of the bacteriocin-like substance at pH 2–11 for 4 h. (B) Thermal stability of the bacteriocin-like substance at different temperatures for 1 h. Each data value is the mean of triplicate experiments.

fish from the G1 and G2 groups were significantly higher than those in fish of the control group. Although the expression levels of G6Pase and PK-L were not significantly different between the control and G1 groups, significantly increased expression levels of these genes were observed in fish from the G2 group. The expression levels of GHR and IGF-1 were also evaluated to indicate the growth status. Although the expression level of GHR was not significantly different between the control and G1 groups, GHR expression was significantly higher in fish from the G2 group than in fish from the control and G1 groups. Fish fed the *C. aquaticum*-supplemented diet exhibited noticeably increased expression of the IGF-1 gene compared with that in fish fed the control diet (Fig. 4).

3.4. Effects of C. aquaticum supplementation on innate immune responses

The expression levels of immune-related genes, including interleukin (IL)-1β, IL-6, IL-10, IL-21, tumor necrosis factor (TNF)-α, nuclear factor (NF)-κB, lysozyme and complement component 3b (C3b), were determined to evaluate the health status of fish. The expression levels of IL-6, IL-10 and IL-21 in fish from the G1 and G2 groups were significantly higher than those in fish from the control group (Fig. 5B, C and D). Although the expression level of the IL-1β gene in fish was not significantly different between the control and G1 groups, it was appreciably higher in the G2 group than in the control and G1 groups (Fig. 5A). The expression levels of TNF-α and NF-κB were significantly increased in fish from the G1 and G2 groups compared with those in fish from the control group. Moreover, the expression levels of TNF-α and NF-κB were significantly different between the G1 and G2 groups (Fig. 5E and F). The expression level of lysozyme was significantly increased in fish from the G2 group compared to that in fish from the control and G1 groups but did not significantly differ between the control and G1 groups (Fig. 5G). The expression level of complement C3b in fish from the G1 and G2 groups was significantly higher than that in fish in the control group (Fig. 5H). The significant increases in the expression levels of immune-related genes in fish from the G1 and G2 groups suggested that the *C. aquaticum*-supplemented diet enhanced innate immune responses in zebrafish.

3.5. Effects of C. aquaticum supplementation on disease resistance

Dietary supplementation with *C. aquaticum* enhanced innate immunity in zebrafish, inspiring us to study the effect of *C. aquaticum* on

Table 2
Antimicrobial potency of the bacteriocin-like substance from *Chromobacterium aquaticum* against various pathogens.

Pathogen	Antimicrobial activity[a]	Origin and source
Aeromonas hydrophila	+ + +	Tilapia [48]
Streptococcus agalactiae	+ + +	tilapia [49]
Vibrio parahaemolyticus[b]	+ +	Chinese mitten crab [50]
Vibrio alginolyticus[b]	+ +	white shrimp [51]
Debaryomyces hansenii	+ +	giant freshwater prawn [52]
Staphylococcus aureus BCRC12991	+ + +	frozen food[c]
Salmonella typhimurium BCRC12947	+	food poisoning in man[c]
Listeria monocytogenes BCRC14932	+ +	food[c]
Methicillin-resistant S. aureus[b] (MRSA)	+ + +	human stool [53]
Pseudomonas aeruginosa BCRC12902	+ + +	sputum of patient[c]
Burkholderia gladioli ATCC19302	+ +	onion bulb rot[c]

[a] Antimicrobial potency was determined by a well diffusion agar assay. + + +, + + and + indicate an inhibition zone diameter of > 1.2 cm, between 1.0 and 1.2 cm and < 1.0 cm, respectively. All assays were performed in triplicate.
[b] Antibiotic-resistant pathogens.
[c] Sources of pathogens were purchased from Bioresource Collection and Research Center (BCRC), Taiwan or American Type Culture Collection (ATCC), USA.

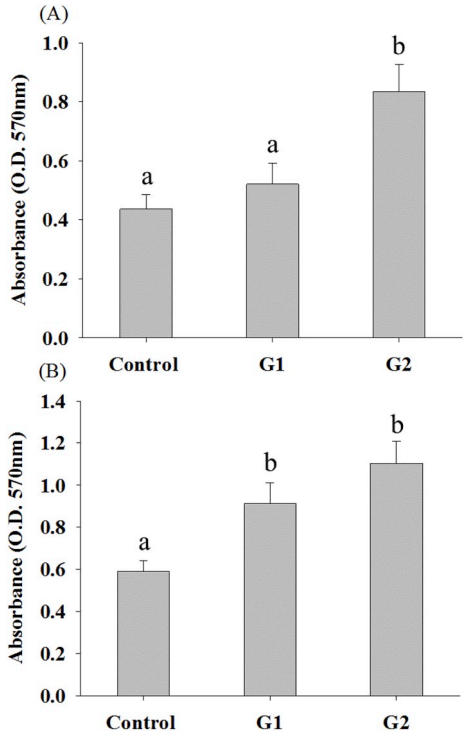

Fig. 3. The level of XOSs derived from xylanase degradation was determined by measuring the absorbance at 570 nm. (A) In vitro and (B) in vivo xylanase activity was evaluated using the DNS method. The data are presented as the mean ± S.E. of six individual samples (n = 6). Values with different letters are significantly different ($p < 0.05$).

disease resistance by challenging zebrafish with *A. hydrophila* and *S. iniae*. As shown in Fig. 6, the survival rate during the first 7 days post infection was 100% for fish fed the control diet and injected with PBS buffer. However, the survival rate in the control group injected with *A. hydrophila* and *S. iniae* was dramatically reduced during the first 4 days and 5 days post infection, respectively, remaining stable at 28.8 ± 7.69% and 17.7 ± 3.85%, respectively, until 7 days post infection. Notably, the fish fed the basal diet containing 10^6 and 10^7 CFU/g *C. aquaticum* exhibited a significantly increased survival rate compared to that of fish fed the control diet. At 7 days after infection with *A. hydrophila* and *S. iniae*, the survival rates were 49.9 ± 3.88% and 53.3 ± 7.69% in fish fed 10^6 CFU/g *C. aquaticum* and 42.2 ± 3.85% and 44.4 ± 3.85% in fish fed 10^7 CFU/g *C. aquaticum*, respectively. The survival rates were not significantly different between fish fed 10^6 or 10^7 CFU/g, suggesting that the dose of 10^6 CFU/g is sufficient to confer disease resistance. This result suggests that supplementation of the fish diet with *C. aquaticum* can enhance resistance to *A. hydrophila* and *S. iniae* infection (Fig. 6).

4. Discussion

Bacteriocins are ribosomally synthesized proteinaceous substances produced by diverse bacteria that exhibit antagonistic activity against closely related or other bacterial species to assist in competing for nutrient sources and establishing predominance in the microbial community. Recently, bacteriocins have attracted attention for their potential development as novel antibacterial agents against pathogens in food preservation, biomedical, cosmetic and agricultural applications due to their heat stability, tolerance to extreme pH conditions, broad-spectrum antimicrobial activity, and lack of drug resistance stemming from differences between their antimicrobial mechanisms and those of antibiotics [23]. Moreover, bacteriocin has been demonstrated to modulate host innate immune responses and improve host protective immunity [24]. For instance, pure nisin (0.0025 μg/fish) injected into turbot (*Scophthalmus maximus* L.) significantly augmented the serum lysozyme concentration and resistance against *Carnobacterium piscicola* [25]. However, the use of pure bacteriocins is impractical in aquaculture due to the high cost and lack of economic benefit. Thus, bacteriocin-producing probiotics are an effective strategy to reveal the advantage of bacteriocin and are an alternative to the use of antibiotics or chemicals in aquaculture. After supplemented bacteriocin-producing probiotics establish a microbial community in the gut of the host, these probiotics can kill pathogens or prevent pathogens from colonizing the intestinal epithelium and can modulate immunity by secreting bacteriocin [26]. In the present study, a potential probiotic *C. aquaticum* with antimicrobial activity was isolated from a lake. The bactericidal activity of the antimicrobial substance was lost in the presence of trypsin, suggesting that the antimicrobial substance is proteinaceous. Thus, the antimicrobial substance is believed to be a bacteriocin-like substance, although its identity was not confirmed in the present study. The bacteriocin-like substance maintained antagonistic activity against *A. hydrophila* at high temperatures (90 °C) and over a pH range of 2–10, suggesting that it was heat-stable and tolerant to pH variation. In addition, this substance exhibited antimicrobial activity against aquacultural, foodborne, clinical, plant and antibiotic-resistant pathogens, suggesting that it has broad-spectrum antimicrobial activity and potential application as a biocontrol agent in diverse industries.

Plant-source feedstuffs are commonly used as protein sources and to partially reduce the use of fish meal in aquaculture. Exogenous protease added to feed can increase dietary protein digestibility and amino acid

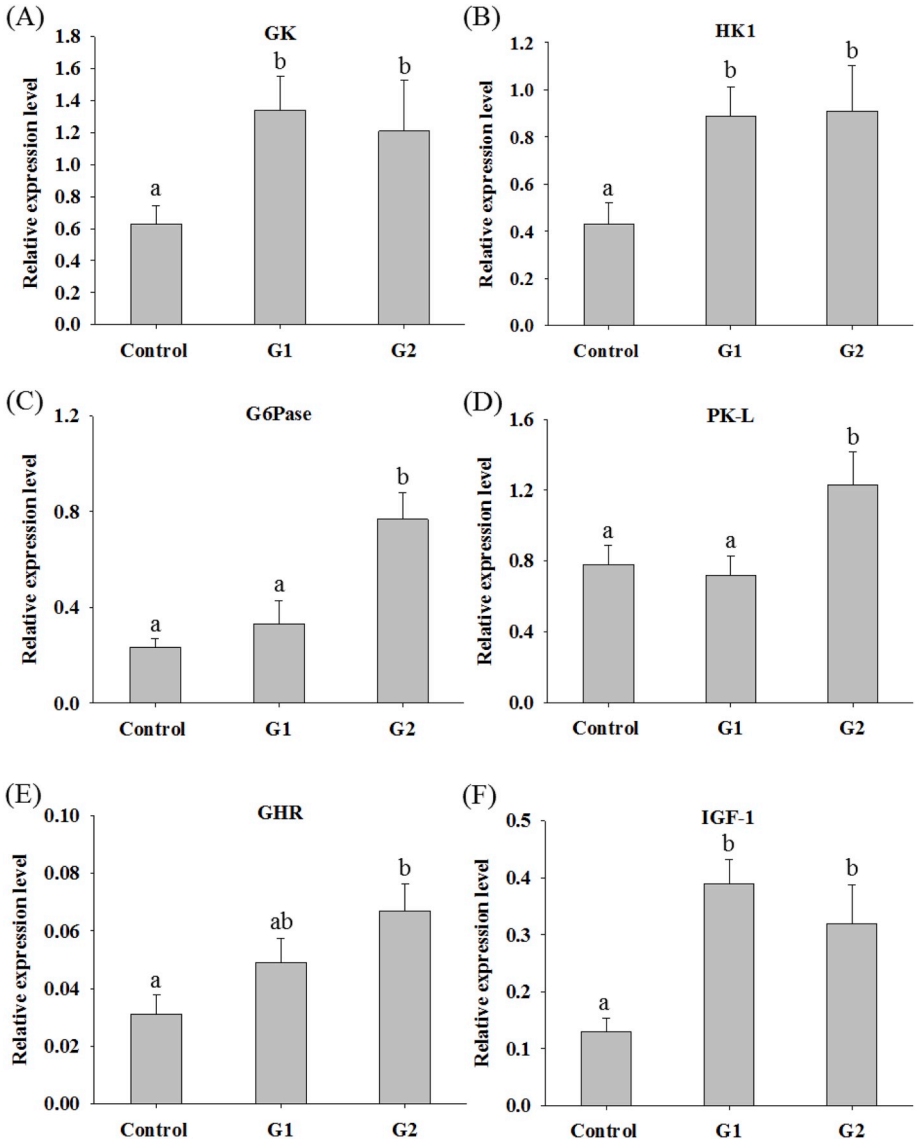

Fig. 4. Quantitative PCR analysis of the hepatic expression levels of the (A) GK, (B) HK1, (C) G6Pase, (D) PK-L, (E) GHR and (F) IGF-1 genes, which are involved in nutrient metabolism and growth, in zebrafish fed 10^6 (G1) or 10^7 CFU/g (G2) of *Chromobacterium aquaticum* for 8 weeks. The values indicated by the bars with different letters are significantly different ($p < 0.05$, one-way ANOVA).

availability in a feedstuff, thus resulting in improved nutrient absorption and utilization. Reports have demonstrated that dietary administration of extracellular protease-producing probiotics can effectively increase nutrient utilization and enhance growth performance in Nile tilapia and *Labeo calbasu* [27,28], suggesting the beneficial effect of exogenous proteases on feed efficiency. However, although proteases increase the digestibility of proteins in feed, the presence of indigestible carbohydrates in plant feedstuffs is the main constraint in nutrient utilization in aquaculture. Indigestible carbohydrates in plants consist primarily of NSPs, which are considered an antinutritional factor that when contained in feed increases intestinal viscosity, thereby impeding the digestive process, nutrient utilization and fish growth. Xylan is a common type of hemicellulose that is a major component of plant cell walls and is the most abundant NSP in plant feedstuffs. Xylanase is an enzyme that can degrade the polysaccharide structures of hemicellulose into XOSs, which are polymers of 2–10 xylose sugar units linked by β-(1 → 4) bonds. Unfortunately, endogenous xylanase is rare in the gastrointestinal tract of fish; hence, administrating xylanase-producing probiotics provides a solution for NSP-induced problems from feed. Recently, reports have shown that dietary supplementation with XOSs as prebiotics not only could significantly improve feed efficiency and growth performance but also can enhance innate immunity against diseases in Nile tilapia, blunt snout bream (*Megalobrama amblycephala*) and European seabass (*Dicentrarchus labrax*) [29–31]. The present study showed that dietary supplementation with xylanase-producing *C. aquaticum* significantly increased the level of XOSs in the diet and in the fish intestine, suggesting that xylanase-producing *C. aquaticum* could significantly reduce the NSP levels in feed and potentially improve growth and immunity. Indeed, Saputra et al. reported that feed supplemented with xylanase-producing or exogenous xylanase-expressing probiotics significantly increased feed efficiency and growth performance in Nile tilapia [11,19]. In the present study, although zebrafish are too small to assess feed efficiency and growth performance, expression levels of specific genes can be used as indicators to evaluate the status of nutrient metabolism and growth. The liver plays a central role in regulating carbohydrate, fat and protein metabolism. GK, HK and PK are rate-limiting enzymes that participate in the first and final step of glycolysis. G6Pase is an enzyme mainly found in the liver that hydrolyzes glucose-6-phosphate to provide free glucose during gluconeogenesis. Hepatic GHR binds growth hormone (GH) from the anterior

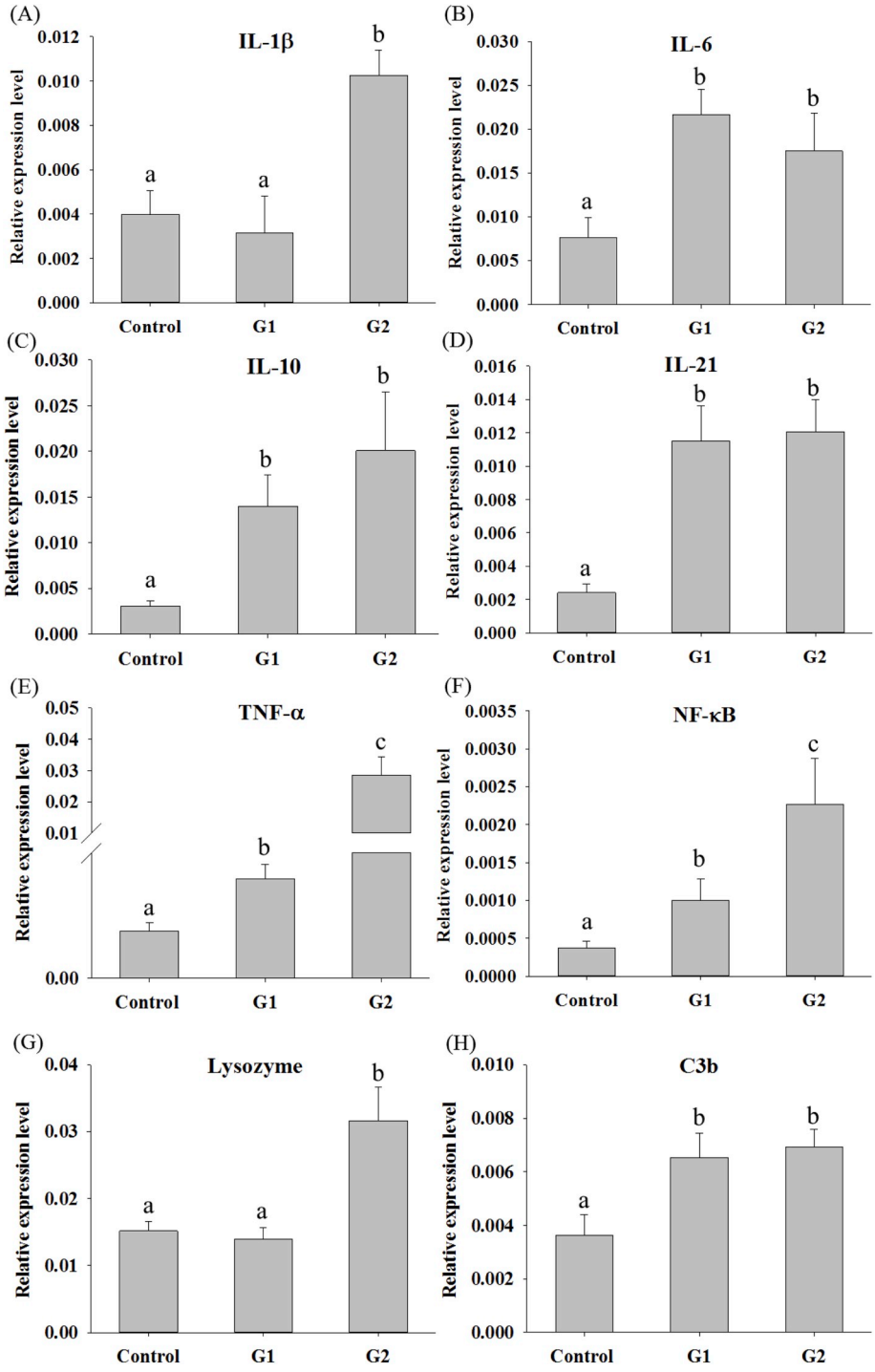

Fig. 5. Quantitative PCR analysis of immune-related (A) IL-1, (B) IL-6, (C) IL-10, (D) IL-21, (E) TNF-α, (F) NF-κb, (G) lysozyme and (H) C3b gene expression in zebrafish fed 10^6 (G1) or 10^7 CFU/g (G2) of *Chromobacterium aquaticum* for 8 weeks. The values indicated by the bars with different letters are significantly different ($p < 0.05$, one-way ANOVA).

pituitary gland and thereby triggers the release of IGF-1 into the circulatory system to stimulate cell proliferation, an important endocrine mechanism for somatic growth. In addition, Lin et al. demonstrated that fish fed xylanase-producing probiotics exhibit enhanced expression levels of genes involved in nutrient metabolism and growth [10,11]. The present study showed that the mRNA expression of HK1, GK, G6Pase, PK-1, GHR and IGF-1 was significantly induced in zebrafish fed protease- and xylanase-producing *C. aquaticum,* supporting the conclusion regarding the beneficial effects of xylanase-producing probiotics on nutrient metabolism and growth performance and suggesting that these characteristics could be enhanced in fish fed a *C. aquaticum*-supplemented diet.

The potency of probiotics to modulate innate immunity is one of the most recognized benefits to the host in aquaculture. In recent decades, immunomodulatory functions that strengthen the host defense against pathogen infections have been shown in diverse bacterial species. However, thus far, no study has addressed the potential of *C. aquaticum* or other members of the genus *Chromobacterium* as probiotics. Indeed, this study is the first to report that the use of *C. aquaticum* as a probiotic is beneficial to fish. These results showed that zebrafish fed a *C.*

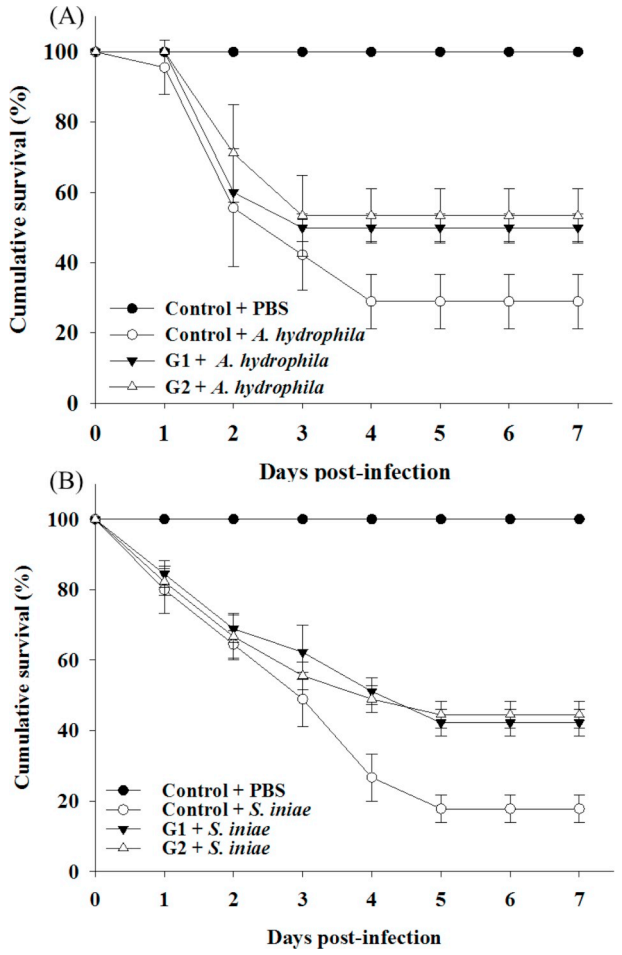

Fig. 6. Cumulative survival rates of zebrafish challenged with (A) *A. hydrophila* and (B) *S. iniae* after feeding with a basal diet only (control) or with a basal diet containing 10^6 CFU/g (G1) or 10^7 CFU/g (G2) of *Chromobacterium aquaticum* for 8 weeks. The cumulative survival rates in the probiotic-fed groups were significantly higher ($p < 0.05$) than those in the corresponding control groups according to Kaplan-Meier analysis.

aquaticum-supplemented diet exhibited a significantly increased survival rate after challenge with *A. hydrophila* and *S. iniae*, suggesting that dietary supplementation with *C. aquaticum* can enhance disease resistance in fish. This result inspired us to further investigate the expression levels of innate immunity indicator genes to demonstrate the immunomodulatory function of *C. aquaticum*. The head kidney is a unique organ that plays an important role in the production of cytokines by immune cells to regulate immunity in response to pathogen invasion in teleosts. Cytokines secreted from immune cells such as lymphoid cells, macrophages and monocytes play critical roles in driving innate immune and inflammatory responses to defend against pathogen infection. IL-1β, IL-6 and TNF-α are early-expressed proinflammatory cytokines that assist the host in responding promptly to pathogen infections and activate immune cells to perform bactericidal functions by inducing a cascade of reactions during phagocytosis. The transcription factor NF-κB serves as a pivotal mediator of proinflammatory signaling pathways to induce cytokine gene expression and regulate innate immune functions. Zhang et al. characterized TNF-α in grass carp (*Ctenopharyngodon idella*) and identified its involvement in the NF-κB pathway to regulate the expression of TNF-α and IL-1β in head kidney leukocytes, suggesting the importance of the link between TNF-α and NF-κB in teleost immunity [32]. Moreover, Kong et al. characterized the IL-6 promoter in flounder and demonstrated IL-6 transcription by the NF-κB subunit, a result suggesting that the NF-κB pathway participates in IL-6 expression [33]. IL-10 is an anti-inflammatory cytokine that plays an important role in limiting excessive inflammation in response to pathogen infection, thereby preventing host damage. Reports have shown that dietary supplementation with probiotics significantly enhances disease resistance and the expression of cytokines such as TNF-α, IL-1β, IL-6, Il-10 and NF-κB in Nile tilapia, crucian carp (*Carassius carassius*), grass carp (*Ctenopharyngodon idellus*), olive flounder (*Paralichthys olivaceus*), sea cucumber (*Apostichopus japonicus*) and *Carassius auratus* [19,34–39]. In fish, the IL-21 cytokine is a critical modulator of the cellular immune response that plays important roles in activating B cells, T cells and natural killer (NK) cells [40]. Reports have shown that IL-21 is expressed in immune tissues and is induced by bacterial infection in trout, suggesting that fish IL-21 plays roles in the immune response against pathogen infection [41]. Consistent with the conclusions of these previous reports, the present study showed an increase in TNF-α, IL-1β, IL-6, IL-10, IL-21 and NF-κB expression in zebrafish fed *C. aquaticum,* suggesting that *C. aquaticum* modulates innate immunity.

Lysozyme is a hydrolytic enzyme that hydrolyzes linkages between N-acetylmuramic acid (NAM) and N-acetyl-D-glucosamine (NAG) residues in the peptidoglycan component of the bacterial cell wall, thereby killing bacterial pathogens. In addition to exerting bactericidal activity, lysozyme can also modulate the host immune system to defend against pathogen infections by the release of degraded bacterial products, which form ligands that bind to pattern recognition receptors and activate the complement system and phagocytosis in the host. Thus, the expression level of lysozyme is considered an important indicator of innate immunity in fish [42]. The complement system, consisting of a series of complement proteins, is a component of the immune system that can clear invaded pathogens by activating phagocytic cells and enhancing the ability of antibodies to attack the cell membrane of pathogens. C3 convertase plays a central role in the activation of the complement system by converting C3 into C3b, which acts as an opsonin, binding to the surface of antigens and mediating pathogen killing by opsonization [43]. Studies have demonstrated that the administration of probiotics can significantly enhance serum lysozyme activity, complement activity, and defensive potency against pathogen infection in rainbow trout (*Oncorhynchus mykiss*), hybrid Hulong grouper, Nile tilapia and *Labeo rohita* [44–47]{Galagarza, 2018 #43}. Similarly, the present results showing increased expression of the lysozyme and C3b genes in zebrafish fed the *C. aquaticum*-supplemented diet suggest that *C. aquaticum* can enhance lysozyme and complement activity in the innate immune system and provide defense against pathogen infection. In addition to beneficial effects (growth promotion and immune modulation) on the host, the absence of resistance is an important criterion to consider when identifying promising probiotic candidates. Although the present study evidenced the beneficial effects of *C. aquaticum* on fish, the antimicrobial resistances of *C. aquaticum* must be further studied before its potential application in the future.

In conclusion, in the present study, a potential probiotic *C. aquaticum* with bacteriocin-like activity and extracellular digestive enzyme activity (protease and xylanase) was isolated. The heat stability, tolerance to pH variation and broad-spectrum bactericidal activity of the bacteriocin-like substance suggested its potential application in diverse industries. Fish fed *C. aquaticum* in the diet exhibited not only increased expression of indicator genes associated with nutrient metabolism, growth performance and the innate immune response but also enhanced resistance to infection with *A. hydrophila* and *S. iniae*. The present study is the first to examine the effects of *C. aquaticum* as a probiotic, and the results suggest that *C. aquaticum* could be developed as a probiotic for use in aquaculture.

Acknowledgments

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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.2019.07.042.

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