Fish and Shellfish Immunology 86 (2019) 410–419

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Dietary administration of *Bacillus amyloliquefaciens* R8 reduces hepatic oxidative stress and enhances nutrient metabolism and immunity against *Aeromonas hydrophila* and *Streptococcus agalactiae* in zebrafish (*Danio rerio*)

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ARTICLE INFO

Keywords:
Bacillus amyloliquefaciens
Zebrafish
Xylanase
Oxidative stress
Innate immunity

ABSTRACT

Zebrafish (*Danio rerio*) are an excellent model for assessing the beneficial effects of probiotics before applying them in aquaculture. This study evaluated the effects on zebrafish of dietary supplementation with the probiotic *Bacillus amyloliquefaciens* R8, which heterologously expresses xylanase from rumen fungi. Nutrient metabolism, hepatic oxidative stress, and innate immunity against pathogen infections were investigated. Treated zebrafish received feed supplemented with *B. amyloliquefaciens* R8 for 30 days and then were compared to zebrafish that were fed a control diet. The treated fish showed significant increases in xylanase activity in the intestines. The livers of the treated fish showed increased mRNA expressions of glycolysis-related genes of hexokinase, glucokinase, glucose-6-phosphatase, and pyruvate kinase; and higher enzyme activities of 3-hydroxyacyl-coenzyme A dehydrogenase and citrate synthase which are associated with fatty acid β-oxidation and mitochondrial integrity. The livers of treated fish also showed decreased mRNA expressions of oxidative stress-related genes (*SOD, Gpx, NOS2,* and *Hsp70*) and an apoptotic gene (*tp53*), as well as increased expression of an anti-apoptotic gene (*bcl-2*). The probiotics-treated fish had increased expression of innate immune-related genes (*IL-1β, IL-6, IL-21, TNF-α, and TLR-1, -3,* and *-4*). Following challenge with *Aeromonas hydrophila* and *Streptococcus agalactiae*, treated fish showed increased a higher survival rate than control fish. Overall, results showed that the administration of xylanase-expressing *B. amyloliquefaciens* R8 can potentially improve nutrient metabolism and hepatic stress tolerance, and enhance immunity and disease resistance against *A. hydrophila* and *S. agalactiae* in zebrafish.

1. Introduction

Problems with diseases result in huge economic losses in aquaculture and have become one of the major issues impeding its development. The diseases with the greatest incidences in fish and shellfish species are caused by microbial pathogens. For example, *Aeromonas hydrophila* is a typical pathogen that causes red fin disease and hemorrhagic septicemia in most freshwater fish species [1]. Gram-positive *Streptococcus agalactiae* primarily results in meningoencephalitis and hemorrhagic septicemia, which produce high mortality in many marine and freshwater fish species [2]. Antimicrobial agents such as antibiotics and disinfectants are commonly used in aquaculture to prevent disease outbreaks. However, the abuse of antimicrobial agents in aquaculture has caused severe issues, such as the rapid spread of antibiotic-resistant pathogens in microbial ecosystems, damage to the natural environment, residual antibiotics in fish products, risks to human health, and reduced fish immunity due to the effects on the gastrointestinal (GI) microflora [3,4]. Thus, alternative biocontrol strategies are required for the sustainable development of aquaculture and to address these issues.

Probiotics are a relatively environmentally friendly approach as an alternative to the use of antimicrobial chemicals to prevent diseases in aquaculture. Probiotics are microorganisms that confer beneficial effects on a host when supplied in adequate amounts. Multiple advantages of using probiotics in aquaculture have been reviewed in

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https://doi.org/10.1016/j.fsi.2018.11.047
Received 8 August 2018; Received in revised form 12 October 2018; Accepted 17 November 2018
Available online 22 November 2018

several reports. Such advantages include providing nutrients and promoting enzymatic digestion, which improve the feed efficiency and enhance growth; stimulating beneficial microflora in the GI tract; competing for adhesion sites with harmful bacteria to inhibit the growth of pathogenic microorganisms; and enhancing the immune defense against pathogen infections [5]. For example, a combination of the probiotics *Bacillus subtilis*, *Lactobacillus casein,* and *Candida utilis* provided a degradation enzyme to detoxify mycotoxins in feed [6]. The administration of the probiotics *Rhodotorula benthica* D30 and *Bacillus amyloliquefaciens* enhanced hydrolytic enzymes such as protease, cellulose, or xylanase in fish to hydrolyze indigestible components and increase feed efficiencies [7–9]. Dietary supplementation with the probiotic *Shewanella putrefaciens* significantly increased the messenger RNA expressions of innate immune response genes in Senegalese sole (*Solea senegalensis*) [10]. Dietary administration of the probiotic *B. licheniformis* also improved mucus and serum immune parameters and reduced the rate of *A. hydrophila* infections in tilapia (*Oreochromis mossambicus*) [11]. Besides lactic acid-producing bacteria, spore-forming *Bacillus* spp. are the most widely used probiotics in the aquaculture industry due to the advantages of long-lasting resistance to environmental stress. Furthermore, their role in enhancing immunity against pathogens has been well studied [12–14]. *B. amyloliquefaciens*, which is closely related to *B. subtilis*, is a potent biocontrol agent against a variety of pathogens and secretes diverse digestion enzymes, such as α-amylase and proteases. However, studies investigating *B. amyloliquefaciens* as a probiotic in aquatic hosts are still rare. Recent reports demonstrated that feeding Nile tilapia (*Oreochromis niloticus*) with *B. amyloliquefaciens* improved their growth performance and increased their immunity against *Yersinia ruckeri* and *Clostridium perfringens* [15]. Dietary supplementation of the probiotic *B. amyloliquefaciens* 54A improved the health status and resistance against *Edwardsiella ictaluri* infection in striped catfish (*Pangasianodon hypophthalmus*) [16]. Feeding Catla (*Catla catla*) with *B. amyloliquefaciens* FPTB16 improved their systemic and mucosal immune responses and their defense against *Edw. tarda* infection [17]. Those studies demonstrated the value of applying *B. amyloliquefaciens* in aquaculture.

Xylanases produced by microorganisms are important hydrolytic enzymes that are capable of cleaving the β-1,4 backbone of the polysaccharide xylan, which is the major hemicellulosic constituent found in cell walls of plants. It has attracted attention as a candidate for improving the efficiency of feed utilization and degrading non-starch polysaccharides in feed ingredients of plant origin [18,19]. Furthermore, xylose and xylooligosaccharides (XOSs) derived from xylanase-degraded xylan can be used as functional foods or feed additives. In the livestock industry, dietary supplementation of a plant-derived diet containing xylanase was demonstrated to provide beneficial effects of improving feed digestibility, ruminal fermentation, energy utilization, and growth performance in cows, pigs, and goats [20–22]. In poultry farming, xylanase supplementation was shown to improve the utilization of nutrients in a wheat-based diet and to significantly increase the growth performance of chickens [23,24]. Reports have shown that the use of XOSs as a prebiotic feed supplement in aquaculture increases digestive enzyme activities in the intestines and enhances the growth performance and innate immune parameters in different fish species, including crucian carp (*Carassius auratus gibelio*), Caspian white fish (*Rutilus frisii kutum*), and European seabass (*Dicentrarchus labrax*) [25–28]. Those reports suggest that using xylanase or XOSs derived from xylanase with plant-derived feed may be an efficient strategy for improving nutrient utilization and immunity in aquaculture. Although potential applications of xylanase have been examined in different industries, less information is available on the use of xylanase in aquaculture. The reason for this limitation may be the cost burden of purified xylanase supplementation of diets. Thus, it is necessary to develop an efficient strategy for the economically sustainable application of xylanase in aquaculture.

The multiple benefits of probiotics in aquaculture have inspired investigations on the isolation of probiotics, and potential applications in diverse fish species are rapidly being developed. However, directly evaluating the functions of a potential probiotic for use in food fish usually requires a large culture space and high costs. Zebrafish provide an excellent animal model to validate the functions of probiotics for aquaculture studies [29]. This study evaluated the effects of the probiotic *B. amyloliquefaciens* R8 on the health status of zebrafish. *Bacillus amyloliquefaciens* R8 expresses the xylanase gene from rumen fungi. The effects on the expression of genes which are associated with hepatic metabolism of nutrients, hepatic stress, and immune status were evaluated. The influence on disease resistance against *A. hydrophila* and *S. agalactiae* was also evaluated. The results could provide reference values for practical applications of the probiotic *B. amyloliquefaciens* R8 in aquaculture.

2. Materials and methods

2.1. Fish and pathogen strains

Three-month old zebrafish (*Danio rerio*) AB strain (around 3.8 cm in length and weighing 0.48 g) were purchased from the Taiwan Zebrafish Core Facility at Academia Sinica (Taipei, Taiwan). The fish were raised in the aquatic laboratory animal facility of National Pingtung University of Science and Technology, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The zebrafish were maintained in a 90-L aquarium at 28 °C with a controlled light cycle (14 h of light/10 h of dark) and fed daily with commercial diet (MeM Prime, BERNAQUA, Olen, Belgium). The experiments were performed in compliance with Taiwan animal welfare regulations. The probiotic *B. amyloliquefaciens* R8 carries the pNZR8 plasmid, which heterologously expresses the xylanase R8 gene from rumen fungi by a lacA promoter [30]. Sources of the pathogens *A. hydrophila* and *S. agalactiae* are described in a previous report [31]. All bacteria were stored in 20% glycerol at −20 °C until use.

2.2. Probiotic preparation and administration

A 0.5-mL aliquot of *B. amyloliquefaciens* or *B. amyloliquefaciens* R8 from the glycerol stock was cultured in 50 mL of tryptic soy broth (TSB) (Difco, Kansas City, MO, USA) in an incubation shaker at 37 °C and 175 rpm for 16 h to obtain a seed culture. The seed culture was then added to a 5-L bioreactor (Winpact FS-02, Taoyuan, Taiwan) containing 3 L of TSB medium and cultured for 24 h at 37 °C. The culture broth was centrifuged at 6000 $\times g$ for 15 min at 4 °C to pelletize the cells. The pellets were collected and washed three times with phosphate-buffered saline (PBS), and then the suspended bacterial sample was freeze-dried to a powder for use in preparing a diet containing *B. amyloliquefaciens* or *B. amyloliquefaciens* R8. The number of viable bacterial cells in the powder was determined by serial dilutions and plate counting on TSB agar plates. The immunomodulatory functions of the probiotic bacterium *B. amyloliquefaciens* supplemented in the diet at levels of 10^6 colony-forming units (CFU) g^{-1} were reported in Nile tilapia [7,15]. Thus an appropriate amount of probiotic powder was added to the basal diet to obtain 2×10^6 CFU g^{-1} of *B. amyloliquefaciens* or *B. amyloliquefaciens* R8 in the present study. The ingredients and proximate composition of the experimental diets are given in Table 1. Adult zebrafish were randomly divided into three groups of 20 fish each, and the experiments were conducted in triplicate. Fish were fed twice daily with the basal diet at an amount equivalent to 2% of their body weight (control group) or the basal diet containing *B. amyloliquefaciens* (Amy group) or *B. amyloliquefaciens* R8 (Amy-R8 group) for 1 month. After 1 month of cultivation, expressions of genes related to metabolism, oxidative stress, and the innate immune response were evaluated along with the enzyme activities of 3-hydroxyacyl-coenzyme A (CoA) dehydrogenase (3-HAD) and citrate synthase (CS). A pathogen challenge test was also conducted.

Table 1
Ingredients used and proximate composition of the formulated diets (g kg^{-1}).

Ingredients	Base diet (Control)	*B. amyloliquefaciens* 2×10^9 CFU (Amy)	*B. amyloliquefaciens* R8 2×10^9 CFU (Amy-R8)
Probiotics	0	0.95	0.87
Fish meal	50	50	50
Soybean meal	360	360	360
Wheat middling	110	110	110
Rice bran	250	250	250
Soybean oil	60	60	60
α-starch	30	30	30
Cellulose	111	111	111
Skim milk	10	9.05	9.13
Mineral mixture[a]	16	16	16
Vitamin mixture[a]	3	3	3
Total	1000	1000	1000
Crude protein[b]	227.1	229.6	232.2
Crude lipid[b]	85.3	84.3	85.1
Moisture[b]	95.4	94.6	96.3
Ash[b]	121.3	123.3	122.8

[a] Mineral mixture (mg kg^{-1} of mixture) and vitamin mixture (mg kg^{-1} of mixture) provided by the Shinta Feed Company, Pingtung.
[b] The proximate composition of experimental diets was determined based on the analysis of AOAC method (AOAC 1997).

2.3. Evaluation of xylanase activity in vitro and in vivo

To evaluate the activity of xylanase *in vitro*, 0.7 g of the unmodified basal diet or basal diet containing *B. amyloliquefaciens* or *B. amyloliquefaciens* R8 was dissolved in 10 mL of sterile water and incubated at 28 °C. After 7 days of cultivation, the solution was centrifuged at 12,000 ×*g* for 15 min at 4 °C, and the supernatant was used to measure xylanase activity. To evaluate xylanase activity *in vivo*, the intestines of zebrafish fed the different diets for 1 month were collected 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 to measure xylanase activity. Xylanase activity was evaluated by determining the amounts of reduced sugars released from xylan according to DNS method described in a previous report [7].

2.4. Evaluation of 3-HAD and CS enzyme activities in the liver

The activities of 3-HAD and CS were measured as previously described with minor modifications [32]. Briefly, the livers of the fish were separated and homogenized for 2 min in 1 mL of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4{\cdot}12H_2O$, and 1.8 mM KH_2PO_4; pH 7.3) using a homogenizer (T10 basic, IKA, Staufen, Germany). Cellular debris was removed by centrifugation at 1000 ×*g* for 5 min, and then the supernatant was diluted five times with PBS buffer for use as follows in assays to assess the enzyme activity of 3-HAD. A 100-μL sample was added to 800 μL of reaction buffer (125 mM triethanolamine, 0.5625 mM NADH, and 6.25 mM EDTA; pH 7.0) in a cuvette (light path, 1 cm; Chrom Technology, Taipei, Taiwan). The cuvette was incubated at 30 °C for 5 min, and the reaction was initiated by adding 100 μL of 1 mM acetoacetyl-CoA. The 3-HAD enzyme activity was measured by the consumption of NADH at a wavelength of 340 nm for 5 min using a spectrophotometer (GENESYS 10S UV–Vis, Thermo Fisher Scientific, Waltham, MA, USA). To examine the activity of CS, supernatant samples described above were diluted ten times with 100 mM Tris-HCl buffer (pH 8.0) and used in assays as follows. A 100-μL assay sample was added to 800 μL of reaction buffer (0.125 mM dithionitrobenzoic acid, 0.375 mM acetyl-CoA, and 125 mM Tris-HCl) in a cuvette (light path, 1 cm). The cuvette was incubated at 30 °C for 5 min, and the reaction was initiated by adding 100 μL of 5 mM oxaloacetate. The CS enzyme activity was determined by the production of thionitrobenzoic acid at 412 nm for 5 min using a spectrophotometer.

Table 2
Primers used for mRNA quantification by real-time PCR.

Gene name	Primer sequence (5′→ 3′)	Accession number
Glycolysis-related genes		
Glucokinase (*GK*)	GCTGTGAAGTCGGCATGATA CTTCAACCAGCTCCACCTTAC	BC122359
Hexokinase 1 (*HK1*)	ACTTTGGGTGCAATCCTGAC AGACGACGCACTGTTTTGTG	BC067330
Glucose-6-phosphatase (*G6Pase*)	TCACAGCGTTGCTTTCAATC AACCCAGAAACATCCACAGC	BC164161
Pyruvate kinase, liver isoform (*PK-L*)	TCCTGGAGCATCTGTGTCTG GTCTGGCGATGTTCATTCCT	BC152219
Immune-related genes		
Interleukin-1β (*IL-1β*)	TGGACTTCGCAGCACAAAATG CACTTCACGCTCTTGGATGA	AY340959
Interleukin-6 (*IL-6*)	TCAACTTCTCCAGCGTGATG TCTTTCCCTCTTTTCCTCCTG	JN698962
Interleukin-21 (*IL-21*)	CATCGAGGAACAACGGTGTACA CACGAGCACAGCAAAGCAAT	NM_ 001128574
Tumor necrosis factor-α (*TNF-α*)	AAGGAGAGTTGCCTTTACCG ATTGCCCTGGGTCTTATGC	BC165066
Lysozyme	CGTGGATGTCCTCGTGTGAAG CCAATGGAGAATCCCTCAAA	NM_139180
Toll-like receptor-1 (*TLR1*)	CAGAGCGAATGGTGCCACTAT GTGGCAGAGGCTCCAGAAGA	AY389444
Toll-like receptor-3 (*TLR3*)	TGGAGCATCACAGGGATAAAGA TGATGCCCATGCCTGTAAGA	AY616582
Toll-like receptor-4a (*TLR4a*)	TTTCAGATGCCACATCAGA TCCACAAGAACAAGCCTTTG	EU551724
Oxidative stress and apoptosis-related genes		
Superoxide dismutase 1 (*SOD1*)	GTCGTCTGGCTTGTGGAGTG TGTCAGCGGGCTAGTGCTT	Y12236
Glutathione peroxidase 1a (*Gpx1a*)	GCTTTGAGGCACAACAGTCA TCTCCCATAAGGGACACAGG	AY216589
Nitric oxide synthase 2a (*NOS2a*)	GGAGATGCAAGGTCAGCTTC GGCAAAGCTCAGTGACTTCC	AY324390
Heat-shock protein (*HSP70*)	AAGCGACGAAGGATGCAGGAG CACGTTGCGCTCTGAGGATT	AF006007
Tumor protein 53 (*tp53*)	GGGCAATCAGCGAGCAAA ACTGACCTTCCTGAGTCTCCA	AF365873
B-cell leukemia/ lymphoma 2 (*Bcl-2*)	AGGAAAATGGAGGTTGGGATG TGTTAGGTATGAAAACGGGTGGA	AY695820
Elongation factor 1α (*EF-1α*)	AACAGCTGATCGTTGGAGTCAA TTGATGTATGCGCTGACTTCCT	AY422992

2.5. Real-time quantitative polymerase chain reaction (PCR)

Zebrafish from the control, Amy, and Amy-R8 groups were sampled, and total RNA was isolated from the liver or whole body of fish. A quantitative (q)PCR was used to determine expression levels of genes related to hepatic glucose metabolism, oxidative stress, apoptosis, the immune response, and *elongation factor* (*EF*)*-1α*. Expression of the *EF-1α* gene was used as an internal control. The specific PCR primers are listed in Table 2. A real-time PCR was performed using SYBR Green PCR reagents and an Applied Biosystems StepOnePlus Real-Time PCR system (Foster city, CA, USA). The cycling profile was as follows: 60 °C for 2 min; 95 °C for 10 min; and 40 cycles of denaturing at 95 °C for 15 s, annealing and primer extension at 60 °C for 1 min. Equal quantities of total RNA from three fish were mixed and examined in triplicate for each condition. Relative expression levels of each group were normalized to *EF-1α* and expressed as the mean ± standard error (SE).

2.6. Challenge test

Aeromonas hydrophila and *S. agalactiae* were separately cultured in TSB at 28 °C for 24 h. Bacterial cells were collected by centrifugation at 6100 ×*g* for 15 min at 4 °C and then resuspended in an appropriate volume of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4{\cdot}12H_2O$, and 1.8 mM KH_2PO_4; pH 7.3). A bacterial challenge experiment was carried out in triplicate by intraperitoneally (IP)

injecting 10 μL of diluted *A. hydrophila* or *S. agalactiae* into zebrafish at a concentration of 10^5 CFU per fish. Experimental zebrafish (15 fish per tank) were kept in a tank containing 10 L of fresh water at 28 °C. There were four groups for each challenge test (negative control, control, Amy, and Amy-R8 groups). Fish fed the control diet and injected with pathogens or with PBS buffer were respectively used as the positive and negative controls. Each group was tested in triplicate. Challenged fish were observed daily, and mortalities were recorded for 7 days.

2.7. Statistical analysis

Relative expression levels of each group were normalized to *ef-1α* and expressed as the means ± SE. Student's *t*-test was used to statistically analyze and compare groups. Multiple-group comparisons were examined using a one-way analysis of variance (ANOVA), and Tukey's test was used to evaluate significant differences between groups. Differences were defined as significant at $p < 0.05$.

3. Results

3.1. Administration of probiotics enhances gene expressions and enzyme activities which are involved in glucose and fatty acid metabolism in zebrafish

Before evaluating the effect of the probiotics on zebrafish metabolism, the *in vitro* and *in vivo* xylanase activities of *B. amyloliquefaciens* R8 were measured. The level of XOSs hydrolyzed from xylan by xylanase was determined by a DNS assay. *In vitro* incubation of the basal diet containing *B. amyloliquefaciens* R8 showed significantly increased XOS levels compared to the control and Amy diets (Fig. 1A). Similarly, the XOS level in the intestines of zebrafish fed the Amy-R8 diet was significantly higher than in those of zebrafish fed the control and Amy diets (Fig. 1B). The XOS level was higher in intestines of zebrafish fed the Amy diet group than that of fish fed the control diet, but the difference was not significant. Genes and enzyme activities involved in glycolysis and mitochondrial fatty acid oxidation were determined to evaluate the nutrient metabolism in zebrafish. A real-time PCR was used to determine mRNA levels of hepatic glycolytic enzymes, such as glucokinase (GK), hexokinase-1 (HK1), glucose-6-phosphatase (G6Pase), and pyruvate kinase (PK-L). Relative mRNA levels of GK, HK1 and G6Pase were significantly increased in zebrafish in the Amy and Amy-R8 groups compared to the control group. Although relative mRNA levels of HK1 and G6Pase did not significantly differ between fish in the Amy and Amy-R8 groups, higher levels of mRNA expression were detected in the Amy-R8 group. Moreover, PK-L expression was significantly higher in the Amy-R8 group compared to the Amy and control groups (Fig. 2). Activities of 3-HAD and CS were higher in the Amy and Amy-R8 groups than in the control group. Moreover, 3-HAD activity was significantly higher in the Amy-R8 group than in the Amy group (Fig. 3).

3.2. Administration of probiotics modulates expression of genes which involved in hepatic stress and apoptosis in zebrafish

The hepatic expression profile was evaluated for zebrafish genes related to oxidative stress, such as *superoxide dismutase* (*SOD*), *glutathione peroxidase 1a* (*Gpx1a*), *nitric oxide synthase 2a* (*NOS2a*), and *heat shock protein 70* (*Hsp70*). There were significantly lower gene expressions of *SOD, Gpx1a,* and *NOS2* in the Amy-R8 group than in the Amy and control groups. Moreover, expressions of these genes were also significantly lower in the Amy group than in the control group (Fig. 4A–C). Expressions of *Hsp70* in the Amy and Amy-R8 groups were significantly lower than that in the control group, but there was no significant difference between the Amy and Amy-R8 groups (Fig. 4D). The reduction in expression of oxidative stress-related genes as a result of *B. amyloliquefaciens* and *B. amyloliquefaciens* R8 administration is also supported by lower expressions of genes involved in apoptotic processes. Expressions of the anti-apoptotic signaling *B-cell leukemia/lymphoma 2* (*Bcl-2*) gene in the livers of zebrafish fed the Amy and Amy-R8 diets were significantly higher than that of fish fed the control diet. Moreover, *Bcl-2* expression in the Amy-R8 group was significantly higher than that in the Amy group (Fig. 4E). In contrast, the proapoptotic *tumor protein p53* (*tp53*) gene was significantly downregulated in the livers of zebrafish fed the Amy and Amy-R8 diets, and its expression in zebrafish fed the Amy-R8 diet was significantly lower than that in fish fed the Amy diet (Fig. 4F).

3.3. Dietary supplementation with probiotics enhances immune-related gene expressions

To determine whether the high level of XOSs derived from xylanase activity of *B. amyloliquefaciens* R8 can effectively enhance innate immune responses, expressions of cytokine genes in the whole body were evaluated after 1 month of dietary supplementation with the Amy and Amy-R8 diets. Expressions of *interleukin* (*IL*)*-1β*, *IL-6*, *toll-like receptor* (*TLR*)*-1,* and *TLR-3* from the whole body of fish in the Amy and Amy-R8 groups were significantly higher than those in the control group, and there were no significant differences between the Amy and Amy-R8 groups. Expression levels of the *IL-21*, *TNF-α*, and *lysozyme* genes in the whole body of zebrafish supplemented with the Amy and Amy-R8 diets

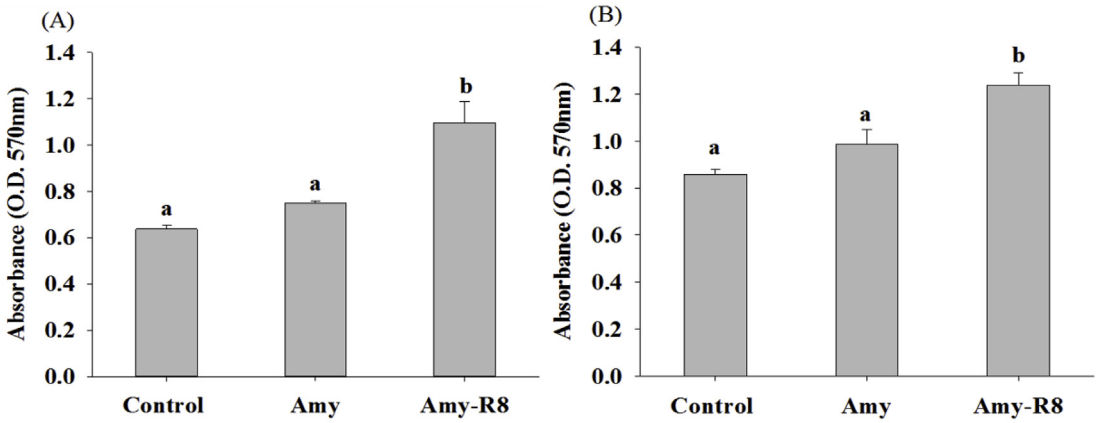

Fig. 1. The level of xylan oligosaccharides (XOS) resulting from xylanase hydrolysis of xylan was determined by measuring absorbance at 570 nm. (A) *In vitro* and (B) *in vivo* xylanase activity was evaluated using the DNS method. The data are presented as the mean ± S.E. from six individual samples (n = 6). Values with different letters indicate a significant difference ($p < 0.05$).

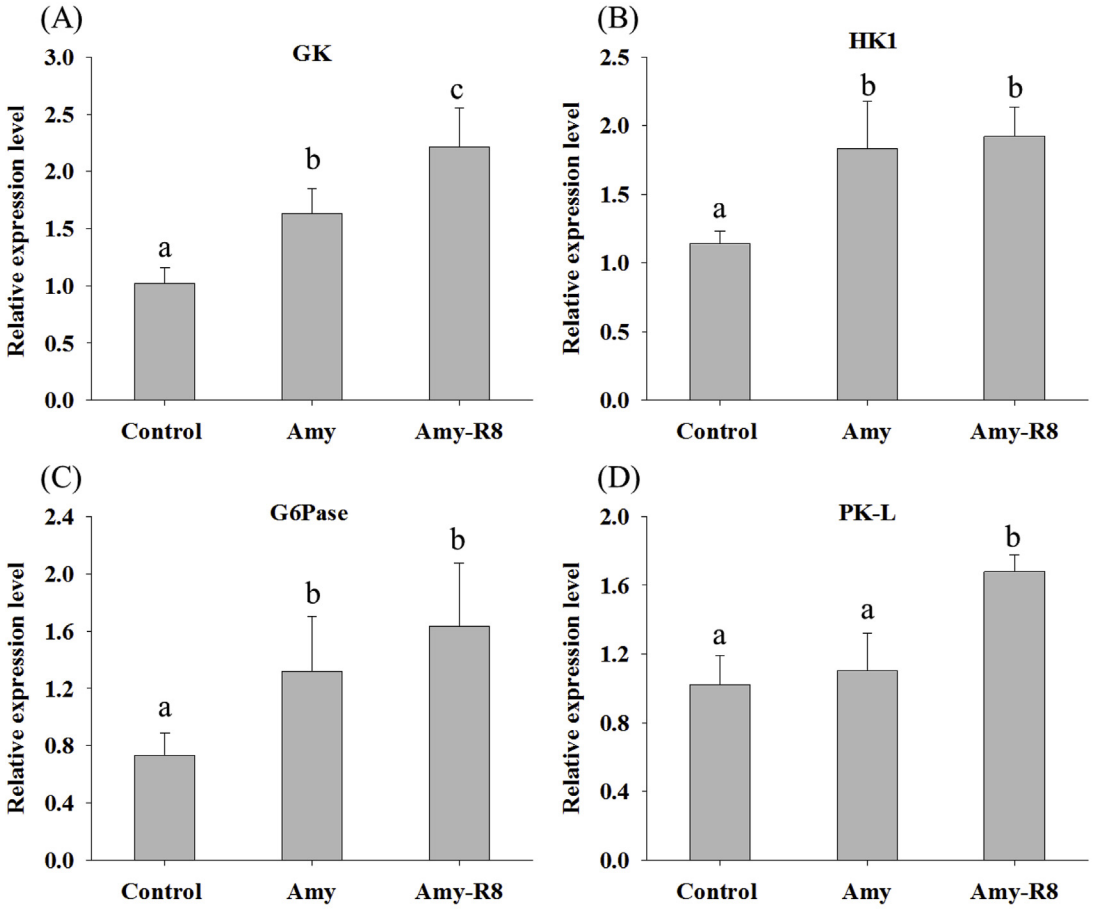

Fig. 2. Relative expression levels of hepatic metabolism genes involved in glycolysis in zebrafish supplemented with basal diet (control), basal diet containing *B. amyloliquefaciens* (Amy), and basal diet containing *B. amyloliquefaciens* R8 (Amy R8) for 1 month: (A) glucokinase (*GK*), (B) hexokinase 1 (*HK1*), (C) glucose-6-phosphatase (*G6Pase*), and (D) pyruvate kinase isoform from the livers (*PK-L*). The data are presented as the mean ± S.E. from six individual samples (n = 6). Bars with different superscripts are significantly different ($p < 0.05$).

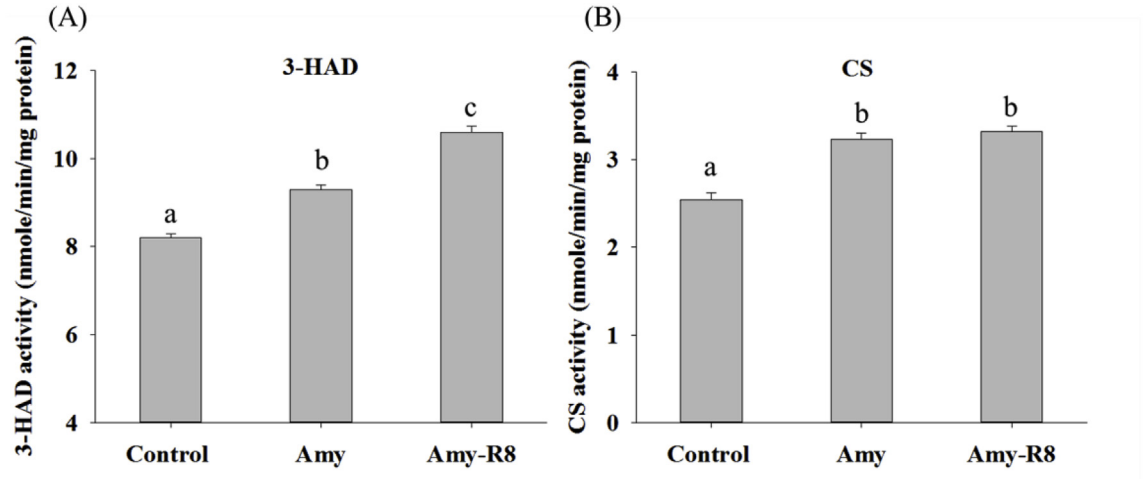

Fig. 3. Enzyme activity levels of (A) 3-HAD and (B) CS determined by spectrophotometry. The data are presented as the mean ± S.E. from six individual samples (n = 6). Values with different letters indicate a significant difference ($p < 0.05$).

for 2 months were higher than in zebrafish fed the control diet. Moreover, there were significant differences in *IL-21*, *TNF-α,* and *lysozyme* gene expressions between the Amy and Amy-R8 groups. The mRNA expression of *TLR-4* in the Amy-R8 group was significantly higher than those in the control and Amy groups (Fig. 5). Significant increases in the cytokine genes of *IL-1β*, *IL-6*, *IL-21*, *TNF-α*, *lysozyme*, *TLR-1,* and *TLR-3* in the Amy and Amy-R8 groups suggest that the heterologous expression of xylanase in *B. amyloliquefaciens* enhanced the immune response in zebrafish.

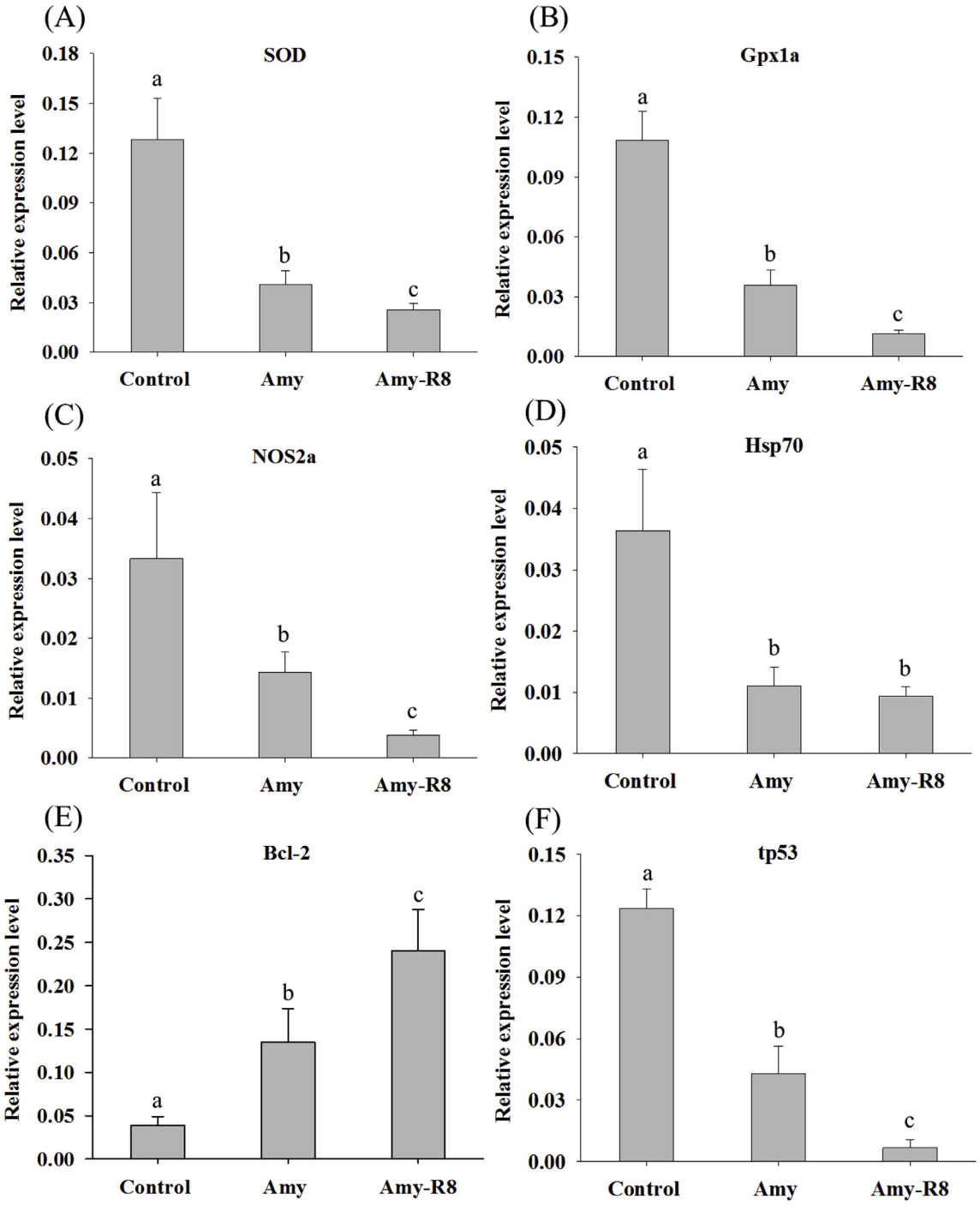

Fig. 4. Relative expression levels of haptic genes in zebrafish supplemented with basal diet (control), basal diet containing *B. amyloliquefaciens* (Amy), or xylanase-expressed *B. amyloliquefaciens* (Amy-R8) for 1 month. Genes involved in oxidative stress: (A) *SOD*, (B) *Gpx1*, (C) *NOS2a,* and (D) *Hsp70*. Genes involved in apoptosis: (E) *Bcl-2* and (F) *tp53*. The data are presented as the mean ± S.E. from six individual samples (n = 6). Bars with different superscripts significantly differences ($P < 0.05$).

3.4. Dietary supplementation of probiotics enhanced defense against A. hydrophila and S. agalactiae in zebrafish

To determine the effect of the probiotic on disease resistance against bacterial infection, we measured survival rates of zebrafish fed the Amy and Amy-R8 diets after being challenged with the Gram-negative *A. hydrophila* and Gram-positive *S. agalactiae*. As shown in Fig. 6, the survival rate of zebrafish at 7 days post-infection was 100% for the group injected with PBS buffer. However, survival rates in the control group injected with *A. hydrophila* or *S. agalactiae* were dramatically reduced during the first 4 days post-infection, and then were respectively maintained at 10% ± 0.0% and 30% ± 10% to 7 days post-infection (Fig. 6). Survival rates in zebrafish in the Amy and Amy-R8 groups were significantly higher than that in fish fed the control diet. Survival rates of the Amy and Amy-R8 groups at 7 days post-infection with *A. hydrophila* were 36.7% ± 5.8% and 56.7% ± 5.8%, respectively (Fig. 6A). Respective survival rates of the Amy and Amy-R8 groups at 7 days post-infection with *S. agalactiae* were 66.7% ± 5.8% and 73.3% ± 5.8% (Fig. 6B). Survival rates for fish supplemented with Amy and Amy-R8 showed no significant difference at 7 days post-

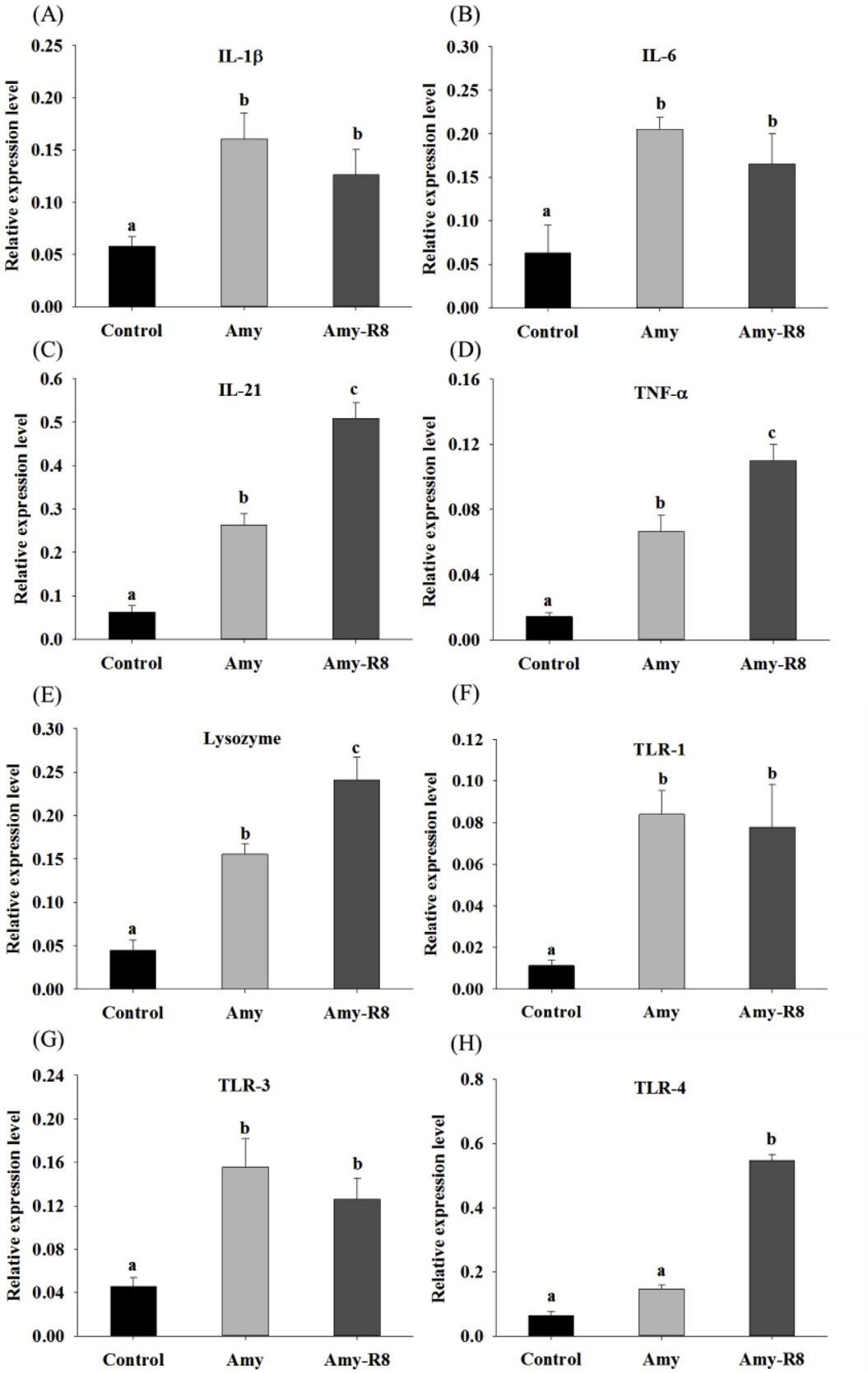

Fig. 5. Relative expression levels of immuno-related genes in zebrafish supplemented with basal diet (control), basal diet containing *B. amyloliquefaciens* (Amy), and basal diet containing *B. amyloliquefaciens* R8 (Amy R8) for 1 month: (A) *IL-1β*, (B) *IL-6*, (C) *IL-21*, (D) *TNF-α*, (E) *Lysozyme*, (F) *TLR-1*, (G) *TLR-3*, and (H) *TLR-4*. The data are presented as the mean ± S.E. from six individual samples (n = 6). Values with different letters indicate a significant difference ($p < 0.05$).

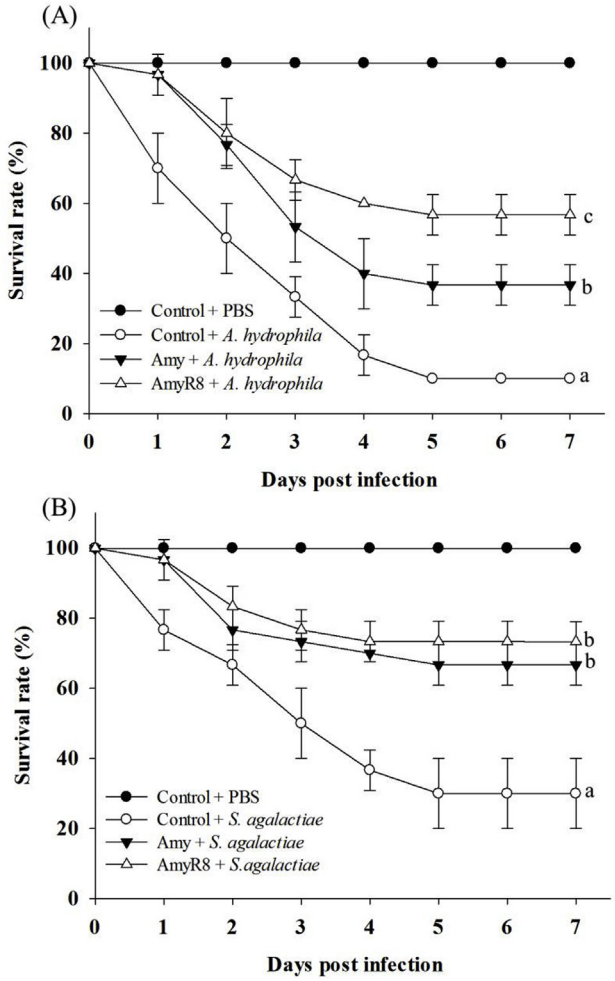

Fig. 6. Survival rates of zebrafish challenged with (A) *A. hydrophila*, and (B) *S. agalactiae*. The daily survival rate of each group was measured in triplicate and is presented as the mean ± standard deviation (SD). Different superscripts represent significant differences ($p < 0.05$).

infection with *Str. agalactiae*. The significant increase in survival suggests that supplementation with *B. amyloliquefaciens* and *B. amyloliquefaciens* R8 enhanced resistance against *A. hydrophila* and *S. agalactiae* infection in zebrafish.

4. Discussion

Bacillus amyloliquefaciens is a popular probiotic species that confers benefits such as growth enhancement and disease resistance in aquaculture according to recent reports [15–17]. Heterologous expressions of functional genes are an efficient approach to strengthen probiotic functions in aquaculture. In the present study, metabolic and immunomodulatory parameters were evaluated with the potential probiotic *B. amyloliquefaciens* R8, which is a recombinant expressing xylanase from rumen fungi. The results showed high levels of XOSs in the Amy-R8 group, suggesting that zebrafish fed a diet containing *B. amyloliquefaciens* R8 exhibited high xylanase activity that resulted in higher levels of XOSs in the intestines. The use of XOSs as a prebiotic supplement in feed was reported to improve the growth, feed efficiency, and health status of fish. However, there are few investigations on the effects of XOSs on the molecular level of nutrient metabolism. The liver is an important metabolic organ, and its metabolic activity is tightly controlled by nutrient metabolism. HK, GK, and PK are rate-limiting enzymes that are involved in regulating glycolysis. G6Pase is an enzyme mainly found in the liver and plays an important role in providing glucose during glycogenolysis. Recently, Guerrriro et al. reported that using XOSs as a prebiotic supplemented in feed increased glucose metabolism in European sea bass (*D. labrax*) [33]. Results of molecular expressions of glycolytic genes, such as *HK1, GK, G6Pase,* and *PK-1,* in zebrafish fed the Amy-R8 diet also support this conclusion. CS is a key enzyme in the mitochondrial Krebs cycle that catalyzes the condensation reaction of acetyl-CoA and oxaloacetate to form citrate, and its activity is often used as a biomarker of intact mitochondria [34]. 3-HAD is a key enzyme involved in the β-oxidation of mitochondrial fatty acids and catalyzes the reduction of 3-hydroxyacyl-CoA to 3-oxoacyl-CoA. Reports show that dietary supplementation with XOSs can improve lipid metabolism in European sea bass (*D. labrax*) and common carp (*Cyprinus carpio*), demonstrating the function of prebiotic XOSs in stimulating fatty acid metabolism [33,35]. Consistently, the induced enzyme activities of 3-HAD and CS suggested higher mitochondrial integrity and lipid β-oxidation in zebrafish fed the Amy-R8 diet. Therefore, the results suggest that supplementation with *B. amyloliquefaciens* R8 possibly increases the utilization of XOSs and enhances liver glucose and lipid metabolism.

This study investigated the effects of the dietary administration of *B. amyloliquefaciens* R8 on hepatic oxidative stress and apoptosis in zebrafish. Oxidative stress is defined as a state in which there is an imbalance of oxidants and antioxidants in cells, resulting in DNA damage, protein denaturation, lipid peroxidation, and apoptosis. Increasing numbers of reports have demonstrated that probiotics can reduce oxidative stress in fish [36–38]. Superoxide anions (O_2^-) and nitric oxide (NO) kill invading pathogens in phagocytes. In macrophages, NO is synthesized by inducible NO synthase (iNOS, NOS2) from L-arginine and oxygen, but O_2^- is thought to be produced mainly by NADPH oxidase. Expressions of hepatic superoxide dismutase (SOD) and glutathione peroxidase (Gpx) are induced in response to oxidative stress to convert O_2^- into H_2O_2, which is then converted into water and oxygen. *Hsp70* plays a vital role in general cellular protection and homeostasis in all living organisms. The induced expression of *Hsp70* mRNA was observed in response to diverse stresses in fish. Thus, *Hsp70* gene expression is used as a sensitive biomarker for monitoring the physical status and aquatic environments of fish. Significantly lower expression levels of hepatic *SOD*, *Gpx1a*, *NOS2a,* and Hsp70 genes in zebrafish fed the Amy and Amy-R8 diets suggest that dietary supplementation with *B. amyloliquefaciens* or *B. amyloliquefaciens* R8 can possibly reduce oxidative stress levels in zebrafish. Reports indicated that oxidative stress can directly induce DNA damage and cell apoptosis [39]. However, the effects of probiotics on the pathways of oxidative stress and cell apoptosis in aquatic species are not well studied. So far, only one study observed decreases in oxidative stress and apoptotic-related genes such as *tp53* in zebrafish given the probiotic *Lactobacillus rhamnosus* [40]. In the present study, the decreased mRNA expression level of *tp53* and increased expression level of the antiapoptotic *bcl2* gene in the liver of zebrafish in the Amy and Amy-R8 groups elucidated that zebrafish supplemented with *B. amyloliquefaciens* and *B. amyloliquefaciens* R8 can potentially prevent hepatic apoptosis in fish. Moreover, expression levels of hepatic *SOD*, *Gpx1a*, *NOS2a*, *bcl2,* and *tp53* significantly differed between fish in the Amy and Amy-R8 groups, suggesting that increased xylanase or XOSs in fish intestines can reduce oxidative stress and apoptosis in fish. To the best of our knowledge, the present study is the first report to demonstrate that dietary supplementation with a xylanase-expressing probiotic or increased XOSs exhibit antioxidative stress and antiapoptotic functions in fish.

Modulation of innate immunity is one of the major benefits that probiotics confer on fish. Increasing evidence has demonstrated that *B. amyloliquefaciens* can enhance the innate immunity of fish against disease infection. Reports demonstrated beneficial effects of dietary *B. amyloliquefaciens* supplementation in increasing the survival rate of Labeo rohu (*L. rohita*) challenged with *Aer. hydrophila* [41]; in striped catfish (*P. hypophthalmus*) challenged with *E. ictaluri* [16]; in catla (*C. catla*) challenged with *E. tarda* [17]; and in Nile tilapia challenged with

A. hydrophila, *Y. ruckeri,* and *C. perfringens* type D [7,15]. Likewise, in the present study, survival rates of zebrafish challenged with *A. hydrophila* and *S. agalactiae* were higher in the Amy and Amy-R8 groups compared to the control group. This suggests that the probiotics *B. amyloliquefaciens* and *B. amyloliquefaciens* R8 enhanced innate immunity against pathogenic infections. Cytokines released from innate immune cells play key roles in regulating immune responses. Thus, we evaluated expressions of cytokines and immune-related genes in zebrafish after administering *B. amyloliquefaciens* and *B. amyloliquefaciens* R8. Results showed significant increases in gene expression levels of *IL-1β, IL-6, IL-21,* and *TNF-α* in the Amy and Amy-R8 groups compared to the control group. Moreover, expressions of *IL-21* and *TNF-α* exhibited significant differences between the Amy and Amy-R8 groups. These results are consistent with those of other reports on the use of probiotic *Bacillus* spp. in fish, in which expressions of cytokine genes were found to have significantly increased after the dietary administration of *B. subtilis* [42–44]. Furthermore, a recent report demonstrated that Nile tilapia (*O. niloticus*) supplemented with dietary *B. amyloliquefaciens* exhibited increased expression levels of *IL-1* and *TNF-α* in the kidneys [15]. Cytokines produced from activated macrophages and monocytes modulate systemic or local immune responses to infection and immunological challenge. Dietary supplementation with *B. amyloliquefaciens* and *B. amyloliquefaciens* R8 induced cytokine expressions in zebrafish, which suggests that the probiotics exhibited an immunomodulatory function in fish. Lysozyme is a cornerstone of host immunity that cleaves to peptidoglycans of bacterial cell walls. In addition to its antimicrobial activity, recent evidence showed that lysozyme activates phagocytosis and a response of the complement system to infection [45].

The present results showed that zebrafish fed *B. amyloliquefaciens* and *B. amyloliquefaciens* R8 exhibited significant increases in expressions of lysozyme genes compared to zebrafish fed the control diet. Similar results were also observed in tilapia fed *B. amyloliquefaciens* and *B. licheniformis,* and in catfish fed *B. aerius* and *B. pumilus* [11,15,16,46]. Recent reports indicated that seabass (*D. labrax*) given dietary XOSs showed enhanced innate immunity and increased lysozyme production against *A. hydrophila* infection [26,47]. In the present study, the Amy-R8 group had higher expression of lysozyme than the Amy group. This suggests that the immunostimulatory function of xylanase from *B. amyloliquefaciens* R8 in zebrafish may act through the effects of XOSs digested from plant ingredients in the feed.

Pattern recognition receptors (PRRs) such as TLRs recognize widely conserved motifs of pathogens and are crucial for activating immune responses against pathogen infections. TLR1 recognizes peptidoglycans and lipoproteins in concert with TLR2 with a specificity for Gram-positive bacteria. TLR4 is well-known for recognizing lipopolysaccharide (LPS), which is a component present in the outer membranes of gram-negative bacteria. Activation of the TRL4 signaling pathway leads to the activation of nuclear factor (NF)-κB and induces the production of proinflammatory cytokines. Although the *in vitro* effects of probiotics on expressions of TLRs have been widely studied, there are fewer *in vivo* studies on fish. Recently, Abid et al. demonstrated that the dietary administration of the synbiotics *Pediococcus acidilactici* and short-chain fructooligosaccharides stimulated innate immune responses and modulated TLR3 expression in the intestines of Atlantic salmon (*Salmo salar*) [48]. The present results showed increased expressions of TLR1 and TLR4 in zebrafish in the Amy-R8 group. This suggests that dietary supplementation with *B. amyloliquefaciens* R8 can enhance immunity against Gram-negative and -positive pathogen infections. TLR3 plays a critical role in innate immunity against viral infections by recognizing double-stranded RNA and inducing the production of type I interferons. Recently, Chai et al. demonstrated that dietary supplementation with the probiotic *Bacillus* PC465 can enhance the innate immunity in white shrimp (*Litopenaeus vannamei*) against infection with white spot syndrome virus [49]. The increased expression of TLR3 in the Amy and Amy-R8 groups suggests that dietary supplementation with *B. amyloliquefaciens* and *B. amyloliquefaciens* R8 may have functions against viral infection, but further investigations are required.

In conclusion, the results provide new insights into the beneficial effects of xylanase-expressing *B. amyloliquefaciens* R8 on improving hepatic glucose and lipid metabolism, and reducing oxidative stress and cell death in a zebrafish model. Furthermore, the probiotic modulated the innate immune responses against *A. hydrophila* and *S. agalactiae* infections. The results showed enhanced effects of the combination of heterologous xylanase and *B. amyloliquefaciens,* suggesting that *B. amyloliquefaciens* R8 can be used as a probiotic and has benefits in aquaculture.

Acknowledgments

This study was supported by a grant from the Ministry of Science and Technology (MOST 106-2313-B-020-007) of Taiwan.

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