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Improvements in the growth performance, immunity, disease resistance, and gut microbiota by the probiotic *Rummeliibacillus stabekisii* in Nile tilapia (*Oreochromis niloticus*)

Herng Yih Tan[a], Sai-Wei Chen[a], Shao-Yang Hu[a,b,*]

[a] Department of Biological Science and Technology, National Pingtung University of Science and Technology, Pingtung, 912, Taiwan
[b] Research Center for Animal Biologics, National Pingtung University of Science and Technology, Pingtung, 912, Taiwan

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ABSTRACT

The application of probiotics as an eco-friendly alternative to antibiotics is an emerging strategy for sustainable aquaculture. In the present study, *Rummeliibacillus stabekisii* was isolated from the gut of Nile tilapia, and the effects of *R. stabekisii* on the growth, innate immunity, disease resistance, and gut microbiota of Nile tilapia (*Oreochromis niloticus*) were investigated. The results showed significantly increased weight gain (WG), feed conversion ratio (FCR), and feed efficiency (FE) in Nile tilapia fed *R. stabekisii* for 8 weeks compared to those in fish fed a control diet. Intestinal digestive enzymes such as protease, cellulase, and xylanase were also significantly increased in the *R. stabekisii*-administered groups. Enhanced cumulative survival was exhibited in fish fed *R. stabekisii* after challenge with *Aeromonas hydrophila* and *Streptococcus iniae*. Immune parameters such as the phagocytic activity, respiratory bursts, and superoxide dismutase of head kidney leukocytes; serum lysozyme activity; and expression of the cytokine genes *interleukin-1β*, *tumor necrosis factor-α*, *transforming growth factor-β*, and *heat shock protein 70* were significantly elevated in fish fed *R. stabekisii*. Administration of *R. stabekisii* considerably increased the abundance of potential probiotics (*Bacillus* and *Lactobacillus* spp.) and reduced abundances of potential pathogenic bacteria (*Streptococcus* and *Staphylococcus* spp.) in fish intestines. The present study indicated that dietary supplementation with *R. stabekisii* improved the growth, immunity, disease resistance, and gut microflora of Nile tilapia. This research is the first study reporting that the genus *Rummeliibacillus* is a potential probiotic in animals, suggesting that *R. stabekisii* can be used as a feed additive to enhance the growth and health status in tilapia.

1. Introduction

Aquaculture is considered the fastest-growing way to produce food to supply animal protein for human consumption. It has the potential to contribute to the global economy and meet some of the increasing demand for seafood worldwide. Tilapia are farmed in more than 100 countries and are the second most cultured fish species worldwide in terms of production yield [1]. However, even though tilapia are easily cultured, grow rapidly, and are relatively tolerant of environmental stresses compared to other fish species, incidences of bacterium-induced diseases still occur frequently and are increasing due to deterioration of water quality resulting from intensive culture in the pursuit of high-yield production [2]. For instance, *Aeromonas hydrophila* and *Streptococcus iniae* are typical pathogens resulting in hemorrhagic septicemia in tilapia [3,4]. Over the past few decades, such diseases have caused significant mortality of cultured fish and substantial economic losses, and thus have become a major potential threat to the sustainable development of tilapia aquaculture. Traditionally, antibiotics and chemicals have been commonly used to treat or prevent disease outbreaks in cultured fish species. However, the abuse of antibiotics and chemicals has led to the rapid spread of drug-resistant pathogens in aquaculture environments and residual antibiotics in aquatic products [5]. Moreover, the use of antibiotics as prophylactic and therapeutic measures causes gut dysbiosis and induces resistant bacterial populations in fish that may result in reduced nutrient metabolism, immunity, and disease resistance [6]. Regarding these issues, alternative strategies for disease control in aquaculture need to be urgently developed.

The application of probiotics as an eco-friendly alternative to antibiotics and chemicals for disease control in aquaculture has attracted increasing attention in the recent years [7]. Unlike the disease control

* Corresponding author. Department of Biological Science and Technology, National Pingtung University of Science and Technology, 1 Hseufu Road, Neipu, Pingtung, 912, Taiwan.
E-mail address: syhu@mail.npust.edu.tw (S.-Y. Hu).

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by antibiotics and chemicals being proposed in aquaculture where most action mechanisms are unilateral, the enormous potential of probiotics as health-promoting to fish relies on their multiple action modes, such as competition with pathogens for adhesion sites, production of antimicrobial substances in the gastrointestinal (GI) lumen to prevent growth of opportunistic pathogenic microorganisms, competition for nutrients that are essential for the growth of pathogens and stimulation of the immune system of hosts [8]. Among the numerous defensive actions of probiotics for fish health, the role of probiotics in modulating the immune system via cytokine action is one of the most commonly purported mechanisms of probiotics. Cytokines secreted from immune cells, such as lymphoid cells, macrophages and monocytes, play important roles in triggering innate immune and inflammatory responses to defend against pathogen infection. The literature indicates that a number of probiotics can effectively modulate immune cells to produce cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and transforming growth factor β (TGF-β) [9]. IL-1β 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. TGF-β is a multipotent cytokine that plays an essential role in the suppression of inflammation. Despite the lack of extensive investigation on the functional role of TGF-β in teleosts, the increased expression of TGF-β in pathogen-infected fish suggests its protective role in preventing excessive inflammation [10]. Fish heat shock protein 70 (HSP70) is a stress protein that is induced in response to various physiological stresses, including pathogen infection. Moreover, HSP70 can also stimulate the production of diverse cytokines, including TNF-α and IL-1β, in immune cells (macrophage, head kidney leukocytes), suggesting its role in fish immunity [11]. These immune-related genes have been widely used as sensitive biomarkers for monitoring the physical status of fish.

Plant-derived proteins such as soybean meal, rice gluten meal, and wheat middlings are commonly used to reduce the use of fish meal in feed. Non-starch polysaccharides (NSPs), such as cellulose and hemicellulose, are the major components of plant cell walls. However, NSPs increase feed viscosity and limit feed availability, which are recognized as anti-nutritional factors and the main cause of limited growth performance in aquaculture. Probiotics yielding hydrolytic enzymes is an important property that supports wide used of probiotics as feed additives to improve nutrient utilization, feed efficiency (FE) and fish growth. For example, proteases can break down complex proteins into oligopeptides or amino acids. Studies have reported that dietary supplementation with protease-producing probiotics could effectively enhance nutrient utilization and growth performance in Nile tilapia and *Labeo calbasu* [12,13]; xylanase is a typical enzyme that hydrolyzes linear polysaccharides and hemicellulose. Dietary supplementation with xylanase-producing probiotics could enhance Nile tilapia growth by improving FE and degrading NSPs in fish feed ingredients of plant origin [14]. These reports demonstrated the improved functions of hydrolytic enzyme-producing probiotics on nutrient utilization and growth performance. In addition to improving immune modulation and feed utilization or FE, probiotics also confer beneficial effects to the host, such as returning a deteriorated microbiota to a normal beneficial status, enhancing a host's defense against diseases, and improving the quality of the culture environment [15]. Because probiotics exhibit multiple advantages, a variety of bacteria have attracted attention as potential probiotics in fish farming sectors in recent years. Among these diverse probiotics, gram-positive spore-forming lactic acid bacteria (mainly of the genera *Lactococcus*, *Lactobacillus,* and *Enterococcus*) and *Bacillus* spp. are the most commonly used biocontrol agents or immunostimulants in aquaculture due to their high tolerance to harsh stresses, antagonistic activities against pathogens, extracellular enzyme production, and ready availability [16]. However, the fundamental principle of beneficial effects of probiotics acting on a host is considered to be species- or even strain-specific [17]; hence, different probiotics for application demands of diverse aquatic animals in aquaculture need to be developed.

The genus *Rummeliibacillus* was originally included in the genus *Bacillus* and was named in honor of the former NASA Planetary Protection Officer Dr. John Rummel, an astrobiologist who brought planetary protection into the public domain. In 2009, the first strains described as belonging to species of *Rummeliibacillus* were isolated from different geographical locations (from the Payload Hazardous Servicing Facility at the Kennedy Space Center in Florida, USA), and they were reclassified into the new genus *Rummeliibacillus* based on phylogenetic analysis of 16S ribosomal RNA gene sequences. *Rummeliibacillus stabekisii* was proposed as the type species of the genus *Rummeliibacillus,* and its whole-genome sequence was reported [18,19]. To date, only two studies that referred to the classification of three species of *Rummeliibacillus,* including *R. stabekisii*, *R. suwonensis,* and *R. pycnus,* have been published, and there are no application studies. In the present study, *R. stabekisii* was isolated from tilapia intestines as a potential probiotic with the ability to produce protease and xylanase. The effects of dietary supplementation with *R. stabekisii* on the utilization of feed nutrients were assessed based on FE and growth performance. Immune parameters such as the phagocytic activity (PA), respiratory bursts (RBs), superoxide dismutase (SOD) activity of head kidney leukocytes, serum lysozyme level and cytokine gene (*interleukin* (*IL*)*-1β, tumor necrosis factor* (*TNF*)*-α*, and *transforming growth factor* (*TGF*)*-β*) expression were determined to evaluate the effects of *R. stabekisii* on immune modulation. The effect of dietary supplementation with *R. stabekisii* on the disease resistance of tilapia against pathogenic infections was evaluated by challenging with *A. hydrophila* and *S. iniae*. Moreover, the effect of *R. stabekisii* on the gut microbiota of tilapia was analyzed by next-generation sequencing (NGS). These validations can provide important information for evaluating the practicality of *R. stabekisii* as a probiotic in tilapia aquaculture.

2. Materials and methods

2.1. Fish and bacteria

Nile tilapia (*Oreochromis niloticus*) fingerlings were purchased from a domestic fish farm and acclimatized in glass aquaria at 28 °C for 10 days in the Aquatic Laboratory Animal Facility of National Pingtung University of Science and Technology, which is certificated by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All fish were fed a basal diet during the acclimatization period and were handled in compliance with local welfare regulations. The sources and culture conditions of the *A. hydrophila* and *S. iniae* pathogens were described in a previous report [20]. All bacteria were cultured in tryptic soy broth (TSB) medium at 28 °C and then stored in 20% glycerol at −20 °C until use.

2.2. Screening, isolation and identification of potential probiotics

Six apparently healthy *O. niloticus* (68.62 ± 15.23 g) were randomly collected from two domestic fish farms in Pingtung. Fish were starved for 72 h before dissection. The abdominal surface was sterilized with 75% alcohol, and the peritoneal cavity was then opened aseptically with a sterile blade. The complete intestine tissue was removed, weighed (average 1.52 ± 0.32 g) and homogenized in 5 mL of sterile water. The total intestinal homogenate was incubated in 250-mL flask containing 50 mL of TSB medium at 28 °C for 24 h to enrich bacterial population. For isolating endospore-producing probiotics, 5 mL of the enriched culture was incubated in a 250-mL flask containing 50 mL of TSB medium at 45 °C for 48 h. Subsequently, the culture was shifted to an 80 °C water bath for 20 min to reduce the population of non-endospore-producing bacteria, and then spread and incubated on TSB agar medium at 28 °C for 24 h. After incubation, each colony was picked and incubated in 50 mL of TSB medium for 72 h. After

centrifugation at 4000 × *g* for 15 min at 4 °C, 2 mL of cell-free supernatant was used to determine protease, cellulase, amylase, and xylanase activities. Based on the hydrolytic enzyme activities, the colony with the strongest protease and xylanase activity was isolated. Isolate identification was performed by the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) using 16S rDNA sequencing and biochemical analysis. A 16S rDNA fragment was amplified by PCR using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARC C-3′). 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 a 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, oxidase, motility, indole production, enzyme activity (arginine dihydrolase, urease, β-glucosidase and β-galactosidase) and carbohydrate fermentation (glucose, arabinose, mannose, mannitol, maltose and N-acetyl-glucosamine) tests.

2.3. Preparation of experimental diet

The protocol for preparing experimental diets was modified in accordance with a previous report [14]. Briefly, culture broth of *R. stabekisii* was centrifuged at 6000 × *g* for 15 min at 4 °C to collect a cell pellet. The pellet was washed three times with phosphate-buffered saline (PBS) and suspended in PBS again. The number of viable bacterial cells in the suspended solution was determined via serial dilution and plate counting on TSB agar plates. An appropriate amount of suspended solution was added to the basal diet, resulting in a diet containing 10^6 colony-forming units (CFU)/g and 10^7 CFU/g. The basal diet comprised the following formulation (w/w): 47% soybean meal, 18% gluten, 10% fish meal, 10% α-starch, 5.7% canola oil, 5.4% corn starch, 1% carboxymethyl cellulose (CMC), 1.9% mineral mixture, and 1% vitamin mixture. Based on the AOAC method (AOAC 1997), the proximate compositions of the experimental diets were approximately 23% crude protein, 8.5% crude lipid, 12.1% ash, and 9.5% moisture [21]. Experimental diets were prepared according to the following procedure. Ingredients were ground in a hammer mill and passed through an 80-mesh screen (150 μm). Then, the diets were prepared by thoroughly mixing the ingredients in a mixer until a stiff dough formed. The dough was then passed through a food grinder (die diameter of 3 mm), and the resulting pellets were cut to the desired particle size (length: ~1 cm) and dried in a drying cabinet using an air blower at 37 °C until the moisture level was approximately 10%. After drying, pellets were stored in plastic bins at 4 °C until used. The viability of probiotic cells was monitored in the supplemented diet by plate counting every two weeks during the feeding experiment period. Accordingly, 1.0 g of the experimental diet was incubated in 9.0 mL of sterile 0.9% saline for 2 min and gently homogenized with a glass pestle. Serial dilutions were cultured on TSB agar at 28 °C for 48 h in triplicate, and CFU were recorded.

2.4. Feeding experiment

In total, 270 Nile tilapia (*O. niloticus*) fingerlings with an average body weight (BW) of 4.1 ± 0.34 g were randomly divided into three groups (C, G1, and G2) and cultured in 120-L tanks. The tanks were supplied with an independent recirculation system, and running fresh water was filtered by a three-layer cotton filter (flow rate 1 L/min) and then passed successively through a tungsten heater. Each group contained 30 fish, and experiments were performed in triplicate. The control group (C) was fed a basal pellet diet. Basal diets containing the probiotic *R. stabekisii* at levels of 10^6 CFU/g (G1) and 10^7 CFU/g (G2) were used as the experimental diets. During the feeding trial, fish were bulk-weighed once every two weeks, and the amount of diet provided was adjusted accordingly. Fish were fed two times daily at 09:00 h and 17:00 h with the diet. The daily feeding rate was approximately 5% of BW. Tanks were cleaned by siphoning the water daily, and uneaten food was collected 1 h after each feeding to determine feed intake. After 8 weeks of cultivation, the growth-related parameters, innate immune parameters, challenge test, and gut microbiota of the fish were analyzed.

2.5. Assessment of growth performance

The weights of all tilapia from each aquarium were determined at the initial and final samplings to evaluate the growth performance. The weight gain (WG), feed conversion ratio (FCR), FE, and survival rate were calculated according to the following formulas: WG = final BW - initial BW; FE = (final BW - initial BW) × (feed intake) - 1; FCR = (total food intake) × (weight gain) - 1; survival rate = 100 × (final number of test fish)/(initial number of test fish).

2.6. Determination of digestive enzyme activities

At the end of the feeding trial, six fish from each group were sacrificed and dissected on ice. The gut (200 mg) of each fish was sampled and homogenized in 5 mL of chilled PBS buffer to create a suspension. The suspension was centrifuged at 6100 × *g* for 15 min at 4 °C, and the cleared supernatant was transferred to a clean 10-mL tube and used for quantitative assays of protease, amylase, cellulase, and xylanase activities. The total protein was measured by the Bradford method using bovine serum albumin (BSA) as a standard [22].

2.6.1. Protease activity assay

The protocol for determining protease activity was modified from that of a report by Ullah [23]. Briefly, 1.0 mL of supernatant from the intestines was mixed with 5.0 mL of a 0.7% (w/v) casein solution and incubated at 37 °C for 15 min. Subsequently, 3 mL of a trichloroacetic acid (110 mM) solution was added and further incubated for 20 min at 37 °C. The resultant solution was centrifuged at 12,000 × *g* for 15 min at 4 °C, and then the supernatant was transferred to a new 10-mL tube. The supernatant (2 mL) was mixed with 1.0 mL of Folin and 0.5 mM Ciolcalteau's reagent and 5 mL of 500 mM Na_2CO_3. The mixture was incubated at 37 °C for 30 min. Tyrosine was used as a standard to establish a calibration curve by measuring the absorbance at 440 nm with an ultraviolet (UV)-visible (Vis) spectrophotometer (CT-2700, Chrom Tech, Taipei, Taiwan). One protease activity unit was defined as the amount of enzyme that releases 1 μmol of tyrosine per minute.

2.6.2. Amylase activity assay

Amylase activity was determined by a modified 3,-5-dinitrosalicylic acid (DNS) method according to a previously described protocol [23]. The supernatant (0.5 mL) from the intestines was mixed with 0.5 mL of a 1% starch solution and incubated at 37 °C for 30 min. Subsequently, 1 mL of DNS reagent was added to the mixture and incubated in boiling water for 5 min. After cooling to ambient temperature, the solution was diluted with an appropriate amount of double-distilled water, and the absorbance was determined at 540 nm with a UV–Vis spectrophotometer. One amylase activity unit was defined as the amount of enzyme that released 1 μg of reducing sugar per minute.

2.6.3. Cellulase activity assay

A protocol described in a previous report [23], with a few modifications was used for determining cellulase activity. Briefly, 1 mL of enzyme supernatant was mixed with 1 mL of 0.1 M citrate buffer containing 1% carboxymethyl cellulose (CMC), and then incubated at 50 °C for 30 min. Subsequently, 3 mL of DNS reagent was added and

incubated in a boiling water bath for 10 min. After boiling, 1 mL of 40% $KNaC_4H_4O_6$ was added, and the mixture was allowed to cool to ambient temperature. Glucose generated from the CMC substrate by cellulase activity was measured at 540 nm with a UV–Vis spectrophotometer. One unit of cellulase activity was defined as the amount of enzyme that released 1 mg of glucose per minute.

2.6.4. Xylanase activity assay

Xylanase activity was evaluated by determining reducing sugars released from xylan according to a report by Saputra [14]. The enzyme supernatant (50 μL) was mixed with 50 μL of a 1% xylan (X-4252, Sigma, St. Louis, MO, USA) substrate solution in a 1.5-mL microcentrifuge tube and incubated at 55 °C for 10 min. Subsequently, 0.2 mL of DNS reagent was added and heated to 100 °C for 10 min. After heating, the reaction was terminated by adding 1 mL of deionized water. After centrifugation at 12,000 × *g* for 1 min, the supernatant was assessed using a microplate reader at 540 nm. One xylanase activity unit was defined as the amount of enzyme that released 1 mg of reducing sugars per minute.

2.7. Assessment of non-specific immune parameters

Different non-specific immune parameters including the PA, RBs, and SOD activity of head kidney leukocytes, and lysozyme activity of serum were measured in probiotic-administered and control fish. Six fish were sampled from each of the control, G1, and G2 groups at the end of the experiment. Blood samples were collected from the caudal vasculature of anesthetized fish using a 5-mL syringe. The blood sample was transferred into a sterilized vial, and then centrifuged at 1500 × *g* and 4 °C for 10 min. Serum was collected and stored at −20 °C until the lysozyme activity assay was conducted. Leukocytes from the head kidneys of the fish were harvested as previously described [20]. The cell concentration of leukocytes was adjusted to 10^5 cells/mL for the assay of RBs, PA, and SOD activity. Detailed procedures for conducting these assays followed previously described protocols [14]. At the end of the probiotic administration trial, total RNA from tilapia head kidneys was extracted using the TRI reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. Approximately 1 μg of total RNA was reverse transcribed into synthesized complementary DNA using an iScript cDNA Synthesis Kit (Bio-Rad, Foster, CA, USA) according to the manufacturer's instructions. The expression levels of IL-1β, TNF-α, TGF-β, HSP70, and β-actin were determined using a quantitative polymerase chain reaction (qPCR). The expression of β-actin was used as an internal control. The specific primers used for detecting each gene are listed in Table 1. The qPCR was performed on an Applied Biosystem StepOnePlus Real-Time PCR system with a KAPA SYBR FAST qPCR Kit (KAPA KR0389, Wilmington, MA, USA). Conditions of the qPCR were conducted according to a previously described protocol [20]. Relative expression levels of each group were normalized to β-actin and are expressed as the mean ± standard error (SE).

2.8. Challenge test

A. hydrophila and *S. iniae* pathogens were individually cultured in TSB at 28 °C for 24 h and then adjusted to graded doses by dilution with appropriate volumes of sterile water. Before the challenge test, graded doses of *A. hydrophila* and *S. iniae* (10^5, 10^6, 10^7, and 10^8 CFU/fish) were intraperitoneally (i.p.) injected into 10 fish to determine the 7-day 50% lethal dose (LD_{50}). At the end of the feeding trial, 15 fish from each group were i. p. injected with 20 μL of PBS buffer containing *A. hydrophila* or *S. iniae* at the respective LD_{50} concentrations of 10^6 and 10^5 CFU per fish. Each group was conducted in triplicate. Fish fed the control diet and injected with PBS buffer were used as a negative control. The survival rate in each group was observed daily, and all mortality was recorded for 7 days.

2.9. Next-generation sequencing analysis of the gut microbiota

At the end of the experiment, intestines of tilapia from the control, G1, and G2 groups (four samples from each group) were sampled, cut open, and rinsed with sterilized water to remove the intestinal contents. Microbial genomic DNA from each intestine was extracted using an EasyPure Genomic DNA Spin Kit (GT-100, Bioman, New Taipei City, Taiwan) according to the manufacturer's instructions. The extracted microbial genomic DNA product was monitored by electrophoresis on 1% agarose gels to confirm the DNA quality. All DNA samples were stored at −80 °C until further analysis. The V3 and V4 regions of the bacterial 16S rRNA gene were amplified by PCR using 5′-barcode-tagged primers 341F 5′-CCTACGGGNGGCWGCAG-3′ and 805R 5′-GACTACHVGGGTATCTAATCC-3′, where each barcode with an eight-base sequence was specific to each sample. The PCR was carried out in a 30-μL mixture containing 15 μL of Phusion High-Fidelity PCR Master Mix (NEB, Ipswich, MA, USA), 0.2 μM forward and reverse primers, and 10 ng of template DNA. The PCR conditions were as follows: 98 °C for 2 min for initiation and denaturation; followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 60 s, with a final extension at 72 °C for 5 min. Amplicons (approximately 400–450 bp each) were separated by 2% agarose gels and purified using a GeneJET Gel Extraction Kit (Thermo Scientific, Maryland, USA) according to the manufacturer's protocol. Sequencing libraries were constructed using an NEB Next Ultra DNA Library Prep Kit (NEB, Ipswich, MA, USA) following the manufacturer's recommendations. The library quality was assessed on a Qubit 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library with paired-end reads (2 × 250 bp) was sequenced on an Illumina HiSeq 2500 platform according to standard protocols. All raw data (fastq files) were analyzed by FastQC software (vers. 0.11.5) followed by MultiQC software (vers. 0.9), and de-multiplexed and quality-filtered with QIIME software (vers. 1.9.0). Operational taxonomic units (OTUs) were clustered with 97% identity using QIIME, and chimeric sequences were identified and used to make an OTU table using the Greengenes 16S rRNA Taxonomy Database (gg_13_8) (http://greengenes.secondgenome.com). Variations in each experimental group were represented by three-dimensional principal components analysis (PCA) plots. The abundances of genera were determined using a web tool for visualizing clustering of multivariate data (http://biit.cs.ut.ee/clustvis/) [24].

2.10. Statistical analyses

A one-way analysis of variance (ANOVA) and Tukey's multiple-comparison tests were used to determine significant differences

Table 1
Primer sequences used for detection of expression of immune-related genes in the head kidneys of Nile tilapia (*O. niloticus*).

Gene name	Primer sequence forward/reverse (5′-3′)	Amplicon size (bp)	Accession no.
TNF-α	F:CCAGAAGCACTAAAGGCGAAGA R:CCTTGGCTTTGCTGCTGATC	82	AY428948.1
IL-1β	F:TGGTGACTCTCCTGGTCTGA R:GCACAACTTTATCGGCTTCCA	86	XM_005457887.1
TGF-β	F:GTTTGAACTTCGGCGGTACTG R:TCCTGCTCATAGTCCCAGAGA	80	XM_003459454.2
HSP70	F:CTCCACCCGAATCCCCAAAA R:TCGATACCCAGGGACAGAGG	95	FJ213839.1
β-Actin	F:TGACCTCAGACTACCTCATG R:TGATGTCACGCACGATTTCC	89	KJ126772.1

($p < 0.05$) in relative gene expression levels and immune parameters between the control and experimental groups. For the results from the challenge test, cumulative survival was analyzed by the Kaplan-Meier method. Data were analyzed using SAS software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Screening and characterization of putative probiotics

In total, 48 bacterial clones were selected from 10^{-3}–10^{-5} dilutions of six intestinal samples of tilapia. Potential probiotics were screened based on the existence of digestive enzyme activities such as protease, cellulase, amylase, and xylanase activities. One isolate with strong protease and xylanase activities (Supplementary Fig. 1S) was isolated, and then genotypically identified by 16S rDNA gene sequence and biochemical characterization. This isolate was found to share 99.8% similarity with *R. stabekisii* by BLAST against known bacterial 16S rDNA sequences from the NCBI GenBank database. Physicochemical analysis revealed that the isolate was a gram-positive, rod-shaped, motile anaerobic bacteria with catalase, arginine dihydrolase and urease activity and the ability to utilize glucose and N-acetyl-glucosamine. Consequently, the protease- and xylanase-producing isolate was identified as *R. stabekisii*.

3.2. Rummeliibacillus stabekisii enhanced nutrient utilization and growth performance

Biosafety issues of putative probiotics must be considered before their application, so different doses of *R. stabekisii* were intraperitoneally injected into tilapia to evaluate its toxicity. Results revealed that no pathology or death occurred during the experimental period at a dosage of 10^6 and 10^7 CFU/fish. A high survival rate (93.3 ± 5.77%) was revealed at 7 days post-injection even though a high dose of 10^8 CFU/fish was injected (Supplementary Fig. 2S). This result indicated that *R. stabekisii* did not exhibit toxicity to the fish's health status. The efficacy of *R. stabekisii* in nutrient utilization and growth performance after 8 weeks of administration was evaluated by determining intestinal digestive enzyme activities and growth parameters of WG, FCR, and FE. Per the results shown in Table 2, WGs of 18.3 ± 3.67 g and 17.0 ± 2.16 g for tilapia fed diets containing 10^6 (G1) and 10^7 CFU/g (G2) *R. stabekisii*, respectively, were significantly higher than that of tilapia fed the control diet (13.4 ± 3.52 g). The survival rates in the control, G1, and G2 groups were 92.2 ± 1.92%, 93.3 ± 3.33%, and 94.4 ± 1.92%, respectively, and exhibited no significant differences. Tilapia in the G1 and G2 groups exhibited significantly better FCRs compared to that of tilapia in the control group. Moreover, the FEs of fish fed 10^6 and 10^7 CFU/g *R. stabekisii*-supplemented diets were significantly higher than that of fish fed the control diet. There were no significant differences in WG, FCR, or FE between fish in the G1 and G2 groups. The increased FEs of tilapia after the 8-week feeding trial in the G1 and G2 groups prompted us to further investigate digestive enzyme activities in the fish gut. The results showed that specific intestinal digestive protease, xylanase, and cellulase activities were significantly enhanced in the G1 and G2 groups compared to those in the control group (Fig. 1). Although intestinal amylase activities in the G1 and G2 groups were higher than that in the control group, there was no significant difference among the three groups. These results suggest that dietary administration of *R. stabekisii* can enhance nutrient utilization and growth performance.

Table 2
Effect of *R. stabekisii* supplementation for 2 months on the growth performance of Nile tilapia (*O. niloticus*).

Parameter	Treatment		
	Control	10^6 CFU/g (G1)	10^7 CFU/g (G2)
Initial weight (g)	2.01 ± 0.56[a]	1.97 ± 0.42[a]	1.90 ± 0.41[a]
Final weight (g)	15.4 ± 4.08[a]	20.2 ± 4.09[b]	18.9 ± 2.58[b]
Weight gain (WG) (g)	13.4 ± 3.52[a]	18.3 ± 3.67[b]	17.0 ± 2.16[b]
Survival rate (%)	92.2 ± 1.92[a]	93.3 ± 3.33[a]	94.4 ± 1.92[a]
Feed conversion ratio (FCR)	1.31 ± 0.12[a]	1.11 ± 0.07[b]	1.16 ± 0.04[b]
Feed efficiency (FE)	0.76 ± 0.06[a]	0.89 ± 0.05[b]	0.86 ± 0.03[b]

Data are expressed as the mean ± standard error from triplicates. Different superscripts in the same row indicate a significant difference ($p < 0.05$).
CFU, colony-forming units.

3.3. Rummeliibacillus stabekisii increased the survival rate of tilapia challenged with pathogens

After the 8-week feeding trial, the efficacy of *R. stabekisii* in disease resistance was evaluated by determining the cumulative survival of tilapia after being challenged with the gram-positive *S. iniae* and gram-negative *A. hydrophila* pathogens. As shown in Fig. 2, no mortality occurred in the control group injected with PBS buffer for 7 days post-infection. In contrast, the cumulative survival of tilapia in the control group infected with *S. iniae* and *A. hydrophila* obviously declined in the first 4 days post-infection and then was maintained at 43.3 ± 5.77% and 33.3 ± 5.77%, respectively, to 7 days post-infection (Fig. 2). Cumulative survival of tilapia in the G1 and G2 groups was significantly higher than that in fish fed the control diet. The cumulative survival rates of fish in the G1 and G2 groups at 7 days post-infection with *S. iniae* were 73.3 ± 5.77% and 63.3 ± 5.77%, respectively (Fig. 2A), and at 7 days post-infection with *A. hydrophila* were 56.7 ± 5.77% and 60.0 ± 10.0%, respectively (Fig. 2B). Cumulative survival between fish in the G1 and G2 groups showed no significant difference. These results suggested that a diet supplemented with *R. stabekisii* induced disease resistance against pathogen infections.

3.4. Rummeliibacillus stabekisii enhanced immune responses

The significantly increased cumulative survival of pathogen-challenged tilapia in the G1 and G2 groups urged us to further investigate the effects of *R. stabekisii* on fish immunity. The PA and RB activity of head kidney leukocytes, and serum lysozyme level of tilapia were significantly increased in the G1 and G2 groups compared to those of fish in the control group. No significant difference in PA, RB, or lysozyme activity was observed between tilapia in the G1 and G2 groups, suggesting that the dose of 10^6 CFU/g was sufficient to achieve immunomodulatory function (Fig. 3A–C). The SOD activity of fish in the G1 group was obviously higher than that of fish in the control group. Although the relative SOD activity of fish in G2 group was higher than that of fish fed the control diet, the SOD activities of fish in the control and the G1 group did not significantly differ (Fig. 3D). The health status of fish after *R. stabekisii* administration was also evaluated by determining the expression of the immune-related genes *IL-β, TNF-α*, *TGF-β* and *Hsp70* in the head kidneys. Expressions of the *IL-β, TNF-α*, *TGF-β,* and *Hsp70* genes in the head kidneys significantly increased in fish fed the diet containing *R. stabekisii* compared to that of fish fed the control diet (Fig. 4). Moreover, *Hsp70* expression in fish of the G2 group was significantly higher than that of fish in the G1 group (Fig. 4D). These results suggested that fish fed an *R. stabekisii*-supplemented diet exhibited enhanced immune modulation.

3.5. Rummeliibacillus stabekisii improves the intestinal microbiota

The effects of a diet containing *R. stabekisii* on alterations of the gut microbiota were analyzed by high-throughput NGS. An average of 57,601 validated nucleotide sequences were retrieved from the three groups ($n = 4$ for each group) through Illumina MiSeq sequencing. The validated nucleotide sequences were clustered into OTUs with a 97%

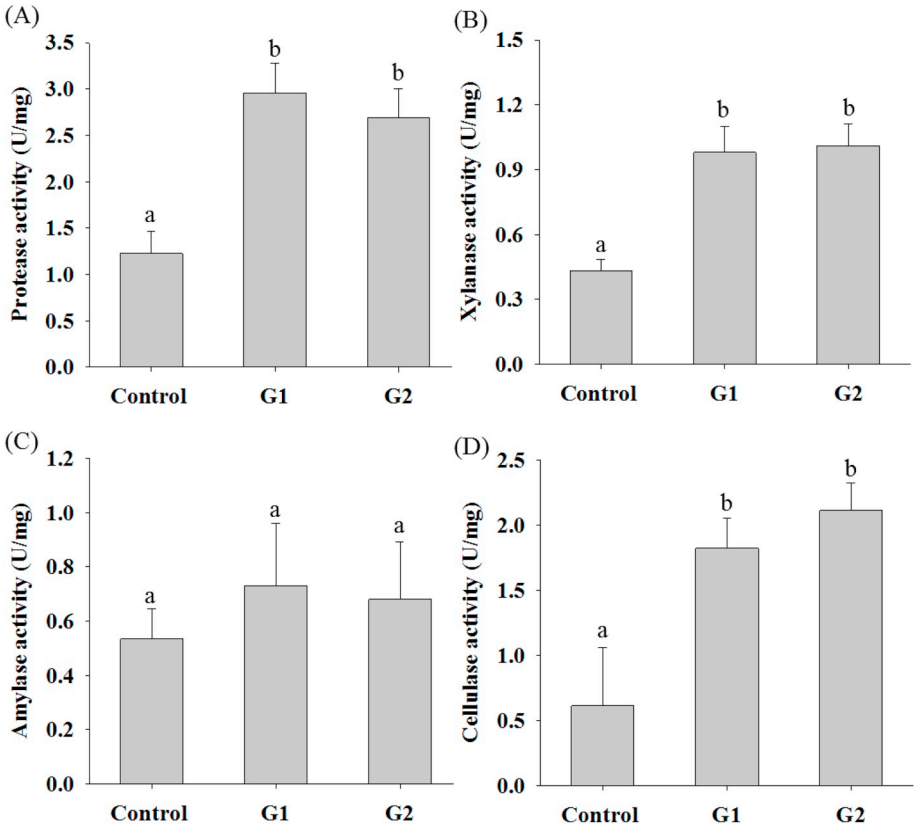

Fig. 1. Effect of *R. stabekisii* administration on the activities of intestinal digestive enzymes of Nile tilapia (*O. niloticus*). (A) Protease activity; (B) xylanase activity; (C) amylase activity; (D) cellulase activity. Bars with different superscripts significantly differ ($p < 0.05$, by one-way ANOVA).

similarity level, which acquired 587 OTUs that were assigned to 14 different phyla. The sample rarefaction curve tended to approach the plateau, and coverage reached 99.9%. The relationship of bacterial communities in each group were evaluated by a PCA. The PCA of the microbiota in tilapia fed the control diet (C) and control diet containing 10^6 CFU/g (G1) or 10^7 CFU/g (G2) of *R. stabekisii* clustered into three distinct groups. The contributions of principal component 1 (PC1), PC2, and PC3 to the total variation were 82.83%, 9.47%, and 6.67%, respectively (Fig. 5). The results of the PCA indicated that the diet with *R. stabekisii* exhibited an impact on the microbial communities. The most abundant genus in Nile tilapia of each group was *Cetobacterium*, which made up 62.8%, 63.3%, and 65.4% of reads in the control, G1, and G2 groups, respectively. In addition, the relative abundances of the predominant genera among the three groups varied. The four predominant genera (with a relative contribution of > 1%) were *Paludibacter* (9.6%), *Plesiomonas* (6.8%), *Algoriphagus* (5.9%), and *Streptococcus* (2.4%) in the control group; *Paludibacter* (8.4%), *Plesiomonas* (8.2%), *Bacillus* (6.0%), and *Leeia* (4.1%) in group G1; and *Plesiomonas* (8.2%), *Bacillus* (5.9%), *Paludibacter* (5.6%), and *Lactobacillus* (2.6%) in group G2 (Fig. 6). Reads of putative probiotic and pathogen genera of Nile tilapia and the *Rummeliibacillus* genus in each group are listed in Table 3. The results showed that abundances of the putative probiotic genera *Bacillus* and *Lactobacillus* in the bacterial community were significantly higher in the G1 and G2 groups than in the control group. Moreover, abundances of the putative pathogen genera, *Staphylococcus* and *Streptococcus,* in the bacterial community were significantly lower in the G1 and G2 groups than in the control. The *Rummeliibacillus* genus investigated in this study also had higher abundances in the G1 and G2 groups than in the control group.

4. Discussion

Several reviews of applications of probiotics in aquaculture had been conducted, showing that they confer advantages such as improved FE, enhanced growth performance, improved beneficial microflora in the fish gut, and modulated immunity against pathogen infections [15,25–27]. However, although a variety of bacterial species have been used as probiotics in aquaculture, a large proportion of probiotic sources are derived from non-natural habitats of the host, and there are only a few investigations using digestive enzyme-producing probiotics. The ability of a bacterium to colonize the gut is an important criterion to evaluate a putative probiotic. Moreover, the actions of probiotics on a host are considered to be species specific or even strain specific [17]. Thus, isolating a putative probiotic from the gut of a natural host and validation of its beneficial functions on the natural host are required to identify suitable probiotics. In the present study, a potential probiotic species, *R. stabekisii,* with properties of protease and xylanase activities was isolated from the gut of Nile tilapia, suggesting that *R. stabekisii* has the ability to colonize the tilapia intestinal epithelium. Starch and plant-based proteins, such as soybean meal, wheat middling, rice gluten meal, sesame seed meal, and linseed meal, are commonly used in fish feed to partially replace fish meal. Proteases are very important digestive enzymes that can improve protein digestibility and FE, resulting in fish growth enhancement. However, NSPs, which are major components of the cell walls of these plants, exhibit anti-nutritional effects by increasing the feed viscosity and limiting feed utilization and growth performance. Cellulose and xylan are abundant NSPs in plant protein-based feedstuffs. Cellulose is a polysaccharide that acts as a major structural substance of plant cell walls and constitutes approximately 33% of all plant materials. Xylan is the major hemicellulosic constituent and represents the major non-cellulosic polysaccharide in the cell walls of plants. Unfortunately, endogenous cellulase and xylanase are scarce

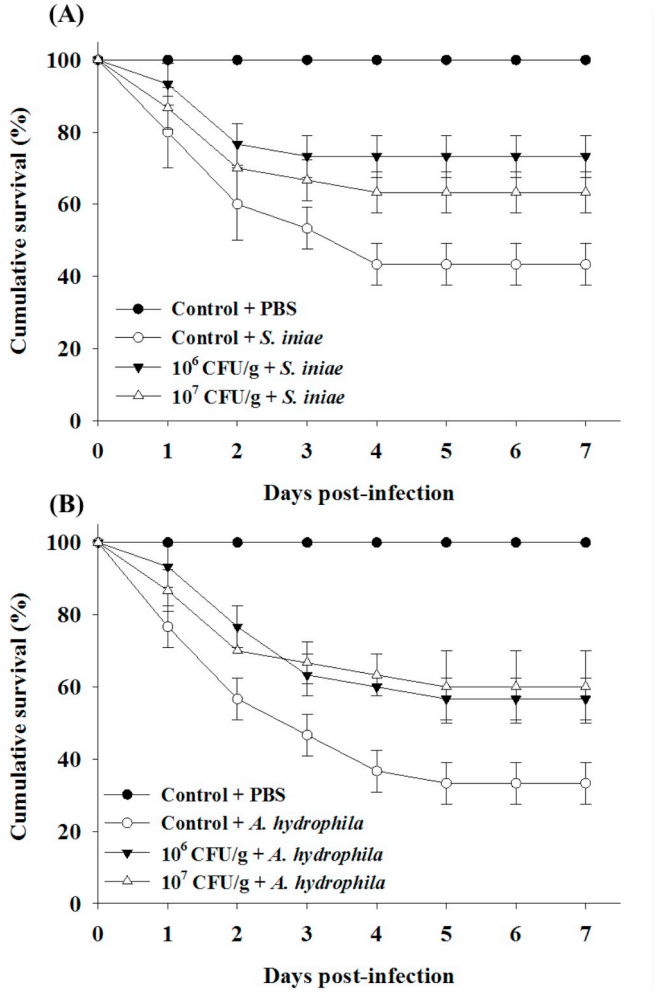

Fig. 2. Cumulative survival of tilapia infected with (A) *S. iniae* or (B) *A. hydrophila* after feeding the basal diet only (control) or the basal diet containing 10^6 colony-forming units (CFU)/g or 10^7 CFU/g *R. stabekisii* for 2 months. The cumulative survival rates in the probiotic groups were significantly higher ($p < 0.05$) than those in the control group according to the Kaplan-Meier method.

in the GI tracts of fish. Thus, supplementation with cellulase- or xylanase-producing probiotics is a potential approach to reduce anti-nutritional impacts on FE and growth performance. Reports have demonstrated that dietary supplementation with protease-, xylanase-, and/or cellulase-producing probiotics could significantly enhance nutrient metabolism, FE, and growth performance in pla-mong (*Pangasius bocourti*), zebrafish (*Danio rerio*), and Nile tilapia (*O. niloticus*) [14,20,28,29]. These studies showed that the administration of digestive enzyme-producing probiotics can promote the growth performance of fish by increasing nutrient utilization. The present study showed that dietary supplementation of protease- and xylanase-producing *R. stabekisii* for 8 weeks significantly improved the WG and FE growth parameters of fish compared to those of fish fed the control diet. Moreover, dietary administration of *R. stabekisii* also increased the endogenous protease, xylanase, and cellulase activities in the GI tract of fish. These results suggest that the mechanism of *R. stabekisii* growth enhancement in fish potentially resulted from improving nutrient utilization by secreting protease- and xylanase enzymes and stimulating the production of intestinal digestive enzymes after *R. stabekisii* administration.

Modulation of immunity and disease resistance is the major function among well-documented benefits claimed for probiotics. In recent decades, a variety of gram-positive and gram-negative bacteria have been widely applied as probiotics in aquaculture to regulate immunity and prevent disease outbreaks. Nevertheless, so far, no study has reported on applying the genus *Rummeliibacillus* as a probiotic in animals or applying *R. stabekisii* in aquaculture. The present study is the first report demonstrating the genus *Rummeliibacillus* as a probiotic that produces beneficial effects in animals. Our results showed that the cumulative survival of tilapia challenged with *A. hydrophila* and *S. iniae* and immune parameters significantly increased in fish administered *R. stabekisii*, suggesting that dietary supplementation with *R. stabekisii* can strengthen innate immunity against pathogen infections. In teleosts, the head kidneys, which contain cytokine-producing lymphoid cells, such as neutrophils, monocytes, and macrophages, are important organs responsible for immune system function. Lymphoid cells have an antibacterial function responsible for innate immunity by directly or indirectly recognizing pathogens and destroying invading pathogens via phagocytosis. During phagocytosis, reactive oxygen species (ROS) such as superoxide anions (O_2^-), hydroxyl free radicals (OH^-), and hydrogen peroxide (H_2O_2), are rapidly released from neutrophils, monocytes, and macrophages to kill invading pathogens through a process called respiratory activity. Thus, the PA and RB activity of lymphoid cells are important parameters indicating the innate immunity of teleosts. Fish lysozymes, which exhibit bactericidal activities through hydrolysis of peptidoglycans of bacterial cell walls, are considered indispensable components of the non-specific defense mechanism. Moreover, the bactericidal function of lysozymes is coupled with an important immunomodulatory function, because components released from lysozyme-mediated bacterial lysis can activate pattern-recognition receptors and act as opsonins in the complement system to modulate host immune responses [30]. In the present study, tilapia fed a diet containing *R. stabekisii* for 8 weeks exhibited significantly higher PA and RB activity in head kidneys and serum lysozyme activity compared to those in fish fed the control diet, suggesting that *R. stabekisii* supplementation in feed provides beneficial effects on immunomodulatory functions in fish. Serum SOD activity, which responds to oxidative stress by catalyzing the dismutation of ROS into O_2 and water, is used as a bioindicator to evaluate a cell's protective ability against potential ROS damage. However, the results were not consistent in studies on the effects of probiotics on SOD activity. For instance, reports have shown that dietary supplementation with *Bacillus licheniformis*, *Bacillus subtilis* HAINUP40, and *Paenibacillus ehimensis* NPUST1 for 1 or 2 months increased the serum SOD activity of tilapia [12,20,31]. However, SOD activity exhibited no significant difference in Nile tilapia fed *Bacillus amyloliquefaciens* and *B. licheniformis* for 8 and 10 weeks, respectively [14,32]. Moreover, reports showed that SOD activity was significantly lower in zebrafish (*Danio rerio*) and *Epinephelus coioides* fed a diet containing *B. amyloliquefaciens,* or *Bacillus pumilus*, *Bacillus clausii,* and *Lactobacillus plantarum* after 1 week, suggesting that the decreased SOD activity may have been due to lower oxidative stress resulting from administering probiotics or from maintaining ROS levels to enhance the pathogen-killing capacity of lymphoid cells [28,33,34]. In the present study, serum SOD activity had obviously increased in head kidneys of tilapia fed *R. stabekisii* at a dose of 10^6 CFU/g compared to that in head kidneys of tilapia fed the control diet, suggesting that *R. stabekisii* stimulates protective defense against oxidative damage.

Cytokine expression by immune system cells, such as macrophages and monocytes, plays important roles in regulating the immune response and protective defense against pathogen infections. Proinflammatory cytokines, such as IL-1β and TNF-α, have been implicated as main bioindicators of immunoeffectors, which are induced in the early stage of pathogen infection and regulate the innate immune system. In teleosts, IL-1β and TNF-α enhance the phagocytic activities of leukocytes. In pathogen-infected fish, IL-1β and TNF-α have been shown to stimulate macrophage survival as well as bactericidal activity by increasing ROS production during phagocytosis [10]. Reports have shown that administration of the probiotics *Lactobacillus pentosus* PL11, *B. velezensis* JW, and *P. ehimensis* NPUST1 significantly increased the expression of IL-1β and TNF-α in the head kidneys of Japanese eels

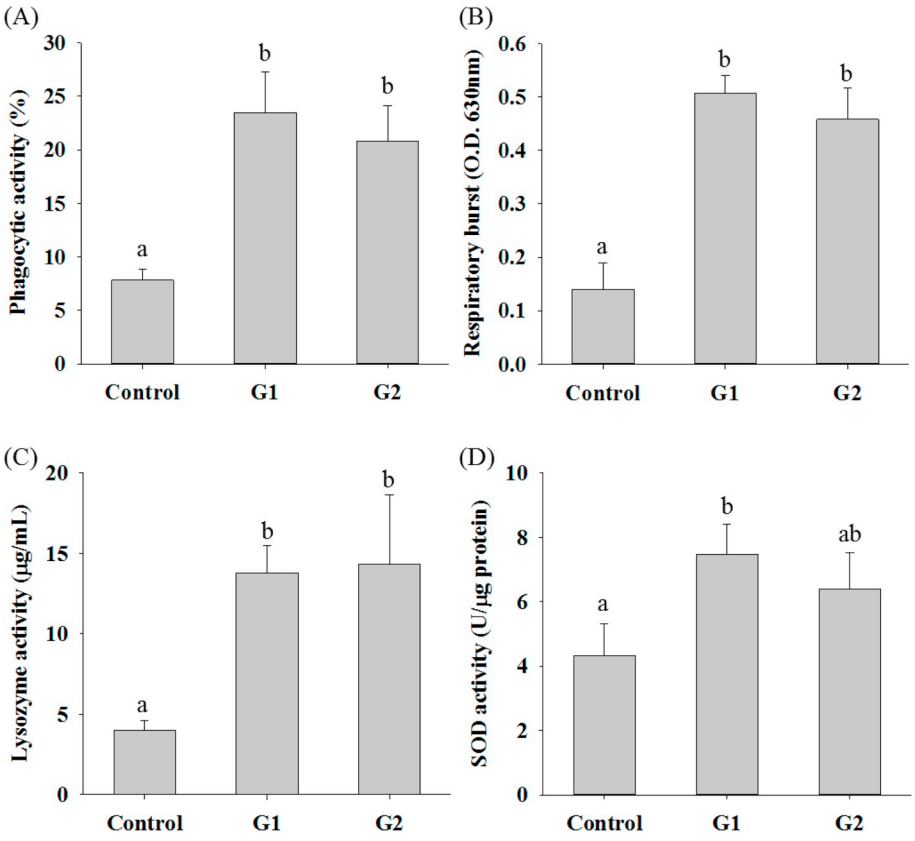

Fig. 3. The effect of probiotic administration on the innate immune response of Nile tilapia fed 10^6 (G1) or 10^7 CFU/g (G2) *R. stabekisii*. (A) Phagocytic activity; (B) respiratory burst activity; (C) lysozyme activity; (D) superoxide dismutase (SOD) activity. Bars with different superscripts significantly differ ($p < 0.05$, by one-way ANOVA).

(*Anguilla japonica*), goldfish (*Carassius auratus*), and Nile tilapia (*O. niloticus*) against *Edwardsiella tarda*, *A. hydrophila,* and *S. iniae* infections, respectively [20,35,36]. The present study showed that tilapia administered *R. stabekisii* exhibited significantly increased expression levels of *TNF-α* and *IL-1β* in head kidneys compared to those in the head kidneys in control diet-treated tilapia, suggesting that *R. stabekisii* can enhance cytokine expression (TNF-α and IL-1β) of the innate immune system and provide protective defense against pathogen infections.

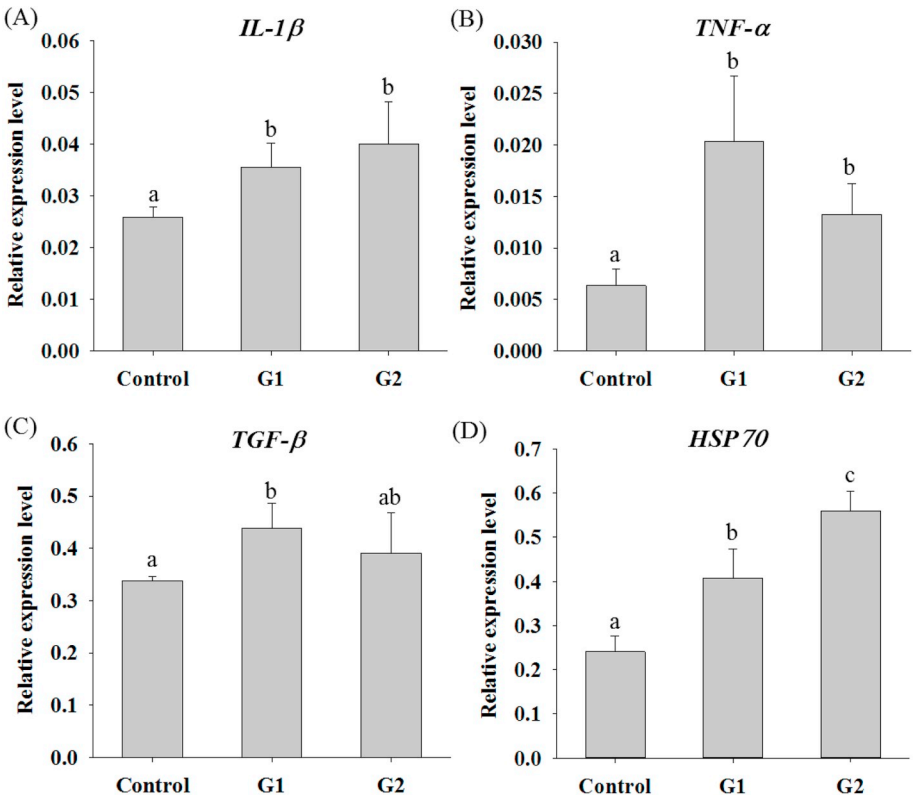

Fig. 4. Relative expression of cytokine and stress-response genes in Nile tilapia after 8 weeks of feeding a diet containing 10^6 (G1) or 10^7 CFU/g (G2) *R. stabekisii*. Gene expression of (A) *interleukin* (*IL*)-*1β*, (B) *tumor necrosis factor* (*TNF*)-*α*, (C) *transforming growth factor* (*TGF*)-*β*, and (D) *heat shock protein 70* (*Hsp70*) by head kidneys. Bars with different superscripts significantly differ ($p < 0.05$, by one-way ANOVA).

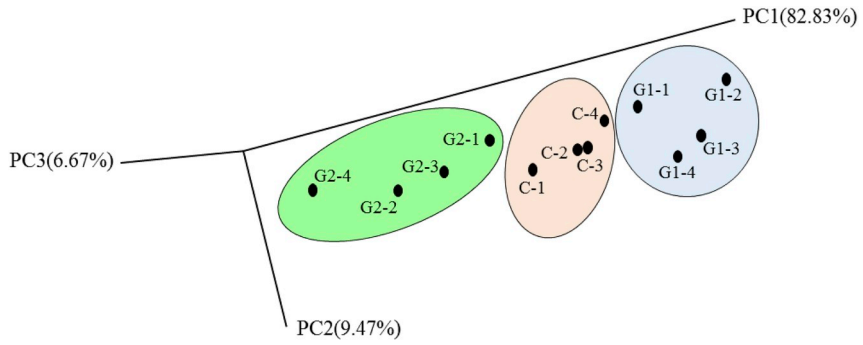

Fig. 5. Principal component analysis of the compositions of microbes in the intestines of Nile tilapia fed the control diet (C), the control diet containing 10^6 CFU/g (G1) *R. stabekisii*, or the control diet containing 10^7 CFU/g (G2) *R. stabekisii.*

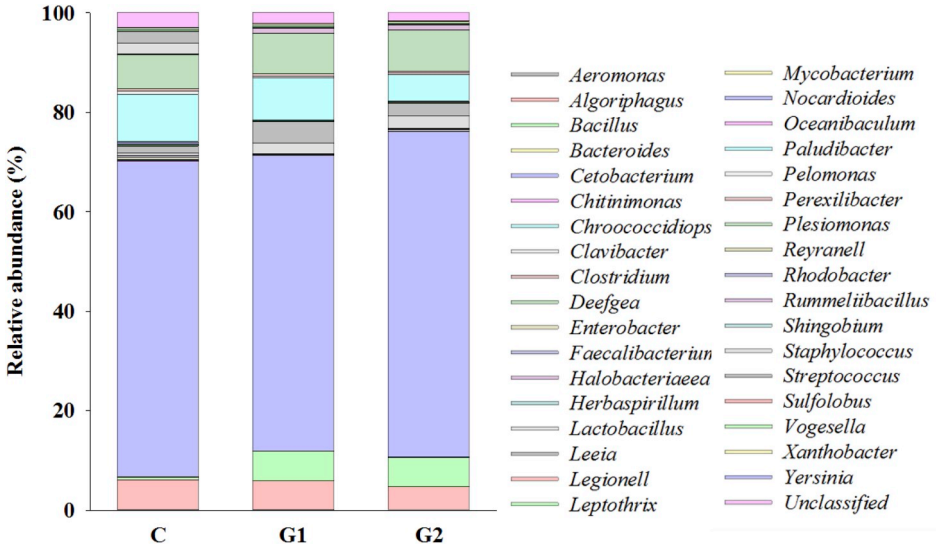

Fig. 6. Relative abundances of different bacterial genera in the intestines of Nile tilapia fed the control diet (C), the control diet containing 10^6 CFU/g (G1) *R. stabekisii*, or the control diet containing 10^7 CFU/g (G2) *R. stabekisii.* Relative abundances of genera of > 1% are presented.

Table 3
Effects of *R. stabekisii* on changes in the bacterial genera of interest.

Genera	Control	G1	G2
Potential probiotics			
Bacillus	541 (0.49%)	8223 (5.9%)	9013 (5.9%)
Lactobacillus	492 (0.44%)	3012 (2.2%)	3892 (2.6%)
Potential pathogens			
Staphylococcus	2378 (2.1%)	121 (0.09%)	69 (0.05%)
Streptococcus	2651 (2.4%)	231 (0.17%)	212 (0.14%)
Rummeliibacillus	94 (0.08%)	1226 (0.89%)	1451 (0.95%)

Numbers represent the reads of a genus present in the bacterial community. The numbers in parentheses are the relative abundances of the bacterial genera in each group.

TGF-β is commonly categorized as an anti-inflammatory cytokine that controls responses of proinflammatory cytokines, such as IL-β and TNF-α, and prevents hosts from damage resulting from inflammation. Variations in TGF-β expression in fish could be a valuable indicator for evaluating immunostimulant or infectious agents in fish immune systems [10]. Despite the role of TGF-β being contrary to that of a proinflammatory cytokine, several studies have shown that TGF-β expression induced coupling with a proinflammatory cytokine after probiotic administration. It was reported that the expression level of IL-1β, TNF-α, and TGF-β were significantly evoked in rainbow trout (*Oncorhynchus mykiss*), grass carp (*Ctenopharyngodon idellus*), Japanese pufferfish (*Takifugu rubripes*), and Nile tilapia (*O. niloticus*) fed *Lactobacillus rhamnosus*, *Enterococcus faecium*, *B. subtilis*, *L. paracasei* spp. *paracasei*, and commercial probiotics, respectively [37–40]. The present study also demonstrated enhanced expression of IL-1β, TNF-α, and TGF-β in tilapia after feeding with *R. stabekisii,* suggesting that fish supplemented with *R. stabekisii* may be able to balance inflammatory cytokines and protect the host from impairment due to excessive inflammation. HSP70 plays a central role in maintaining cellular homeostasis in all living organisms. Similar to its mammalian counterpart, fish HSP70 is induced in response to a variety of physiological and environmental stresses, such as temperature variations, toxic chemicals, and bacterial infections. Recently, Zhang et al. showed that HSP70 exhibited the potential to increase the release of the cytokines IL-1β and TNF-α by head kidney leukocytes of Chinese grass carp (*C. idella*), suggesting a regulatory role of HSP70 in fish immunity [11]. The upregulation of HSP70 expression strengthening the immune system in fish hosts was shown by administration of a variety of probiotics [41–45]. In the present study, significantly higher expression levels of the HSP70 gene in head kidneys of tilapia fed *R. stabekisii* than in the head kidneys of control tilapia suggest that dietary supplementation with *R. stabekisii* can modulate cytokine gene (IL-1β and TNF-α) expression and innate immunity against pathogen infections. These results suggest that the mechanism of *R. stabekisii* improving fish immunity potentially resulted from increasing phagocytic, lysozyme, and respiratory burst activity and the expression of cytokines (IL-1β, TNF-α, and TGF-β). Moreover, reviews have indicated that a higher dose does not result in a greater level of protection, and overdosage administrations of probiotics can induce negative immunosuppression of continuous responses of

nonspecific immune systems. Appropriate probiotic levels depend on the fish species, their physiological status, and rearing conditions and the specific goal of the applications [46,47]. In the present study, the effects of two doses of *R. stabekisii* on the growth and immunity of fish showed no significant difference, suggesting that 10^6 CFU/g is an effective and sufficient dose for providing beneficial effects to tilapia.

The intestinal microbiota of fish plays important roles in mediating immunity, nutrient metabolism, and energy homeostasis [48]. Diet is considered a major factor affecting the intestinal microbiota. Several reports showed that probiotics supplemented in the fish diet can effectively modulate the intestinal microbiota, thereby further improving growth, immunity, and disease resistance in tilapia [42,49–52]. In the present study, the predominant genera detected in tilapia were *Cetobacterium*, *Paludibacter*, *Plesiomonas*, *Algoriphagus*, *Bacillus*, *Lactobacillus,* and *Leeia* (with abundances of > 1%). In general, these results are consistent with data presented in reports that also described *Cetobacterium, Bacillus,* and *Lactobacillus* [53]; *Cetobacterium*, *Bacillus,* and *Plesiomonas* [52]; *and Cetobacterium* and *Plesiomonas* [54] as components of the core microbiome observed in tilapia. Notably, the abundance of the *Rummeliibacillus* genus significantly increased in the G1 and G2 groups compared to that in the control group, suggesting that *R. stabekisii* was actually already colonized in the fish intestines. Probiotics belonging to the *Bacillus* and *Lactobacillus* genera are widely used for providing benefits to tilapia aquaculture [12,14,31,41,42,55–57]. Pathogens belonging to the *Streptococcus* and *Staphylococcus* genera, such as *S. iniae*, *S. agalactiae*, and *S. aureus*, are major pathogenic genera in tilapia [58–60]. The present study revealed that *Bacillus* and *Lactobacillus* genera had higher abundances and the *Streptococcus* and *Staphylococcus* genera had lower abundances in the G1 and G2 groups than in the control group, suggesting that *R. stabekisii* can modulate the intestinal microbiota of Nile tilapia and contribute to growth enhancement, immunomodulation, and disease resistance.

In conclusion, the present study isolated a potent probiotic, *R. stabekisii,* from the tilapia gut. Dietary supplementation with *R. stabekisii* in Nile tilapia for 8 weeks not only enhanced growth performance and FE, but also increased endogenous digestion, including protease, cellulase, and xylanase activities, in the intestines of tilapia. Tilapia supplemented with *R. stabekisii* revealed significant increases in parameters of innate immunity against *A. hydrophila* and *S. iniae* infection. Administration of *R. stabekisii* was helpful in improving the intestinal microbiota, and the mechanism of alteration of the intestinal microbiota provided beneficial effects of immunomodulation, which are worth exploring in future studies. This report is the first study to investigate the effects of *R. stabekisii* as a probiotic, and the results suggest that *R. stabekisii* can be used in tilapia aquaculture to improve growth performance, feed utilization, the intestinal microbiota, and immunity against diseases.

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.2019.06.027.

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