CHAPTER III
MATERIALS AND METHODS

3.1 Ethics statement

All animal experiments were conducted in compliance with the regulations and approved by the Ethics Committee of Suranaree University of Technology (SUT), Animal Care and Use Committee (approval no. SUT-IACUC-0012/2023).

3.2 The effects of dietary recombinant probiotic *B. subtilis* expressing *GULO* supplementation in normal fish

3.2.1 Experimental design

The experiment was conducted using a completely randomized design comprising four treatment groups, each with three replicates. A total of 120 healthy Nile tilapia (approximately 75 g each) were distributed into twelve 700-liter fiberglass tanks containing clean freshwater with continuous aeration. Each tank held ten fish that underwent a two-week acclimation before the experiment commenced. Throughout the experimental period, all treatment groups were fed *ad libitum* twice daily with their respective experimental diets. The treatments were as follows:

Treatment 1: a commercial diet + 0.85% NaCl

Treatment 2: a commercial diet + vitamin C 500 mg kg^{-1}

Treatment 3: a commercial diet + wild-type isolated *B. subtilis*

Treatment 4: a commercial diet + recombinant isolated *B. subtilis* expressing *GULO*.

3.2.2 Diet preparation

Prior to the experiment, wild-type *B. subtilis* and recombinant *B. subtilis* expressing *GULO* were cultured, aliquoted, and stored in glycerol stocks at –80°C. For the preparation of each experimental diet, aliquots of the wild-type and recombinant strains were streaked onto LB agar and LB agar containing 100 µg/mL kanamycin, respectively. Plates were incubated at 37°C for 16–18 hours. Subsequently, a single

colony of each strain was inoculated into 5 mL of LB broth and LB broth containing kanamycin, as described above, and incubated in a shaking incubator at 37°C for 18–24 hours. The resulting starter cultures were then transferred into 2-liter flasks containing 800 mL of culture medium and further incubated with shaking at 180 rpm and 37°C for another 18–24 hours. After cultivation, bacterial suspensions were harvested by centrifugation at 5,000 ×g for 5 minutes, washed twice with sterile 0.85% NaCl, and re-suspended in sterile 0.85% NaCl. Each bacterial suspension was adjusted to 1×10^8 CFU/mL in a final volume of 100 mL and thoroughly mixed with 500 g of a commercial diet containing 30% protein to achieve a final probiotic concentration of 1×10^8 CFU/kg of feed. The control diet was prepared using 100 mL of 0.85% sterile NaCl per 500 g of the same diet. The mixed diets were coated with 2.5% (v/w) sterile squid oil and air-dried at room temperature for 3 hours. After that, the experimental diets were aliquoted for daily use before being stored at 4 °C until feeding the fish. In the vitamin C supplementation group, 500 mg kg^{-1} of vitamin C (Stay C-35™, F. Hoffmann-La Roche, Basel, Switzerland) was prepared by mixing in the fish diet using the same method described above for the probiotic supplementation. The major chemical compositions of a commercial diet were analyzed according to the standard method of the Association of Official Analytical Chemists (AOAC) (1990), which includes 30% crude protein, 12% moisture, 8% fiber, and 3% fat. No significant difference was observed among the experimental diets ($p \geq 0.05$).

3.2.3 Growth performance

The individual body weight and length of fish from each tank were measured at 0, 30, 60, and 90 days. Growth performance, including weight gain (WG), specific growth rate (SGR), average daily gain (ADG), feed conversion ratio (FCR), protein efficiency ratio (PER), and relative growth rate (RGR),

Weight gain (WG) = Final weight - Initial weight

Average daily gain (ADG) = (Final weight - Initial weight)/experimental days

Specific growth rate (SGR %/day) = 100×[(lnFW−lnIW)/experimental days]

Feed conversion ratio (FCR) =dry feed fed/wet weight gain

Protein Efficiency Ratio (PER) = Weight gain/ protein intake

Relative growth rate (RGR) = (Final weight - Initial weight)/Initial weight*100

where FWis final weight, and IW is initial weight.

3.2.4 Blood collection

Blood samples were collected from one fish per tank (three fish per dietary treatment) via the caudal vein of anesthetized fish on days 30 and 90. The blood was kept at ambient temperature for 2 hours, after which the serum was harvested by centrifugation at 3,500 ×g at 25°C for 30 minutes and stored at –80°C until use.

3.2.5 Determination of vitamin C in Nile tilapia serum using HPLC analysis

To analyze the concentration of vitamin C in experimental fish, the serum was collected from the experimental fish after 30 and 90 days of the feeding trial. The HP 1100 series reversed-phase high-performance liquid chromatography (HPLC) system (Agilent Technologies, Waldbronn, Germany) with a C18 HPLC column, 5 µm, 250 × 4.0 mm, was used in this experiment according to the method described by Pitaksong et al. (2013). The mobile phase was used with a flow rate of 0.8 mL min^{-1}. The serum was centrifuged at 10,000 rpm for 10 min to remove debris. The clear supernatant was filtered through a 0.45 µm syringe filter. Twenty microliters of clear filtrate were injected into the HPLC system. High-purity vitamin C (Sigma, St. Louis, MO, USA) was used as the reference standard for quantifying vitamin C in fish serum. Each experiment was conducted in triplicate.

3.2.6 Lysozyme activity

Lysozyme activity was measured according to the method of Siwicki et al. (1987). Briefly, lysozyme standards at concentrations of 0, 2.5, 5, 10, 15, and 20 µg/mL were prepared in 6 M phosphate-citrate buffer with NaCl (PCB-NaCl, pH 6.0) to generate a standard curve. Then, 10 µL of Nile tilapia serum and each reference standard concentration was added in triplicate to the wells of a 96-well flat-bottom

plate. To assess serum lysozyme activity, 190 µL of a 0.3 mg/mL suspension of *Micrococcus lysodeikticus* (ATCC 4698; Sigma-Aldrich, St. Louis, MO, USA) was quickly added to each well. The reaction was carried out at 25°C, and the optical density (OD) was measured at 450 nm at 0, 30, and 60 minutes using a microplate reader Epoch BioTek instruments (Agilent Technologies). Lysozyme concentrations (µg/mL) were determined using the generated standard curve.

3.2.7 Total immunoglobulin (Ig)

The total immunoglobulin (Ig) concentration (mg/mL) in Nile tilapia serum was determined using a total protein kit (Biuret method; Erba, Mannheim, Germany), following the method of Nakharuthai et al. (2023). Briefly, 10 µL of Nile tilapia serum was added to a 1.5 mL microcentrifuge tube, followed by an equal volume of 12% polyethylene glycol (PEG) solution (Sigma-Aldrich, St. Louis, MO, USA). The mixture was incubated at 25°C for 30 minutes and then centrifuged at 12,500 rpm at 4°C for 10 minutes. Following centrifugation, the supernatant (non-immunoglobulin proteins) and pellet (total immunoglobulins) were separated. Subsequently, 4 µL of the supernatant, untreated serum (total protein), and bovine serum albumin standard (Sigma-Aldrich, St. Louis, MO, USA) were added in triplicate to a 96-well flat-bottom plate. Protein concentration was determined according to the manufacturer's instructions, and absorbance was measured at 546 nm using a microplate reader Epoch BioTek instruments (Agilent Technologies). Total Ig concentration was calculated by subtracting the non-Ig protein concentration from the total protein concentration. Protein concentrations were determined using a standard curve constructed with bovine serum albumin.

3.2.8 Alternative complement pathway 50% hemolytic activity (ACH_{50})

ACH_{50} activity was determined following the method described by Milla et al. (2010). Briefly, 50 µL of Nile tilapia serum was subjected to a two-fold serial dilution using EGTA-GVB buffer (gelatin veronal-buffered saline containing 10 mM ethylene glycol-bis(ß-aminoethyl ether)-N,N′-tetraacetic acid). An equal volume of a 5×10^7 cells/mL suspension of goat red blood cells (GRBCs) was then added to each diluted serum sample. The mixture was incubated at 25°C for 90 minutes and subsequently centrifuged at 12,000 ×g for 10 minutes at 4°C. The optical density (OD)

of the supernatant was measured at 415 nm using a microplate reader Epoch BioTek instruments (Agilent Technologies). The ACH_{50} value, defined as the volume of serum producing 50% hemolysis of GRBCs, was calculated. ACH_{50} activity was expressed in units/mL and determined in triplicate for each experimental group.

3.2.9 Phagocytic activity analysis

The percentage of phagocytic activity (PA) was determined with modifications to the method described by Puangkaew et al. (2004). Peripheral blood leukocytes (PBLs) were used in this assay. Briefly, 1 mL of whole blood was withdrawn from the caudal vein of each fish using a sterile syringe coated with K_2EDTA anticoagulant. The collected blood was transferred to a 15 mL conical tube containing 2 mL of RPMI medium, gentle mixed, and then layered onto 3 mL of Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO, USA) in a separate 15 mL tube. This two-layer mixture was centrifuged at 500 ×g for 30 minutes at 25°C using a swing rotor centrifuge.

Approximately 3 mL of the opaque interface containing PBLs was carefully transferred into a new 15 mL tube, and an equal volume of phosphate-buffered saline (PBS, pH 7.4) was added. The mixture was gently mixed and centrifuged twice at 250 ×g for 10 minutes. The cell pellet was resuspended in PBS (pH 7.4), and the leukocyte concentration was adjusted to 1×10^6 cells/mL. For the phagocytosis assay, PBL_s from each group were placed onto 22 × 22 mm coverslips and incubated for 2 hours to allow for cell adhesion. All assays were performed in triplicate. Adherent cells were then incubated at room temperature for 1.5 hours with 1×10^7 latex beads (Sigma-Aldrich, St. Louis, MO, USA) suspended in 200 µL of PBS (pH 7.4). Non-adherent cells and excess beads were removed by washing the coverslips three times with PBS. The cells and beads were then stained using Diff-Quick staining dye (Fisher Scientific, Waltham, MA, USA). At least 300 cells (phagocytic and non-phagocytic) were examined under 100× magnification using light microscopy to determine PA. The percentage of phagocytic activity (PA) was calculated using the following formula, as described by Nakharuthai et al. (2016).

PA (%) = (Number of phagocytic cells/Total number of counted cells) × 100

3.2.10 Activity of antioxidant enzymes

The activities of catalase (CAT), total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) levels were measured using a commercial assay kit (Abbkine Corporation, Atlanta, GA, USA), according to the manufacturer's instructions.

3.2.11 Expression of *GULO* mRNA in normal fish via qRT-PCR

3.2.11.1 Total RNA extraction

Total RNA was extracted from Nile tilapia intestine using TRIzol reagent (Gibco BRL, USA). Briefly, 1 mL of TRIzol was added to a 1.5 mL microcentrifuge tube containing 100 mg of tissue, and the sample was homogenized using mini-beadbeater-16 (Thermo Fisher Scientific, Waltham, MA, USA). The homogenate was incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Next, 0.3 mL of chloroform per 1 mL of TRIzol was added, and the tube was shaken vigorously by hand for 15 seconds, then incubated at room temperature for 3 minutes. The sample was centrifuged at 12,500 rpm at 4°C for 15 minutes. After centrifugation, three phases formed: a lower red phenol-chloroform phase, an interphase, and an upper colorless aqueous phase. The aqueous phase was carefully transferred to a new tube, and RNA was precipitated by adding an equal volume of isopropyl alcohol. The mixture was incubated on ice for at least 30 minutes and centrifuged at 12,000 rpm at 4°C for 30 minutes. The supernatant was discarded, and the RNA pellet was washed with 1 mL of 80% ethanol, vortexed briefly, and centrifuged at 8,000 rpm at 4°C for 5 minutes. After removing the ethanol, the RNA pellet was air-dried for 5–10 minutes on ice and then dissolved in DEPC-treated water. To eliminate genomic DNA contamination, the RNA solution was treated with RNase-free DNase I (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. The integrity of the RNA was confirmed by agarose gel electrophoresis stained with SafeRed nucleic acid staining solution (Vivantis Technologies Sdn Bhd., Selangor, Malaysia). The Nanodrop 2000™ spectrophotometer (Thermo Fisher Scientific) was used to measure the quantity and quality of RNA.

3.2.11.2 First strand cDNA synthesis

First-strand cDNA was synthesized using the ImProm-II™ Reverse Transcription System Kit (Promega Corporation, Madison, WI, USA). Briefly, 0.5 µg of oligo(dT) and 1 µg of total RNA were incubated at 70°C for 5 minutes. A master mix was prepared to bring the total reaction volume to 15 µL, consisting of 4 µL of ImProm-II™ 5X reaction buffer, 4.8 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTP mix, 0.5 µL of RNase inhibitor, 1 µL of reverse transcriptase, and 3.7 µL of nuclease-free water. The final volume was adjusted to 20 µL and incubated at 25°C for 10 minutes, followed by 42°C for 90 minutes, and then 72°C for 15 minutes to complete the reaction. The synthesized first-strand cDNA was kept at –20°C in a freezer until use.

3.2.11.3 Construction of cDNA plasmid standards for qRT-PCR

To construct cDNA plasmid standards for qRT-PCR, each target gene of interest was cloned to evaluate the mRNA expression levels in Nile tilapia after feeding the experimental diets. Primer sets used for qRT-PCR analysis are listed in Table 2. PCR products of the expected sizes were purified using FavorPrep™ GEL/PCR Purification Kit (Farvogen® Biotech Corp, Ping Tung, Taiwan), following the manufacturer's instructions. The purified DNA fragments were then ligated into the pGEM®-T Easy plasmid vector (Promega Corporation, Madison, WI, USA). The recombinant plasmids were sequenced Macrogen sequencing service (Macrogen Inc., Seoul, Republic of Korea) to confirm the insertion, and the confirmed plasmids were kept at –20°C in a freezer until use.

3.2.11.4 Real-time PCR analysis

One microliter of first-strand cDNA was used for quantitative real-time PCR (qRT-PCR) analysis, performed in triplicate using the CFX Opus Real-Time PCR System (Bio-Rad, Hercules, CA, USA). Each reaction was carried out in a final volume of 10 µL, containing 1 µL of first-strand cDNA, 5 µL thunderbird SYBR® qPCR master mix (TOYOBO, Osaka, Japan), 2 µL of distilled water (dH_2O), and 1 µL of each gene-specific primer, as listed in Table 3.1. The qRT-PCR thermal cycling conditions were as follows: an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s and annealing/extension at 55-59°C for 30 s. A DNA melting curve analysis was performed at the end of the amplification to confirm the specificity of the primers.

Table 3.1 The list of oligonucleotide sequences used in this study for qRT-PCR.

Primer name	5′ to 3′ Nucleotide Sequences	Product size (bp)	Annealing temperature (°C)	Accession No.
GULO-qPCRF	ACAGGGACGCACAACACTGG	172	59	XM_015285218
GULO-qPCRR	TGACGGTGAGCACAACACCC			
ß-actinF	ACAGGATGCAGAAGGAGATCACAG	155	55	KJ126772.1
ß-actinR	GTACTCCTGCTTGCTGATCCACAT			
*On*CC-F	ACAGAGCCGATCTTGGGTTACTTG	229	55	KJ535436.1
*On*CC-R	TGAAGGAGAGGCGGTGGATGTTAT			
*On*TNF-αF	GAGGCCAATAAAATCATCATCCC	161	55	NM_001279533
*On*TNF-αR	CTTCCCATAGACTCTGAGTAGCG			

3.3 The effects of dietary supplementation with probiotic *B. subtilis* expressing *GULO* after a challenge with *S. agalactiae* in Nile tilapia

3.3.1 Experimental design

A total of 60 healthy Nile tilapia were used in this experiment following one month of the feeding trial. The fish were randomly distributed into twelve 500-liter fiber tanks, with three replicate tanks assigned to each dietary treatment group. Each tank contained five individual fish. The experimental diets were described in Section 3.2.3.

3.3.2 Preparation of *S. agalactiae* and challenge test

The virulent strain of *S. agalactiae*, previously isolated by our research group from infected Nile tilapia (Nakharuthai & Srisapoome, 2020), was used in the challenge experiment following a 30-day feeding trial. A single colony of *S. agalactiae* was inoculated into 5 mL of tryptic soy broth (TSB, Merck KGaA, Darmstadt, Germany) and incubated at 37°C for 16–18 hours with shaking. After cultivation, the bacterial suspension was harvested by centrifugation at 5,000 ×g for 5 minutes. The resulting pellet was washed twice with sterile 0.85% NaCl and re-suspended in the same solution. The concentration of live *S. agalactiae* was adjusted to 1×10^8 CFU/mL, corresponding to an optical density of 1.0 at 600 nm.

After one month of the trial, fish in each experimental group were intraperitoneally injected with 0.1 mL of a *S. agalactiae* suspension at a concentration of 1×10^8 CFU/mL, corresponding to 0.1 mL per 100 g of fish body weight. At 0 hours, 6 hours, 12 hours, 24 hours, and 48 hours post-challenge, the liver, spleen, and blood were collected from three fish in each group for analysis of innate immune responses and gene expression, as described below.

3.3.3 Immune parameters

After the challenge, serum from the injected fish was collected at 0 hours, 6 hours, 12 hours, 24 hours, and 48 hours. The serum samples were analyzed for immune parameters, with measurements performed in triplicate for each experimental group. Lysozyme activity (LZM), total immunoglobulin (total Ig), and alternative complement activity (ACH_{50}) were assessed as described in Sections 3.2.6, 3.2.7, and 3.2.8, respectively.

3.3.4 Gene expression

Total RNA was extracted from various fish tissues, including the liver and spleen, using the same method described in section 3.2.10.1. First-strand cDNA synthesis, the construct cDNA plasmid standards for qRT-PCR, and the expression of pro-inflammatory genes CC chemokine and tumor necrosis factor alpha (TNFα) in the liver and spleen of challenged fish were assessed using qRT-PCR, as previously described in Sections 3.2.11.2, 3.2.11.3, and 3.2.11.4. The primer sets for qRT-PCR analysis used in challenged fish are shown in table 3.1.

3.4 Statistical analysis

The statistical analysis using the SPSS software version 25 (SPSS Inc., Chicago, IL, USA). The data were analyzed using a one-way analysis of variance followed by the post hoc Tukey's test to assess the significance of differences between the groups. A paired-sample t-test was conducted to evaluate the difference between 30 and 90 days after the feeding trial within immune parameters and the expression of *GULO* mRNA. The difference between groups in comparative experiments was determined by statistical significance at $p < 0.05$.