EFFECTS OF DIETARY SUPPLEMENTATION WITH RECOMBINANT *BACILLUS SUBTILIS* EXPRESSING L-GULONOLACTONE OXIDASE ON GROWTH PERFORMANCE, ANTIOXIDANT ACTIVITY, AND IMMUNE RESPONSE AGAINST *STREPTOCOCCUS AGALACTIAE* IN NILE TILAPIA, *OREOCHROMIS NILOTICUS*

JIRAWADEE KAEWDA

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science Program in Biotechnology for Aquaculture
Suranaree University of Technology
Academic Year 2024

ผลของการเสริมรีคอมบิแนนท์โปรไบโอติก *Bacillus subtilis* ที่แสดงออกเอนไซม์ L-gulonolactone oxidase ในอาหาร ต่อสมรรถนะการเจริญเติบโต ความสามารถในการต้านอนุมูลอิสระ และการตอบสนองทางภูมิคุ้มกันต่อเชื้อ *Streptococcus agalactiae* ในปลานิล (*Oreochromis niloticus*)

นางสาวจิราวดี แก้วดา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพสำหรับการผลิตสัตว์น้ำ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2567

EFFECTS OF DIETARY SUPPLEMENTATION WITH RECOMBINANT *BACILLUS SUBTILIS* EXPRESSING L-GULONOLACTONE OXIDASE ON GROWTH PERFORMANCE, ANTIOXIDANT ACTIVITY, AND IMMUNE RESPONSE AGAINST *STREPTOCOCCUS AGALACTIAE* IN NILE TILAPIA, *OREOCHROMIS NILOTICUS*

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's degree.

Thesis Examining Committee

(Prof. Dr. Nopadon Pirarat)
Chairperson

(Asst. Prof. Dr. Chatsirin Nakharuthai)
Member (Thesis Advisor)

(Prof. Dr. Surintorn Boonanuntanasarn)
Member

(Assoc. Prof. Dr. Amonrat Molee)
Member

(Asst. Prof. Dr. Pakanit Kupittayanant)
Member

(Assoc. Prof. Dr. Yupaporn Ruksakulpiwat)
Vice Rector for Academic Affairs
and Quality Assurance

(Prof. Dr. Neung Teaumroong)
Dean of Institute of Agricultural
Technology

จิราวดี แก้วดา : ผลของการเสริมรีคอมบิแนนท์โปรไบโอติก *Bacillus subtilis* ที่แสดงออกเอนไซม์ L-gulonolactone oxidase ในอาหาร ต่อสมรรถนะการเจริญเติบโต ความสามารถในการต้านอนุมูลอิสระ และการตอบสนองทางภูมิคุ้มกันต่อเชื้อ *Streptococcus agalactiae* ในปลานิล (*Oreochromis niloticus*) (EFFECTS OF DIETARY SUPPLEMENTATION WITH RECOMBINANT *BACILLUS SUBTILIS* EXPRESSING L-GULONOLACTONE OXIDASE ON GROWTH PERFORMANCE, ANTIOXIDANT ACTIVITY, AND IMMUNE RESPONSE AGAINST *STREPTOCOCCUS AGALACTIAE* IN NILE TILAPIA, *OREOCHROMIS NILOTICUS*)
อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. ฉัตรศิรินทร์ นาคหฤทัย, 68 หน้า.

คำสำคัญ: แอล-กูโลโนแลคโตนออกซิเดส/สารต้านอนุมูลอิสระ/*Bacillus subtilis*/ความสามารถในการต้านเชื้อ/ปลานิล/*Streptococcus agalactiae*

ปลานิล ไม่สามารถสังเคราะห์วิตามินซีได้เอง เนื่องจากขาดเอนไซม์แอล-กูโลโนแลกโตนออกซิเดส (L-gulonolactone oxidase; *GULO*) จึงจำเป็นต้องได้รับวิตามินซีจากอาหารในปริมาณที่เพียงพอ เพื่อเสริมสร้างคุณสมบัติในการต้านอนุมูลอิสระและผลที่เกี่ยวข้องกับวิตามินซี งานวิจัยนี้มีวัตถุประสงค์เพื่อทดสอบประสิทธิภาพของโปรไบโอติกชนิดรีคอมบิแนนท์ *Bacillus subtilis* ที่มีการแทรกยีน *GULO* ไว้บนโครโมโซม โดยใช้เป็นสารเสริมในอาหารสัตว์น้ำ ในการทดลอง ปลานิลถูกแบ่งออกเป็น 4 กลุ่มทดลอง ได้แก่ กลุ่มควบคุมที่ได้รับอาหารพื้นฐาน (CON) กลุ่มที่ได้รับอาหารพื้นฐานเสริมวิตามินซี (VC) กลุ่มที่ได้รับอาหารพื้นฐานเสริม *B. subtilis* สายพันธุ์ปกติ (BS) และกลุ่มที่ได้รับอาหารพื้นฐานเสริม *B. subtilis* ชนิดรีคอมบิแนนท์ที่มีการแทรกยีน *GULO* (BS+GULO) หลังจากเลี้ยงเป็นระยะเวลา 90 วัน พบว่ากลุ่ม BS+GULO มีการเจริญเติบโตที่ดีที่สุด โดยมีค่าน้ำหนักสุดท้าย การเพิ่มน้ำหนัก อัตราการเจริญเติบโตจำเพาะ น้ำหนักเฉลี่ยที่เพิ่มขึ้นต่อวัน และอัตราการเจริญเติบโตสัมพัทธ์ สูงกว่ากลุ่มอื่นอย่างมีนัยสำคัญทางสถิติ กลุ่ม VC, BS และ BS+GULO มีระดับอิมมูโนโกลบูลินรวม และกิจกรรมของเอนไซม์ไลโซไซม์เพิ่มขึ้นเมื่อเปรียบเทียบกับกลุ่มควบคุม อย่างไรก็ตาม พบว่าเฉพาะกลุ่ม VC และ BS+GULO เท่านั้นที่มีระดับของระบบคอมพลีเมนต์ทางเลือก กิจกรรมการกลืนกินเชื้อโรคและสิ่งแปลกปลอมของเซลล์เม็ดเลือดขาว และค่าพารามิเตอร์ด้านสารต้านอนุมูลอิสระสูงขึ้นอย่างมีนัยสำคัญ ผลการวิเคราะห์โดยใช้เทคนิค HPLC และ qRT-PCR พบว่าปลานิลในกลุ่ม BS+GULO มีระดับวิตามินซีในซีรัม และการแสดงออกของยีน *GULO* ในลำไส้เพิ่มขึ้น นอกจากนี้ การทดสอบความสามารถในการต้านทานต่อเชื้อ *Streptococcus agalactiae* พบว่ากลุ่ม BS+GULO มีการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการอักเสบและการตอบสนองทาง

ภูมิคุ้มกันเพิ่มขึ้น ซึ่งสะท้อนถึงความสามารถในการต้านเชื้อ *S. agalactiae* ได้ดีกว่ากลุ่มที่ได้รับ *B. subtilis* สายพันธุ์ปกติ

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา ศิราวดี แก้วดา
ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา

JIRAWADEE KAEWDA : EFFECTS OF DIETARY SUPPLEMENTATION WITH RECOMBINANT *BACILLUS SUBTILIS* EXPRESSING L-GULONOLACTONE OXIDASE ON GROWTH PERFORMANCE, ANTIOXIDANT ACTIVITY, AND IMMUNE RESPONSE AGAINST *STREPTOCOCCUS AGALACTIAE* IN NILE TILAPIA, *OREOCHROMIS NILOTICUS*. THESIS ADVISOR : ASST. PROF. CHATSIRIN NAKHARUTHAI, Ph. D., 68 PP.

Keyword: L-gulonolactone oxidase/Antioxidant/*Bacillus subtilis*/Antagonistic activity/ Nile tilapia/*Streptococcus agalactiae*

Due to its lack of the L-gulonolactone oxidase (*GULO*) enzyme, Nile tilapia is unable to synthesize vitamin C; thus, it requires an adequate level of exogenous vitamin C in its diet. To enhance antioxidant properties and vitamin C-related effects, this study aimed to evaluate the efficacy of recombinant probiotic *Bacillus subtilis* with the *GULO* gene integrated into its chromosome. In this study, fish were divided into four groups: those fed with a basal diet (CON), a basal diet + vitamin C (VC), a basal diet + wild-type *B. subtilis* (BS), and a basal diet + recombinant *B. subtilis* (BS+GULO). After 90 days of the feeding trial, the BS+GULO groups showed the highest improvements in final weight, weight gain, specific growth rate, average daily gain, and relative growth rate. The VC, BS, and BS+GULO groups exhibited increased total immunoglobulin and lysozyme activity; however, only the VC and BS+GULO groups showed elevated alternative complement 50 levels, phagocytic activity and improved antioxidant parameters compared to the control group. HPLC and qRT-PCR analyses revealed elevated serum vitamin C and intestinal *GULO* mRNA levels in the BS+GULO group. A challenge test showed increased pro-inflammatory gene expression and immune response against *Streptococcus agalactiae* in the BS+GULO group, indicating improved antagonistic activity over wild-type *B. subtilis*.

School of Animal Technology and Innovation Student's Signature Jirawadee Kaewda
Academic Year 2024 Advisor's Signature Chatsirin Nakharuthai

ACKNOWLEDGEMENT

This dissertation would not have been possible without the invaluable guidance and continuous support of Assistant Professor Dr. Chatsirin Nakharuthai, my academic advisor, whose dedication, insightful suggestions, and meticulous revisions were instrumental to the successful completion of this research.

I am very grateful to my advisory committee members, Professor Dr. Nopadon Pirarat from Chulalongkorn University, as a defense chairperson, Professor Dr. Surintorn Boonanuntanasarn, Associate Professor Dr. Amonrat Molee, and Assistant Professor Dr. Pakanit Kupittayanant from Suranaree University of Technology, as a defense committee, for their constructive comments, beneficial suggestions, and productive advice to my research.

I would also like to express my sincere gratitude to Mr. Sunai Plaimee and the aquaculture team from the University Farm for their kind assistance, cooperation, and practical advice throughout the fieldwork and experimental phases. Their contributions were vital to the progress and outcomes of this study.

My heartfelt thanks extend to all individuals and organizations who supported this research in any capacity. Your encouragement and generosity have played a significant role in my academic journey and are deeply appreciated.

Finally, I would like to express my deepest gratitude to my family for their guidance, understanding, encouragement, and support throughout my education until its successful completion.

Jirawadee Kaewda

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

%	=	Percent
r	=	Relative centrifugal force
°C	=	Degree celsius
µg	=	Microgram
µL	=	Microliter
µm	=	Micrometre
ACH_{50}	=	Alternative Complement pathway 50% hemolytic activity
ADG	=	Average daily gain
APC	=	Antigen presenting cell
ASC	=	Ascorbate
bp	=	Base pair
BS	=	Wild-type *Bacillus Subtilis*
BS+GULO	=	Recombinant *Bacillus Subtilis*
CAT	=	Catalase
cDNA	=	Complementary DNA
CFU	=	Colony forming unit
CON	=	Control
COVID-19	=	Coronavirus disease starting in 2019
DEPC	=	Diethyl Pyrocarbonate
DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
dNTP	=	Deoxyribonucleoside Triphosphate
e.g.	=	Exempli gratia
etc.	=	Et cetera
FCR	=	Feed conversion ratio
Fe^{2+}	=	Iron
g	=	Gram
GI	=	Gastrointestinal

LIST OF ABBREVIATIONS (Continued)

GMO	=	Genetically modified organism
GR	=	Glutathione reductase
GRBCs	=	Goat red blood cells
GSH	=	Glutathione
GSH-Px	=	Glutathione peroxidase
GULO	=	L-gulonolactone oxidase
H_2O_2	=	Hydrogen peroxide
HPLC	=	High-performance liquid chromatography
IFNγ	=	Interferon gamma
Ig	=	Immunoglobulin
K_2EDTA	=	Potassium Ethylenediaminetetraacetic acid
kg	=	kilogram
L	=	Liter
LB	=	Luria–Bertani
LH	=	Lipid
LOO•	=	Lipid radical
LOOH	=	Lipid hydroperoxides
LZM	=	Lysozyme activity
m	=	Milli
M	=	Molar
MAC	=	Membrane attack complex
MDA	=	Malondialdehyde
mg	=	Milligram
mL	=	Milliliter
mm	=	Millimetre
mM	=	Millimolar
N/A	=	Not Available
NaCl	=	Normal saline
NK	=	Natural killer

LIST OF ABBREVIATIONS (Continued)

nm	=	Nanometre
O_2^-	=	Superoxide radical
O_2	=	Oxygen
OD	=	Optical density
OH•	=	Hydroxyl radical
PA	=	Phagocytic activity
PBLs	=	Peripheral blood leukocytes
PBS	=	Phosphate-buffered saline
PCB-NaCl	=	Phosphate-citrate buffer with sodium chloride
PCR	=	Polymerase chain reaction
PEG	=	Polyethylene glycol
PER	=	Protein efficiency ratio
pH	=	Potential of hydrogen ion
PUFAs	=	Polyunsaturated fatty acids
qRT-PCR	=	Quantitative real-time PCR
RGR	=	Relative growth rate
RNA	=	Ribonucleic Acid
RNase	=	ribonuclease
ROS	=	Reactive oxygen species
RPM	=	Revolutions per minute
RPMI	=	Roswell Park Memorial Institute
SGR	=	Specific growth rate
SOD	=	Superoxide dismutase
sp.	=	Species
spp.	=	Species pluralis
SUT	=	Suranaree University of Technology
TAC	=	Total antioxidant capacity
TLR	=	Toll-like receptor
TNF	=	Tumor necrosis factor

LIST OF ABBREVIATIONS (Continued)

TNFα	=	Tumor necrosis alpha
Toc	=	Tocopherol
Toc•	=	Oxidized tocopherol
TSB	=	Tryptic soy broth
v/w	=	Volume/weight
VC	=	Vitamin C
WG	=	Weight gain

CHAPTER I

INTRODUCTION

1.1 Background and Significance

In recent decades, the production of Nile tilapia has steadily shifted towards intensive culture systems (Assefa & Abunna, 2018). However, the advancement of intensive culture has led to the deterioration of water quality, facilitating the proliferation of pathogens in aquatic environments (Debnath et al., 2023). In such situations, coupled with the impact of climate change, fish are more susceptible to stress, leading to impaired growth performance and a weakened immune system. To address this issue, fish farmers have prioritized fish health maintenance by implementing effective management practices, supplying high-quality nutritional feed, and administering immunostimulants (Nayak, 2010; Munguti et al., 2022). Among the immunostimulant agents, probiotics *Bacillus subtilis* and vitamin C have attracted research interest for application in intensive culture systems (Aly et al., 2008; Liu et al., 2012; Liu et al., 2013; Liu et al., 2017; Cui et al., 2018; Harsij et al., 2020; Laosam et al., 2024). The probiotic *B. subtilis* is one of the most commonly used dietary supplements in various fish species owing to its numerous positive effects on the gut microbiota, growth performance, disease resistance, health status of aquatic animals, and water quality (Aly et al., 2008; Liu et al., 2012; Liu et al., 2017; Suwanangul et al., 2023). Moreover, it is generally recognized as safe for humans, animals, and the environment under specified conditions of use. Additionally, it is widely considered an ideal bacterial factory for producing heterologous proteins (Liu et al., 2013; Cui et al., 2018).

In modern fish farming, vitamin C is a crucial exogenous micronutrient and immunostimulant in aquafeed, as natural levels are often insufficient to support normal body functions in fish, particularly under intensive aquaculture conditions. Due to vitamin C's pivotal function as an enzyme cofactor, it plays a crucial role in facilitating many physiological processes that involve biosynthesis, protein metabolism (Harsij et al., 2020), iron metabolism (Zafar & Khan, 2020), lipid metabolism (John et

al., 1979), immune response (Barros et al., 2014), stress (Caxico et al., 2018), and physiological antioxidant activity (Gasco et al., 2018; Gouda et al., 2020). In fish, vitamin C deficiency has various adverse consequences, including impaired growth and survival rate, increased susceptibility to stress, depressed immune status, reduced reproductive performance, skeletal alterations, impaired collagen formation, slow wound healing, and anemia (Jauncey et al., 1985; Mæland & Waagbø, 1998; Zehra & Khan, 2021).

On the other hand, adequate vitamin C intake has been widely shown to have beneficial effects on the growth and health of fish. For example, dietary supplementation with the optimal level of vitamin C markedly improved growth performance (Lin & Shiau, 2005; Roosta et al., 2014; Xu et al., 2022) and serum antioxidant activities (Xu et al., 2022). It also enhanced several immune responses, including phagocytic activity, phagocytic index, alternative complement activity (ACH_{50}), and lysozyme activity (LZM) (Ai et al., 2004; Lin & Shiau, 2005; Abo-Al-Ela et al., 2017). In addition, dietary vitamin C increment has also been proven to enhance the proliferation of spermatogonia and hematocrit value in Japanese eel broodstock (*Anguilla japonica*) (Shahkar et al., 2015).

In aquaculture conditions, vitamin C is naturally derived from plants found in aquatic environments. However, in intensive commercial operations, natural plant-based food sources are usually inadequate to meet the required amounts for fish. Moreover, more advanced teleosts, including Nile tilapia, are incapable of synthesizing vitamin C de novo due to a lack/mutation of the L-gulonolactone oxidase (*GULO*), an enzyme necessary for the last step of ascorbic acid biosynthesis (Eo & Lee, 2008). In contrast, amphibians, reptiles, mammals (such as mice, sheep, and dogs), birds, chickens, primitive lobe-finned fish, cartilaginous fish species (such as shark species and white sturgeon), and almost all plants possess the ability to synthesize vitamin C due to the presence of the functional *GULO* gene (Smirnoff, 2001; Drouin et al., 2011; Shanaka et al., 2021).

As a result, more advanced teleost species must obtain vitamin C through dietary supplementation to ensure their optimum growth and health, especially in intensive culture conditions where limited natural foods are available (Wang et al., 2003). Unfortunately, the stability of vitamin C as a dietary component often makes it inadequate at proper levels for aquatic animals due to its rapid oxidation. The loss of vitamin C is accelerated in inappropriate environmental conditions during the

commercial manufacturing process of aquafeed, storage, handling, and feeding. The loss rate depends on various factors, including temperature, oxygen, UV irradiation, light, pH levels, and transition metal ions (Sheraz et al., 2015; Yin et al., 2022).

Various approaches have emerged to ensure that animals receive a sufficient amount of vitamin C and to enhance its stability and bioavailability. They include the shielding of vitamin C through encapsulation (Comunian et al., 2014), the development of chemical vitamin C derivatives (Liu et al., 2020), genomic integration of L-gulonolactone oxidase (Toyohara et al., 1996; Shanaka et al., 2021), utilization of exogenous 2-keto-L-gulonic acid supplementation (Shi et al., 2023), and so on. In this study, we aimed to evaluate the effect of recombinant *B. subtilis* expressing L-gulonolactone oxidase and its effectiveness on growth performance, antioxidant activity, and immune response in Nile tilapia. Since the ascorbate biosynthesis pathway, starting with D-glucose-1-phosphate as the initial precursor and progressing until L-gulonate, is conserved in all animal species (Crawford, 1982), it may be possible to re-establish this pathway by integrating the *GULO* gene into probiotic *B. subtilis* using recombinant probiotic technology.

The advancement of recombinant technology has facilitated the production of heterologous proteins using potential probiotics as expression systems. Recombinant probiotics offer a promising approach to delivering the specific traits and functionalities of heterologous proteins. Among these, the genus *Bacillus* has gained recognition as a reliable biofactory for producing heterologous proteins, serving both basic research and industrial applications (Cutting et al., 2009; Amal & Zamri-Saad, 2011; Nakharuthai et al., 2023). Employing *Bacillus* spp. presents numerous advantages, including their capacity for rapid and high-yield product synthesis, ease of genetic modification, and suitability for the expression and delivery of target genes.

In our previous study, we isolated and characterized the potential probiotic *B. subtilis* B29 from the intestinal microbiota of Nile tilapia based on its biological functions (Nakharuthai et al., 2023). Our investigation elucidated its advantageous properties, emphasizing antagonistic activity as the main criterion for selection, along with bile salts and pH tolerance, protease-producing capacity, antibiotic susceptibility, and results from pathogenicity tests. It is noteworthy that the probiotic *B. subtilis* B29 exhibited antagonistic activity against the three primary pathogenic bacteria in Nile tilapia, namely *Aeromonas hydrophila*, *Streptococcus iniae*, and *Streptococcus agalactiae*,

with higher efficacy against *S. agalactiae*.

In Thailand, *S. agalactiae*, a Gram-positive pathogenic bacterium, is frequently encountered in Nile tilapia. This bacterium causes the disease known as 'streptococcosis', which is characterized by several clinical symptoms in Nile tilapia, including unilateral or bilateral exophthalmia, erratic swimming, hemorrhaging in both external and internal organs, and septicemia (Amal & Zamri-Saad, 2011; Chen et al., 2012). Currently, it is recognized for causing significant mortality, typically occurring over a brief timeframe, particularly in intensive Nile tilapia farms (Dangwetngam et al., 2016). Therefore, the application of recombinant probiotic *B. subtilis* expressing *GULO* may provide a possible alternative option to achieve the combined effect of probiotic *B. subtilis* and vitamin C supplementation.

1.2 Research objectives

The objectives of this study were:

1.2.1 To investigate the effects of dietary supplementation of recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase on growth performance, innate immune responses, antioxidant status, and gene expression in Nile tilapia.

1.2.2 To investigate the effects of dietary supplementation with recombinant *B. subtilis* expressing L-gulonolactone oxidase on the resistance to *S. agalactiae* infection in Nile tilapia.

1.3 Research hypothesis

1.3.1 Recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase can improve growth performance, antioxidant activity, and immune response in Nile tilapia

1.3.2 Recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase can improve disease resistance against S. agalactiae infected in Nile tilapia.

1.4 Scope and limitation of this study

This study primarily investigates the effect of dietary supplementation with recombinant *B. subtilis* expressing L-gulonolactone oxidase (*GULO*), through the chromosomal integration of a promising heterologous gene into a conventional probiotic strain. Nile tilapia were assigned to four dietary groups: a commercial diet

supplemented with 0.85% NaCl (control), a commercial diet supplemented with vitamin C, a commercial diet supplemented with wild-type *B. subtilis*, and a commercial diet supplemented with recombinant *B. subtilis* expressing *GULO*. Following 30 days of feeding, the fish were challenged with *S. agalactiae*, and immune parameters were assessed to evaluate the response to the pathogen, with a particular focus on key immune-related genes and mechanisms. After 90 days of feeding, growth performance, innate immune response, and antioxidant activity were evaluated to assess the potential of the recombinant probiotic for future application in aquaculture.

1.5 Expected results

From this study, recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase can improve growth performance, antioxidant activity, immune response, and disease resistance against *S. agalactiae* infected in Nile tilapia

CHAPTER II

LITERATURE REVIEW

2.1 Biology of Nile tilapia (*Oreochromis niloticus*)

Nile tilapia (*Oreochromis niloticus*), a member of the Cichlidae family, is a freshwater fish species with economic significance on a global scale. It is indigenous to the African continent and is commonly found in rivers, estuary areas, marshes, and lakes throughout several countries such as Sudan, Uganda, Tanzania, and Kenya (Njiru et al., 2006). This species can thrive in both freshwater and brackish water environments with a rapid growth rate due to its adaptability to diverse environmental conditions; as a result, it is cultivated extensively for agricultural purposes. In addition, Nile tilapia's meat is versatile and has a delectable taste, making it a favored choice for a variety of cuisines.

In Thailand, a total of 50 Nile tilapia were first introduced into Thailand on March 25, 1965, as a gift from Emperor Akihito, His Royal Highness the Crown Prince of Japan to King Bhumibol Adulyadej of Thailand. Subsequently, His Majesty King Bhumibol Adulyadej graciously granted permission for an experimental cultivation of Nile tilapia in the ponds of Chitralada Royal Palace and many fry fish were produced more than 5 months later. The fish were transferred from the old pond to 6 new ponds and their growth and behavior were closely monitored by the Department of Fisheries technical staff. In 1966, His Majesty King Bhumibol Adulyadej bestowed the local name of this fish "Pla nin" prior to its distribution to the Thai people for widespread breeding and consumption (Belton et al., 2009).

Taxonomy of Nile tilapia

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Perciformes

Family: Cichlidae

Genus: *Oreochromis*

Species: *O. niloticus*

Binomial name: *Oreochromis niloticus* (Linnaeus, 1758)

Common name: Nile tilapia

2.2 Characteristic of Nile tilapia

Nile tilapia is a freshwater bony fish with distinctive features such as a terminal mouth, compressed body, and one nostril on each side of the head. Within their small mouths, both jaw and pharyngeal teeth of varying sizes are located on the mandible and maxilla. The first gill arch consists of 15-27 gill rakers. The cheek is covered with scales arranged in 3-4 horizontal rows. The long dorsal fin, anal fin, and caudal fin exhibit distinct white spots and intersecting black lines. The dorsal fin is composed of 15-18 spines and 12-14 soft rays, while the anal fin has 3 spines and 9-10 soft rays. Along an interrupted lateral line, there are typically 21-23 scales above the line and 14-18 scales below the line. Nile tilapia is an excellent candidate species for farming because it can survive in water with poor quality, grow quickly, is efficiently reproductive, can withstand a wide range of pH, and is resistant to low levels of dissolved oxygen. The optimal temperature range for tilapia growth is between 27 and 31°C, while the lower and upper lethal temperature are 11-12°C and 42°C, respectively (FAO, 2021). In addition, Nile tilapia can tolerate dissolved oxygen concentrations as low as 3-4 mg/l, albeit at a slower growth rate. The optimal pH and ammonia ranges for Nile tilapia growth are 7-8 and 0.05 mg/L, respectively (El-Sherif & El-Feky, 2009).

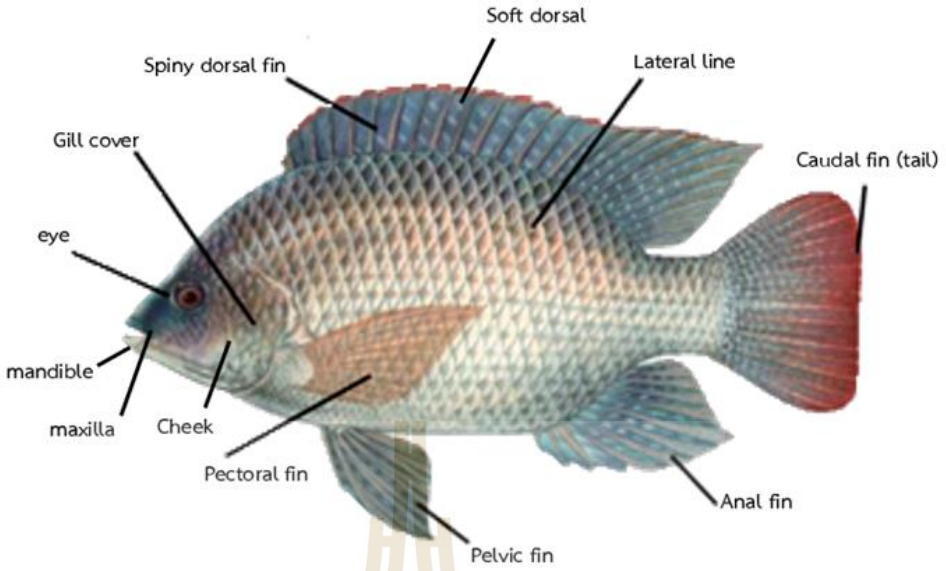


Figure 2.1 Shape and composition of tilapia.
Reference: Getnet et al. (2024).

2.3 Economic significance

Over the past three decades, aquaculture production and its respective industries have grown continuously to support the increased demand of the world population. Aquaculture products have continued to be the preferred choice of protein source and generate significant revenue for many countries. According to a market report published by the Food and Agriculture Organization (FAO) in 2022, global production of aquatic animals was estimated at 178 million tonnes in 2020, showing a slight decrease from the all-time record of 179 million tonnes in 2018 (Figure 2.2). Capture fisheries contributed 90 million tonnes and aquaculture 88 million tonnes. Of the total production, 112 million tonnes were harvested in marine waters and 37 percent in inland waters. Among freshwater fish species, Nile tilapia has become the third-largest source of freshwater fish products, following carp and salmon. After surviving the COVID-19 pandemic with a slight decrease in market value, the global Nile tilapia production is anticipated to increase by 2% to 4% in 2022 and to continue rising annually because of its consistent availability of products with low prices (FAO, 2022). Interestingly, the increasing demand for Nile tilapia products in North America and the European Union is partially due to the rising prices of other seafood products

on the global market. In addition, the expansion of Nile tilapia farming in Asia and Latin America is also expected to further expand the trade market, with a focus not only on the United States but also on other regions (FAO, 2023).

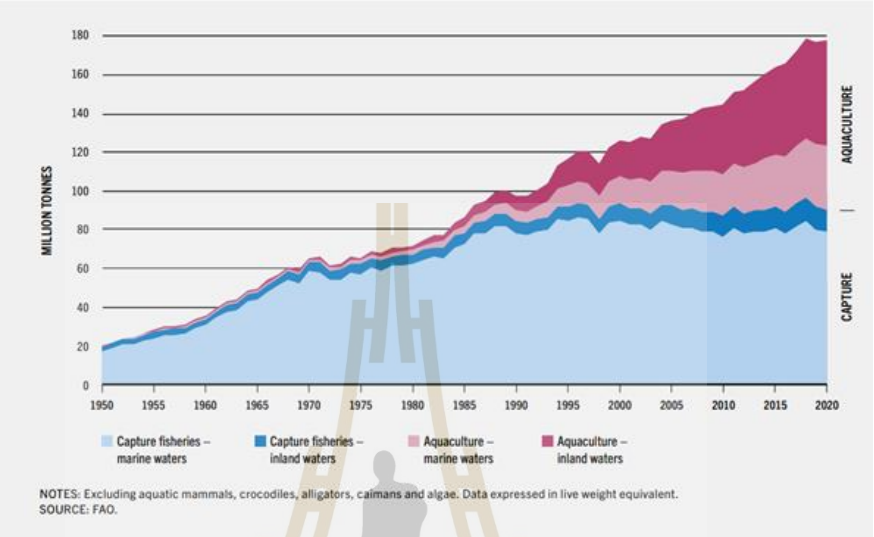


Figure 2.2 World Capture Fisheries and Aquaculture Production.
Reference: Food and Agriculture Organization (2022).

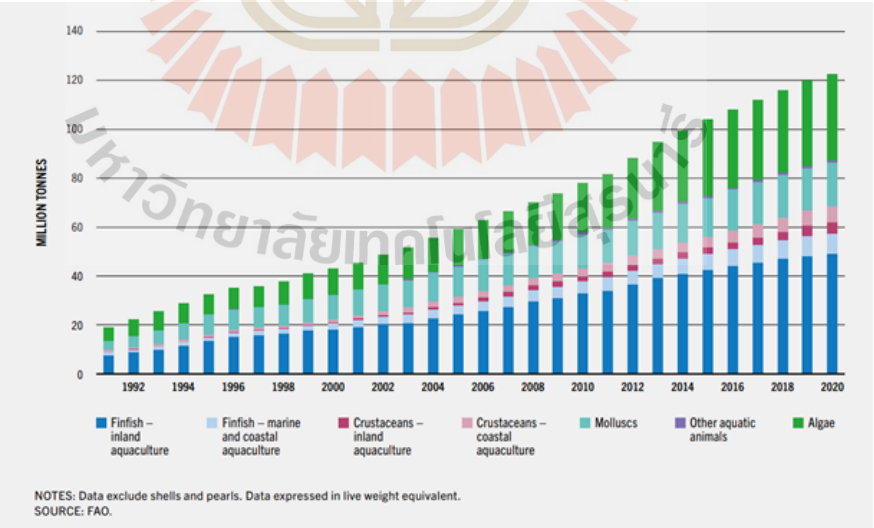


Figure 2.3 World aquaculture production of aquatic animals, 1991-2020.
Reference: Food and Agriculture Organization (2022).

In Thailand, Nile tilapia is one of the most important freshwater fish species in Thailand's aquaculture industry for the same reasons mentioned previously. Consequently, intensive production of Nile tilapia has been rapidly developed to increase their yield for commercial purposes and household consumption.

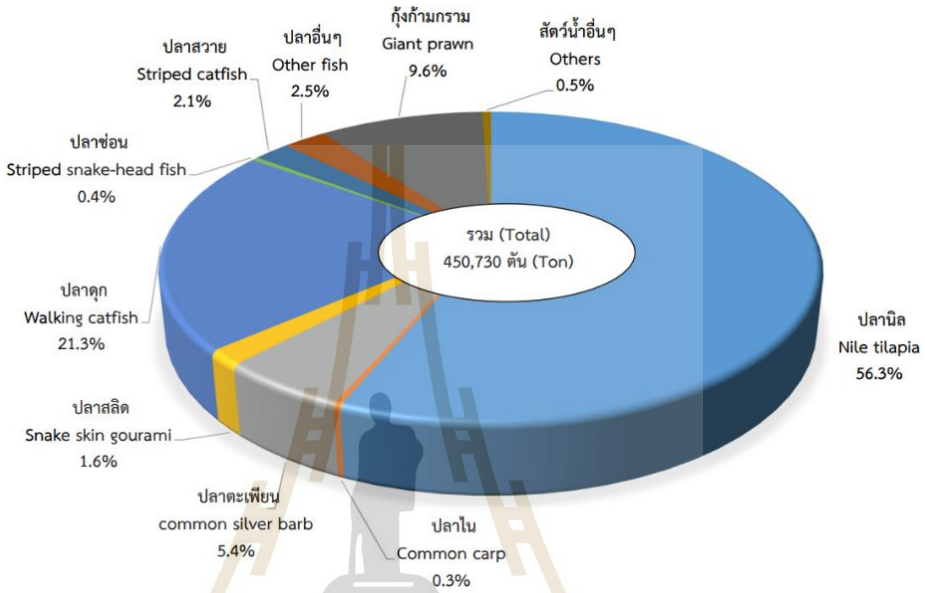


Figure 2.4 Freshwater Aquaculture Production by Species, 2021.
Reference: Fisheries statistics of Thailand (2021).

2.4 Nile tilapia culture system and its constraints in Thailand

In the past, the Nile tilapia culture system was an extensive culture for primary household consumption. In this culture system, fish rely on natural food sources available within the pond, are raised in low-density with low crop yield, and require a lengthy period per crop. Nowadays, the Nile tilapia culture system in Thailand has transformed into a semi-intensive and intensive culture system. The small-scale farmers commonly practice a semi-intensive culture of Nile tilapia for household consumption and income generation within the country. In this approach, the stocking density and fish production are higher than in an extensive system. Meanwhile, the large-scale Nile tilapia farming industry (intensive culture system) commonly approaches mainly aimed at commercial production to meet domestic and international consumer demand,

emphasizing high stocking densities, and providing formulated feed meeting total nutritional requirements as the main source of nutrition. In this system, the farmer typically provides a commercial diet of high quality and quantity, which, if it exceeds the fish's actual dietary requirements, can result in poor water quality due to the accumulation of leftover feed on the bottom pond. This is regarded as a threat to fish because the bioremediation process requires oxygen and excessive organic matter can lead to dissolved oxygen deficiency and the development of anaerobic bacteria, a concern in intensive aquaculture. This condition causes stress in fish due to the accumulation of toxins such as ammonia, nitrate, and nitrite. In addition, residual organic matter in the water serves as a nutrient source for pathogens. At that time, plasma cortisol and metabolic responses are elevated to maintain the fish's homeostasis. Nonetheless, maladaptive responses may negatively affect fish health, increasing their susceptibility to pathogens.

2.5 Diseases of Nile tilapia

Currently, Nile tilapia farming is primarily conducted through intensive culture systems, which are characterized by elevated stocking densities. This practice can increase fish stress, leading to an elevated FCR, and suppress their immune response, making them more susceptible to disease. Similar to other animal species, Nile tilapia diseases can be broadly categorized into two main types: non-infectious diseases and infectious diseases with predisposing factors mostly related to stress in intensive culture systems. Non-infectious diseases are those that result from unsuitable environmental conditions, nutritional deficiencies, genetic abnormalities, etc. Non-infectious diseases cannot be transmitted and there are usually no medicines for treatment. Therefore, the application of hygienic procedures and preventative environmental measures contributes to the improvement of fish farms' biosecurity. Examples of non-infectious diseases in Nile tilapia include physical deformities, gas bubble disease, alkalosis, acidosis, etc. (Ibrahim, 2020).

Infectious diseases, by contrast, are caused by pathogens such as viruses, bacteria, parasites, and fungi, which are commonly present in the environment and in carrier hosts. Contagious diseases can be readily spread from infected fish to other healthy fish, resulting in mass mortality and morbidity throughout the aquaculture

production system. Managing and controlling these diseases remains one of the most significant challenges in intensive aquaculture (Yanong et al., 2021).

2.5.1 Parasitic disease

Parasitic diseases, especially *Ichthyophthirius multifiliis*, *Trichodina* sp., *Dactylogyrus* sp., *Gyrodactylus* sp., louse, and isopod, are commonly encountered in the epithelial tissue of gills, skin, and fins of Nile tilapia (Shinn et al., 2023). Adult fish are typically unaffected by the presence of a single or small number of individual parasites. However, the presence of many parasites in larval and fry stages may result in mass mortality. Fish infected with a high number of parasites may exhibit a variety of symptoms, such as pale or dark skin, erratic swimming behavior, weight loss, and irritation. To eliminate parasites, infected fish typically produce an excessive amount of mucus which contains innate immune components against parasite invasion. Different chemicals, such as Dipterex (Organophosphate insecticide), hydrogen peroxide, potassium permanganate, formalin, and salt, can be used to treat parasites.

2.5.2 Fungal disease

Fungal infections in Nile tilapia are primarily attributed to *Saprolegnia* spp. (Zahran et al., 2017). The spores of these fungi are commonly found in aquatic environments, and infections often occur, especially in poorly hatched eggs. These fungal infections are typically considered secondary infections during or after the presence of pathogenic bacteria, viruses, and parasites, which can exacerbate the condition.

2.5.3 Viral disease

Certain diseases in Nile tilapia are caused by viral infections, which are often difficult to diagnose using conventional laboratory methods. No specific antiviral drugs are available for the treatment of viral diseases in tilapia culture. Additionally, several viral diseases have been identified in Nile tilapia, including Iridovirus, Aquatic Birnavirus, Rhabdovirus, Betanodavirus, and tilapia Lake Virus (Sunarto et al., 2022).

2.5.4 Bacterial disease

Among the significant diseases that pose a threat to the tilapia aquaculture industry in Thailand are those mostly caused by bacterial infections, especially *S. agalactiae*, *Aeromonas hydrophila*, and *Flavobacterium columnare* (Nakharuthai et al., 2016).

2.5.4.1 *Streptococcus agalactiae*

Streptococcus agalactiae, the most common pathogenic bacteria found in tilapia culture, is a Gram-positive bacterium belonging to the *Streptococcaceae* family. This bacterium can cause the disease known as "Streptococcosis", which affects both aquatic and terrestrial animals, such as humans, cattle, horses, pigs, dogs, and cats (El-Noby et al., 2021). It is an opportunistic pathogen that can cause disease in fish when their immune systems are compromised or when they are stressed due to poor water quality or other factors. Infected fish may exhibit various clinical signs, including lethargy, loss of appetite, abnormal swimming behavior, and external lesions (Abdallah et al., 2024). The disease can progress rapidly leading to high mortality rates among infected fish. *S. agalactiae* can be transmitted through various routes, including direct contact between infected and healthy fish, contaminated water, and contaminated equipment or materials used in aquaculture operations (Pretto-Giordano et al., 2010).

2.5.4.2 *Aeromonas hydrophila*

A. hydrophila is a Gram-negative bacterium that can be commonly found in freshwater. It can act as an opportunistic pathogen, causing disease when fish are under stress or have a compromised immune system. Nile tilapia infected with *A. hydrophila* may display a range of clinical signs, including fin rot, skin ulcers, abdominal swelling, lethargy, loss of appetite, and abnormal swimming behavior (Janda & Abbott, 2010). The bacterium can be transmitted through a variety of routes, including waterborne transmission, direct contact between infected and healthy fish, and through contaminated aquaculture equipment or materials.

2.5.4.3 *Flavobacterium columnare*

F. columnare is a Gram-negative, long rod-shaped bacterium belonging to the genus *Flavobacterium* within the family *Flavobacteriaceae*. Infected fish may exhibit varying degrees of external lesions such as erosion, ulcers, fin rot, lamellar gills, and holes in the head (Declercq et al., 2013). This bacterium can cause diseases known as "columnaris, cotton-wool, cotton-mouth, flexibacter, and mouth fungus disease ". The bacterium is transmissible via direct contact between infected and healthy fish, contaminated water, and contaminated aquaculture equipment or

materials. Stressors such as poor water quality, overcrowding, and temperature fluctuations can increase the susceptibility of fish to infection.

The intensive fish culture increases disease susceptibility because of poor water quality, high stocking densities, and rapid alterations in culture conditions. These factors have the potential to increase fish stress, thereby facilitating the spread of numerous infectious diseases. Therefore, the farmers usually apply antibiotics and other drugs and chemicals to control the disease outbreak. Nevertheless, the overuse of antibiotics and improper treatment can accelerate the spread of antibiotic-resistant bacteria in the environment and in fish products (Serwecińska, 2020). To reduce the use of drugs, numerous researchers are developing alternative preventative and therapeutic strategies applicable to Nile tilapia farming. The application of live biotherapeutic agents, such as probiotics, for controlling disease outbreaks in diverse commercial aquatic animals, is one method to reduce drug usage (Panigrahi et al., 2004).

2.6 The overview of the immune system in Nile tilapia

The immune system is the body's complex network that defends and protects itself from infection. In all vertebrates, including fish, the immune system is composed of non-specific or innate immune responses and specific or adaptive immune responses that are activated upon infection or invasion by pathogens (Castro & Tafalla, 2015).

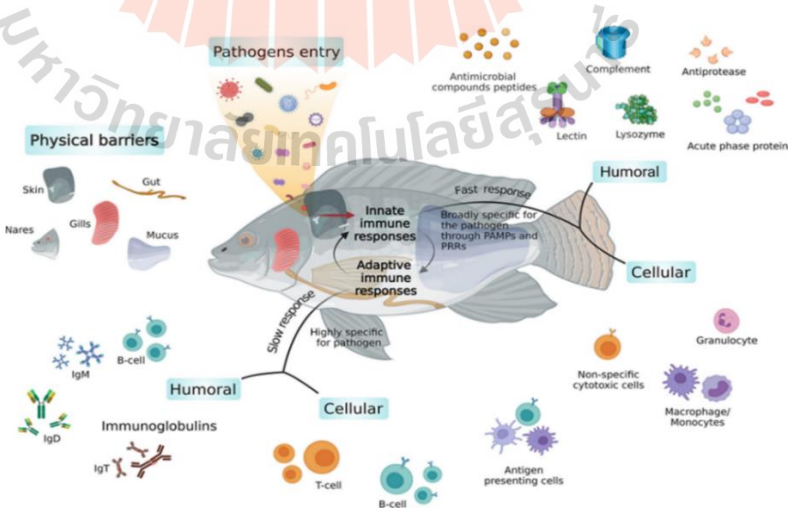


Figure 2.5 Overview of fish immunity.

Reference: Wang et al. (2023).

2.6.1 Innate immune response

The innate immune response is the first line of defense against pathogens found in vertebrates and invertebrates. It is a non-specific response with limited memory that is active against a variety of microbial antigens. The three compartments of innate immunity are epithelial barriers, cellular immune responses, and humoral immune responses.

2.6.1.1 Epithelial barriers

Physical barriers include the skin and scale that are the outer body coverings of many fish to prevent the entry of pathogens, and leakage of water, solutes, or nutrients.

Chemical barriers include mucus, sweat, tears, and saliva, which contain enzymes that kill pathogens. In fish, mucus is secreted by epithelial cells and covers most of the external surface, including the gills and skin. Many pathogens are easily trapped in the mucus layer.

Biological barriers are living organisms on the skin and in the urinary, reproductive, and gastrointestinal tracts that help protect the body.

2.6.1.2 Cellular innate immune responses

When pathogens can enter the fish's body, the cellular innate immune response is the subsequent mechanism for pathogen elimination. There are several cells that work together in this mechanism, including:

Phagocytosis is the cellular response to microbial invasion and/or tissue injury leading to the local accumulation of leukocytes and fluid, regulated by cytokines. In fish, macrophages are the professional phagocytic cells that ingest and kill bacteria through the production of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide and hydroxyl radicals in the oxygen-dependent bactericidal mechanism (Ellis, 1999). Phagocytes can ingest and kill invading pathogens, including bacteria and viruses.

Nonspecific cytotoxic cells (Natural killer cells; NK cells) are a type of cytotoxic lymphocyte critical to the innate immune system, providing rapid responses to virus-infected cells, cancer, and tumor formation. NK cells will secrete cytokines such as IFNγ and TNFα, which act on other immune cells like macrophages and dendritic cells to enhance the immune response (Subramani et al., 2016).

Inflammation is a common response of the innate immune system to infection and injury. The inflammatory reaction is controlled by several mediators, including cytokines, which can recruit other immune cells to the site of infection and enhance the overall immune response (Abdel-Latif et al., 2022).

2.6.1.3 Humoral innate immune responses

The complement system is a complex system composed of several plasma proteins that can be activated in response to infection in a sequential cascade. The complement system is a component of the immune system that enhances the ability of phagocytes to combat pathogens through antibody opsonization. It is innate and remains constant throughout an individual's lifespan.

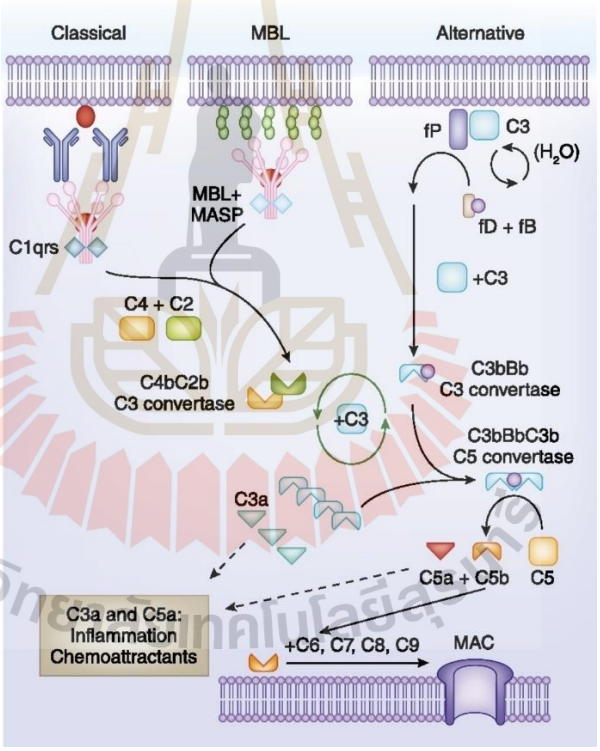


Figure 2.6 Scheme of the complement system

Reference: Mathern & Heeger (2015).

Figure 2.6 illustrates the three major pathways of complement activation: classical, MBL (mannose-binding lectin), and alternative. All three converge at the formation of C3 convertase, which cleaves C3 into C3a and C3b. In the classical

pathway, C1qrs is activated by antigen–antibody complexes, leading to the cleavage of C4 and C2 to form C4bC2b (C3 convertase). In the MBL pathway, MBL binds to mannose on microbial surfaces and activates MASP, which similarly forms C3 convertase. The alternative pathway is initiated by the spontaneous hydrolysis of C3 and further amplification involving factor B and factor D, forming C3bBb as the C3 convertase. These convertases cleave more C3, leading to the formation of C5 convertase (e.g., C4b2b3b or C3bBbC3b), which cleaves C5 into C5a and C5b. C5a and C3a act as chemoattractants to recruit immune cells and promote inflammation, while C5b initiates the assembly of the membrane attack complex (MAC) with C6–C9, forming pores in the pathogen membrane and causing cell lysis.

Lysozyme is an enzyme that can hydrolyze the bacterial cell wall peptidoglycan. Lysozyme is present in the leucocytes and other body fluids of Nile tilapia, including the head kidney, spleen, skin, gill, and gastrointestinal tract, protecting against bacterial infections.

Cytokines are small, secreted proteins that mediate and regulate immunity, inflammation, and hematopoiesis. They generally (although not always) act over short distances and have a short half-life, functioning at very low concentrations. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumor necrosis factors (TNF).

2.6.2 Adaptive immune response

The adaptive immune response is the second line of defense following the innate immune system. Unlike the innate response, which is immediate and non-specific, the adaptive immune system is specific to particular pathogens and has the ability to remember previous encounters, providing long-lasting immunity. The immune response upon subsequent exposure to the same antigen is faster and stronger than the initial response. Adaptive immunity is divided into two components, including.

2.6.2.1 Humoral immunity

Humoral immunity refers to the antibody-mediated immune response carried out by soluble proteins and other factors present in blood and extracellular fluids.

B lymphocytes (B cells) are responsible for the production of antibodies (immunoglobulins). When B cells encounter a pathogen, they differentiate

into plasma cells, which secrete antibodies specific to that pathogen. The antibodies produced by B cells neutralize the pathogen and facilitate its elimination.

Antigen-presenting cells (APCs) function primarily by processing antigenic material and presenting it on their surface to T cells, thereby initiating the adaptive immune response. They act as messengers between the innate and the adaptive immune systems.

2.6.2.2 Cell-mediated immune response

T lymphocytes (T cells) have diverse roles, including assisting B cells in antibody production (helper T cells), directly attacking infected cells (cytotoxic T cells), and regulating immune responses (regulatory T cells). T cells recognize antigens presented by antigen-presenting cells (APCs) like dendritic cells.

2.7 Probiotics

Probiotics are defined as beneficial microorganisms which, when administered in adequate amounts, confer positive effects on the overall health status of the host (Nayak, 2010). They have a variety of uses and multiple benefits for the host, including boosting economic growth, providing nutrients and enzymatic digestion including alginate lyases, amylases, and proteases, and absorption that improves metabolism and enhances growth, stimulating beneficial microflora in the GI tract (Balcázar et. al., 2006). In addition, probiotics can improve water quality and functional feed development (Verschuere et al., 2000; Rinkinen et al., 2003). They prevent the spread of infectious diseases by competing for adhesion sites with harmful bacteria to inhibit the growth of pathogenic microorganisms and enhance the host's innate immunity against pathogen infection (Pereira et al., 2022). Probiotic microorganisms applied in aquaculture have included specific strains of yeast, algae, and especially bacteria, such as *Bacillus* sp., *Lactococcus* sp., *Micrococcus* sp., *Lactobacillus* sp., and *Streptococcus* sp., all of which have been widely considered safe (Gheziel et al., 2019).

2.7.1 *Bacillus* sp.

Bacillus group belongs to the phylum Firmicutes, class Bacilli, order Bacillales, and family Bacillaceae (Ciccarelli et al., 2006; Wu et al., 2009). *Bacillus* sp. is a Gram-positive bacterium with a rod shape. It can grow in both aerobic and facultative

anaerobic environments. *Bacillus* sp. can produce endospores within its cells, with one spore formed per cell. It is commonly found in nature and can thrive in various environmental conditions due to the resilience of its endospores. *Bacillus* sp. can adapt to different surroundings, including those that are not conducive to its growth. Furthermore, it can grow well in various types of medium at normal temperatures and neutral pH levels, ability to produce a multitude of enzymes/digestive enzymes, such as proteases, lipases, and amylases (Moriarty, 1999; Hong et al., 2005). *B. subtilis* exhibits several beneficial effects on intestinal health and function. It has been shown to improve the villus-crypt architecture, which is a critical site for enterocyte proliferation and differentiation, thereby enhancing villus growth and overall nutrient absorption (Uni, 2006). Additionally, *B. subtilis* supports the recovery of gut epithelial barriers, reduces epithelial cell shedding, and improves intestinal absorptive efficiency (Al-Fataftah & Abdelqader, 2014). This probiotic also promotes the proliferation of other beneficial Gram-positive bacteria in the gut, such as *Lactobacillus*, though its role in promoting the growth of *Enterococcus faecalis* remains to be fully clarified (Kuebutornye et al., 2019). Unlike *B. subtilis*, *E. faecalis* has the unique ability to form biofilms and adhere to the gut mucosa, providing a protective barrier against harmful substances. This suggests that *B. subtilis* and *E. faecalis* may possess complementary functions that could synergistically enhance gut health (Belkaaloul et al., 2015). Currently, several members of the *Bacillus* species are available in aquaculture. Among others, *B. subtilis* is the most studied and widely used species (Wu et al., 2012; Keysami & Mohammadpour, 2013).

2.7.2 Mode of action of probiotics

Probiotics' beneficial effects come from several mechanisms. They can compete for dietary ingredients as growth substrates for pathogenic. They release digestive enzymes that help break down macronutrients and improve the host's ability to absorb nutrients. They can act by blocking pathogens due to competition for space and nutrients, by stimulating the immune system (without the presence of disease), and via the production of antimicrobial substances (such as lactic acid and bacteriocins). They can lead to death via pore formation, preventing the action of peptidoglycan transporters and, consequently, cell wall synthesis, and via damage to genetic material and protein synthesis. Probiotics, bacteriocins, and the host's nutritional improvement contribute to pathogens elimination and disease control (Pereira et al., 2022).

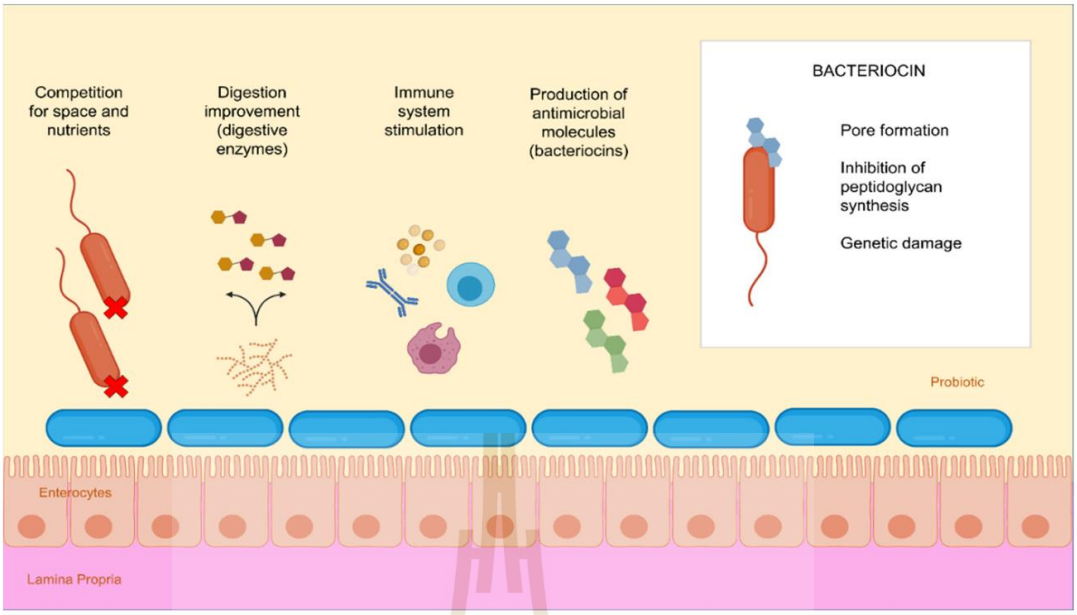


Figure 2.7 The mechanism of probiotics.
Reference: Pereira et al. (2022).

Table 2.1 Effects of dietary probiotic *Bacillus* sp. supplementation on fish health or against aquaculture pathogenic bacteria.

Probiotic	Pathogen or Challenge	Clinical Impact	Reference
B. subtilis	N/A	Improvement of immune response	Nakharuthai et al. (2023)
B. subtilis	*A. hydrophila*	Improvement of immune response and antimicrobial activity	Kuebutornye et al. (2020)
B. subtilis and B. licheniformis	N/A	Enhanced immunological parameters and improced growth and feed utilization	Elsabagh et al. (2018)

2.8 Vitamin C

Vitamin C, also named L-ascorbic acid, is an essential micronutrient for aquatic animals, with pleiotropic functions related to their ability to donate electrons. Dietary vitamin C supplementation is reported to enhance growth performance and feed utilization in aquatic animals. The improvement of growth performance by feeding vitamin C mostly appears to result from an increase in the feed efficiency of the diet. Vitamin C might be helpful for proper nutrient utilization because ascorbic acid plays an important role in certain aspects of protein metabolism (Agwu et al., 2023). Vitamin C probably is considered an essential nutrient due to its potent antioxidant properties and immunomodulatory effects in aquatic animals. Aquatic animals require vitamin C (ascorbic acid or ascorbate) to maintain optimal health. The immune responses, including macrophage infiltration, cell proliferation, natural killer cell activity, complement activity, lysozyme levels, phagocytic activity of leucocytes, development of cytokines, and antibody concentrations (Carr & Maggini, 2017; Gęgotek & Skrzydlewska, 2022). The ascorbate biosynthesis pathway starts with D-glucose. D-glucose is first converted into D-glucuronate. This intermediate is then reduced to L-gulonate, which undergoes further conversion to L-gulono lactone. The enzyme GULO (L-gulono lactone oxidase) catalyzes the final step, transforming L-gulono lactone into L-ascorbic acid, is conserved in all animal species. This pathway is crucial for synthesizing vitamin C, an essential nutrient for various metabolic processes in animals (Gad & Sirko, 2024). Nile tilapia cannot synthesize vitamin C from scratch because they lack the enzyme L-gluconolactone oxidase. As a result, they must obtain their vitamin C from external sources (Fracalossi et al., 2001). However, vitamin C can be easily degraded and very sensitive to various external factors, especially high temperature, oxygen, light (Wang et al., 2017). The structural formula is shown in Figure 2.8.

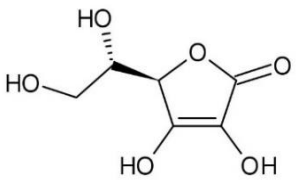


Figure 2.8 The structural formula of vitamin C.

2.8.1 Vitamin C–mediated antioxidant defense

Vitamin C (ascorbic acid) is a strong antioxidant capable of scavenging reactive oxygen species (Bae et al., 2012). Stressors disrupt the antioxidative balance due to the high generation of reactive oxygen metabolites (ROS), hydrogen peroxide, and peroxide radicals (Wang et al., 2017). These free radicals induce lipid peroxidation and lead to DNA and cell damage. Enzymatic and nonenzymatic antioxidants play crucial roles in eliminating excessive free radicals and ROS. The antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GSH-Px) and the nonenzymatic antioxidants, including Vitamin C (ascorbic acid), Vitamin E (tocopherols and tocotrienols), glutathione (GSH), carotenoids (ß-carotene, astaxanthin, lycopene) and ascorbate (ASC), have been proven to be significantly affected by oxidative stress that cause damage to the cell (Gęgotek & Skrzydlewska, 2022).

Oxygen (O_2) undergoes partial reduction, which results in the formation of the superoxide radical (O_2^-). According to the widely accepted model of enzymatic removal of ROS, as shown in figure 2.9 (Mechanism of antioxidant), SOD catalyzes the conversion of O_2^- into hydrogen peroxide (H_2O_2), thereby mitigating oxidative stress. Subsequently, CAT facilitates the decomposition of H_2O_2 into water and molecular oxygen, preventing the generation of hydroxyl radicals (OH•). GSH-Px utilizes GSH to reduce H_2O_2 and lipid hydroperoxides (LOOH) into non-toxic forms. In the absence of neutralization, H_2O_2 reacts with iron (Fe^{2+}), resulting in the production of highly reactive OH•, which induce severe oxidative damage. Lipid peroxidation is initiated when ROS interacts with lipids (LH), resulting in the formation of LOOH, which can subsequently damage nucleic acids and proteins. Malondialdehyde (MDA) is a highly reactive aldehyde and a widely utilized biomarker for assessing oxidative stress and lipid peroxidation. It is generated as a byproduct of the oxidative degradation of polyunsaturated fatty acids (PUFAs) induced by ROS (Pehlivan, 2017).

Non-enzymatic antioxidants, such as vitamin C (ascorbic acid, Asc), function as redox catalysts capable of reducing and thereby neutralizing ROS, including H_2O_2. Additionally, vitamin C converts lipid radicals (LOO•) into non-reactive forms and facilitates the regeneration of oxidized vitamin E (Toc•) to its active state (Toc). This antioxidant defense system is essential for maintaining cellular integrity,

mitigating oxidative stress, and promoting fish health in response to environmental stressors.

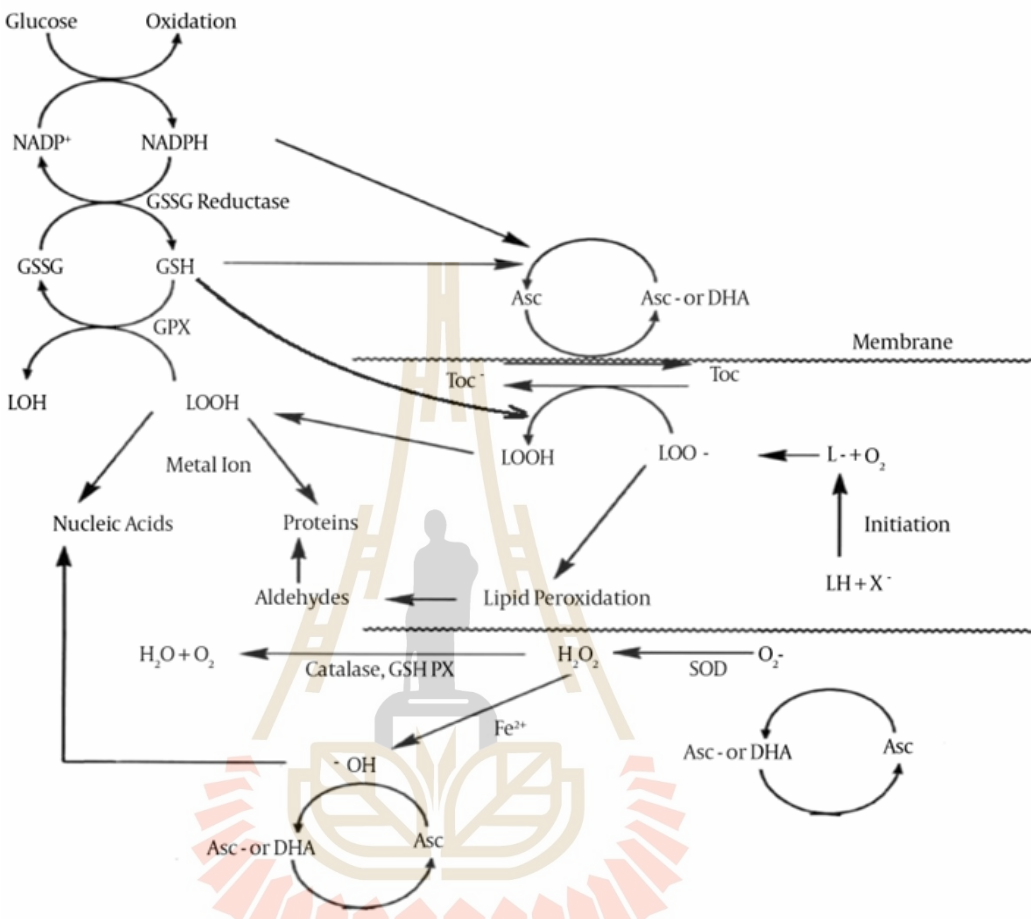


Figure 2.9 Relationships Among Radical Reactions, Fenton Reaction, Lipid Peroxidation and Antioxidant Properties of Vitamin C.

Reference: Akbari et al. (2017).

2.8.2 The role of vitamin C in aquatic animals

Vitamin C is an essential micronutrient for aquatic animals, particularly fish, as they lack the enzyme L-gulonolactone oxidase necessary for its synthesis. Therefore, they must obtain vitamin C through their diet. Vitamin C is crucial for collagen synthesis, which supports the structural integrity of tissues, bones, and skin. Adequate levels enhance growth performance and feed efficiency in various species, including Nile tilapia, red sea bream, and largemouth bass (Zhu et al., 2024). Vitamin

C deficiency in fish can result in a range of health problems, including skeletal deformities such as scoliosis, lordosis, and opercular abnormalities, internal and external hemorrhaging like bleeding gums and skin lesions; delayed wound healing due to impaired collagen formation, weakened immune response leading to higher vulnerability to infections; and growth retardation with poor feed conversion efficiency (Dosedělet al., 2021; Chandra et al., 2024). To prevent or rectify vitamin C deficiency, dietary supplementation with stabilized forms of ascorbic acid is essential. Moreover, the dosage of vitamin C should be tailored to each aquatic species, considering variables such as species type, developmental stage, and environmental factors to sustain optimal health and performance (Omoniyi et al., 2018). Maintaining adequate vitamin C levels is crucial for optimal health, growth, and disease resistance in aquaculture species. It may be possible to re-establish this pathway by integrating the *GULO* gene into probiotic *B. subtilis* using recombinant probiotic technology.

2.9 Genetic engineering

Genetic engineering is the process of genetic modification of organisms through transferring genetic material from one organism to another to change an organism's characteristics to the desired traits or produce novel traits in the recipient living organism. Genetic modification (GM) is the area of biotechnology that concerns itself with the manipulation of the genetic material in living organisms, enabling them to perform specific functions (Zhang et al., 2016). A genetically modified organism (GMO) is an organism (plant, animal, or microorganism) whose genetic material has been altered using gene or cell techniques of modern biotechnology (Ssekyewa & Muwanga, 2009). Recombinant proteins are proteins that are produced through genetic engineering techniques, by inserting a piece of DNA or gene of interest that codes for the desired protein into a host cell. A prokaryotic or eukaryotic expression host system, such as *Bacillus* bacteria, is used to make DNA in a laboratory. The cell's machinery can be used to produce the protein of interest. This is different from the traditional method of protein production, which involves isolating the protein from its natural sources, such as an animal or plant (Rai & Padh, 2001). Recombinant probiotics offer a promising approach to delivering the specific traits and functionalities of heterologous

proteins. Among these, the genus *Bacillus* has gained recognition as a reliable biofactory for producing heterologous proteins, serving both basic research and industrial applications (Yang et al., 2020; Nakharuthai et al., 2023). Employing *Bacillus* spp. presents numerous advantages, including their capacity for rapid and high-yield product synthesis, ease of genetic modification, and suitability for the expression and delivery of target genes.

In aquaculture, Nile tilapia cannot synthesize vitamin C from scratch because they lack the enzyme L-gulonolactone oxidase oxidase. Vitamins C are organic substances that are necessary for life because they are required in trace levels for appropriate growth, reproduction, and health. Vitamin C is one of the essential vitamins. Dietary vitamin C supplementation has been shown to improve fish development and immunological response. Therefore, recombinant probiotic expressing L-gulonolactone oxidase enzyme is an interesting alternative to address this issue.

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℃ for 30 minutes and stored at –80℃ 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 $p < 0.05$.

CHAPTER IV
RESULTS

4.1 Growth performance

The results of growth performance in Nile tilapia fed with experimental diets are presented in Table 4.1. After the 30-day feeding trial, no significant differences were observed in final weight, final length, weight gain, FCR, ADG, SGR, and RGR ($p \geq 0.05$). However, the PER in both the wild-type *B. subtilis* and *B. subtilis* expressing *GULO* groups was significantly different ($p < 0.05$) compared to the control and vitamin C groups. After 90 days of feeding trial, final weight, weight gain, FCR, ADG, SGR, and RGR of the *B. subtilis* expressing *GULO* group were significantly higher ($p < 0.05$) when compared to the control, vitamin C and wild-type *B. subtilis* groups.

4.2 The ascorbic acid levels in Nile tilapia serum were examined using High-performance liquid chromatography (HPLC)

The accumulation of serum ascorbic acid in Nile tilapia after 90 days of feeding with experimental diets showed that the vitamin C group had significantly higher levels than the *B. subtilis* expressing *GULO* group, both of which were significantly higher compared to the control group ($p < 0.05$).

Table 4.1 Effects of dietary supplementation with recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase on growth performance.

Diet	Initial weight (g)	Final weight (g)	Initial length (cm)	Final length (cm)	Weight gain (g)	FCR	ADG (g day^{-1})	SGR (% day^{-1})	RGR	PER
30 days										
CON	75.11±5.81	141.47±13.78	15.97±0.58	19.40±0.51	66.36±8.78	1.52±0.06	2.21±0.29	0.91±0.06	88.23±7.43	2.14±0.07^{a}
VC	73.16±2.39	138.80±6.24	15.71±0.19	19.19±0.25	65.64±4.90	1.55±0.02	2.19±0.16	0.93±0.05	89.74±6.13	2.15±0.02^{a}
BS	80.18±1.34	151.29±10.30	16.30±0.13	19.73±0.46	71.11±10.24	1.44±0.07	2.57±0.07	0.97±0.04	95.94±4.78	2.32±0.12^{b}
BS+GULO	78.56±3.50	158.64±3.55	16.21±0.45	19.99±0.09	80.09±0.50	1.41±0.01	2.67±0.02	1.02±0.03	102.09±4.56	2.37±0.02^{b}
90 days										
CON	75.11±5.81	268.75±7.19^{a}	15.97±0.58	25.15±1.74	195.72±0.73^{a}	1.60±0.07^{b}	2.17±0.01^{a}	0.63±0.03^{a}	268.99±22.79^{a}	2.23±0.09
VC	73.16±2.39	312.17±13.12^{b}	15.71±0.19	25.83±0.35	237.73±14.47^{b}	1.52±0.09^{ab}	2.64±0.16^{b}	0.69±0.03^{a}	319.60±24.91^{a}	2.20±0.13
BS	80.18±1.34	327.06±12.45^{b}	16.30±0.13	25.94±0.49	246.88±12.08^{b}	1.54±0.03^{ab}	2.74±0.13^{b}	0.68±0.02^{a}	307.93±14.78^{a}	2.17±0.05
BS+GULO	78.56±3.50	381.25±8.13^{c}	16.21±0.45	27.46±0.41	302.75±3.18^{c}	1.42±0.00^{a}	3.36±0.04^{c}	0.76±0.02^{b}	386.31±20.30^{b}	2.34±0.00

Means with a different superscript in each column differed significantly ($p < 0.05$). Values are means ± SD of ten replicates. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

Table 4.2 Accumulation of serum ascorbic acid in Nile tilapia fed with experimental diets for 90 days.

Diet	Ascorbic Acid Level (μg mL^{-1})
CON	5.88 ± 1.21^{a}
VC	20.29 ± 2.91^{c}
BS	5.92 ± 0.66^{a}
BS+GULO	10.43 ± 1.20^{b}

Significant differences among diet groups are denoted by different letters ($p < 0.05$). Values are means ± SD of three replicates. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

4.3 Examination of vitamin C by using qRT-PCR

The *GULO* mRNA expression in Nile tilapia fed a diet supplemented with recombinant *B. subtilis* for 30 and 90 days, the result showed that the level of *GULO* mRNA expression in the intestine was significantly higher at day 90 compared to day 30 ($p < 0.05$).

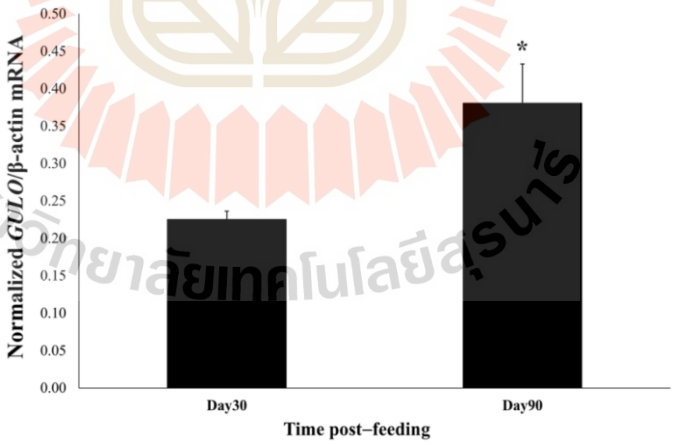
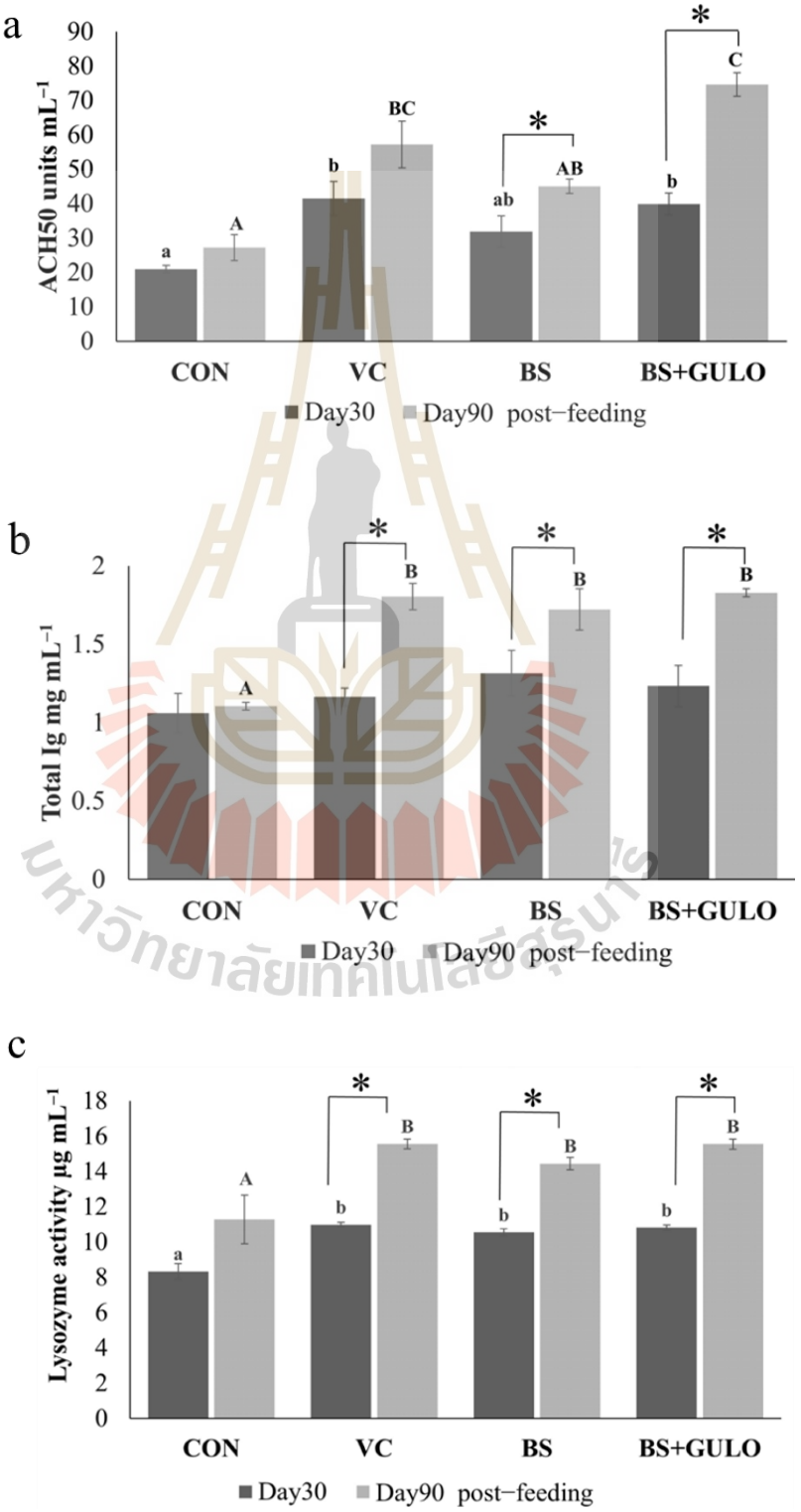


Figure 4.1 The intestinal *GULO* mRNA expression levels in Nile tilapia fed a diet supplemented with recombinant *B. subtilis* were compared between the 30- and 90-day feeding periods. The asterisk indicates significant statistical differences ($p < 0.05$).

4.4 Effects of dietary supplementation with recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase on innate immune responses in normal Nile tilapia



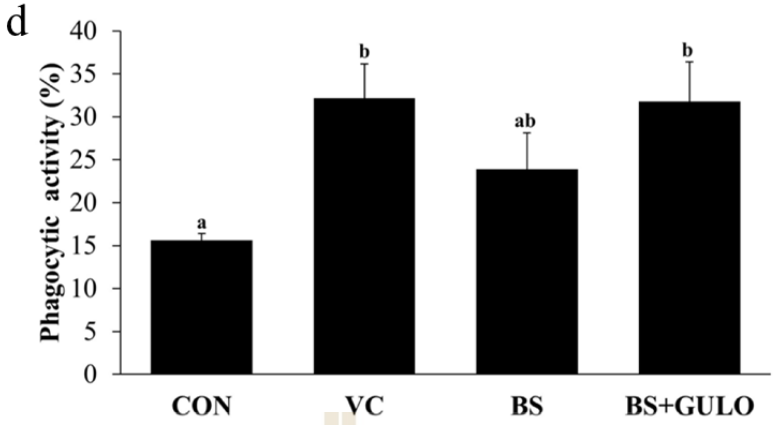


Figure 4.2 Immune parameters of Nile tilapia fed experimental diets for 30 days and 90 days of a feeding trial. ACH_{50} (a); total Ig (b); LZM (c). Bars with asterisks indicate significant differences between day 30 and day 90 of the feeding trial, whereas bars labeled with different lowercase letters denote significant differences for day 30 of the feeding trial, and bars labeled with uppercase letters indicate significant differences for day 90 of the feeding trial, respectively ($p < 0.05$). Phagocytic activity (%) of phagocytic cells in PBLs of Nile tilapia fed experimental diets for 90 days of the feeding trial (d). Bars with different letters indicate significant differences ($p < 0.05$). Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

As shown in figure 4.2. The innate immune responses after feeding. After 30 and 90 days of the trial, the ACH_{50} levels results showed that vitamin C and recombinant *B. subtilis* expressing *GULO* groups were significantly higher ($p < 0.05$) compared to the control group. Notably, only the wild-type and recombinant *B. subtilis* groups exhibited significant increases in ACH_{50} levels between day 30 and day 90 ($p < 0.05$) (Figure 4.2a).

Total immunoglobulin of fish fed all supplemented diets after 30 days of feeding were not significantly different ($p > 0.05$). However, by day 90, the vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* expressing *GULO* groups showed significant increased total Ig levels compared to the control group, with levels rising markedly between days 30 and 90 (Figure 4.2b).

In the case of lysozyme activity, significant increases were observed on both day 30 and day 90 in the vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* groups compared to the control, with levels increasing significantly over time ($p < 0.05$) (Figure 4.2c).

Furthermore, the phagocytic activity of Nile tilapia fed experimental diets for 90 days of the feeding trial was significantly elevated only in vitamin C and recombinant *B. subtilis* expressing *GULO* groups compared to the control group ($p < 0.05$) (Figure 4.2d).

4.5 Effects of dietary supplementation with recombinant probiotic *B. subtilis* expressing L-gulonolactone oxidase on antioxidant activity of Nile tilapia

After 90 days of the feeding trial, antioxidant activity in the serum of Nile tilapia was assessed. The results showed that serum TAC, SOD, GSH-Px, and CAT levels were significantly higher in the vitamin C and recombinant *B. subtilis* expressing *GULO* groups compared to the control group ($p < 0.05$). Serum MDA levels in vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* groups were significantly lower compared to the control group ($p < 0.05$).

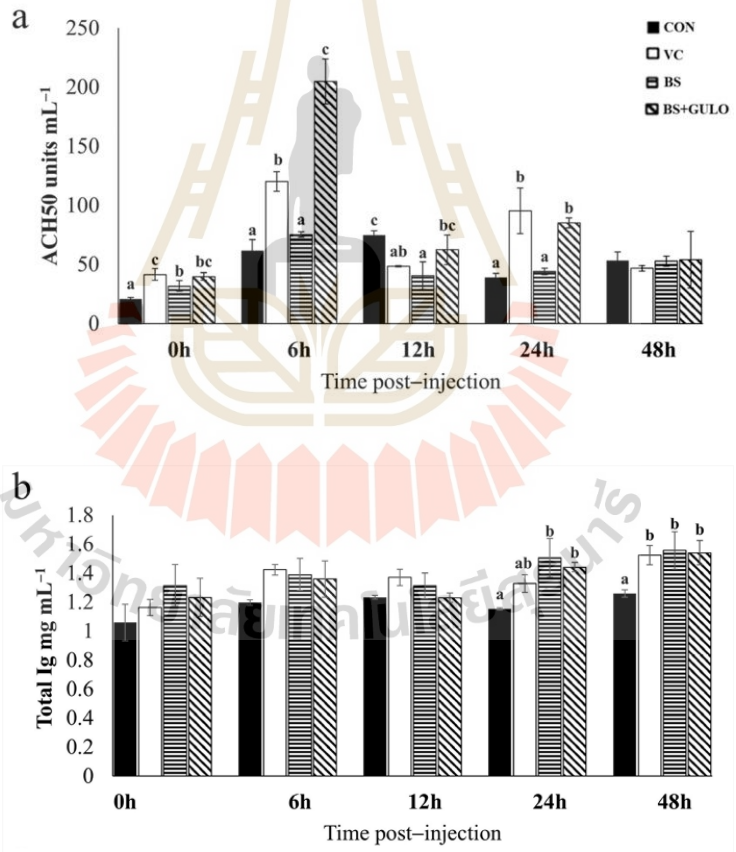
Table 4.3 Antioxidant parameters of Nile tilapia fed experimental diets for 90 days.

Diet	TAC μmol mL^{-1}	SOD U mL^{-1}	MDA nmol mL^{-1}	GSH-Px U mL^{-1}	CAT nmol min^{-1} mL^{-1}
CON	28.16±0.91^{a}	3.32±0.10^{a}	0.36±0.006^{c}	0.068±0.001^{a}	10.39±0.83^{a}
VC	38.85±1.32^{b}	4.34±0.34^{b}	0.23±0.025^{a}	0.121±0.013^{b}	31.27±2.59^{c}
BS	32.03±1.02^{ab}	4.04±0.12^{ab}	0.31±0.002^{b}	0.088±0.007^{ab}	17.29±1.32^{ab}
BS+GULO	36.01±1.78^{b}	4.69±0.32^{b}	0.28±0.006^{b}	0.117±0.016^{b}	20.34±4.16^{b}

Means with a different superscript in each column differed significantly from each other ($p< 0.05$). Values are means ± SD of three replicates. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

4.6 Immune parameter after *S. agalactiae* injection

Following the challenge test, ACH_{50} levels showed a rapid and significant upregulation at 6 hours post-injection in the groups supplemented with vitamin C and recombinant *B. subtilis* expressing *GULO* compared to the control group ($p < 0.05$) (Figure 4.3a). For total Ig, significant increases were observed at 24- and 48-hours post-injection in the wild-type *B. subtilis* and recombinant *B. subtilis* expressing *GULO* groups ($p < 0.05$), but these increases were not significantly different from those in the vitamin C group (Figure 4.3b). In the case of lysozyme activity, all experimental groups exhibited significantly higher levels compared to the control group at all measured time points ($p < 0.05$) (Figure 4.3c).



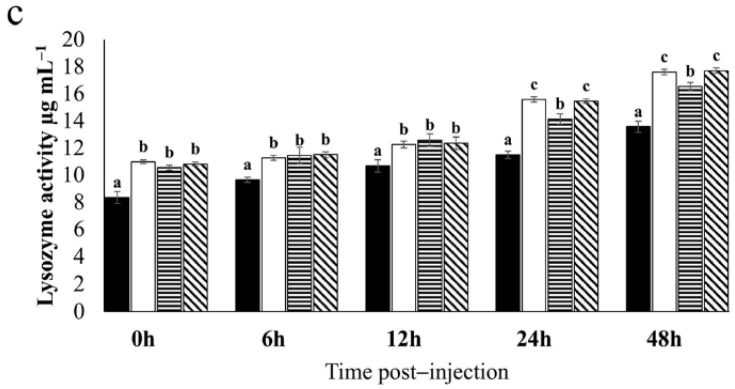


Figure 4.3 Immune parameters of Nile tilapia in response to *S. agalactiae* at different time points following the 30-day feeding trial. ACH_{50} (a); total Ig (b); LZM (c). Bars with different letters indicate significant differences ($p < 0.05$). Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

4.7 Pro-inflammatory gene expression after *S. agalactiae* injection

After the challenge test, the vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* expressing *GULO* groups exhibited rapid and significant upregulation of CC chemokine mRNA levels in only the spleen at 6 hours compared to the control group. However, CC chemokine mRNA expression in the recombinant *B. subtilis* expressing *GULO* group peaked at 12 hours in both the liver and spleen, followed by a decline at 48 hours across all experimental diet groups (Figure 4.4).

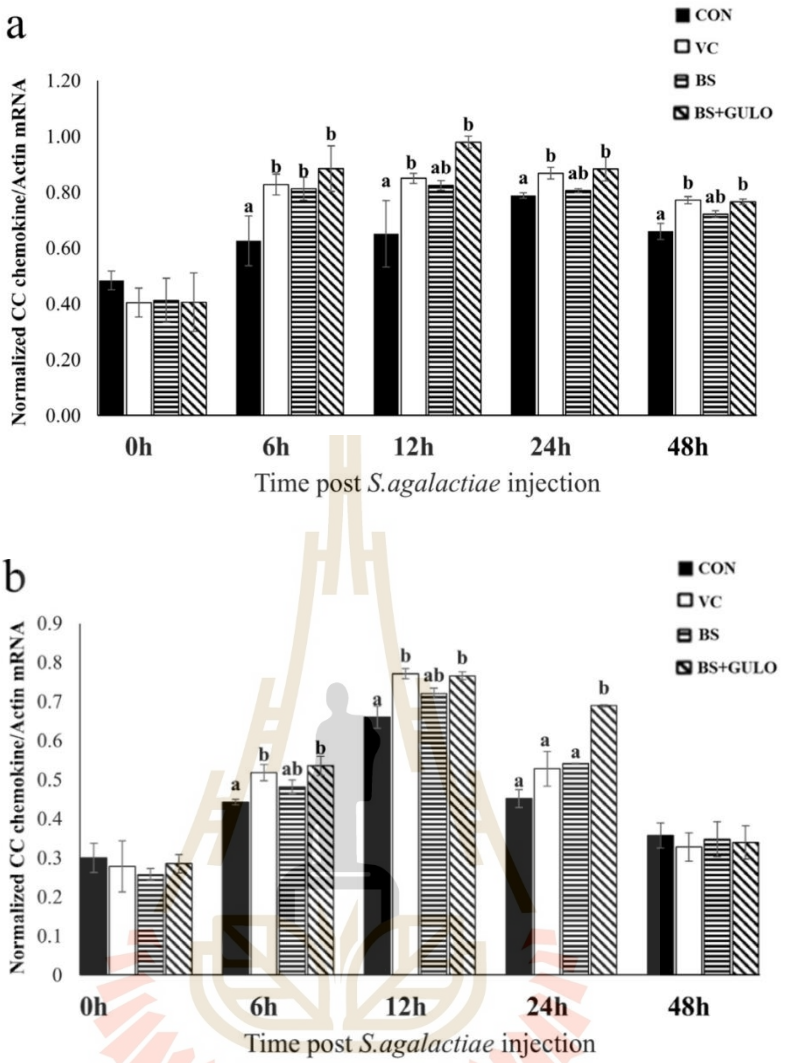


Figure 4.4 Quantitative real-time PCR analysis of CC chemokine expression in the spleen (a) and liver (b) of Nile tilapia in response to *S. agalactiae* at different time points following the 30-day feeding trial. The different letters on each bar indicate significant differences at $p < 0.05$. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

In the case of TNF**α**, significant mRNA upregulation was observed in the vitamin C, wild-type *B. subtilis*, and recombinant *B. subtilis* expressing *GULO* groups at 6 hours only in the liver compared to the control group, persisting until 48 hours post-injection. In the spleen, significant increases in TNF**α** mRNA levels were detected at 12 hours

post-injection only in the recombinant *B. subtilis* expressing *GULO* group compared to the control group, followed by gradual decreases at 24- and 48-hours (Figure 4.5).

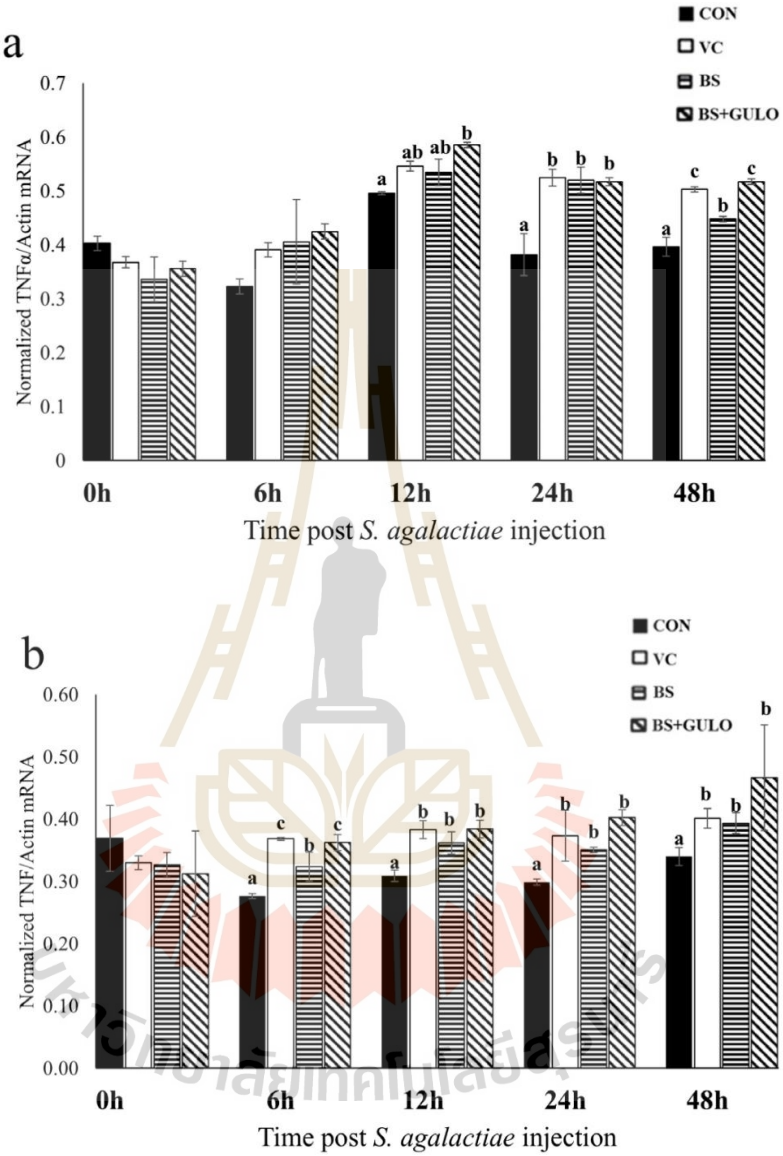


Figure 4.5 Quantitative real-time PCR analysis of tumor necrosis factor **α** expression in the spleen (a) and liver (b) of Nile tilapia in response to *S. agalactiae* at different time points following the 30-day feeding trial (n = 3). The different letters on each bar indicate significant differences at $p < 0.05$. Abbreviations: a basal diet (CON); a basal diet + vitamin C (VC); a basal diet + wild-type *B. subtilis* (BS); and a basal diet + recombinant *B. subtilis* (BS+GULO).

CHAPTER V
DISCUSSION

Advances in biotechnology have led to novel approaches to alleviate the vulnerability associated with the application of dietary vitamin C supplementation in aquaculture (Shanaka et al., 2021; Bremus et al., 2006; Jiménez-Fernández et al., 2014; Tian et al., 2022). In this study, a recombinant probiotic *B. subtilis* expressing *GULO* was successfully constructed and administered as a dietary supplement to Nile tilapia. To evaluate its potential as a growth promoter and immunostimulant, the expression of L-gulonolactone oxidase produced by probiotic *B. subtilis* as a dietary supplement. After 30 days of the feeding trial, the fish fed a diet supplemented with either wild-type or recombinant probiotic *B. subtilis* expressing *GULO* showed a significantly improved PER, correlating with an increase in fish weight gain. The significant difference may have been due to the presence of the protease-producing capacity of isolated *B. subtilis* to enhance the digestibility of protein content, as reported in our previous study (Nakharuthai et al., 2023). In contrast, no significant differences were observed in the final weight, weight gain, FCR, ADG, SGR, and RGR among the experimental groups. This may be due to the probiotic dose of approximately 10^8 CFU/mL in the Nile tilapia diet, which might not have been sufficient to enhance growth performance within the 30-day feeding period. Similarly, consistent with the findings of Han et al. (2015) and Zhao et al. (2016), the probiotic-supplemented groups in their studies also showed no significant differences compared to the control group after 30 days of probiotic feeding. In addition, a study by Panase et al. (2023) reported that supplementation with *B. subtilis* at a concentration of 1–5 × 10^9 CFU/g for 56 days in Nile tilapia resulted in no significant differences compared to the control group. In contrast, Liu et al. (2021) observed significant improvements in growth performance following 42 days of feeding with *B. subtilis* and *E. faecalis* at a higher dose of 2 × 10^{11} CFU/g in Nile tilapia. The possible reasons for the observed differences in growth performance may be attributed to several factors, including variations in probiotic activity, interactions with existing gut

microbiota, the amount and composition of probiotic strains or species, their viability, as well as differences in feed formulation, feeding duration, probiotic dosage, and overall experimental conditions.

Interestingly, after 90 days of the feeding trial, the fish fed a diet supplemented with recombinant *B. subtilis* expressing *GULO* showed the highest improvements in final weight, weight gain, SGR, ADG, and RGR. This phenomenon may be attributed to the incorporation function of probiotics and vitamin C in enhancing the digestibility and absorption of nutrients within the fish's body. Numerous studies have reported that dietary supplementation with *B. subtilis* can enhance intestinal digestive enzyme activities, thereby leading to an improvement in the growth performance of the fish (Won rt al., 2020). Meanwhile, several pieces of evidence have supported the positive impact of vitamin C on nutrient utilization within metabolic processes and protein synthesis, resulting in a beneficial influence on the growth performance of aquatic animals (Dawood & Koshio, 2018). Nevertheless, the effect of dietary supplementation with vitamin C can vary based on fish species, age, and size; the form of vitamin C; and differences in experimental conditions, as well as the health status and stress levels of the fish (Drouin et al., 2011; Wang et al., 2017).

In this study, HPLC and qRT-PCR analyses were conducted to validate and confirm the role of L-gulonolactone oxidase, produced by probiotic *B. subtilis*, in the biosynthesis of vitamin C. This was supported by the significant increase in serum vitamin C of fish fed recombinant *B. subtilis* expressing *GULO* for 90 days compared to the control group. The increase in serum ascorbic acid levels in fish fed recombinant *B. subtilis* expressing *GULO* corresponded to their growth performance results. This result aligns with several previous studies that have documented the advantageous effects of using both *B. subtilis* and vitamin C as supplements in aquafeed, aiming to improve the overall growth of fish (Chen et al., 2004; Nayak et al., 2007; Lim et al., 2010; Dawood & Koshio, 2018; Zafar & Khan, 2020; Xu et al., 2022).

Beyond their role in enhancing growth performance, vitamin C and probiotics are recognized as immunomodulators that elicit immune responses in fish. According to our previous study (Nakharuthai et al., 2023), the probiotic *B. subtilis* isolated from the intestine of Nile tilapia demonstrates substantial tolerance to the hostile environment of the gastrointestinal (GI) tract, thus increasing its chances of survival and colonization

on the internal surfaces of the GI tract. Like other probiotics, the presence of probiotic *B. subtilis* in the GI tract could activate the immune system of Nile tilapia through signaling by toll-like receptors (TLRs) on intestinal epithelial cells and antigen-presenting cells (APCs) (Fong t al., 2016). Meanwhile, the concentration of vitamin C in leukocytes and tissues has been reported to stimulate the activity of innate immune responses (Verlhac et al., 1996).

In this study, fish fed a diet supplemented with vitamin C, wild-type *B. subtilis*, and *B. subtilis* expressing *GULO* showed a significant increase in LZM following 30 and 90 days of the feeding trial. The results of LZM activity confirmed the vital role of probiotic *B. subtilis* and vitamin C in enhancing innate immunity through the mechanism of this enzyme. Similarly, several studies have stated that the supplementation with both vitamin C and probiotic *B. subtilis* in fish diets could stimulate LZM activity by activating myeloid cells (macrophages, monocytes, and neutrophils) (Adorian et al., 2019; Zafar & Khan, 2020; Medagoda et al., 2023). In fish, LZM has emerged as a powerful innate defense that exerts antimicrobial activity directly against Gram-positive bacteria or indirectly against Gram-negative bacteria after disrupting the bacterial cell wall through the action of complements and other enzymes.

Regarding total Ig, a significant difference was detected only at day 90, suggesting that both vitamin C and probiotics may require prolonged administration to induce a measurable adaptive immune response. The lack of significant differences on day 30 may be attributed to the time needed for these interventions to optimize immunomodulation (Magnadottir, 2010). Interestingly, total Ig and LZM levels at day 30 were significantly higher than those at day 90 in the groups of fish fed a diet supplemented with vitamin C, *B. subtilis* expressing *GULO*, and the wild-type *B. subtilis*. This result demonstrated the immunostimulatory function of vitamin C and probiotic *B. subtilis* to stimulate the total Ig and LZM in Nile tilapia, which could enhance immune response. This improvement is attributed to factors such as the stabilization of gut microbiota, cumulative probiotic effects, physiological adaptation, and enhanced immunological responses over time (Haque et al., 2021).

In the case of ACH_{50}, a significant difference in ACH_{50} levels between day 30 and day 90 of the feeding trial was observed only in the groups of fish fed, a diet

supplemented with *B. subtilis* expressing *GULO* and the wild-type *B. subtilis*. This finding indicates that the continuous administration of *B. subtilis* could enhance the ACH_{50} activity of Nile tilapia, consistent with evidence from previous studies (Aly et al., 2008; Liu et al., 2012). The continuous administration of probiotics led to an increase in complement component 3 (C3) through the stimulation of cytokines following recognition by TLRs, as described above (Chen et al., 2010; Panase et al., 2023). Moreover, C3 is a central component in three complement pathways (classical, alternative, and lectin pathway). It interacts with other proteins in the complement cascade to form the membrane attack complex (MAC), ultimately killing pathogens. In addition, previous studies have demonstrated that supplementation with an appropriate amount of vitamin C can enhance complement activity in fish (Li & Lovell, 1985; Hardie et al., 1991; Ai et al., 2004).

In addition to the function described above, LZM and complement components (C1q, C3b, and Bb) also act as an innate opsonin that binds bacteria to accelerate and facilitate phagocytic activity in fish. This is evident in our phagocytic activity results, where fish fed with dietary supplementation of vitamin C and recombinant probiotic *B. subtilis* expressing *GULO* exhibited significantly higher phagocytic activity. In general, phagocytes generate ROS as a key component of their pathogen-killing mechanism. Consequently, an enhanced antioxidant system protects these cells from self-inflicted oxidative damage, thereby maintaining their effectiveness in eliminating infections.

In teleosts, SOD, MDA, GSH-Px, and CAT are the main antioxidant enzymes that protect fish from oxidative stress damage caused by free radicals. In this study, dietary supplementation with vitamin C and recombinant *B. subtilis* expressing *GULO* led to higher contents of TAC, SOD, CAT, and GSH-Px and lower levels of MDA in the serum of Nile tilapia compared to control group. In the wild-type *B. subtilis* group, a significant decrease was only observed in MDA levels, indicating decreased lipid peroxidation and a reduction in oxidative damage to cellular membranes (Garcia et al., 2020). These findings could primarily be attributed to the supplementation of vitamin C in the fish diet rather than probiotics. The enhancement of antioxidant enzymes possibly occurs because of vitamin C's ability to readily donate electrons, aligning with previous findings in several teleost species (Siwicki & Studnicka, 1987; Dawood wt al., 2020; Xu et al., 2022; Medagoda et al., 2023). In an intensive culture system, Nile tilapia

frequently encounters periods of stress at any time. The stress condition can cause an imbalance between reactive oxygen species and endogenous antioxidants in cells and tissues, potentially leading to cell and tissue damage. Hence, the continuous supply of exogenous antioxidants, such as vitamin C supplementation in fish diets, is necessary to counteract the adverse effects of oxidative stress.

In Thailand, *S. agalactiae* has emerged as a major pathogenic bacterium, causing severe economic losses in tilapia farming (Suanyuk et al., 2008). To investigate the effect of dietary supplementation with recombinant probiotic *B. subtilis* expressing *GULO* on immune response following a challenge with *S. agalactiae*, Nile tilapia were intraperitoneally injected with this bacterium after a 30-day feeding trial. The results showed that the ACH_{50} level rapidly increased at 6 hours post-injection in fish fed vitamin C and recombinant probiotics compared to the control group. Meanwhile, total Ig levels were subsequently elevated at 24 and 48 hours post-injection in the same groups.

The rapid increase in ACH_{50} indicates its ability to attenuate/limit the spread of invading pathogens, a consequence of activation by either recombinant probiotic *B. subtilis* or vitamin C. The elevation of total Ig at 24 and 48 hours post-injection could result from the opsonization facilitated by immune genes such as cytokines, phagocytes, and complement components, leading to the activation of the phase of adaptive immune responses. In addition, a significant increase in total Ig levels after the injection of *S. agalactiae* suggests a more robust humoral immune response, with increased production of antibodies that play a vital role in pathogen recognition and neutralization (Chan et al., 2023). In the challenge test, LZM showed a significant elevation in levels at all time points in fish fed with vitamin C, wild-type, and recombinant probiotics compared to the control group. These results reflect the enhanced ability of lysozyme, due to vitamin C and probiotic *B. subtilis*, to eliminate *S. agalactiae* in Nile tilapia. Probiotic *B. subtilis* is recognized for its role in regulating the fish gut's immune response, while vitamin C is notable for reinforcing the immune response and disease resistance, probably attributable to its antioxidant and immunostimulatory properties (Nayak et al., 2010; Santos-Sánchez et al., 2019).

Under normal conditions, the continuous application of probiotic *B. subtilis* in fish feed influences the TLR4 triggering, which serves as the pattern-recognition receptor that initiates the activation of the immune cascade. Additionally, dietary

supplementation with vitamin C not only modulates the production of fish immune cells, contributing to maintaining immune homeostasis, but also plays a role in disease resistance by activating the expression of inflammatory cytokines under stress conditions (Carr & Maggini, 2017). In the challenge test, mRNA levels of pro-inflammatory cytokines, including CC chemokine and TNFα, in response to *S. agalactiae*, were analyzed among the experimental fish after a 30-day feeding trial using qRT-PCR.

The results indicate a significant and rapid increase in CC chemokine mRNA expression at 6 hours post *S. agalactiae* injection in both the liver and spleen of fish fed diets supplemented with vitamin C and recombinant probiotic *B. subtilis* expressing *GULO*, compared to the control group. A similar pattern was observed for TNFα in the liver of fish fed dietary supplementation with vitamin C, wild-type *B. subtilis*, and recombinant probiotic *B. subtilis* expressing *GULO*. These findings suggest that both vitamin C and probiotic *B. subtilis* may potentially contribute to enhancing the production and chemoattractant activity of CC chemokine and TNFα.

Moreover, only the recombinant probiotic *B. subtilis* expressing *GULO* group exhibited a significant increase in TNFα expression in the spleen at 12 h. The rapid upregulation of inflammatory gene expression facilitated the recruitment of white blood cells to the site of infection during the initial stage (Nakharuthai & Srisapoome, 2020). Furthermore, our previous in vitro study confirmed that the potential probiotic *B. subtilis* used in this study exhibits antibacterial activity and effectively antagonizes pathogenic *S. agalactiae* (Yin et al., 2022). Together, these findings suggest that the enhanced antagonistic activity against *S. agalactiae* in recombinant *B. subtilis* may result from the combined effect of *B. subtilis* and vitamin C, modulating both innate and adaptive immunity in Nile tilapia.

CHAPTER VI
CONCLUSION

In conclusion, based on the overall results, dietary supplementation with recombinant probiotic *B. subtilis* expressing *GULO* could be considered for prophylactic and therapeutic applications, owing to the combined effects of vitamin C and probiotic *B. subtilis*. In prophylactic roles, supplementation with recombinant *B. subtilis* expressing *GULO* in normal fish resulted in improvements in growth performance, antioxidant activity, and immune responses. In addition, it may demonstrate therapeutic potential in the early stages of *S. agalactiae* infections due to its ability to enhance immune responses and pro-inflammatory cytokine production and to exhibit antagonistic properties against *S. agalactiae*. These could help to reduce the prevalence of the disease in Nile tilapia, particularly in the intensive aquaculture industry, which often relies on the application of drugs and chemicals. However, this study was primarily conducted under strictly controlled laboratory conditions. Further research is needed to assess long-term effects, optimize the dosage under field conditions, and improve signal peptides for enhanced recombinant *B. subtilis* protein expression.

REFERENCES

Abdallah, E. S. H., Metwally, W. G. M., Abdel-Rahman, M. A. M., Albano, M., & Mahmoud, M. M. (2024). *Streptococcus agalactiae* Infection in Nile Tilapia (*Oreochromis niloticus*): A Review. **Biology**, 13(11), 914.

Abdel-Latif, H. M., Shukry, M., & Abd-Elaziz, R. A. (2022). Clinico-pathological findings and expression of inflammatory cytokines, apoptosis, and oxidative stress-related genes draw mechanistic insights in Nile tilapia reared under ammonia-N exposure and *Aeromonas hydrophila* challenge. **Fish & Shellfish Immunology**, 127, 1-12.

Abo-Al-Ela, H. G., El-Nahas, A. F., Mahmoud, S., & Ibrahim, E. M. (2017). Vitamin C modulates the immunotoxic effect of 17α-methyltestosterone in Nile tilapia. **Biochemistry**, 56(14), 2042-2050.

Adorian, T. J., Jamali, H., Farsani, H. G., Darvishi, P., Hasanpour, S., Bagheri, T., & Roozbehfar, R. (2019). Effects of probiotic bacteria *Bacillus* on growth performance, digestive enzyme activity, and hematological parameters of Asian sea bass, *Lates calcarifer* (Bloch). **Probiotics and antimicrobial proteins**, 11, 248-255.

Agwu, E., Ezihe, C., & Kaigama, G., (2023). Antioxidant roles/functions of ascorbic acid (vitamin C). **In Ascorbic Acid-Biochemistry and Functions**. IntechOpen

Ai, Q., Mai, K., Zhang, C., Xu, W., Duan, Q., Tan, B., & Liufu, Z. (2004). Effects of dietary vitamin C on growth and immune response of Japanese seabass, *Lateolabrax japonicus*. **Aquaculture**, 242(1-4), 489-500.

Akbari, A. (2016). An overview of the characteristics and function of vitamin C in various tissues: relying on its antioxidant function. **Zahedan Journal of Research in Medical Sciences**, 18(11), e4037.

Al-Fataftah, A. R., & Abdelqader, A. (2014). Effects of dietary *Bacillus subtilis* on heat-stressed broilers performance, intestinal morphology and microflora composition. **Animal feed science and technology**, 198, 279-285.

Aly, S. M., Ahmed, Y. A. G., Ghareeb, A. A. A., & Mohamed, M. F. (2008). Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. **Fish & shellfish immunology**, 25(1-2), 128-136.

Amal, M. N. A., & Zamri-Saad, M. (2011). Streptococcosis in tilapia (*Oreochromis niloticus*): a review. **Pertanika Journal of Tropical Agricultural Science**, *34*, 195–206.

Assefa, A., & Abunna, F. (2018). Maintenance of fish health in aquaculture: review of epidemiological approaches for prevention and control of infectious disease of fish. **Veterinary medicine international**, *2018*(1), 5432497.

Association of Official Analytical Chemists (AOAC). (1990). **Official methods of analysis of the association of official analytical chemists**. (14th ed.). AOAC: Arlington, VA, USA.

Bae, J. Y., Park, G.H., Yoo, K. Y., Lee, J. Y., Kim, D. J., & Bai, S.C. (2012). Re-evaluation of the optimum dietary vitamin C requirement in juvenile eel, *Anguilla japonica* by using L-ascorbyl-2-monophosphate. **Asian-Australasian journal of animal sciences**, 25(1), 98.

Balcázar, J. L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., & Múzquiz, J. L. (2006). The role of probiotics in aquaculture. **Veterinary microbiology**, 114(3-4), 173-186.

Barros, M. M., Falcon, D. R., de Oliveira Orsi, R., Pezzato, L. E., Fernandes Jr, A. C., Guimarães, I. G., ... & Sartori, M. M. P. (2014). Non-specific immune parameters and physiological response of Nile tilapia fed β-glucan and vitamin C for different periods and submitted to stress and bacterial challenge. **Fish & Shellfish Immunology**, 39(2), 188-195.

Belkaaloul, K., Haertlé, T., Chobert, J. M., Merah, R., Taibi, K., El Hachemi, H. S., ... & Kheroua, O. (2015). Protective effect of *Enterococcus faecalis* DAPTO 512 on the intestinal tract and gut mucosa: milk allergy application. **Beneficial Microbes**, 6(5), 679-686.

Belton, B., Turongruang, D., Bhujel, R., & Little, D. C. (2009). The history, status, and future prospects of monosex tilapia culture in Thailand. **Aquaculture Asia**, 14(2), 16-19.

Bremus, C., Herrmann, U., Bringer-Meyer, S., & Sahm, H. (2006). The use of microorganisms in L-ascorbic acid production. **Journal of biotechnology**, 124(1), 196-205.

Carr, A. C., & Maggini, S., (2017). Vitamin C and immune function. **Nutrients**, 9(11), 1211.

Castro, R., & Tafalla, C. (2015). Overview of fish immunity. **In Mucosal health in Aquaculture**, 3-54.

Caxico Vieira, C. A. S., Vieira, J. S., Bastos, M. S., Zancanela, V., Barbosa, L. T., Gasparino, E., & Del Vesco, A. P. (2018). Expression of genes related to antioxidant activity in Nile tilapia kept under salinity stress and fed diets containing different levels of vitamin C. **Journal of toxicology and environmental health, part A**, 81(1-3), 20-30.

Chan, J., Carmen, L. C. P., Lee, S. Q., & Prabakaran, M. (2023). Identification and characterization of immunoglobulin tau (IgT) in Asian Seabass (*Lates calcarifer*) and mucosal immune response to nervous necrosis virus. **Frontiers in Immunology**, 14, 1146387.

Chandra, G., Saini, V. P., Kumar, S., & Fopp‐Bayat, D. (2024). Deformities in fish: A barrier for responsible aquaculture and sustainable fisheries. **Reviews in Aquaculture**, 16(2), 872-891.

Chen, M., Daha, M. R., & Kallenberg, C. G. (2010). The complement system in systemic autoimmune disease. **Journal of autoimmunity**, 34(3), J276-J286.

Chen, M., Li, L. P., Wang, R., Liang, W. W., Huang, Y., Li, J., ... & Gan, X. (2012). PCR detection and PFGE genotype analyses of streptococcal clinical isolates from tilapia in China. **Veterinary microbiology**, 159(3-4), 526-530.

Chen, R., Lochmann, R., Goodwin, A., Praveen, K., Dabrowski, K., & Lee, K. J. (2004). Effects of dietary vitamins C and E on alternative complement activity, hematology, tissue composition, vitamin concentrations and response to heat stress in juvenile golden shiner (*Notemigonus crysoleucas*). **Aquaculture**, 242(1-4), 553-569.

Ciccarelli, F. D., Doerks, T., Von Mering, C., Creevey, C. J., Snel, B., & Bork, P. (2006). Toward automatic reconstruction of a highly resolved tree of life. **Science**, 311(5765), 1283-1287.

Comunian, T. A., Abbaspourrad, A., Favaro-Trindade, C. S., & Weitz, D. A. (2014). Fabrication of solid lipid microcapsules containing ascorbic acid using a microfluidic technique. Food **chemistry**, 152, 271-275.

Crawford, T.C. (1982). Synthesis of L-ascorbic acid. **ACS Publications**, 200, 1–36.

Cui, W., Han, L., Suo, F., Liu, Z., Zhou, L., & Zhou, Z. (2018). Exploitation of *Bacillus subtilis* as a robust workhorse for production of heterologous proteins and beyond. **World Journal of Microbiology and Biotechnology**, 34, 1-19.

Cutting, S. M., Hong, H. A., Baccigalupi, L., & Ricca, E. (2009). Oral vaccine delivery by recombinant spore probiotics. **International reviews of immunology**, 28(6), 487-505.

Dangwetngam, M., Suanyuk, N., Kong, F., & Phromkunthong, W. (2016). Serotype distribution and antimicrobial susceptibilities of *Streptococcus agalactiae* isolated from infected cultured tilapia (*Oreochromis niloticus*) in Thailand: Nine-year perspective. **Journal of Medical Microbiology**, 65(3), 247-254.

Dawood, M. A., & Koshio, S. (2018). Vitamin C supplementation to optimize growth, health and stress resistance in aquatic animals. **Reviews in Aquaculture**, 10(2), 334-350.

Dawood, M. A., Zommara, M., Eweedah, N. M., Helal, A. I., & Aboel-Darag, M. A. (2020). The potential role of nano-selenium and vitamin C on the performances of Nile tilapia (*Oreochromis niloticus*). **Environmental Science and Pollution Research**, 27, 9843-9852.

Debnath, S. C., McMurtrie, J., Temperton, B., Delamare-Deboutteville, J., Mohan, C. V., & Tyler, C. R. (2023). Tilapia aquaculture, emerging diseases, and the roles of the skin microbiomes in health and disease. **Aquaculture International**, 31(5), 2945-2976.

Declercq, A.M., Haesebrouck, F., Van den Broeck, W., Bossier, P., & Decostere, A. (2013). Columnaris disease in fish: A review with emphasis on bacterium-host interactions. **Veterinary Research**, 44, 27.

Doseděl, M., Jirkovský, E., Macáková, K., Krčmová, L. K., Javorská, L., Pourová, J., ... & Oemonom. (2021). Vitamin C—sources, physiological role, kinetics, deficiency, use, toxicity, and determination. **Nutrients**, 13(2), 615.

Drouin, G., Godin, J. R., & Pagé, B. (2011). The genetics of vitamin C loss in vertebrates. **Current genomics**, 12(5), 371-378.

Ellis, A. E. (1999). Immunity to bacteria in fish. **Fish & shellfish immunology**, 9(4), 291-308.

El-Noby, G., Hassanin, M., El-Hady, M., & Aboshabana, S. (2021). Streptococcus: a review article on an emerging pathogen of farmed fishes. **Egyptian Journal of Aquatic Biology and Fisheries**, 25(1), 123-139.

Elsabagh, M., Mohamed, R., Moustafa, E. M., Hamza, A., Farrag, F., Decamp, O., ... & Eltholth, M. (2018). Assessing the impact of *Bacillus* strains mixture probiotic on water quality, growth performance, blood profile and intestinal morphology of Nile tilapia, *Oreochromis niloticus*. **Aquaculture nutrition**, 24(6), 1613-1622.

El-Sherif, M. S., & El-Feky, A. M. I. (2009). Performance of Nile tilapia (*Oreochromis niloticus*) fingerlings. I. Effect of pH. **International journal of Agriculture and Biology**, 11(3), 297-300.

Eo, J., & Lee, K. J. (2008). Effect of dietary ascorbic acid on growth and non-specific immune responses of tiger puffer, *Takifugu rubripes*. **Fish & shellfish immunology**, 25(5), 611-616.

FAO, (2021). **Cultured Aquatic Species Information Programme: *Oreochromis niloticus* (Linnaeus, 1758)**. Food Agric. Organ, United Nations.

Fisheries Development Policy and Planning Division Department of Fisheries. (2022). **Fisheries statistics of Thailand 2021**. Retrieved from https://www4.fisheries.go.th/local/file_document/20221file.pdf.

Fong, F. L. Y., Shah, N. P., Kirjavainen, P., & El-Nezami, H. (2016). Mechanism of action of probiotic bacteria on intestinal and systemic immunities and antigen-presenting cells. **International Reviews of Immunology**, 35(3), 179-188.

Food and Agriculture Organization (FAO). (2022). **The State of World Fisheries and Aquaculture 2022**. Retrieved from https://openknowledge.fao.org/server/api/core/bitstreams/a2090042-8cda-4f35-9881-16f6302ce757/content.

Food and Agriculture Organization (FAO). (2023). **Demand for tilapia resumes growth despite higher prices**. Retrieved from https://www.fao.org/in-action/globefish/news-events/news/news-detail/Demand-for-tilapia-resumes-growth-despite-higher-prices-/en.

Food and Agriculture Organization of the United Nations Rome. (2022). **Thailand GLOBEFISH Market Profile 2018**. Retrieved from https://www.fao.org/3/cb5866en/cb5866en.pdf.

Fracalossi, D. M., Allen, M. E., Yuyama, L. K., & Oftedal, O. T. (2001). Ascorbic acid biosynthesis in Amazonian fishes. **Aquaculture**, 192(2-4), 321-332.

Gad, A. A. M., & Sirko, A. (2024). L-gulono-**γ**-lactone Oxidase, the Key Enzyme for L-Ascorbic Acid Biosynthesis. **Current Issues in Molecular Biology**, 46(10), 11057-11074.

Garcia, D., Lima, D., da Silva, D. G. H., & de Almeida, E. A. (2020). Decreased malondialdehyde levels in fish (*Astyanax altiparanae*) exposed to diesel: Evidence of metabolism by aldehyde dehydrogenase in the liver and excretion in water. **Ecotoxicology and Environmental Safety**, 190, 110107.

Gasco, L., Gai, F., Maricchiolo, G., Genovese, L., Ragonese, S., Bottari, T., ... & Caruso, G. (2018). Supplementation of vitamins, minerals, enzymes and antioxidants in fish feeds. **Feeds for the Aquaculture Sector: Current Situation and Alternative Sources**, 63-103.

G**ę**gotek, A., & Skrzydlewska, E., (2022). Antioxidative and anti-inflammatory activity of ascorbic acid. **Antioxidants**, 11(10), 1993.

Getnet, M. A., Mekonnen, M. Y., Yimam, H. M., Berihun, A. M., & Malede, B. A. (2024). Histopathology based study of Nile tilapia fish (*Oreochromis niloticus*) as a biomarker for water pollution evaluation in the southern gulf of Lake Tana, Ethiopia. **BMC Veterinary Research**, 20(1), 409.

Gheziel, C., Russo, P., Arena, M. P., Spano, G., Ouzari, H. I., Kheroua, O., ... & Capozzi, V. (2019). Evaluating the probiotic potential of *Lactobacillus plantarum* strains from Algerian infant feces: towards the design of probiotic starter cultures tailored for developing countries. **Probiotics and antimicrobial proteins**, 11, 113-123.

Gouda, A., Amer, S. A., Gabr, S., & Tolba, S. A. (2020). Effect of dietary supplemental ascorbic acid and folic acid on the growth performance, redox status, and immune status of broiler chickens under heat stress. **Tropical Animal Health and Production**, 52, 2987-2996.

Han, B., Long, W. Q., He, J. Y., Liu, Y. J., Si, Y. Q., & Tian, L. X. (2015). Effects of dietary *Bacillus licheniformis* on growth performance, immunological parameters, intestinal morphology and resistance of juvenile Nile tilapia (*Oreochromis niloticus*) to challenge infections. **Fish & shellfish immunology**, 46(2), 225-231.

Haque, M. M., Hasan, N. A., Eltholth, M. M., Saha, P., Mely, S. S., Rahman, T., & Murray, F. J. (2021). Assessing the impacts of in-feed probiotic on the growth performance and health condition of pangasius (*Pangasianodon hypophthalmus*) in a farm trial. **Aquaculture reports**, 20, 100699.

Hardie, L. J., Fletcher, T. C., & Secombes, C. J. (1991). The effect of dietary vitamin C on the immune response of the Atlantic salmon (*Salmo salar* L.). **Aquaculture**, 95(3-4), 201-214.

Harsij, M., Kanani, H. G., & Adineh, H. (2020). Effects of antioxidant supplementation (nano-selenium, vitamin C and E) on growth performance, blood biochemistry, immune status and body composition of rainbow trout (*Oncorhynchus mykiss*) under sub-lethal ammonia exposure. **Aquaculture**, 521, 734942.

Hong, H. A., Duc, L. H., & Cutting, S. M. (2005). The use of bacterial spore formers as probiotics. **FEMS microbiology reviews**, 29(4), 813-835.

Ibrahim, T. (2020). Diseases of Nile tilapia with special emphasis on water pollution. **Journal of Environmental Science and Technology**, 13, 29-56.

Janda, J.M., & Abbott, S.L. (2010). The genus *Aeromonas*: Taxonomy, pathogenicity, and infection. **Clinical Microbiology Reviews**, 23(1), 35–73.

Jauncey, K., Soliman, A., & Roberts, R. J. (1985). Ascorbic acid requirements in relation to wound healing in the cultured tilapia *Oreochromis niloticus* (Trewavas). **Aquaculture Research**, 16(2), 139-149.

Jiménez-Fernández, E., Ruyra, A., Roher, N., Zuasti, E., Infante, C., & Fernández-Díaz, C. (2014). Nanoparticles as a novel delivery system for vitamin C administration in aquaculture. **Aquaculture**, 432, 426-433.

John, T. M., George, J. C., Hilton, J. W., & Slinger, S. J. (1979). Influence of dietary ascorbic acid on plasma lipid levels in the rainbow trout. *International Journal for Vitamin and Nutrition research. Internationale Zeitschrift fur Vitamin-und Ernahrungsforschung.* **Journal International de Vitaminologie et de Nutrition**, 49(4), 400-405.

Keysami, M. A., & Mohammadpour, M. (2013). Effect of *Bacillus subtilis* on *Aeromonas hydrophila* infection resistance in juvenile freshwater prawn, *Macrobrachium rosenbergii* (de Man). **Aquaculture International**, 21, 553-562.

Kuebutornye, F. K., Abarike, E. D., & Lu, Y. (2019). A review on the application of *Bacillus* as probiotics in aquaculture. **Fish & shellfish immunology**, 87, 820-828.

Kuebutornye, F. K., Wang, Z., Lu, Y., Abarike, E. D., Sakyi, M. E., Li, Y., ... & Hlordzi, V. (2020). Effects of three host-associated *Bacillus* species on mucosal immunity and gut health of Nile tilapia, *Oreochromis niloticus* and its resistance against *Aeromonas hydrophila* infection. **Fish & shellfish immunology**, 97, 83-95

Laosam, P., Luasiri, P., Nakharuthai, C., Boonanuntanasarn, S., Suwanangul, S., Sarnthima, R., ... & Sangsawad, P. (2024). Enzymatic hydrolysis of duck blood protein produces stable bioactive peptides: Pilot-scale production, identification, and stability during gastrointestinal and plasma digestion. **International journal of biological macromolecules**, *283*, 137864.

Li, Y., & Lovell, R. T. (1985). Elevated levels of dietary ascorbic acid increase immune responses in channel catfish1. **The Journal of nutrition**, 115(1), 123-131.

Lim, C., Yildirim‐Aksoy, M., Welker, T., Klesius, P. H., & Li, M. H. (2010). Growth performance, immune response, and resistance to *Streptococcus iniae* of Nile tilapia, *Oreochromis niloticus*, fed diets containing various levels of vitamins C and E. **Journal of the world aquaculture society**, 41(1), 35-48.

Lin, M. F., & Shiau, S. Y. (2005). Dietary L-ascorbic acid affects growth, nonspecific immune responses and disease resistance in juvenile grouper, *Epinephelus malabaricus*. **Aquaculture**, 244(1-4), 215-221.

Liu, C. H., Chiu, C. H., Wang, S. W., & Cheng, W. (2012). Dietary administration of the probiotic, *Bacillus subtilis* E20, enhances the growth, innate immune responses, and disease resistance of the grouper, *Epinephelus coioides*. **Fish & shellfish immunology**, 33(4), 699-706.

Liu, H., Wang, S., Cai, Y., Guo, X., Cao, Z., Zhang, Y., ... & Zhou, Y. (2017). Dietary administration of *Bacillus subtilis* HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, *Oreochromis niloticus*. **Fish & shellfish immunology**, 60, 326-333.

Liu, L., Liu, Y., Shin, H. D., Chen, R. R., Wang, N. S., Li, J., ... & Chen, J. (2013). Developing *Bacillus* spp. as a cell factory for production of microbial enzymes and industrially important biochemicals in the context of systems and synthetic biology. **Applied Microbiology and Biotechnology**, 97, 6113-6127.

Liu, Q., Wen, L., Pan, X., Huang, Y., Du, X., Qin, J., ... & Lin, Y. (2021). Dietary supplementation of *Bacillus subtilis* and *Enterococcus faecalis* can effectively improve the growth performance, immunity, and resistance of tilapia against *Streptococcus agalactiae*. **Aquaculture Nutrition**, 27(4), 1160-1172.

Liu, Y., Liu, C., & Li, J. (2020). Comparison of vitamin C and its derivative antioxidant activity: evaluated by using density functional theory. **ACS omega**, 5(39), 25467-25475.

Mæland, A., & Waagbø, R. (1998). Examination of the qualitative ability of some cold water marine teleosts to synthesise ascorbic acid. **Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology**, 121(3), 249-255.

Magnadottir, B. (2010). Immunological control of fish diseases. **Marine biotechnology**, 12, 361-379.

Mathern, D. R., & Heeger, P. S. (2015). Molecules great and small: the complement system. **Clinical Journal of the American Society of Nephrology**, 10(9), 1636-1650.

Medagoda, N., Chotikachinda, R., Hasanthi, M., & Lee, K. J. (2023). Dietary supplementation of a mixture of nucleotides, β-glucan and vitamins c and e improved the growth and health performance of olive flounder, *Paralichthys olivaceus*. **Fishes**, 8(6), 302.

Milla, S., Mathieu, C., Wang, N., Lambert, S., Nadzialek, S., Massart, S., ... & Kestemont, P. (2010). Spleen immune status is affected after acute handling stress but not regulated by cortisol in Eurasian perch, *Perca fluviatilis*. **Fish & shellfish immunology**, 28(5-6), 931-941.

Moriarty, D. J. (1999, August). Disease control in shrimp aquaculture with probiotic bacteria. ***In Proceedings of the 8th international symposium on microbial ecology*** (pp. 237-243). Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Munguti, J. M., Nairuti, R., Iteba, J. O., Obiero, K. O., Kyule, D., Opiyo, M. A., ... & Ogello, E. O. (2022). Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) culture in Kenya: Emerging production technologies and socio-economic impacts on local livelihoods. **Aquaculture, Fish and Fisheries**, 2(4), 265-276.

Nakharuthai, C., & Srisapoome, P. (2020). Molecular identification and dual functions of two different CXC chemokines in Nile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae* and *Flavobacterium columnare*. **Microorganisms**, 8(7), 1058.

Nakharuthai, C., Areechon, N., & Srisapoome, P. (2016). Molecular characterization, functional analysis, and defense mechanisms of two CC chemokines in Nile tilapia (*Oreochromis niloticus*) in response to severely pathogenic bacteria. **Developmental & Comparative Immunology**, 59, 207-228.

Nakharuthai, C., Boonanuntanasarn, S., Kaewda, J., & Manassila, P. (2023). Isolation of potential probiotic *Bacillus* spp. from the intestine of Nile tilapia to construct recombinant probiotic expressing CC chemokine and its effectiveness on innate immune responses in Nile tilapia. **Animals**, 13(6), 986.

Nayak, S. K. (2010). Probiotics and immunity: a fish perspective. **Fish & shellfish immunology**, 29(1), 2-14.

Nayak, S. K., Swain, P., & Mukherjee, S. C. (2007). Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham.). **Fish & shellfish immunology**, 23(4), 892-896.

Njiru, M., Ojuok, J. E., Okeyo‐Owuor, J. B., Muchiri, M., Ntiba, M. J., & Cowx, I. G. (2006). Some biological aspects and life history strategies of Nile tilapia

Oreochromis niloticus (L.) in Lake Victoria, Kenya. **African Journal of Ecology**, 44(1), 30-37.

Omoniyi, A. D., Ovie, I. A. (2018). Vitamin C: An important nutritional factor in fish diets. **Journal of Agriculture and Ecology Research International**, 16(2), 1-7.

Panase, A., Thirabunyanon, M., Promya, J., & Chitmanat, C. (2023). Influences of *Bacillus subtilis* and fructooligosaccharide on growth performances, immune responses, and disease resistance of Nile tilapia, *Oreochromis niloticus*. **Frontiers in Veterinary Science**, 9, 1094681.

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S., & Sugita, H. (2004). Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. **Veterinary immunology and immunopathology**, 102(4), 379-388.

Pehlivan, F. E. (2017). Vitamin C: An antioxidant agent. **Vitamin C**, 2, 23-35.

Pereira, W. A., Mendonça, C. M. N., Urquiza, A. V., Marteinsson, V. Þ., LeBlanc, J. G., Cotter, P. D., ... & Oliveira, R. P. (2022). Use of probiotic bacteria and bacteriocins as an alternative to antibiotics in aquaculture. **Microorganisms**, 10(9), 1705.

Pérez-Sánchez, T., Mora-Sánchez, B., & Balcázar, J. L. (2018). Biological approaches for disease control in aquaculture: advantages, limitations and challenges. **Trends in microbiology**, 26(11), 896-903.

Pitaksong, T., Kupittayanant, P., & Boonanuntanasarn, S. (2013). The effects of vitamins C and E on the growth, tissue accumulation and prophylactic response to thermal and acidic stress of hybrid catfish. **Aquaculture Nutrition**, 19(2), 148-162.

Pretto-Giordano, L. G., Müller, E. E., Freitas, J. C. de., & Silva, V. G. da. (2010). Evaluation on the Pathogenesis of *Streptococcus agalactiae* in Nile Tilapia (*Oreochromis niloticus*). **Brazilian Archives of Biology and Technology**, 53(1), 87–92.

Puangkaew, J., Kiron, V., Somamoto, T., Okamoto, N., Satoh, S., Takeuchi, T., & Watanabe, T. (2004). Nonspecific immune response of rainbow trout (*Oncorhynchus mykiss* Walbaum) in relation to different status of vitamin E and highly unsaturated fatty acids. **Fish & shellfish immunology**, 16(1), 25-39.

Rai, M., & Padh, H. (2001). Expression systems for production of heterologous proteins. **Current science**, 1121-1128.

Rinkinen, M., Jalava, K., Westermarck, E., Salminen, S., & Ouwehand, A. C. (2003). Interaction between probiotic lactic acid bacteria and canine enteric pathogens: a risk factor for intestinal *Enterococcus faecium* colonization? **Veterinary microbiology**, 92(1-2), 111-119.

Roosta, Z., Hajimoradloo, A., Ghorbani, R., & Hoseinifar, S. H. (2014). The effects of dietary vitamin C on mucosal immune responses and growth performance in Caspian roach (*Rutilus rutilus caspicus*) fry. **Fish physiology and biochemistry**, 40, 1601-1607.

Santos-Sánchez, N. F., Salas-Coronado, R., Villanueva-Cañongo, C., & Hernández-Carlos, B. (2019). Antioxidant compounds and their antioxidant mechanism. **IntechOpen**.

Serwecińska, L. (2020). Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and public health. **Water**, 12(12), 3313.

Shahkar, E., Yun, H., Kim, D. J., Kim, S. K., Lee, B. I., & Bai, S. C. (2015). Effects of dietary vitamin C levels on tissue ascorbic acid concentration, hematology, non-specific immune response and gonad histology in broodstock Japanese eel, *Anguilla japonica*. **Aquaculture**, 438, 115-121.

Shanaka, K. A. S. N., Jung, S., Janson, N. D., Jayasingha, J. R. P., Madushani, K. P., Kim, M. J., & Lee, J. (2021). Growth and antioxidant-related effects of the reestablished ascorbic acid pathway in Zebrafish (*Danio rerio*) by genomic integration of L-gulonolactone oxidase from cloudy catshark (*Scyliorhinus torazame*). **Frontiers in Physiology**, 12, 685595.

Sheraz, M. A. L. I., Khan, M. F., Ahmed, S. O. F. I. A., Kazi, S. H., & Ahmad, I. Q. B. A. L. (2015). Stability and stabilization of ascorbic acid. **Household and Personal Care Today**, 10, 22-25.

Shi, M., Gao, M., Sun, H., Yang, W., Zhao, H., Zhang, L., & Xu, H. (2023). Exogenous 2-keto-L-gulonic acid supplementation as a novel approach to enhancing L-ascorbic acid biosynthesis in zebrafish (*Danio rerio*). **Animals**, 13(15), 2502.

Shinn, A. P., Avenant-Oldewage, A., Bondad-Reantaso, M. G., Cruz-Laufer, A. J., García-Vásquez, A., Hernández-Orts, J. S., ... & Deveney, M. R. (2023). A global review of problematic and pathogenic parasites of farmed tilapia. **Reviews in Aquaculture**, 15, 92-153.

Siwicki, A., & Studnicka, M. (1987). The phagocytic ability of neutrophils and serum lysozyme activity in experimentally infected carp, *Cyprinus carpio* L. **Journal of Fish Biology**, 31, 57-60.

Smirnoff, N. (2001). L-ascorbic acid biosynthesis. **Vitamins & Hormones**, 61, 241-266.

Ssekyewa, C. & Muwanga, M.K. (2009). Biotechnology in Organic Agriculture in Africa: Myth or Oversight. **Journal of Science and Sustainable Development**, 2(1), 33-38.

Suanyuk, N., Kong, F., Ko, D., Gilbert, G. L., & Supamattaya, K. (2008). Occurrence of rare genotypes of *Streptococcus agalactiae* in cultured red tilapia *Oreochromis* sp. and Nile tilapia *O. niloticus* in Thailand—relationship to human isolates? **Aquaculture**, 284(1-4), 35-40.

Subramani, P. A., Narasimha, R. V., Balasubramanian, R., Narala, V. R., Ganesh, M. R., & Michael, R. D. (2016). Cytotoxic effects of *Aeromonas hydrophila* culture supernatant on peripheral blood leukocytes of Nile tilapia (*Oreochromis niloticus*): Possible presence of a secreted cytotoxic lectin. **Fish & Shellfish Immunology**, 58, 604-611.

Sunarto, A., Grimm, J., McColl, K. A., Ariel, E., Nair, K. K., Corbeil, S., ... & Holmes, B. (2022). Bioprospecting for biological control agents for invasive tilapia in Australia. **Biological Control**, 105020.

Suwanangul, S., Jaichakan, P., Narkprasom, N., Kraithong, S., Narkprasom, K., & Sangsawad, P. (2023). Innovative Insights for Establishing a Synbiotic Relationship with *Bacillus coagulans*: Viability, Bioactivity, and *In Vitro*-Simulated Gastrointestinal Digestion. **Foods**, 12(19), 3692.

Tian, Y. S., Deng, Y. D., Zhang, W. H., Xu, J., Gao, J. J., Fu, X. Y., ... & Yao, Q. H. (2022). Metabolic engineering of *Escherichia coli* for direct production of vitamin C from D-glucose. **Biotechnology for Biofuels and Bioproducts**, 15(1), 86.

Toyohara, H., Nakata, T., Touhata, K., Hashimoto, H., Kinoshita, M., Sakaguchi, M., ... & Ozato, K. (1996). Transgenic expression of L-gulono-γ-lactone oxidase in medaka (*Oryzias latipes*), a teleost fish that lacks this enzyme necessary for L-ascorbic acid biosynthesis. **Biochemical and Biophysical Research Communications**, 223(3), 650-653.

Uni, Z. (2006). Early development of small intestinal function. Perry GC, (Ed). **28th Poultry Science Symposium of the World's Poultry Science Association** (pp. 29-42). Wallingford UK: CABI.

Verlhac, V., Gabaudan, J., Obach, A., Schüep, W., & Hole, R. (1996). Influence of dietary glucan and vitamin C on non-specific and specific immune responses of rainbow trout (*Oncorhynchus mykiss*). **Aquaculture**, 143(2), 123-133.

Verschuere, L., Rombaut, G., Sorgeloos, P., & Verstraete, W. (2000). Probiotic bacteria as biological control agents in aquaculture. **Microbiology and Molecular Biology Reviews**, 64, 655–671.

Wang, B., Thompson, K. D., Wangkahart, E., Yamkasem, J., Bondad-Reantaso, M. G., Tattiyapong, P., ... & Surachetpong, W. (2023). Strategies to enhance tilapia immunity to improve their health in aquaculture. **Reviews in Aquaculture**, 15, 41-56.

Wang, J., Law, C.L., Mujumdar, A.S., & Xiao, H.W. (2017). The degradation mechanism and kinetics of vitamin C in fruits and vegetables during thermal processing. Nema, PK, Kaur, BP and Mujumdar, (Eds). **AS Fundamentals & applications (Part III)**, 227-253.

Wang, L., Chen, D., Lou, B., Zhan, W., Chen, R., Liu, F., & Mao, G. (2017). The effects of dietary vitamin C on growth performance, serum enzymes activities and resistance to *Vibrio alginolyticus* challenge of yellow drum *Nibea albiflora*. **Aquaculture Research**, 48(9), 4684-4695.

Wang, X., Kim, K. W., Bai, S. C., Huh, M. D., & Cho, B. Y. (2003). Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). **Aquaculture**, 215(1-4), 203-211.

Won, S., Hamidoghli, A., Choi, W., Park, Y., Jang, W. J., Kong, I. S., & Bai, S. C. (2020). Effects of *Bacillus subtilis* WB60 and *Lactococcus lactis* on growth, immune

responses, histology and gene expression in Nile tilapia, *Oreochromis niloticus.* **Microorganisms**, 8(1).

Wu, D., Hugenholtz, P., Mavromatis, K., Pukall, R., Dalin, E., Ivanova, N. N., ... & Eisen, J. A. (2009). A phylogeny-driven genomic encyclopaedia of Bacteria and Archaea. **Nature**, 462(7276), 1056-1060.

Wu, Z. X., Feng, X., Xie, L. L., Peng, X. Y., Yuan, J., & Chen, X. (2012). Effect of probiotic *Bacillus subtilis* Ch9 for grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844), on growth performance, digestive enzyme activities and intestinal microflora. **Journal of Applied Ichthyology**, 28(5), 721-727.

Xu, C. M., Yu, H. R., Li, L. Y., Li, M., Qiu, X. Y., Fan, X. Q., ... & Shan, L. L. (2022). Effects of dietary vitamin C on the growth performance, biochemical parameters, and antioxidant activity of coho salmon *Oncorhynchus kisutch* (Walbaum, 1792) Postsmolts. **Aquaculture nutrition**, 2022(1), 6866578.

Yang, Y., Zhang, G., Wu, J., Chen, X., Tong, D., Yang, Y., ... & Du, A. (2020). Recombinant HcGAPDH protein expressed on probiotic *Bacillus subtilis* spores protects sheep from *Haemonchus contortus* infection by inducing both humoral and cell-mediated responses. **Msystems**, 5(3), 10-1128.

Yanong, R. P. E., Francis-Floyd, R., & Petty, B. D. (2021). **Infectious diseases in aquaculture**. Retrieved from https://www.msdvetmanual.com/exotic-and-laboratory-animals/aquaculture/infectious-diseases-in-aquaculture.

Yin, X., Chen, K., Cheng, H., Chen, X., Feng, S., Song, Y., & Liang, L. (2022). Chemical stability of ascorbic acid integrated into commercial products: A review on bioactivity and delivery technology. **Antioxidants**, 11(1), 153.

Zafar, N., & Khan, M. A. (2020). Effects of dietary iron on growth, haematology, oxidative stress and hepatic ascorbic acid concentration of stinging catfish *Heteropneustes fossilis*. **Aquaculture**, 516, 734642.

Zahran, E., Hafez, E. E., Mohd Altaf Hossain, F., Elhadidy, M., & Shaheen, A. A. (2017). Saprolegniosis in Nile tilapia: identification, molecular characterization, and phylogenetic analysis of two novel pathogenic *Saprolegnia* strains. **Journal of Aquatic Animal Health**, 29(1), 43-49.

Zehra, S., & Khan, M. A. (2021). Dietary vitamin C requirement based on growth performance, non-specific immune response, antioxidant capacity, and liver

vitamin C concentration of fingerling *Channa punctatus* (Bloch). **Animal Feed Science and Technology**, 280, 115058.

Zhang, C., Wohlhueter, R. & Zhang, H. (2016). Genetically modified foods: A critical review of their promise and problems. **Food Science and Human Wellness**, 5(3), 116-123.

Zhao, Y., Yuan, L., Wan, J., Sun, Z., Wang, Y., & Sun, H. (2016). Effects of potential probiotic *Bacillus cereus* EN25 on growth, immunity and disease resistance of juvenile sea cucumber *Apostichopus japonicus*. **Fish & Shellfish Immunology**, 49, 237-242.

Zhu, C. B., Ren, H. C., Wu, Y. J., Yang, S., & Fei, H. (2024). Benefits and applications of vitamin C in farmed aquatic animals: An updated review. **Aquaculture International**, 32(2), 1295-1315.

BIOGRAPHY

Miss Jirawadee Kaewda was born on November 16, 1999, in Ubon Ratchathani Province, Thailand. She received her Bachelor of Science degree in Animal Production Technology from Suranaree University of Technology (SUT) in 2022. In 2023, she began her Master's studies in Biotechnology for Aquaculture at the Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand, under the supervision of Assistant Professor Dr. Chatsirin Nakharuthai.