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).

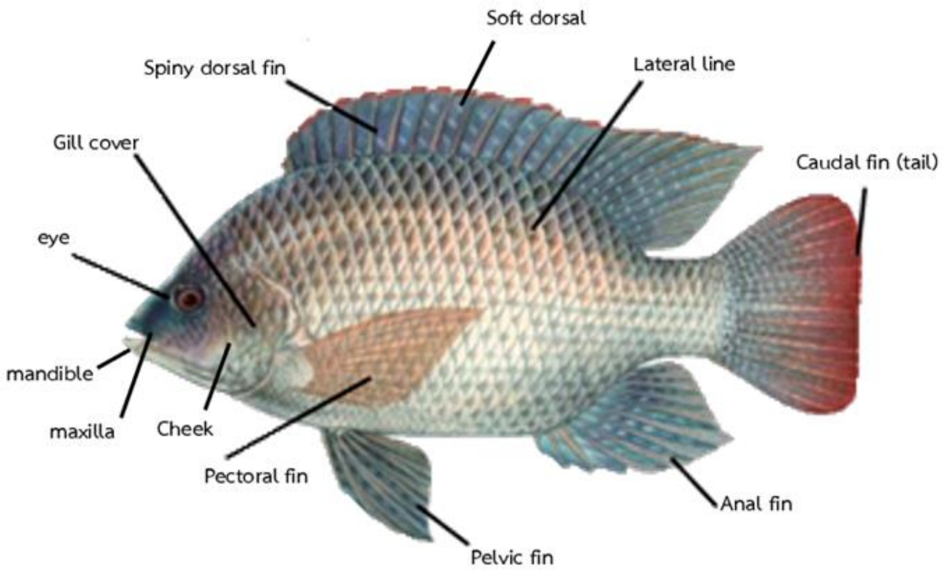

Figure 2.1 Shape and composition of tilapia.

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).

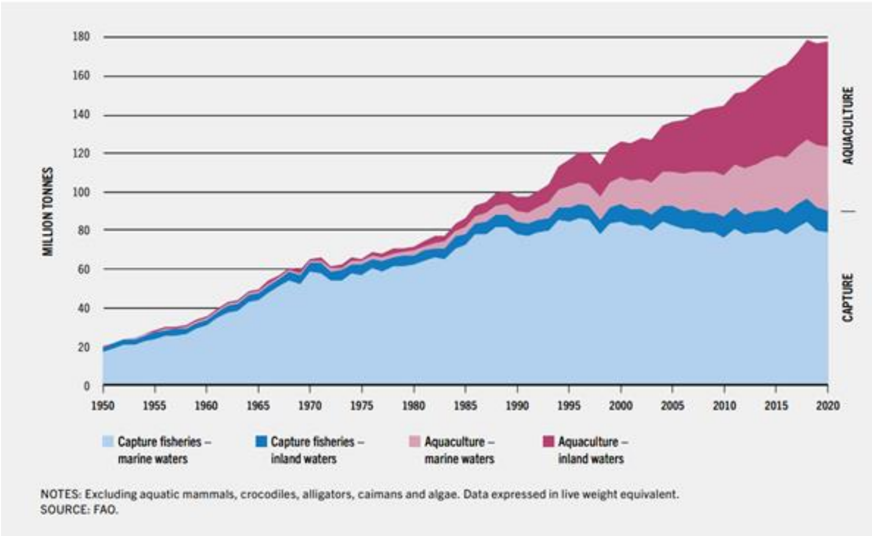

Figure 2.2 World Capture Fisheries and Aquaculture Production.

Reference: Food and Agriculture Organization (2022).

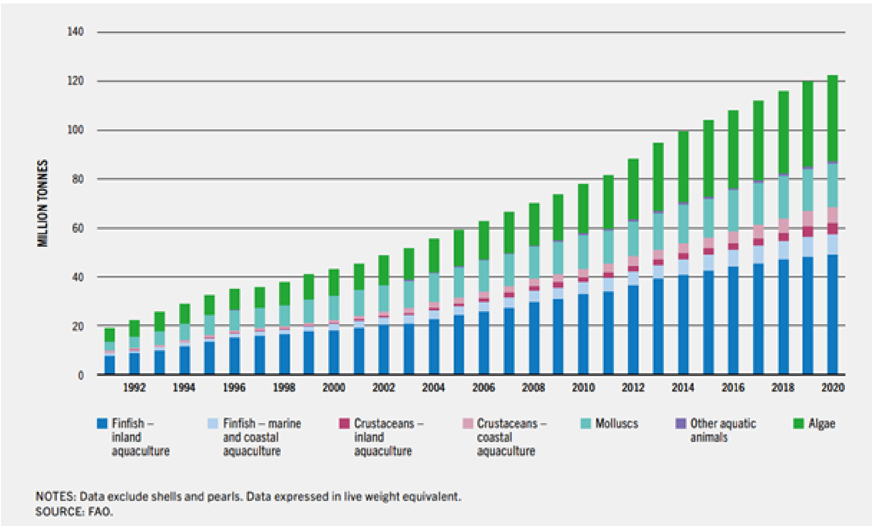

Figure 2.3 World aquaculture production of aquatic animals, 1991-2020.

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.

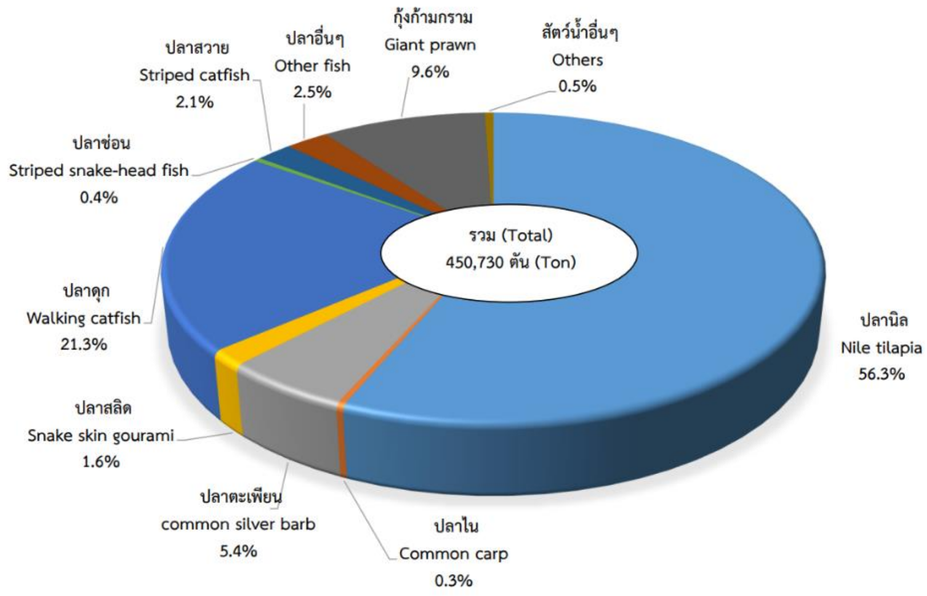

Figure 2.4 Freshwater Aquaculture Production by Species, 2021.

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).

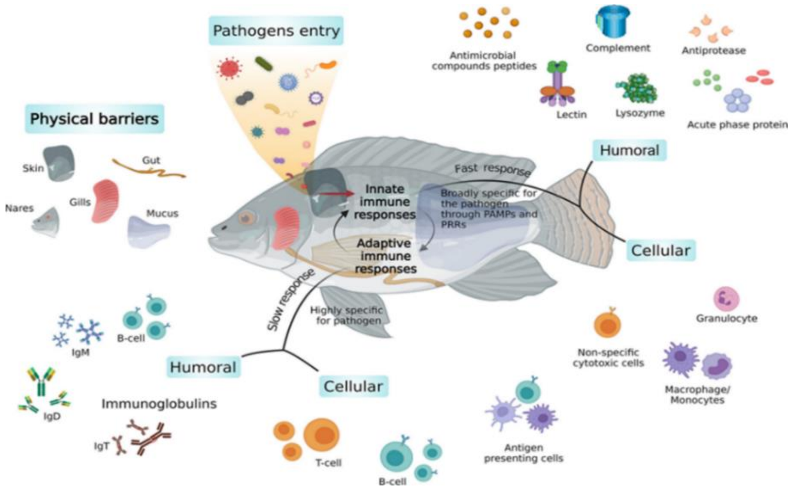

Figure 2.5 Overview of fish immunity.

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.

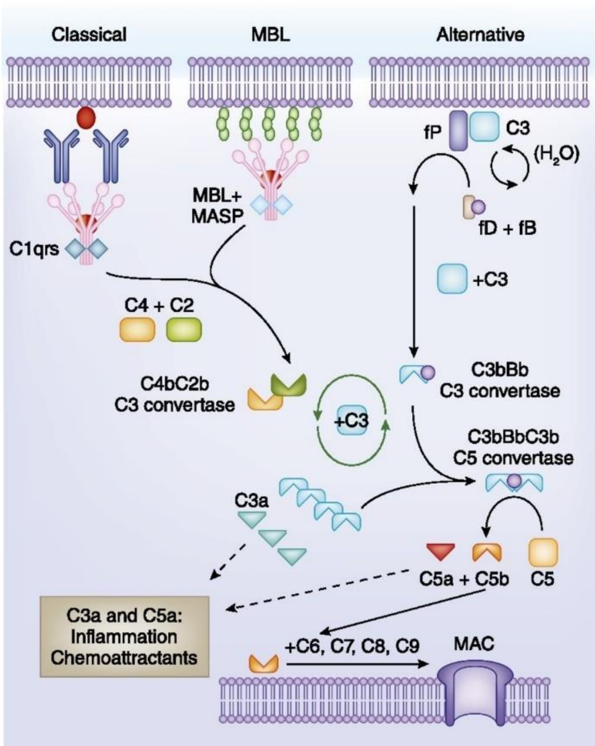

Figure 2.6 Scheme of the complement system

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).

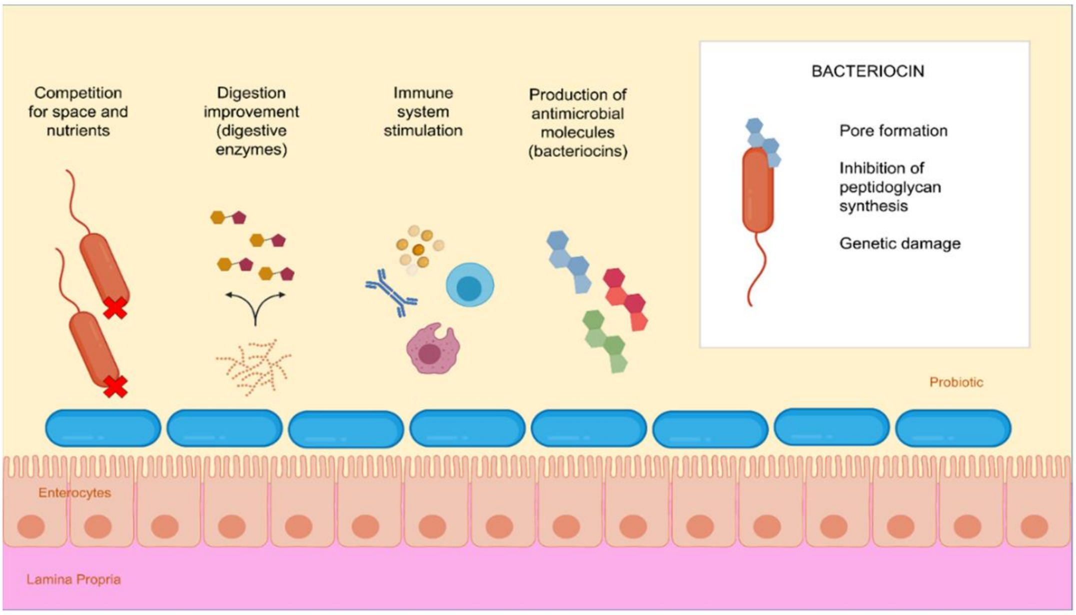

Figure 2.7 The mechanism of probiotics.

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.

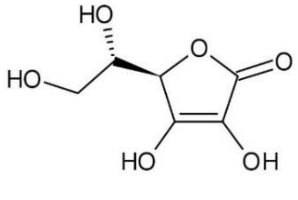

Figure 2.8 The structural formula of vitamin C.

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.

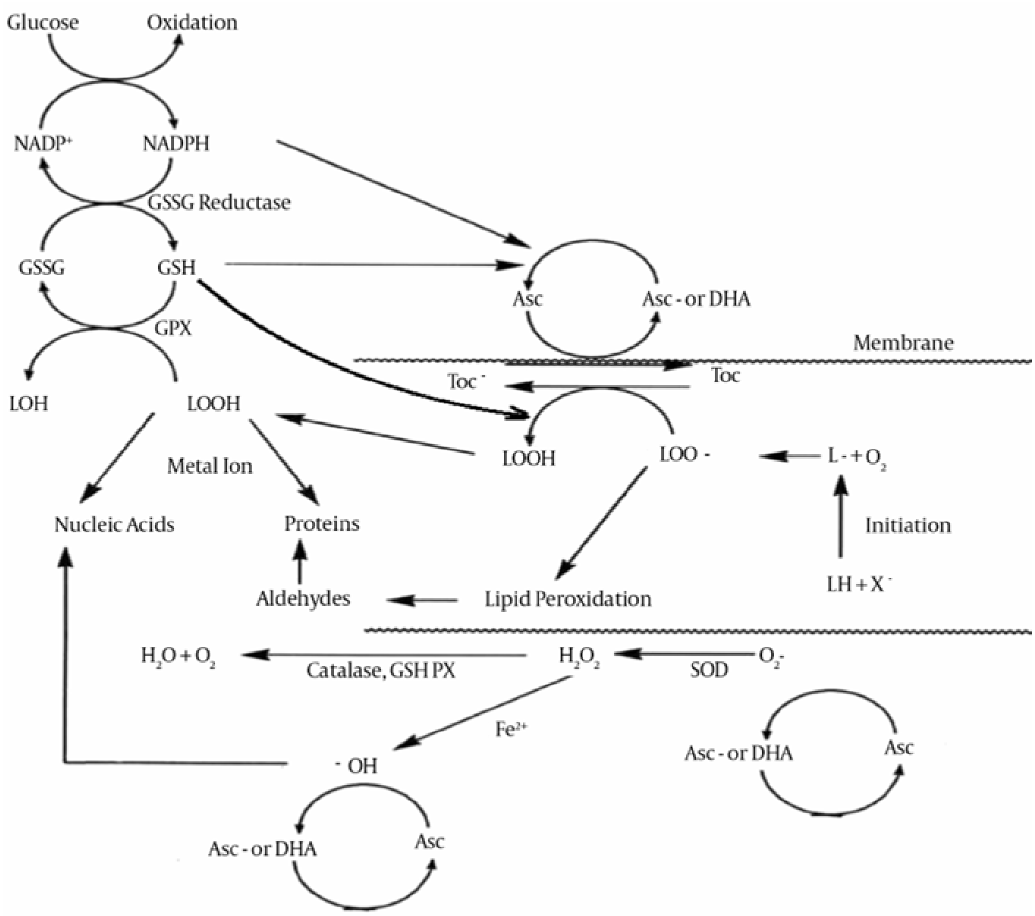

Figure 2.9 Relationships Among Radical Reactions, Fenton Reaction, Lipid Peroxidation and Antioxidant Properties of Vitamin C.

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 (Doseděl et 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.