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