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.