CHAPTER V
DISCUSSION

Protein hydrolysates from animal blood byproducts have gained attention as promising aquaculture feed additives (Chang et al., 2007; Wang et al., 2008; Gisbert et al., 2012; Zheng et al., 2018). Duck plasma hydrolysate exhibited strong antioxidant properties and a beneficial amino acid composition (Yang et al., 2020). However, industrial-scale production faces challenges such as anticoagulant contamination, which increases sodium levels, and the need for strict temperature control during plasma separation, complicating the process and raising costs. This study introduces an innovative approach using WB without anticoagulants. We employed thermal denaturation (90°C for 30 min) to inactivate fibrinogen and reduce microbial load, followed by Neutrase-mediated hydrolysis at pH 7. This process eliminates the need for pH adjustment, avoiding sodium chloride contamination typically associated with acid–base neutralization. Ultrafiltration with a 10 kDa cut-off membrane yielded a low molecular weight of the DBPH, with approximately 32% of peptides below 3 kDa. These small peptides (<3 kDa) demonstrate superior antioxidant activity compared to larger peptides (Czelej et al., 2022), enhancing DPPH performance, metal chelating, and hydroxyl radical scavenging activities (Agrawal et al., 2017). They also exhibit remarkable bioavailability and efficient intestinal absorption, similar to fish protein hydrolysates (Chalamaiah et al., 2018). Notably, peptides below 1.4 kDa show significant ABTS radical scavenging ability (Czelej et al., 2022). Given these advantageous characteristics, our low molecular weight DBPH presents a promising bioactive feed additive for aquaculture. Its unique properties and potential benefits warrant further investigation and development for commercial applications.

In the feeding trial, the fish-fed vitamin C supplementation group was used as a positive control. Previous studies indicate that vitamin C and protein hydrolysates exert their effects through similar biological pathways, such as enhancing antioxidant activity and modulating immune responses (Yang et al., 2020; Dawood and Koshio, 2018).

Therefore, using vitamin C as a positive control aids in clarifying the specific contributions of DBPH in this study. In addition, vitamin C is less stable due to its chemical structure and susceptibility to environmental factors. Consequently, DBPH may represent an alternative source of novel and potent antioxidants. After a 30-day feeding trial, the fish that were fed a diet supplemented with 2% DBPH exhibited superior weight gain and humoral immune response (lysozyme, ACH_{50}, and total Ig) to those fed diets containing 0.5% and 1% DBPH, as did the control groups. Furthermore, adding 2% DBPH in the diet showed no significant difference in the positive control group. In this sense, a higher concentration of low molecular weight bioactive peptides could enhance nutrient absorption, resulting in improved weight gain. Dietary supplementation with 2% DBPH could improve the lysozyme and ACH_{50} activities, as well as the total Ig levels in the flowerhorn fish compared to the control group. In addition, no significant difference was observed when compared to the group supplemented with vitamin C (positive control). Low molecular weight DBPH could enhance the bioavailability and absorption of nutrients, which are essential for the synthesis of humoral immune protein. Consequently, their increased availability contributes to improving the immune function and acts as a defense against pathogens (Tang et al., 2008; Pascual et al., 2014). Additionally, undergoing hydrolysis, DBPH may possess bioactive activities, such as antimicrobial or immunomodulatory effects, which can directly support or enhance the humoral immune response in flowerhorn fish (Tang et al., 2008; Duarte et al., 2006). These findings could confirm the bioactive activity of DBPH.

Regarding antioxidant activity, our previous study (Laosama et al., 2014) identified antioxidant peptides in DBPH, including WMHVR, YAHVR, MPFKY, PDDPR, and NKVHF. These peptides exhibited strong ABTS radical scavenging activity with IC50 values ranging from 0.47 to 5.82 mg/mL, which can reduce oxidative stress-related damage by scavenging free radicals. In our present study, dietary supplementation with 1% and 2% DBPH could enhance the ability of antioxidant enzymes including CAT, SOD, and MDA, with no significant difference observed compared to the group supplemented with powerful antioxidant vitamin C. CAT and SOD function as antioxidant enzymes that aid in neutralizing reactive oxygen species (ROS) within cells, while MDA is a marker of lipid peroxidation, indicative of oxidative stress. These enzymes play a crucial role in the antioxidant defense system of cells. Bioactive peptides derived from hydrolysis can directly scavenge ROS within cells and regulate gene expression related to antioxidant enzymes, leading to increased production and activity of CAT and SOD. In addition, DBPH may possess the ability to inhibit lipid peroxidation, thereby, reducing the formation of MDA. Overall, humoral immune response and antioxidant activity results demonstrated that 2% DBPH is the optimal level of supplementation as a bioactive feed additive for enhancing both antioxidant activity and humoral immune response in flowerhorn fish under these experimental conditions.

Similar to livestock animals, dietary nutrient composition in aquafeed is one of the most important criteria in shaping the composition and function of the gut microbiota in fish, which in turn influences the immune system. To further improve the overall health of fish, the interaction between gut microbiota and fish immunity has garnered interest from nutritional researchers. The intestine provides a living environment for a diverse community of microorganisms, known as the gut microbiota, as well as a large number of immune cells that defend against harmful substances. Evidence has been reported that the optimal amount of protein hydrolysate and its bioactive compounds can improve gut microbiota and enhance immune system function (Bui et al., 2014; Wei et al., 2023). In this study, fish fed with 2% DBPH exhibited a reduction in the variety of intestinal microbiota as indicated by OTUs and the alpha diversity analysis, including Chao1 and Shannon. These results correlated with those reported for turbot (*Scophthalmus maximus*) (Wei et al., 2023) and largemouth bass (*Micropterus salmoides*) (Fan et al., 2022), suggesting that low molecular weight DBPH could contain the antimicrobial peptides (AMPs). However, further studies are needed to characterize the sequences of peptides in DBPH that possess antimicrobial activity. In this context, AMPs, which play a crucial role in defending against pathogens, could potentially reduce the biodiversity of microorganisms in the intestines of fish fed with 2% DBPH. This reduction may occur through the disruption of cell membranes or interference with essential microbial functions. In the present study, the dominant phyla in both the unsupplemented DBPH group and the group supplemented with 2% DBPH were proteobacteria, fusobacteriota, firmicutes, bacteroidota, and actinobacteriota. These findings were consistent with the previous studies on turbot (*S. maximus*) (Wei et al., 2023), and largemouth bass (*M. salmoides*) (Fan et al., 2022; Sheng et al., 2022). At the genus level, fish fed with a 2% DBPH-supplemented group exhibited a significantly higher abundance of cetobacterium, romboutsia, and shewanella, but lower levels of crenobacter compared to the control group. Within the fish gut, the genus *Cetobacterium* plays a role in nutritional utilization by enhancing the digestion and metabolism of both carbohydrates and proteins, especially those molecules that are difficult to break down (Tao et al., 2022). In addition, it has been well-demonstrated that cetobacterium is a producer of short-chain fatty acids (butyrate), and vitamin B12. Some studies suggest that certain species of cetobacterium may produce antimicrobial compounds that could potentially help modulate the microbial community in the gut environment by inhibiting the colonization and proliferation of pathogenic species (Finegold et al., 2003). Hence, the abundance of cetobacterium in the fish gut could be an indicator of the overall health and well-being of the fish (Tao et al., 2022; Li et al., 2017). The genus *Romboutsia* is capable of breaking down complex carbohydrates and fermenting amino acids in the gut environment (Gerritsen et al., 2019). These processes contribute to the metabolic activity of the gut microbiota and may influence various aspects of host health and physiology. The genera *Shewanella* and *Crenobacter* are occasionally found in the gut microbiota of fish, while commonly found in aquatic environments. However, the exact role of these genera in the fish gut microbiota and their impact on host health and physiology are still not fully understood. Their effects depend on several factors, such as diet, environmental conditions, and the species of fish. Some species of Shewanella have been reported as probiotics in fish (Cámara-Ruiz et al., 2020). however, other species have been associated with fish diseases (Li et al., 2017).

To investigate the role of low molecular weight DBPH as an immunomodulator during pathological conditions, the experimental fish were intraperitoneally injected with *S. agalactiae*. In this study, the mRNA expressions of antioxidant genes (SOD and CAT) and inflammatory cytokines (IL-1β, IL-6, CC, and CXC chemokine) were determined at the 30-day feeding trial (prechallenge) and 24 hr (postchallenge) after the *S. agalactiae* injection. At the prechallenge stage, significantly higher expression levels of SOD and CAT were observed in the 0.1% vitamin C and 2% DBPH-supplemented groups compared to the negative control group. This suggests that both vitamin C and DBPH may promote antioxidant activity and strengthen cellular defenses against oxidative stress.

During bacterial infection, significantly higher upregulation of antioxidant gene expressions was found in all experimental groups (negative control, 0.1% vitamin C, and 2% DBPH). This demonstrated the crucial role of CAT and SOD genes in immune responses to eliminate ROS during bacterial infection. Interestingly, the expression levels of CAT and SOD were significantly higher in the groups supplemented with 0.1% vitamin C and 2% DBPH compared to the negative control group. This could be attributed to the antioxidant properties of DBPH and vitamin C that support the innate immune response of fish.

In innate immunity, cytokines are critical for initiating immune defense by recruiting the immune cells to the infection site and coordinating subsequent immune responses as a link between innate and adaptive immune responses. In this study, at the prechallenge stage, only the expression of the CC chemokine gene was significantly higher in the spleen of fish-fed dietary vitamin C and DBPH supplementation compared to the control group. This could suggest the ability of vitamin C and DBPH to influence CC chemokine production in the spleen of flowerhorn fish. According to current information, the spleen plays a crucial role in modulating homeostatic conditions through the circulation of CC chemokines (Nakharuthai et al., 2016). Given that the spleen is the predominant lymphatic tissue in teleost fish, it serves as the site for phagocytic, lymphocytic, and dendritic cells, which are sources of CC chemokines. In addition, other inflammatory genes, including IL-1β, IL-6, and CXC chemokine, also exhibited constitutive expression patterns in both the liver and spleen, which are lymphoid organs in flowerhorn fish. This finding demonstrates the functionality of these cytokine genes to continuously balance immune responses under normal conditions. The constitutive expression of cytokines involves their steady-state production and is expressed at relatively constant levels without requiring an inducer or stimulus under normal physiological conditions (Nakharuthai and Srisapoome, 2020; Wang et al., 2020). Similarly, in other fish species, the constitutive expression of these cytokine genes has also been observed primarily in immune organs. This indicates their role in immune surveillance and maintaining tissue homeostasis in flowerhorn fish. At the postchallenge stage, the significantly higher mRNA upregulation levels of IL-1β, CC, and CXC chemokine persisted in both the liver and spleen among the vitamin C and DBPH-supplemented groups compared to the control group. In the case of IL-6, a significantly higher expression persisted only in the spleen at 24 hr, indicating that this organ plays a vital role in the regulation of IL-6 expression in response to *S. agalactiae* in flowerhorn fish. As part of the innate immune response, the upregulation of cytokines occurs rapidly, ranging from hours to days following bacterial injection, to recruit the immune cells to the infection site. In this context, DBPH may enhance pro-inflammatory gene expression in response to pathogenic bacteria in this study. Similarly, the supplementation of other protein hydrolysates at suitable levels has been convincingly demonstrated to enhance disease resistance in Japanese sea bass (Liang et al.,2006), turbot (Wei et al., 2023), European sea bass (Kotzamanis et al., 2007), red sea bream (Bui et al., 2014), and barramundi (Siddik et al., 2019). Furthermore, a significant increase in the mRNA expression level between pre- and postchallenge was observed for CC and CXC chemokine in both the spleen and liver across all treatments. The significance of upregulation revealed the important role of CC and CXC chemokine in response to *S. agalactiae* infection in these organs of flowerhorn fish. A significantly higher expression of IL-1β was only observed in the liver in all treatments, suggesting that the liver plays a crucial role in the regulation of IL-1β production during *S. agalactiae* infection. The significance of upregulation revealed the important role of these inflammatory cytokines in response to *S. agalactiae* infection in the liver and spleen of flowerhorn fish. The liver and spleen are the primary source of immune cells in the defense against bacterial infection of teleost fish as well as the flowerhorn fish. The spleen acts as a secondary lymphoid organ involved in filtering blood and coordinating immune responses, while the liver contributes by producing acute-phase proteins and modulating inflammatory cytokines and chemokines by hepatocytes and Kupffer cells (Nakharuthai et al., 2016).