CHAPTER IV

RESULTS

4.1 Determining the Molecular Weight of DBPH and Peptide Distributions

The protein profiles of WB and DBPH samples were analyzed using SDS-PAGE, as shown in Figure 4.1A. In the whole duck blood (WB) lane, prominent proteins, such as hemoglobin monomer, globulin, albumin, and fibrinogen were observed. In contrast, the DBPH lane exhibited no discernible protein bands, indicating complete protein degradation. In this instance, the peptides had a molecular weight below 10 kDa. The molecular weight profile of peptides obtained from the DBPH sample via size exclusion chromatography is depicted in Figure 4.1B, while the corresponding calculated molecular weight distribution is presented in Figure 4.1C. Upon analyzing the distribution of molecular weights, it was evident that the most common sizes fell within the range of 3-7 kDa (39.68%), followed by >7 kDa (20.69%), 1-3 kDa (23.03%), and <1 kDa (9.00%).

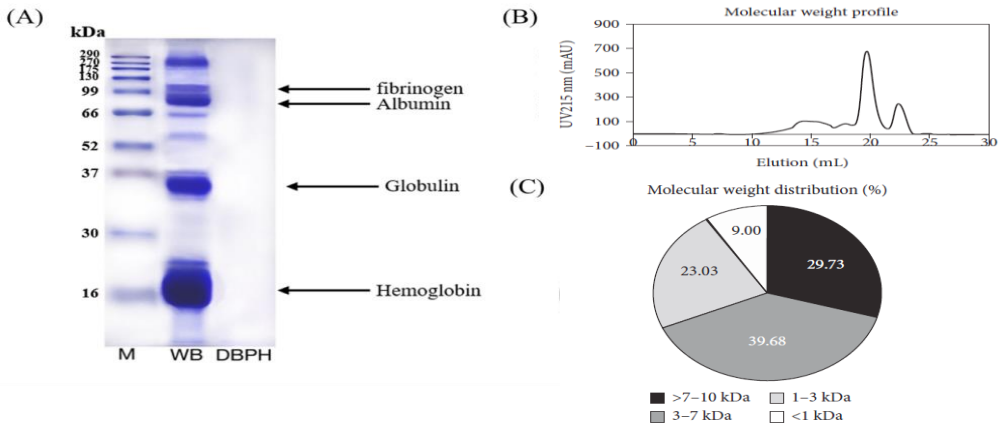

Figure 4.1 The SDS-PAGE profile (12.5% acrylamide) of samples containing 20 µg of protein (A). The samples include protein standard markers (M), whole duck blood (WB), and low molecular weight duck blood protein hydrolysate (DBPH). Additionally, size exclusion chromatography was used to obtain the molecular weight profile (B) and molecular weight distribution (C) of DBPH peptides.

4.2 Growth and Survival Rate

At the end of the feeding trial, the results showed that weight gain had significantly increased in the fish that were fed diets supplemented with 2% DBPH and 0.1% vitamin C (*P<0.05*) compared to the control group, while final body length did not differ among the experimental groups (Figure 4.2). The survival rate of all experimental groups was 100%

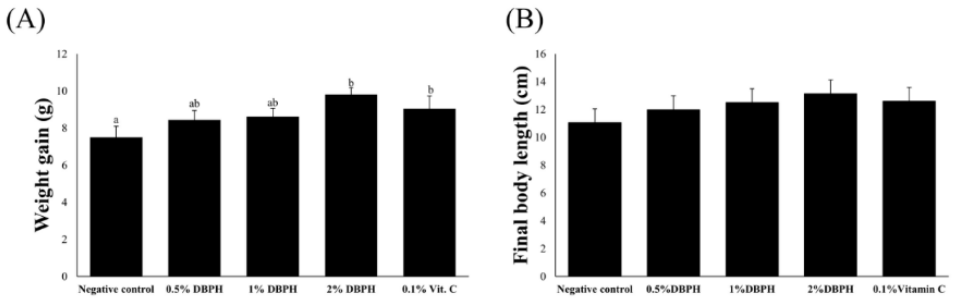

Figure 4.2 Weight gain (A) and Final body length (B) of flowerhorn fed with different experimental diets for 30 days. Means ± S.D. (n = 8) with different superscript letters is significantly different ($P < 0.05$).

4.3 Immune responses

Only the group supplemented with 2% DBPH in the diet showed a significant increase in ACH_{50}, lysozyme activity, and total Ig levels compared to the negative control group. In addition, there was no significant difference between the group supplemented with 2% DBPH and the group supplemented with 0.1% vitamin C (positive control), as shown in Table 4.1

Table 4.1 Immune parameters of flowerhorn fish fed experimental diets for 30 days.

Parameters	Treatments				
	Control	0.1% Vitamin C	0.5% DBPH	1% DBPH	2% DBPH
ACH_{50} (units mL^{-1})	23.67±0.68[a]	29.28±0.35[b]	25.27±0.44[a]	25.51±0.72[a]	30.18±1.02[b]
Lysozyme activity (μg mL^{-1})	15.41±0.12[a]	16.63±0.05[b]	16.80±0.19[b]	16.31±0.19[b]	16.51±0.23[b]
Total Ig (mg mL^{-1})	1.29±0.01[a]	1.51±0.04[b]	1.33±0.01[a]	1.51±0.07[b]	1.64±0.07[b]

Abbreviations: DBPH = low molecular weight duck blood protein hydrolysate; ACH50 = alternative complement activity. Total Ig = Total immunoglobulin. Data are presented as mean±SEM. Means with different superscripts in each row differ significantly (*P < 0.05*).

4.4 Antioxidant activity

Overall, the groups supplemented with 1% and 2% DBPH in the diet exhibited a significant increase in SOD and CAT values, while the MDA value also significantly decreased in the same groups compared to the negative control group. In addition, there was no significant difference between the group supplemented with 1% and 2% DBPH and the group supplemented with 0.1% vitamin C (positive control), as shown in Table 4.2.

Table 4.2 Antioxidant parameters of flowerhorn fish fed experimental diets for 30 days.

Parameters	Treatments				
	Control	0.1% Vitamin C	0.5% DBPH	1% DBPH	2% DBPH
SOD (U mL^{-1})	2.63±0.07[a]	4.61±0.41[b]	3.74±0.19[ab]	3.82±0.22[b]	4.12±0.25[b]
MDA (nmol mL^{-1})	4.14±0.53[b]	1.15±0.15[a]	4.39±0.36[b]	2.18±0.26[a]	1.24±0.11[a]
CAT (nmol min^{-1} mL^{-1})	17.74±0.36[a]	23.81±0.84[b]	23.59±1.35[b]	24.50±1.66[b]	23.99±1.29[b]

Abbreviations: DBPH = low molecular weight duck blood protein hydrolysate; SOD = superoxide dismutase; MDA = malondialdehyde; CAT = catalase. Data are presented as mean±SEM. Means with different superscripts in each row differ significantly (*P < 0.05*).

4.5 Intestinal microbiota and diversity analysis

A total of 480,439 raw reads were generated from six libraries, comprising three libraries of the 2% DBPH group and another three libraries in the control group. The average raw read, clean read, and tags of each group are displayed in Table 4.3. The 2% DBPH group possessed significantly fewer unique OTUs than the control group. The alpha diversity indexes, including Chao1 and Shannon, of the microbiota in flower horn fed with dietary supplementation of 2% DBPH were significantly lower than those of the control group, while the variation in the Simpson index was not significantly different.

The relative abundance (%) at the phylum level is shown in Figure 4.3A the result demonstrated that *Proteobacteria* (Control = 63.84%±9.20 and 2% DBPH = 62.47%±1.00), *Fusobacteriota* (Control = 14.63%±3.55 and 2% DBPH = 26.75%±0.26), *Firmicutes* (Control = 10.34%±5.01 and 2% DBPH = 7.03%±0.54), *Bacteroidota* (Control = 4.36%±2.34 and 2% DBPH = 1.42%±0.12), and *Actinobacteriota* (Control = 0.78%±0.18 and 2% DBPH = 0.22%±0.01) phyla were the most abundant in these experimental groups. *Aeromonas*, *Cetobacterium*, *Romboutsia*, Clostridium_sensu_stricto_1, *unclassified_Barnesiellaceae, unclassified_Peptostreptococcaceae, Crenobacter, Plesiomonas, Terrisporobacter*, and *Shewanella* were the most plentiful at the genus level (Figure 4.3B). In addition, four genera, including *Cetobacterium, Romboutsia, Crenobacter*, and *Shewanella*, showed significant differences between the control group and the 2% DBPH group, as shown in Figure 4.3C. The beta diversity analysis presented by PCA plot illustrates the variation in microbial community composition in flowerhorn cichlids fed different diets, comparing the control group (C) with the treatment group (T) fed diets supplemented with DBPH. PC1 (Principal Component 1): Explains 74.77% of the variation. PC2 (Principal Component 2): Explains 24.73% of the variation. Group Clustering Control Group (C), represented by blue, orange, and green dots. Encircled by a red ellipse. Shows considerable dispersion along both PC1 and PC2 axes, indicating variability within the control samples. Treatment Group (T), represented by red, purple, and brown dots. Encircled by a green ellipse. Clusters tightly together, indicating less variability and a distinct microbial community composition compared to the control group. The separation between the control and treatment groups along PC1 suggests significant differences in their microbial communities.

The group DBPH diet forms a distinct cluster, indicating a more uniform and consistent microbial composition in the gut of fish fed DBPH. The PCA plot demonstrates that the dietary inclusion of defatted DBPH significantly alters the gut microbiota of flowerhorn cichlids, resulting in a distinct and more uniform microbial community compared to the control group show in Figure 4.4

Table 4.3 Effects of flowerhorn fish fed 2% DBPH for 30 days on the diversity of the microbiome in the intestinal tract.

	Treatments	
	Control	**2% DBPH**
Raw read	80137±125.08	80009±91.26
Clean read	72201±305.31	72084±362.86
Tags	71203±382.78	71558±362.41
OTU	562.00±35.25[a]	316.67±15.86[b]
Chao1	563.95[a]	318.20[b]
Shannon	4.15[a]	3.14[b]
Simpson	0.82	0.74

Abbreviations: DBPH = low molecular weight duck blood protein hydrolysate. Data are presented as mean±SEM. Means in the same row sharing different superscripts were significantly different as determined by an independent-sample t-test at the significance level accepted at ($P < 0.05$).

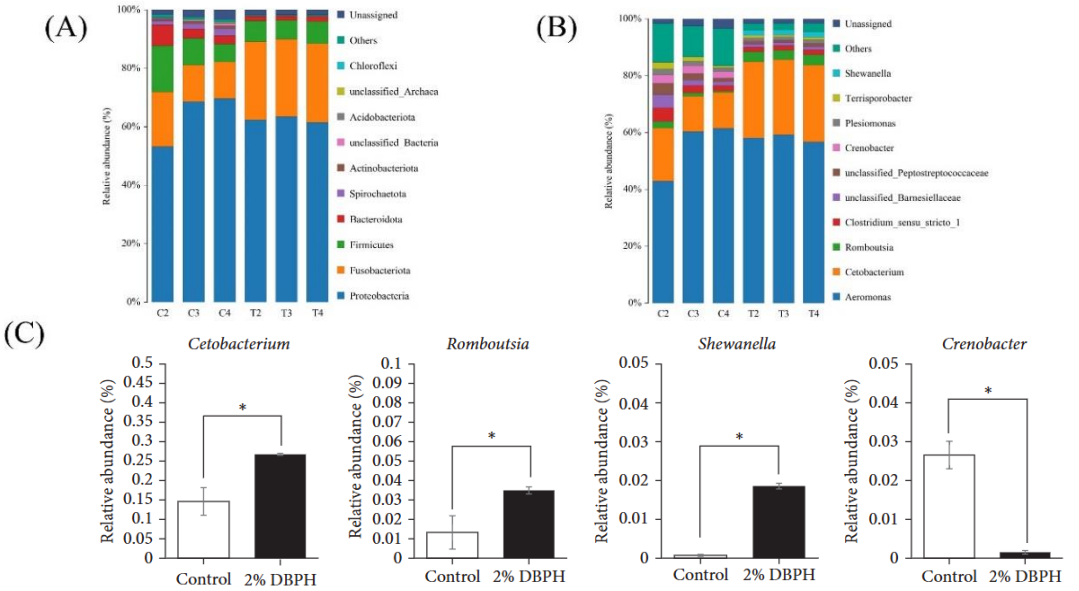

Figure 4.3 Intestinal microbiota composition of flowerhorn fish fed with the control diet and 2% DBPH for 30 days. Taxonomic distribution at phylum level (A), Taxonomic distribution at genus (B), and Abundance of significant intestinal bacteria communities at the genus level (C).

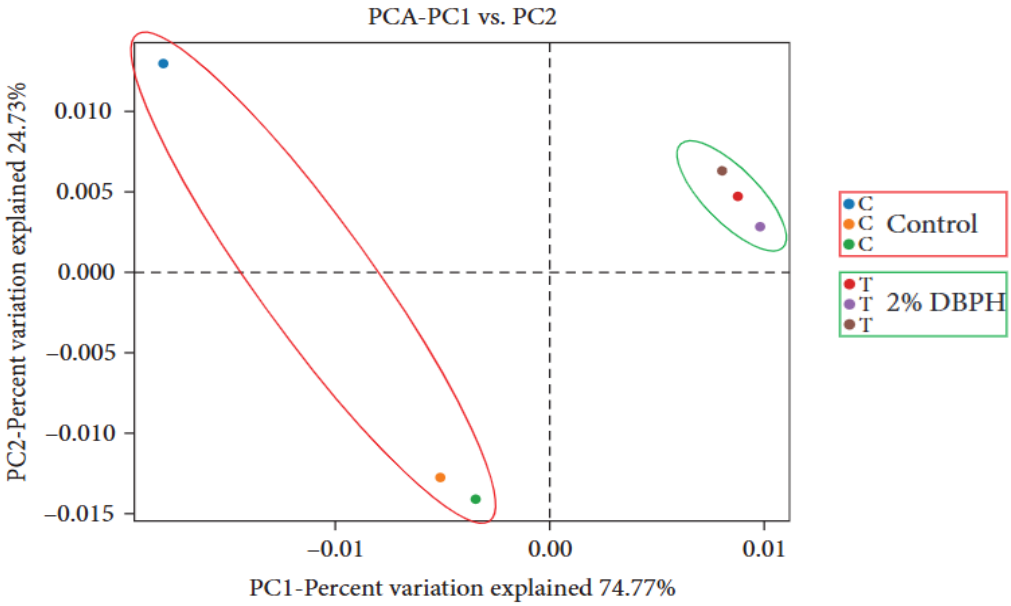

Figure 4.4 Principal component analysis (PCA) based on distances of intestinal bacteria communities of flowerhorn fish fed with the control diet and 2% DBPH for 30 days.

4.6 Antioxidant gene expression in response to *S. agalactiae*

Regarding antioxidant gene expression, catalase (CAT) and superoxide dismutase (SOD) mRNA levels in the liver of fish fed with dietary supplemen-tation with 2% DBPH for 30 days (prechallenge) increased incomparison to the negative control group. Moreover, at 24 hr postinjection, the expression of CAT and SOD in response to the infection was still significantly higher than in the control group. However, the expression levels of SOD and CAT mRNA in the liver in all groups were significantly increasedat 24 hr postinjection compared to the baseline (prechallenge; Figure 4.5 A, B)

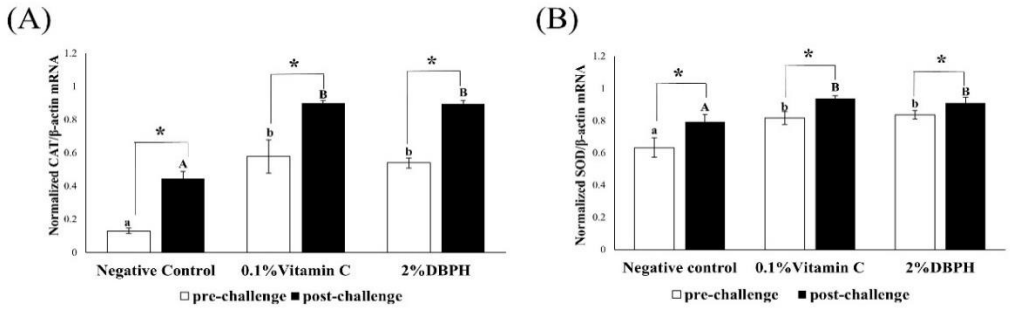

Figure 4.5 Quantitative real-time PCR analysis of CAT (A) and SOD (B) expression in the liver of flowerhorn fish after the 30-day feeding trial. Bars with asterisks indicate significant differences between pre- and post-challenge. Bars labeled with different lowercase letters denote significant differences at the pre-challenge stage, while bars labeled with uppercase letters indicate significant differences at 24 h post-challenge ($P < 0.05$).

4.7 Inflammatory gene expression in response to *S. agalactiae*

After 30 days of the feeding trial (pre-challenge), the expression of inflammatory genes, including IL-1β, IL-6, CC, and CXC chemokine, was investigated Figure 4.6. There was no significant difference in the mRNA expression levels in the liver and spleen between the experimental groups except for CC chemokine in the spleen, which showed higher up-regulation in 2% DBPH and vitamin C groups compared to the negative control group Figure 4.6H.

After 24 hr post-challenge, IL-1β, CXC, and CC chemokine mRNA expression levels increased in both spleen and liver tissues, with greater up-regulation in the 2% DBPH and vitamin C groups compared to the negative control group. However, the IL-6 expression

level was upregulated only in the spleen Figure 4.6D. In comparing individual treatments between pre-and post-challenge, significantly higher expression levels of IL-1β, CXC, and CC chemokine were found in both the spleen and liver across all treatments (Figure 4.6A, E–H), except for IL-1 β levels in the negative control group (Figure 4.6B). However, no significant difference was found in IL-6 levels in all treatments in the liver (Figure 4.6C).

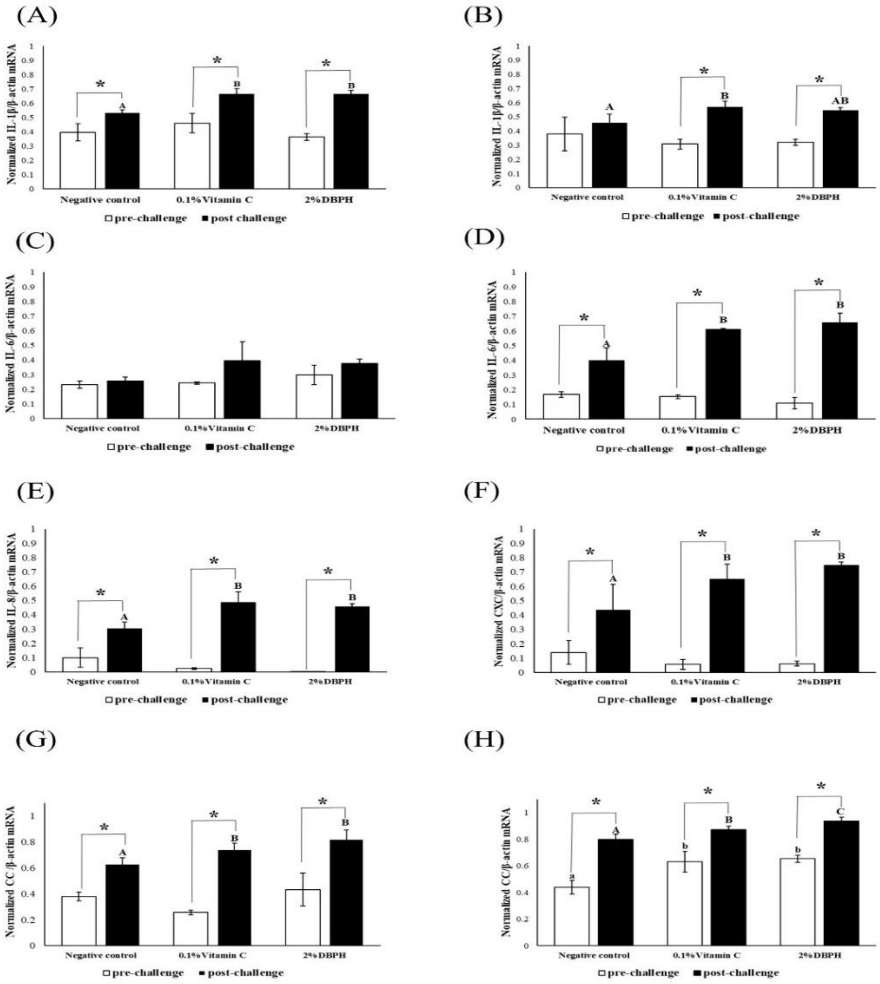

Figure 4.6 Quantitative real-time PCR analysis of IL-1β, IL-6, CC, and CXC chemokine expression in the liver (A, C, E, G) and IL-1β, IL-6, CC, and CXC chemokine expression in the spleen (B, D, F, H) of flowerhorn fish after the 30-day feeding trial. Bars with asterisks indicate significant differences between pre- and post-challenge. Bars labeled with different lowercase letters denote significant differences at the pre-challenge stage, while bars labeled with uppercase letters indicate significant differences at 24 h post-challenge ($P < 0.05$).