EFFECTS OF LOW MOLECULAR WEIGHT DUCK BLOOD PROTEIN HYDROLYSATE AS A FEED ADDITIVE ON THE INTESTINAL MICROBIOME, ANTIOXIDANT ACTIVITY, AND HUMORAL IMMUNE RESPONSES IN FLOWERHORN FISH

PIMPISUT MANASSILA

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ผลของการเสริมโปรตีนไฮโดรไลเสตจากเลือดเป็ดที่มีน้ำหนักโมเลกุลต่ำในอาหารต่อประชากรจุลินทรีย์ในลำไส้ กิจกรรมต้านอนุมูลอิสระ และการตอบสนองภูมิคุ้มกันของปลาหมอสีฟลาวเวอร์ฮอร์น

นางสาวพิมพ์พิสุทธิ์ มนัสศิลา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพสำหรับการผลิตสัตว์น้ำ
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's degree.

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พิมพ์พิสุทธิ์ มนัสศิลา : ผลของการเสริมโปรตีนไฮโดรไลเสตจากเลือดเป็ดที่มีน้ำหนักโมเลกุลต่ำในอาหารต่อประชากรจุลินทรีย์ในลำไส้ กิจกรรมต้านอนุมูลอิสระ และการตอบสนองภูมิคุ้มกันของปลาหมอสีฟลาวเวอร์ฮอร์น (EFFECTS OF LOW MOLECULAR WEIGHT DUCK BLOOD PROTEIN HYDROLYSATE AS A FEED ADDITIVE ON THE INTESTINAL MICROBIOME, ANTIOXIDANT ACTIVITY, AND HUMORAL IMMUNE RESPONSES IN FLOWERHORN FISH) อาจารย์ที่ปรึกษา: ผู้ช่วยศาสตราจารย์ ดร.ฉัตรศิรินทร์ นาคหฤทัย, 76 หน้า.

คำสำคัญ: เลือดเป็ด/โปรตีนไฮโดรไลเสต/ไมโครไบโอม/ระบบภูมิคุ้มกัน/สารต้านอนุมูลอิสระ

เปปไทด์ออกฤทธิ์ทางชีวภาพที่ได้จากอาหารสามารถใช้เป็นวัตถุดิบหรือสารเสริมในอาหารสัตว์ได้ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาคุณสมบัติในการส่งเสริมสุขภาพของโปรตีนไฮโดรไลเสตจากเลือดเป็ดที่มีน้ำหนักโมเลกุลต่ำ ซึ่งทำการแยกขนาดด้วยการกรองผ่านเมมเบรนขนาดโมเลกุล 10 กิโลดาลตัน ในปลาหมอสีฟลาวเวอร์ฮอร์น ผลการวิเคราะห์การกระจายตัวของน้ำหนักโมเลกุลพบว่า โปรตีนไฮโดรไลเสตจากเลือดเป็ดส่วนใหญ่อยู่ในช่วง 3-7 กิโลดาลตัน (39.68%) รองลงมาคือ >7-10 กิโลดาลตัน (20.69%), 1-3 กิโลดาลตัน (23.03%) และ <1 กิโลดาลตัน (9.00%) หลังจากการทดลองให้อาหารเป็นเวลา 1 เดือน พบว่าปลาที่ได้รับอาหารเสริมโปรตีนไฮโดรไลเสตจากเลือดเป็ดที่มีน้ำหนักโมเลกุลต่ำที่ระดับ 2% ของน้ำหนักอาหาร มีการเจริญเติบโต การแสดงออกของเอนไซม์ต้านอนุมูลอิสระ และการตอบสนองของภูมิคุ้มกันเพิ่มขึ้นภายใต้สภาวะปกติ นอกจากนี้การวิเคราะห์ไมโครไบโอมยืนยันว่า โปรตีนไฮโดรไลเสตจากเลือดเป็ดที่ระดับ 2% มีฤทธิ์ต้านจุลชีพ โดยพบว่าหน่วยอนุกรมวิธานปฏิบัติการ (Operational taxonomic unit; OTUs) และดัชนีประเมินความหลากหลายทางชีวภาพของจุลชีพภายในกลุ่ม (alpha diversity) ได้แก่ Chao1 และ Shannonลดลงอย่างมีนัยสำคัญ เมื่อเปรียบเทียบกับกลุ่มควบคุมพบว่าปลาที่ได้รับโปรตีนไฮโดรไลเสตจากเลือดเป็ดที่มีน้ำหนักโมเลกุลต่ำที่ระดับ 2% ในอาหาร มีการเพิ่มขึ้นของแบคทีเรียสกุล *Cetobacterium* และ *Romboutsia* อย่างมีนัยสำคัญ ซึ่งสามารถใช้เป็นดัชนีบ่งชี้ด้านสุขภาพโดยรวมที่ดีของปลาได้หลังจากการทดสอบความต้านทานต่อเชื้อ *Streptococcus agalactiae* พบว่าปลาที่ได้รับอาหารเสริมโปรตีนไฮโดรไลเสตจากเลือดเป็ดที่ระดับ 2% ในอาหารมีความสามารถในการควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการอักเสบ ได้แก่ IL-1β, IL-6, CC และ CXC chemokine รวมถึงยีนที่เกี่ยวข้องกับเอนไซม์ต้านอนุมูลอิสระ (SOD และ CAT) ได้ดีขึ้น ดังนั้นการเสริมโปรตีนไฮโดรไลเสตจากเลือดเป็ด 2% ในอาหาร

สามารถส่งเสริมสุขภาพโดยรวมของปลาหมอสีฟลาวเวอร์ฮอร์นได้ โดยช่วยกระตุ้นภูมิคุ้มกัน ลดความเครียดจากปฏิกิริยาออกซิเดชัน และเพิ่มความต้านทานต่อเชื้อ *S. agalactiae*

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา _____

PIMPISUT MANASSILA : EFFECTS OF LOW MOLECULAR WEIGHT DUCK BLOOD PROTEIN HYDROLYSATE AS A FEED ADDITIVE ON THE INTESTINAL MICROBIOME, ANTIOXIDANT ACTIVITY, AND HUMORAL IMMUNE RESPONSES IN FLOWERHORN FISH. THESIS ADVISOR : Asst. PROF. CHATSIRIN NAKHARUTHAI, Ph. D., 76 PP.

Keyword: Duck blood/Protein hydrolysate/Microbiome/Immune response/Antioxidant

Food-derived bioactive peptides could serve as feed ingredients and/or feed additives. We investigated the health-promoting properties of low molecular weight duck blood protein hydrolysate (DBPH), fractionated by ultrafiltration with a 10 kDa molecular weight cut-off membrane in flowerhorn fish. The analysis of molecular weight distribution revealed that the most common sizes of DBPH fell within the range of 3-7 kDa (39.68%), followed by >7-10 kDa (20.69%), 1-3 kDa (23.03%), and <1 kDa (9.00%). After one month of the feeding trial, fish fed with diets supplemented with 2% DBPH exhibited the highest growth, antioxidant activity, and humoral immune response enhancement under normal conditions. In addition, microbiome analysis confirmed that 2% DBPH possesses antimicrobial activity, as evidenced by the significant decrease in operational taxonomic units (OTUs) and alpha diversity indexes, including Chao1 and Shannon. Compared to the control group, fishes that were fed with diets supplemented with 2% DBPH exhibited a significantly higher abundance of the genera *Cetobacterium* and *Romboutsia*, which could serve as indicators of the overall health and well-being of the fish. After a *Streptococcus agalactiae* challenge, fish fed with diets supplemented with 2% DBPH exhibited an enhanced ability to modulate inflammatory genes, including IL-1β, IL-6, CC, and CXC chemokine as well as antioxidant gene expression (SOD and CAT). Overall, dietary supplementation with 2% DBPH could improve the overall health of the flowerhorn fish by ameliorating humoral immune response, alleviating oxidative stress and strengthening resistance against *S. agalactiae*.

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LIST OF ABBREVIATIONS

%	=	Percent
×g	=	Relative centrifugal force
°C	=	Degree celsius
µ	=	Micro
ABTS	=	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
DPPH	=	1,1-diphenyl-2-picrylhydrazy
AMPs	=	Antimicrobial Peptides
B cell	=	B lymphocyte
CAT	=	Catalase
CC	=	Chemokine (C–C motif)
cDNA	=	Complementary DNA
CXC	=	Chemokine (C–X–C motif)
DBPH	=	Duck blood Protein hydrolysates
DEPC	=	Diethyl pyrocarbonate
DNA	=	Deoxyribonucleic acid
EDTA	=	Ethylenediaminetetraacetic acid
hr	=	Hour
Ig	=	Immunoglobulin
IgA	=	Immunoglobulin A
IL-1	=	Interleukin-1
IL-6	=	Interleukin-6
m	=	Milli
M	=	Molar
MAD	=	Malondialdehyde
min	=	Minute
mRNA	=	Messenger RNA
N.S	=	Not Available
NaCl	=	Sodium saline

LIST OF ABBREVIATIONS (Continued)

NLRs	=	NOD-like Receptors (Nucleotide-binding Oligomerization Domain-like Receptors)
OTUs	=	Operational Taxonomic Units
PAMPs	=	Pathogen-associated molecular patterns
PCR	=	Polymerase chain reaction
pH	=	Potential of hydrogen ion
PRRs	=	Pattern Recognition Receptors
q	=	Quantitative
qRT-PCR	=	Quantitative real-time PCR
RGR	=	Relative growth rate
RNA	=	Ribonucleic acid
RNase	=	ribonuclease
ROS	=	Reactive oxygen species
rRNA	=	Ribosomal RNA
SCFAs	=	Short-Chain Fatty Acids
SD	=	Standard deviation
Seq	=	Sequencing
SR	=	Survival rate
SOD	=	Superoxide Dismutase
spp.	=	Species pluralis
SUT	=	Suranaree University of Technology
T cell	=	T lymphocyte
TLRs	=	Toll-like Receptors
TNFα	=	Tumor necrosis alpha
WGR	=	Weight gain
β-actin	=	Beta-actin
MHC	=	major histocompatibility complex
O_2^-	=	Superoxide anion
H_2O_2	=	Hydrogen peroxide

LIST OF ABBREVIATIONS (Continued)

O_2		molecular oxygen
Cu/Zn-SOD	=	Cytosolic
Mn-SOD	=	Mitochondria

CHAPTER I

INTRODUCTION

1.1 Background and Significance

Recently, the price of feed ingredients has begun an unprecedented escalation, leading to an unprecedented rise in feed costs. Using alternative feed materials, particularly food and feed by-products, would enable the reduction of feed costs. Food-derived bioactive peptides could provide feed ingredients and/or feed additives. In Thailand, the duck meat production yield was approximately 74,700 metric tons in 2022 (Statista Search Department, 2023), with duck blood being one of the main byproducts. Duck blood contains a richness of essential amino acids, heme iron, and other macro- and micronutrients and can be utilized as an inexpensive protein source with a high percentage of protein when compared to chicken and bovine sources (Sorapukdee and Narunatsopanon, 2017). For these reasons, duck blood byproduct is considered a beneficial protein source whose value increases through hydrolysis as a feed additive in animal diets.

Protein hydrolysates derived from animal byproducts have been reported to be a feasible alternative source of high-quality protein in the diets of the livestock and aquaculture industry sectors (Hou et al., 2017; Nikoo et al., 2023). In addition to providing essential nutrient sources and growth factors, protein hydrolysate is also recognized as a value-added product due to its functionalities. The hydrolysis process breaks down proteins into peptides of varying sizes, making them easier to absorb compared to native proteins. Peptides are indeed common products of protein digestion that can enter enterocytes through the peptide transport system according to metabolic physiology. In addition, some peptides also generate a bioactive activity by stimulating the gastrointestinal tract and the immune system to further exert a broad spectrum of functions, including immunomodulatory, antioxidant, anti-inflammatory, and antimicrobial properties depending on their sequence and amino acid composition (Kiewiet et al., 2018; Siddik et al., 2020).

Typically, bioactive peptides refer to low molecular weight peptides ranging in size from 2 to 20 amino acid residues, although they can sometimes be larger. In general, it is commonly reported in the literature that potent bioactive peptides typically have a molecular weight below 10 kDa. Since protein hydrolysate consists not only of valuable functional ingredients but also possesses health-enhancing properties, it has been well-demonstrated that it boosts productivity and performance, disease resistance, and immune responses of many fish species, such as common carp (Carvalho et al., 1997), Japanese sea bass (Liang et al., 1997), large yellow croaker (Tang et al., 2008), Japanese flounder (Zheng et al., 2014), barramundi (Chaklader et al., 2020), Gilthead sea bream (Gisbert et al., 2021), and Nile tilapia (Rahman et al., 2023). Although the hydrolysis process increases the price of value-added protein hydrolysate products, it still reached the break-even point for the ornamental fish business due to the high market value of the ornamental fish industry.

The global ornamental fish market is valued at approximately USD 15–30 billion each year and is expected to increase continuously (Evers et al., 2019). Consequently, demand for feed additives for ornamental fish has increased. Among ornamental fish species, the flowerhorn fish has emerged as one of the most popular aquarium ornamental fish in the world since it first appeared in 1996 (Lin et al. 2008). The price of flowerhorn fish is determined by the uniqueness of the type of fish regarding its size, color, attributes, rarity, individual consumer preferences, healthiness, and its demand in different regional markets. Generally, flowerhorn fish are highly territorial and aggressive, especially in confined spaces. They are known to attack other fish, making them difficult to keep in a community tank. Indeed, flowerhorn fish often suffer from disease, and the requirement of dietary prophylaxis in flowerhorn fish is necessary.

Recently, numerous studies have investigated the effects of protein hydrolysate on the health of fish, as well as its effect on gut microbiota. Some research has reported that protein hydrolysates can interact with the microbiota in the fish gut, thus enhancing the epithelial barrier and nutrient absorption, and exerting antimicrobial activity by promoting the release of mucus in the intestinal tract (Kiewiet et al., 2017; Gao et al., 2023). Moreover, the administration of protein hydrolysate can improve the development of the immune system, augmenting immunoglobulin and cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and IL-10 production in

juvenile barramundi Lates calcarifer (Siddik et al., 2020). To date, several scientific studies have demonstrated that antimicrobial and antioxidant activities were found in animal blood protein hydrolysates, such as those from bovine, chicken, and porcine sources (Chang et al., 2007; Wang et al., 2008). Research on low molecular weight duck blood protein hydrolysate and their application in the ornamental aquaculture field is required. The development of duck blood byproducts as a source of protein and bioactive peptides in the ornamental fish industry through the application of protein hydrolysate technology is a promising and reliable strategy for promoting the health and immunity of fish.

Therefore, this study aimed to investigate the optimal level of DBPH as a feed additive in a commercially practical diet and its effects on the intestinal microbiome, humoral immune response, and antioxidant activity in flowerhorn fish. In addition, after one month of feeding trials, the fish were intraperitoneally injected with *S. agalactiae*, a significant bacterial pathogen affecting various freshwater fish species, particularly under stress conditions. The expression of antioxidant and inflammatory genes was then analyzed to evaluate the immune response's effectiveness in combating the harmful bacterial infection.

1.2 Research objectives

The objectives of this study were:

1.2.1 To investigate the effects of low molecular weight duck blood protein hydrolysate as a feed additive on growth, innate immune responses, antioxidant status, inflammatory response, and gene expression in flowerhorn fish.

1.2.2 To investigate the effects of low molecular weight duck blood protein hydrolysate as a feed additive on resistance to *Streptococcus agalactiae* infection in flowerhorn fish.

1.2.3 To investigate the effects of low molecular weight duck blood protein hydrolysate as a feed additive on the intestinal microbiome in flowerhorn fish.

1.3 Research hypothesis

1.3.1 Dietary supplementation with low molecular weight DBPH enhances innate immune responses, antioxidant activity, and modulates gene expression related

to immunity and inflammation in flowerhorn fish.

1.3.2 Dietary supplementation with DBPH improves resistance to *Streptococcus agalactiae* infection in flowerhorn fish.

1.3.3 Dietary supplementation with DBPH positively alters the composition and diversity of the intestinal microbiome in flowerhorn fish, promoting beneficial bacterial populations and enhancing gut health.

1.4 Scope and limitation of this study

This research focuses on increasing the value of underutilized poultry byproducts, specifically duck blood from the poultry processing industry. The study explored the development of duck blood protein hydrolysate as a valuable feed additive in aquaculture, aiming to enhance the value of this resource and promote zero-waste practices. Flowerhorn fish were fed a commercial diet + 0.85% NaCl as a control group, a commercial diet + DBPH at levels of 0.5%, 1%, and 2%, the fish were challenged with *S. agalactiae*. Immune-related parameters were examined to understand the genes associated with crucial immune responses and the antioxidant activity of fish.

1.5 Expected benefits

This study is expected to demonstrate that dietary supplementation with 2% low-molecular-weight duck blood protein hydrolysate (DBPH) can enhance the overall health of flowerhorn fish. Anticipated benefits include improved growth performance, stronger immune and antioxidant responses, and beneficial changes in gut microbiota specifically increased *Cetobacterium* and *Romboutsia*. The fish are also expected to show greater resistance to *S. agalactiae* infection. These results would support the application of DBPH as a functional feed additive for promoting health and disease resistance in ornamental aquaculture species.

CHAPTER II

LITERATURE REVIEW

2.1 Biology of Flowerhorn Cichlid

Figure 2.1 Flowerhorn fish Aquarium. (2024, August 20). Flowerhorn Cichlid Care Retrieved from https://www.blessingsaquarium.com/post/flowerhorn-cichlid-care.

Taxonomic Classification of Flowerhorn Fish

Kingdom: Animalia

Phylum Chordata

Class Actinopterygii

Order Cichliformes

Family Cichlidae (Cichlids)

Subfamily Cichlinae

Genus Cichlasoma

Species Flowerhorn Cichlid (Sanders, 2022)

Common name: Flowerhorn Cichlid

Flowerhorn cichlid *(Amphilophus hybrid*), a member of the cichlidae family, has been one of the most popular ornamental fish over the past decade. In 1993, the

flowerhorn cichlid was first successfully bred as a hybrid ornamental fish species by a fish enthusiast in Malaysia. It is believed that the flowerhorn originated from the crossbreeding of a blood parrot cichlid (*Amphilophus citrinellus* × *Vieja melanurus*) (Nico et al. 2007; McMahan 2010) and a red devil cichlid (*Amphilophus labiatus*), as these species exhibit similar morphological features and originate from the same geographic region. However, this origin has not been conclusively established. The name “flowerhorn” is derived from 2 words: “flower” refers to the dark spots that lay along its body's lateral lines and “horn” refers to the nuchal hump located on its head. The first generation of flowerhorn crossbreeding is hua luo han cichlids, also known as luohans, followed by the emergence of other flowerhorn crossbreeding cichlids with strikingly different patterns depending on their unique type, such as king kong parrot, super red monkey, golden monkey, kamfa, short body, zhen zhu, thai silk, and albino flowerhorn. Subsequently, the flowerhorn crossbreed was initially introduced to Asia, including Malaysia, Thailand, Taiwan, and China, before being brought to America and Europe. since then, it has gained considerable economic importance.

2.2 Characteristics of Flowerhorn Cichlid

Flowerhorn is a well-known ornamental fish belonging to the Cichlidae family with vivid color, nuchal humps (Kok) on the head (more prominent in males), and a black dotted pattern (marking) on the body's middle. The flowerhorn color has a variety of patterns, including plain, bicolored, and multicolored forms, with red, pink, orange, yellow, and white being the most prominent colors, especially from the middle of the body to the head. It is a tropical freshwater species that has a round or oval shape, a symmetrical body, a dorsal fin with a hard spine and a long soft running along the back, a relatively long anal fin, and a fan-shaped caudal fin. Pectoral fins are small, soft rays, and two ventral fins are composed of a hard and a soft ray (Sari et al., 2023). It has teeth in the mouth and the throat that help to break down food. The expected lifespan ranges from 10 to 12 years. The length of an adult flowerhorn can reach 14 to 16 inches, with variations observed among different strains. The preferred water temperature of flowerhorn is between 26 and 30°C, and a neutral pH value of 6-8. It is an omnivorous fish that can feed on a wide range of food types such as phytoplankton,

zooplankton, detritus, aquatic plants, and prefers a type of live feed such as brine shrimp, bloodworms, insect larvae, etc. The appropriate aquarium size for flowerhorn is at least 55 gallons (approximately 200 liters), although the required volume may vary depending on the size of the fish. Due to their strong territorial behavior and aggressive nature, flowerhorns are not suitable for community aquariums. Flowerhorns should be housed individually, or a tank divider should be installed if they must share the same aquarium. Males and females can be distinguished by several characteristics; for example, males typically have a prominent horn on the head, are larger in size, and display more vivid colors than females. In addition, the male's vent or genital papillae has a V-shaped opening, whereas the females have a U-shaped opening.

2.3 The Global Ornamental Fish

Ornamental fish are species reared primarily for decorative purposes in aquariums and ponds due to their attractive colors, patterns, and distinctive characteristics. Cichlids, guppies, goldfish, and bettas are among the most commonly kept ornamental fish species, reflecting the global popularity of fishkeeping as a recreational activity. For this reason, the ornamental fish industry represents a multimillion-dollar global market encompassing breeding, production, and distribution processes. Currently, ornamental aquatic animals are considered a growing trend in pet ownership, and the global trade in aquarium species has continued to increase annually, as illustrated in Figure 2.2 (Selvarasu and Sankaran 2012; Allen et al. 2017; Novak et al., 2020).

The number of ornamental freshwater fish species (over 6,500 species) recorded up to 2019 was categorized according to the standard classification outlined in the review by Novak et al. (2020), as shown in Table 2.1.

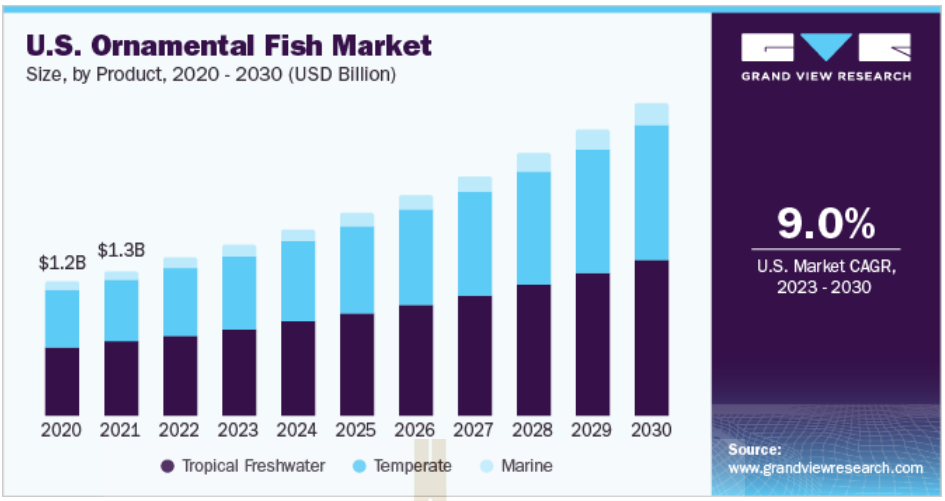

Figure 2.2 The Global Ornamental Fish Market. FQA (2021). The Global Ornamental Fish Market. Retrieved from https://www.grandviewresearch.com/industryanalysis/ornamental-fish-market.

Table 2.1 Classification of ornamental freshwater fish species.

Ordinal Number	Species	Order
i	'catfish'	Siluriformes
ii	'characids'	Characiformes
iii	'cichlids'	Cichliformes
iv	'cyprinids'	Cypriniformes
v	'killifish'	Both egg-laying taxa from orders Cyprinodontiformes: families Aplocheilidae, Cyprinodontidae, Fundulidae, Nothobranchiidae, Profundulidae, Rivulidae, Valenciidae; and Beloniformes: family Adrianichthyidae
vi	'labyrinth fish'	Taxa with an additional developed respiratory organ, known as a labyrinth, from the order Anabantiformes: families Anabantidae, Channidae, Helostomatidae, Osphronemidae
vii	'livebearers'	Both viviparous and ovoviviparous taxa from orders Cyprinodontiformes: families Anablepidae, Goodeidae, Poeciliidae; and Beloniformes: family Hemiramphidae
viii	'rainbowfish'	Atheriniformes: families Bedotiidae, Melanotaeiniidae, Pseudomugilidae, Telmatherinidae);
ix	'Other fish'	Taxa from groups not mentioned above

In 2022, the global trade value of ornamental fish reached USD 5.88 billion, with tropical freshwater ornamental fish making up more than 90% of that total (Ornamental Fish Market Size and Share Analysis Report, 2022). Most of them are captive-bred, although some are harvested from the wild (Raghavan et al., 2013). Japan is the top ornamental fish exporting country with the highest value in the world at 41.2 million US dollars, followed by Singapore, Indonesia, Sri Lanka, and Thailand as shown in Figure 2.3. In Thailand, the number of aquatic ornamental animals exported reached 85,406,621 units in 2019, with a total export value of 680,083,889 baht and the top importers of ornamental fish from Thailand are the United States, the United Kingdom, Germany, China, and Japan (Department of Fisheries, 2019).

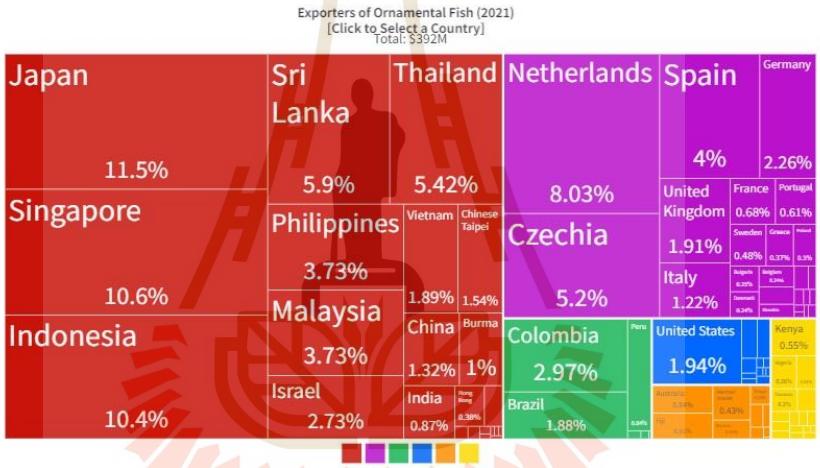

Figure 2.3 Exporters of Ornamental Fish. OEC. (2025, May 26). The Observatory of Economic Complexity. Retrieved from https://oec.world/en/profile/hs/freshwater-ornamental-fish.

Among the ornamental freshwater fish, flowerhorn is one of the most popular aquarium ornamental fish among ornamental freshwater fish. The price of flowerhorn could depend on the uniqueness of the type of fish, sizes, colors, attributes, rarity, individual consumer preferences, and the consumer's demand in the different regional markets. Generally, the rounded and large size of the nuchal hump, vivid colors, and well-balanced body are the most desirable to consumers and can increase their price, whereas deformed-shaped fish are typically less valuable (Mutia et al. 2007; Nico et al.

2007; Ng 2016). In China, for instance, flowerhorn is regarded as a symbol of good luck, fortune, health, and prosperity, particularly when they display markings on their bodies that resemble Chinese lettering, which can increase their value depending on their meaning. In 2009, the most expensive flowerhorn cichlid in the world was recorded at 600,000 USD in Malaysia. Currently, the price of flowerhorn ranges from 30 to 300 USD, with an average online cost of approximately 100 USD.

2.4 Nutritional requirements and demand in ornamental fish

In recent years, the ornamental fish farming industry has become increasingly professionalized. Consequently, 50-70 percent of the production chain's costs are attributable to aquafeed for supporting the exponential growth of this industry. This business relies heavily on high-quality protein from commercial and live feed, which serve as major nutrient sources essential for the development, growth, and health of ornamental fish. Similar to livestock species, proteins serve as the basic building elements for cellular architecture, the regulation of a variety of biological processes, and molecular transport (Gatta, 2022). The State of World Fisheries and Aquaculture 2022. In FAO. It is one of the most vital components with the highest value in aquafeed, therefore, it is an important factor in determining the cost of an ornamental fish's diet. Fishmeal has long been the preferred protein source in the commercial diets of various aquatic animals (De Silva et al., 2011). Because it is rich in essential bioactive compounds such as choline, taurine, and anserine which serve vital functions such as stabilizing protein structures, safeguarding cells against osmotic stresses, preventing oxidative damage, and enhancing the immune system in animals including swine, poultry, and fish (Kuzmina et al., 2010; Yun et al., 2011). However, due to climate change, overfishing, and diminishing ocean fishery stocks, fishmeal as a raw material protein source in commercial aquafeed has steadily decreased, resulting in a limited supply of raw material protein sources and an increase in fishmeal prices.

In addition, it is crucial to note that fish larvae fed a commercial diet face numerous limitations due to their inefficient nutrient digestion and assimilation. In the first month after hatching, larvae encounter challenges as their stomachs are not fully developed, and the digestive enzyme activity of their pancreas remains low compared to adult fish (Yúfera and Darias, 2007).

Table 2.2 The size of live feed organisms.

Species	Dimension
Artemia	0.2-0.5 mm
Rotifers	0.04-0.5 mm
Copepods	0.038–0.22 mm
commercial feed	0.1-1 mm

Retrieved from: (Wuller et al., 2009; Stottrup et al., 2003).

Live feed is an important diet for newly hatched fish after the absence of the yolk sac because it is a natural living nutrition containing high protein and other essential nutrients for fish larvae. The movement of live feed in the water column can constantly stimulate the feeding behavior of fish larvae (David 2003). In addition, the mouth size of fish larvae is a significant factor in determining the feed sizes that can be eaten. In their digestive system, the larvae are still lacking in enzymes for digestion. Therefore, they are not fully able to break down food for absorption into the bloodstream. Although live feeds have been shown to significantly enhance the growth and survival of fish, their availability is often limited during certain seasons. Furthermore, if not properly sanitized, live feeds may serve as vectors for pathogens. In addition, commercial production of live feed is currently unavailable due to the increasing demand associated with intensive aquaculture. Therefore, additional research on alternative protein sources is necessary to create opportunities and sustainability for their further practical application.

2.5 The digestion and absorption of proteins in teleost

Protein digestion involves the breakdown of large protein molecules into smaller peptide chains through luminal digestion by proteolytic enzymes. Digestion begins in the stomach since teleost fish do not have salivary glands like mammals or birds. Consequently, the fish's mouth serves primarily a mechanical role in capturing. Different chemicals, such as Dipterex (Organophosphate insecticide), hydrogen peroxide, potassium permanganate, formalin, and salt, can be used to treat parasites. and processing food rather than in digestion (Digestive Enzymes in Fish Veterinaria Digital, 2020). In teleost, the stomach serves crucial functions through both mechanical (contraction-expansion) and

chemical processes. It grinds and breaks down large feed particles through these processes. In fish stomachs, a unique type of secretory cell within the gastric glands carries out both pepsinogen and acid-secreting functions. This contrasts with mammals, where separate cells are specialized for these functions (Wilson and Castro, 2011).

Proteins are initially broken down into polypeptides by proteases. Endopeptidases such as trypsin, chymotrypsin, and elastase further hydrolyze polypeptides into smaller peptides, including oligopeptides containing fewer than ten amino acids, in the intestine. This enzymatic digestion occurs in a slightly alkaline environment maintained by pancreatic secretions of bicarbonate, while bile acids from the gall bladder primarily aid in fat digestion (Deguara et al., 2003; Fard et al., 2007) in Table 2.3. Constrained by the limited space of the coelomic cavity, the intestinal surface area is optimized to enhance nutrient absorption. This optimization is primarily achieved through the amplification of the apical plasma membrane and the folding of the mucosa, both of which substantially increase the absorptive surface area The main cell types found in the fish gut include enterocytes, mucous cells, and enteroendocrinecells across all species. Additionally, rodlet cells are present in Most teleost, whereas ciliated and zymogen cells are typically observwd in elasmobranchs (Wilson and Castro, 2011; Alesci et al., 2022).

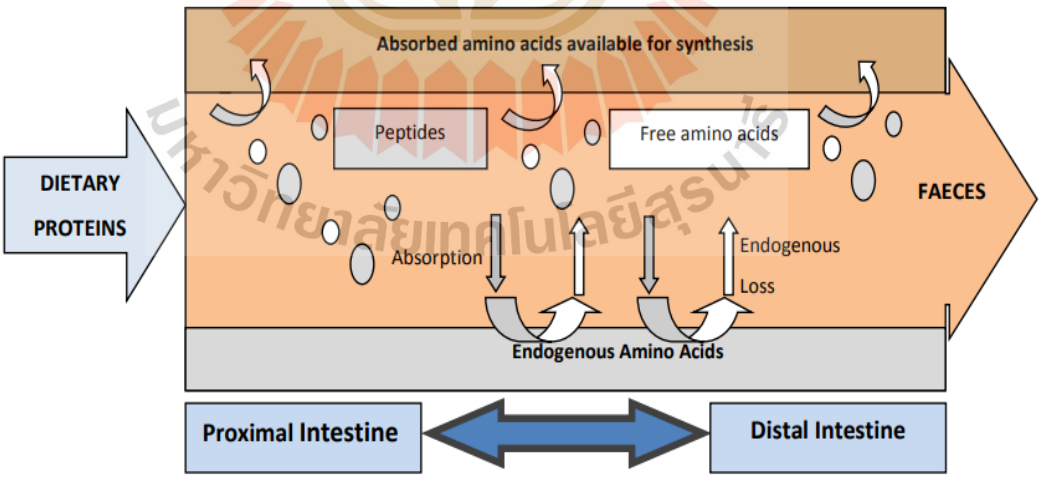

Figure 2.4 A conceptual diagram of digestion and absorption of dietary proteins in intestine of fish. (Chowdhury 2012).

Table 2.3 Major enzymes in fish for the digestion of protein, peptides, amino acids and other non-nitrogenous compounds.

Organ source	Enzyme	Substrate	Specificity
Stomach	Pepsins (pepsinogens)	Proteins and polypeptides	Peptide bonds adjacent to aromatic AA
Exocrine pancrease	Trypsin (trypsinogens)	Proteins and polypeptides	Peptide bonds adjacent to arginine or lysine
	Chymotrypsins (chymotrypsinogens)	Proteins and polypeptides	Peptide bonds adjacent to aromatic AA
	Elastase (proelastase)	Elastin, some other proteins	Peptide bonds adjacent to aliphatic AA
	Carboxypeptidase A (procarboxypeptidase A)	Proteins and polypeptides	Carboxy terminal AA with aromatic or branched aliphatic side chains
	Carboxypeptidase B (procarboxypeptidase B)	Proteins and polypeptides	Carboxy terminal AA with basic side chains
	Ribonuclease	RNA	Nucleotides
	Deoxyribonuclease	DNA	Deoxyribonucleic acid

Table 2.3 Continue.

Organ source	Enzyme	Substrate	Specificity
	Intestinal mucosa	Enterokinase	Trypsinogen
		Aminopeptidases	Polypeptides
		Dipeptidases	Dipeptides
		Nucleases	Nucleic acids
	Cytoplasm of mucosal cells	Various peptidases	Di-, tri- and tetrapeptides
		Deoxyribonuclease	DNA

Retrieved from: Chowdhury 2012; Ganong, 2009; Krogdahl and Sundby, 1999; Lauff and Hofer, 1984.

2.6 Utilization of animal by-products

Utilization of animal by-products from slaughterhouses has been regarded as a crucial industrial strategy to alleviate the livestock and aquaculture industry's protein resource shortage. Animal by-products refer to parts or materials derived from animals that are not intended for human consumption. These by-products, including blood, feathers, bones, offal, skin, and meat trimmings, are typically obtained from abattoirs during the processing of livestock into meat (Alao et al., 2017). Each year, a large amount of animal by-products is generated. If left unused, these by-products can pose significant problems for humans, animals, and the environment. Fortunately, animal by-products also provide a high nutritional and functional value. Consequently, their utilization has increased every year, particularly as a feasible alternative source of protein in livestock and aquaculture feed formulations (Hamilton, 2004; Jin et al., 2020; Gómez-Juárez et al., 1999). The global animal by-products market has become of growing interest, with an estimated 26,190 million USD in 2021 and projected to be 31,130 million USD by 2028 (Industry Research, 2022). Because protein hydrolysate can serve diverse purposes, functioning as flavor enhancers, valuable functional ingredients, or rich sources of essential amino acids (Cho et al., 2010; Guérard et al., 2010; Kumar et al., 2012; Qiao et al., 2011; Zhang et al., 2013). Moreover, these hydrolysates consist of peptides that are believed to have health-enhancing properties, positioning them as promising nutraceuticals for both food and pharmaceutical applications (Khan et al., 2011; Lasekan et al., 2013; Rustad et al., 2011; Senevirathne and Kim, 2012; Toldrá et al., 2012).

In recent years, many researchers have been interested in the bioactivity and functionality of specific peptides derived from animal byproducts. Consequently, animal by-products have been well-demonstrated to enhance productivity and performance, disease resistance, and immune response of many fish species, such as common carp (Carvalho et al., 1997), Japanese sea bass (Liang et al., 2006), large yellow croaker (Tang et al., 2008), Japanese flounder (Zheng et al., 2013), Barramundi (Chaklader et al., 2020), Gilthead Sea Bream (Gisbert et al., 2021), and Nile Tilapia (Ameret al., 2022). Several studies have demonstrated the extraction of bioactive peptides, particularly those with antioxidant properties, from porcine plasma using

enzymes such as trypsin (Wei and Chiang, 2009), pepsin (Xu et al., 2009), chymotrypsin (Wei and Chiang, 2009), papain (Xu et al., 2009), or Alcalase (Liu et al., 2010). These findings indicate a promising avenue for maximizing the value of animal by-products, including blood, by converting them into functional components. This transformation not only contributes to a more sustainable animal farming industry but also enhances its economic viability.

Animal blood, accounting for approximately 3 to 5 percent of total body mass, is a common by-product generated during the slaughtering of livestock. It consists of 60-80% liquid (blood plasma) and 20-40% solid (blood cells; red blood cells, white blood cells, and platelets) components (Madruga et al., 2007; Leoci, 2014; Tarté, 2011). In the part of the solid component, hemoglobin is the primary protein found in red blood cells, accounting for approximately 70% of total blood proteins (Leoci, 2014). Blood plasma is a light-yellowish or straw yellow and contains 91% water and 9% other solid organic (albumin, globulin, heme iron, fibrinogen, glucose fatty acids, cholesterol, triglycerides, hormones, vitamins urea, and amino acids), and 1% inorganic compounds (carbon dioxide, magnesium, calcium, potassium, and phosphorus) (Przybylski et al., 2016; Kowalski et al., 2017). Owing to the functional properties of its well-characterized proteins and other nutrients, animal blood is of considerable interest and is commonly processed into blood meal for use in the animal feed industry. In Thailand, the duck industry produces approximately 34.31 million ducks per year. Duck blood is the most prevalent byproduct of the duck meat industry, with an annual production of around 6,850 tons (Duck Meat Production Thailand 2012-2021, 2022). By converting this waste into an aquafeed protein source, duck blood can be used as a source of inexpensive protein rich in essential amino acids, heme iron, and other macro and micronutrients. The cost per kilogram of duck blood is between 10 and 13 baht. Therefore, the development of duck blood as a source of protein in aquafeed through the application of protein hydrolysate technology is a promising and reliable strategy for promoting the health and immunity of fish.

2.7 Protein hydrolysate

Protein hydrolysate, also known as hydrolyzed protein, is produced through hydrolysis, a process that breaks down complex protein molecules into smaller peptides and amino acids using acids or enzymes. This method is recognized for its safety, high production efficiency, and relatively low cost (McCarthy et al., 2013; Martínez-Alvarez et al., 2015). The hydrolysis process cleaves multiple amide bonds (–RCO–NHR′–) within peptides, resulting in the breakdown of these bonds and the formation of smaller peptide fragments or free amino acids. The smaller peptide derived from this process can accelerate protein digestion and facilitate more efficient nutrient absorption. Current research has revealed the antimicrobial, antioxidant, and immunomodulatory properties of protein hydrolysates derived from animal protein sources (Lewandowski et al., 2013; Chakka et al., 2015; Hou et al., 2017; Chaklader et al., 2020; Zou et al., 2021; Gisbert et al., 2022). Consequently, protein hydrolysates derived from various animal by-products have been incorporated into aquafeeds as a viable protein source to enhance fish growth and health, particularly during larval development.

2.7.1 Type of hydrolysis

Hydrolysis is a chemical reaction in which water is used to break down a compound into its constituent parts. The mechanism of hydrolysis depends on the type of compound being hydrolyzed (Nikhita and Sachindra., 2021).

2.7.1.1 Acid hydrolysis: Acid hydrolysis is a type of hydrolysis reaction in which an acid is used to break down a compound. In this mechanism, a proton (H+) is donated by the acid to the functional group of the compound, which then reacts with water to form an alcohol and a carboxylic acid. This is commonly observed in the hydrolysis of esters, amides, and acetal groups.

2.7.1.2 Enzymatic hydrolysis: Enzymatic hydrolysis is a type of hydrolysis reaction that is catalyzed by enzymes. In this mechanism, the enzyme binds to the substrate and orients it to facilitate the nucleophilic attack of water molecules on the functional group, resulting in bond cleavage and the formation of the hydrolysis products. This is commonly observed in the hydrolysis of proteins, carbohydrates, and

lipids. Common enzymes are Neutrase, Alcalase, Papain, Trypsin, Pepsin, and Flavourzyme.

2.7.1.3 Alkaline Hydrolysis: Alkaline hydrolysis is a chemical method used to break down complex molecules-such as proteins, esters, or even biological tissues-by using a strong base (typically sodium hydroxide (NaOH) or potassium hydroxide (KOH) in an aqueous solution. In protein hydrolysis, it specifically targets peptide bonds between amino acids. Can lead to destruction of certain amino acids (e.g., serine, threonine) (Kristinsson & Rasco (2000).

2.7.1.4 Autolysis: Autolysis (from Greek "auto" = self, "lysis" = breaking) refers to the self-digestion or self-degradation of cells or tissues by their own endogenous enzymes-especially proteases. In the context of protein hydrolysis or biotechnology, autolysis is used to break down proteins into smaller peptides and amino acids without adding external enzymes (Chalamaiah et al., 2012).

2.7.1.5 Microbial fermentation hydrolysis: Microbial fermentation hydrolysis is a biological process in which microorganisms-such as bacteria, fungi, or yeasts-break down proteins into peptides and amino acids through the action of enzymes they naturally produce during fermentation (Elavarasan et al., 2022). This method combines two processes:

1. Fermentation the growth of microbes under specific conditions.
2. Enzymatic hydrolysis breakdown of proteins by microbial proteases during fermentation.

2.8 Industrial production of protein hydrolysates

The method used to produce protein hydrolysates depends on the source of the protein. For example, proteins from feathers, bristles, horns, beaks, or wool contain keratin structures and are typically hydrolyzed using acidic or alkaline treatments or bacterial keratinases. On the other hand, animal and plant-based proteins are often hydrolyzed using general enzymatic or microbial methods. Protein hydrolysates are produced by hydrolyzing proteins using cell-free proteases, microorganisms, acids, or bases. Hydrolysis times can vary from 4 to 48 hours, depending on the method employed. If bacteriostatic or bactericidal preservatives are used during prolonged

hydrolysis, the hydrolysis is usually stopped by heating to deactivate the enzyme or enzyme systems. The insoluble fractions are then separated from the protein hydrolysates using a centrifuge filter or microfiltration system. The filtration process is repeated several times to achieve the desired color and clarity of the solution. Charcoal powder is typically used to decolorize and remove haze-forming components. If low salt concentrations are required, the filtrate can be subjected to ion exchange chromatography to remove salts. After filtration, the protein hydrolysate product undergoes pasteurization to eliminate or reduce microorganisms. Finally, the product is dried and packaged.

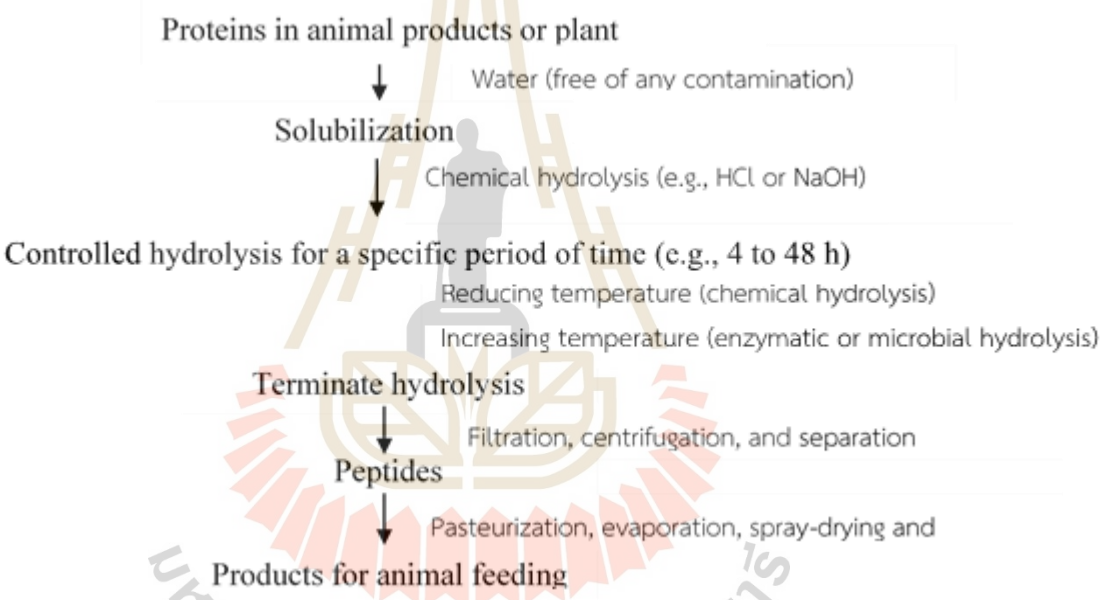

Figure 2.5 General procedures to produce peptides from animal and plant proteins. (Hou et al., 2017).

2.8.1 Applications of protein hydrolysates in aquafeed

The commercial development of aquaculture and the increasing demand for fish have led to the intensification of fish farming, exposing fish to a high risk of infectious diseases caused by pathogens. The use of antibiotics to combat these diseases may lead to the development of drug-resistant bacteria, environmental contamination, and fish residues. Therefore, it is of great interest to look for alternative strategies to reduce the use of these chemicals. In this sense, some studies have shown

that some protein hydrolysates can enhance non-specific immunity in fish and are interesting alternatives to antibiotics for controlling the spread of infectious diseases. The effect of protein hydrolysates on fish immunity was first observed *in vitro* by Khosravi et al.(2014), Lorenz et al.(2017), Gildberg et al.(1996) and Bøgwald et al. (1996) reported that intraperitoneal injection of cod muscle hydrolysate (molecular weight in the range 500–3000 Da) into Atlantic salmon stimulated the production of reactive oxygen metabolites in head kidney leukocytes by Gildberg et al., 1996 found an increase in the respiratory burst activity of leukocytes in the kidneys of Atlantic salmon exposed to small amounts of hydrolysates from the cod stomach in the culture medium.

Protein hydrolysate is now widely used in the animal feed industry as an adjuvant in nutrient absorption and growth (Hou et al., 2017). The study in rainbow trout indicated that diets supplemented with size-fractionated fish hydrolysate resulted in improved growth performance compared to those on high plant protein diets without the hydrolysate (Aksnes et al., 2006). This improvement could include increased weight gain and enhanced overall growth rates of rainbow trout. The positive effects observed in rainbow trout are likely attributable to the presence of essential nutrients in the hydrolysate, including amino acids and micronutrients (Aksnes et al., 2006). It is important to note that these diets are highly digestible and facilitate the fast passing and absorption of peptides and amino acids through the intestinal membrane (Aksnes et al., 2006; Wilson and Castro, 2010; Zheng et al., 2012).

Table 2.4 Effects of animal protein hydrolysates on aquaculture fish.

Tested fish	Source of hydrolysate	Enzyme used for preparing hydrolysate	Inclusion level	Duration of growth trial	Response	Reference
Barramundi (*Lates calcarifer*)	Yellowtail kingfish Seriola lalandi Carp hydrolysate Cyprinus carpio Bluefin tuna Thunnus Maccoyii	Alcalase	10% with PBM		(↑) Survival (↔) Final body weight, specific growth rate, feed conversion ratio and feed intake	Chaklader et al., 2020
Asian Sea bass	Tuna viscera	Alcalase	1–4%	28 days	(↑) Growth performance	Chotikachinda et al. 2013

Table 2.4 (Continued).

Tested fish	Source of hydrolysate	Enzyme used for preparing hydrolysate	Inclusion level	Duration of growth trial	Response	Reference
Turbot (*Scophthalmus Maximus*)	By-products of pollock Theragra chalcogramma	N.S.	UF: 5, 10, 15 and 20%	68 days	(↓) Final weight, specific growth rate and protein efficiency ratio at 20% (↔) Feed intake, condition factor and survival	Wei et al. 2016
European sea bass (*Dicentrarchus labrax*)	White shrimp Litopenaeus vannamei and Nile tilapia *Oreochromis niloticus*	N.S.	5%	70 days	(↑) Final body weight, specific growth rate compared to low FM diet (↔) Feed intake and survival	Leduc et al. 2018
Nile tilapia	poultry liver	N.S.	0, 10, 20, 40%	45 days	(↑) Productive performance, (↑) gill oxidative status	Gomes et al., 2023

2.9 Overview of the Immune System

The immune system in teleost fish serves as a vital defense mechanism against a wide array of pathogens present in the aquatic environment. Unlike mammals, fish rely heavily on innate immunity for immediate protection due to their poikilothermic nature, although they also possess functional adaptive immune components. Understanding the fish immune system is essential for disease control and health management in aquaculture, especially as the industry moves toward sustainable practices involving immunostimulants, probiotics, and functional feed additives.

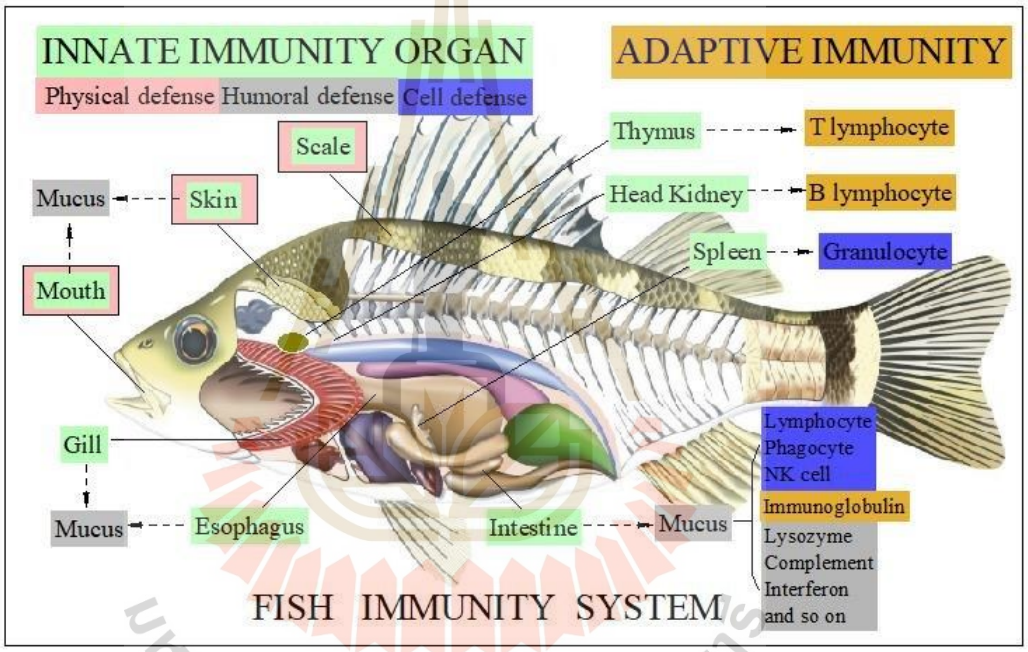

Figure 2.6 The innate immune system of fish. (Le et al., 2022).

2.9.1 Innate immune response

The innate immune system serves as the first line of defense against pathogens in fish and is critical for immediate, non-specific responses. It provides rapid recognition and neutralization of pathogens and plays a crucial role in initiating and regulating adaptive immunity. This defense system is evolutionarily conserved and includes physical barriers, humoral components, and cellular immune mechanisms (Magnadóttir, 2006).

2.9.1.1 Epithelial barriers

2.9.1.1.1 Physical Barriers

The skin and scales of fish form a continuous protective layer that acts as a mechanical barrier to external threats. These structures not only help prevent the invasion of pathogenic microorganisms but also inhibit the loss of water, ions, and nutrients-functions especially important in osmoregulation for fish living in aquatic environments (Tort et al., 2003). The epidermis, which contains living cells unlike that of mammals, also plays an active role in immune defense by secreting mucus and other immune related molecules.

2.9.1.1.2 Chemical Barriers

The mucus layer is secreted by epithelial goblet cells and covers most external surfaces of the fish, including the skin, gills, and fins. This mucus serves as a chemical shield, rich in bioactive compounds such as lysozyme, antimicrobial peptides (AMPs), immunoglobulins, proteases, and lectins (Salinas, 2015; Esteban, 2012). These molecules can neutralize or kill bacteria, viruses, and parasites. Mucus also physically traps pathogens, preventing their attachment and entry into the body. Moreover, mucus acts as a medium for signaling molecules and supports the colonization of beneficial microbiota that outcompete potential pathogens, contributing to immune surveillance (Lazado & Caipang, 2014).

2.9.1.1.3 Biological Barriers

Fish depend on biological barriers which include resident microbiota that settle on their skin and gastrointestinal tract and gills and mucosal surfaces. The microbial community's function in a symbiotic manner through three main mechanisms: The microbial communities fight pathogens for both food resources and attachment locations. The host immune system receives regulation from these biological processes. The production of antimicrobial compounds occurs through these biological processes. The microbiota found in these environments plays a crucial role in developing and operating both innate and adaptive immune responses (Gomez et al., 2013). A normal gut microbiome helps build mucosal defenses while making the body more resistant to intestinal pathogens.

2.9.1.2 Cellular innate immunity

consists of macrophages and polymorphonuclear cells. The main innate immune cells in fish are macrophages, neutrophils, dendritic cells, and eosinophils. These cells participate in phagocytosis, antigen presentation, and cytokine and reactive oxygen species (ROS) secretion (Whyte et al., 2007). Pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are immediately produced in response to pathogen recognition to recruit leukocytes and activate them (Secombes and Wang, 2012). The acute-phase response involves the activation of complement, opsonization, and lysis of pathogens to ensure effective early defense.

Phagocytosis is a fundamental cellular response to microbial invasion or tissue damage, involving the accumulation of leukocytes and fluid at the site of infection, regulated by cytokines. In teleost fish, macrophages serve as the primary professional phagocytes. These cells actively ingest and destroy pathogens through an oxygen-dependent killing mechanism mediated by reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and hydroxyl radicals (Ellis and Castro, 1999). Phagocytes play a vital role in the early clearance of both bacterial and viral pathogens.

Nonspecific cytotoxic cells (Natural Killer cells; NK cells). are cytotoxic lymphocytes integral to innate immunity, particularly in responding to virus-infected cells and tumorigenic processes. NK cells function by releasing cytokines such as IFN-γ and TNF-α, which stimulate macrophages and dendritic cells, amplifying the immune response (Zhang et al., 2018).

Inflammation is a hallmark innate response to infection or injury. This process is orchestrated by multiple mediators, especially cytokines, which recruit immune cells to the site of infection and enhance immune activity. Inflammatory responses contribute significantly to pathogen containment and elimination (Zou and Secombes, 2016).

2.9.1.3 Humoral Innate Immunity

The humoral component of the innate immune system in fish consists of soluble molecules present in body fluids such as blood plasma, mucus, and other secretions. These molecules play a critical role in the recognition,

neutralization, and elimination of pathogens, acting as a first line of defense in both systemic and mucosal immunity (Uribe et al., 2011).

2.9.1.3.1 Complement System

The complement system is one of the most important humoral factors in fish innate immunity. It is composed of a series of plasma proteins that, once activated, enhance phagocytosis (opsonization), directly lyse pathogens (via the membrane attack complex), and stimulate inflammation (Boshra et al., 2006). Fish possess all three known complement activation pathways—classical, alternative, and lectin pathways although the alternative pathway appears to play a more prominent role in innate responses due to its ability to be activated without antibodies (Sunyer & Lambris, 1998).

2.9.1.3.1.1 Classical Pathway: The classical pathway is initiated when C1q (first protein of the cascade) binds to the IgM or IgG antigen/antibody complexes. In addition, some other danger signals can also activate the classical pathway with antibody-independence, such as C-reactive protein, viral proteins, polyanions, apoptotic cells, and amyloid. The classical pathway acts as the link between the effectors of the innate and adaptive immunity.

2.9.1.3.1.2 Lectin Pathway: The lectin pathway is initiated when either mannose-binding lectin (MBL) or ficolin bind to mannose residues on the surfaces of pathogens. Once activated, the lectin pathway proceeds through the C4 and C2 to activate other complement proteins down in the cascade. The biological activities and the regulatory proteins of the lectin pathway are like the classical pathway.

2.9.1.3.1.3 Alternative Pathway: This pathway can be activated by when the exogenous viruses, fungi, bacteria, parasites, cobra venom, immunoglobulin A, and polysaccharides invade the organism, the component C3b will bind to factor B to start the alternative pathway. It is an important part of the defense mechanism independent of the immune response.

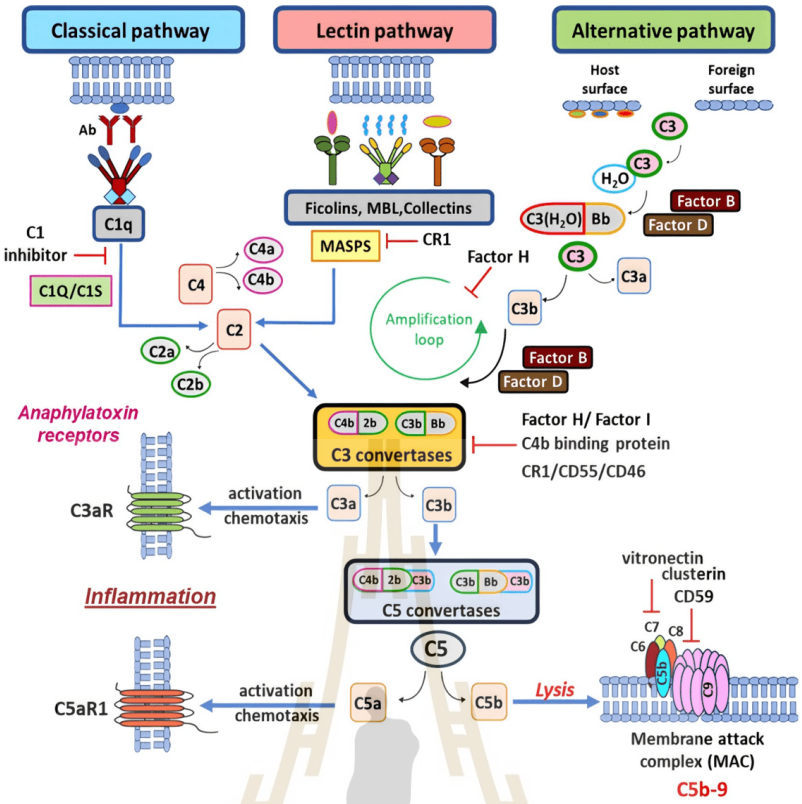

Figure 2.7 The complement cascade pathways (Detsika et al., 2024).

Involve the activation of C1s and C1r, which cleaves circulating C2 and C4 molecules into active C2a, C2b, and C4a, C4b molecules. The lectin pathway recognizes microbial carbohydrates and binds to mannan binding lectin serine peptidases (MASPs), leading to the formation of C3 convertase. The alternative pathway is triggered by spontaneous C3 hydrolysis, forming a fluid phase C3 convertase. The C5 convertase cleaves circulating C5 molecules into active C5a and C5b molecules, forming the membrane attack complex (MAC). The presence of complement regulatory proteins (CRPs) prevents overactivation of the complement cascade pathways and dysregulation of the complement system. (Detsika et al., 2024).

2.9.1.3.2 Lysozyme is an antimicrobial enzyme present in various fish tissues and secretions, including the head kidney, spleen, skin, gill, and gastrointestinal tract. It exerts bactericidal activity by hydrolyzing the peptidoglycan layer of bacterial cell walls, thereby offering a vital line of defense against bacterial invasion.

2.9.1.3.3 Cytokines are signaling molecules that regulate immune cell communication and function. These include interleukins, chemokines, interferons, lymphokines, and tumor necrosis factors (TNFs). Cytokines are produced in response to infection and play critical roles in mediating inflammation, cell recruitment, and the activation of adaptive immune responses.

2.9.2 Adaptive Immune Responses

The adaptive immune system in fish provides specific and long-lasting protection against pathogens through antigen recognition, clonal expansion, and the development of immunological memory. Although less complex than that of mammals, the adaptive immune system of teleost fish includes functional T and B lymphocytes, major histocompatibility complex (MHC) molecules, and immunoglobulins, which together form the basis of acquired immunity (Zapata et al., 2006; Uribe et al., 2011).

2.9.2.1 Humoral Immune Response

B lymphocytes (B cells) are essential for antibody-mediated immunity. Upon antigen recognition, B cells differentiate into plasma cells that secrete specific immunoglobulins (antibodies). These antibodies neutralize pathogens and facilitate their elimination through mechanisms such as opsonization and complement activation (Magadan et al., 2015).

Antigen-presenting cells (APCs), including macrophages and dendritic cells, process and present antigens to T lymphocytes. This presentation is crucial for the initiation and regulation of adaptive immune responses, linking innate and adaptive immunity.

2.9.2.2 Cell-Mediated Immune Response

T lymphocytes (T cells) perform multiple immune functions. Helper T cells (Th) support B cell differentiation and antibody production. Cytotoxic T cells (Tc) directly target and eliminate infected or abnormal cells. Regulatory T cells (Tregs) help maintain immune homeostasis and prevent excessive immune responses. T cells recognize antigens through interaction with major histocompatibility complex (MHC) molecules on APCs, initiating targeted immune responses against specific pathogens (Yamaguchi and Dijkstra, 2019).

2.10 Antioxidant enzyme activities

Fish, like other aerobic organisms, constantly produce reactive oxygen species (ROS) as by-products of normal cellular metabolism, particularly during mitochondrial respiration. Under physiological conditions, ROS play roles in cell signaling and immune responses. However, excessive ROS can lead to oxidative stress, damaging lipids, proteins, and nucleic acids, thereby impairing cellular functions and health (Lushchak, 2011). To mitigate oxidative damage, fish possess an effective antioxidant defense system comprising both enzymatic and non-enzymatic components.

Superoxide dismutase (SOD) is the first line of enzymatic defense against ROS. It catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). SOD exists in different isoforms, such as cytosolic Cu/Zn-SOD and mitochondrial Mn-SOD, each playing vital roles in different cellular compartments (Bagnyukova et al., 2006).

Glutathione peroxidase (GPx) reduces hydrogen peroxide and organic hydroperoxides to water and corresponding alcohols, using reduced glutathione (GSH) as a substrate. This enzyme plays a crucial role in protecting membrane lipids and oxidative stress in biological systems. Lipid peroxidation refers to the oxidative degradation of polyunsaturated fatty acids (PUFAs) in cell membranes, which occurs through a free radical chain reaction involving three main stages: initiation, propagation, and termination (Ayala et al., 2014). In the initiation phase, reactive oxygen species (ROS), such as hydroxyl radicals (•OH), abstract hydrogen atoms from the methylene groups of PUFAs, generating lipid radicals (L•). During the propagation phase, these lipid radicals react with molecular oxygen to form lipid peroxyl radicals (LOO•), which can further attack neighboring lipid molecules, forming lipid hydroperoxides (LOOH) and additional lipid radicals. This cycle continues, leading to extensive membrane damage. Finally, in the termination phase, the chain reaction is halted either through the reaction of two radicals to form a non-radical product or via antioxidant activity.

MDA is generated primarily during the decomposition of lipid hydroperoxides in the later stages of lipid peroxidation. It results from the fragmentation of certain per oxidized fatty acids, particularly arachidonic acid and docosahexaenoic acid (DHA) (Del Rio et al., 2005). The accumulation of MDA is toxic to cells leading to impaired function and potential mutagenesis.

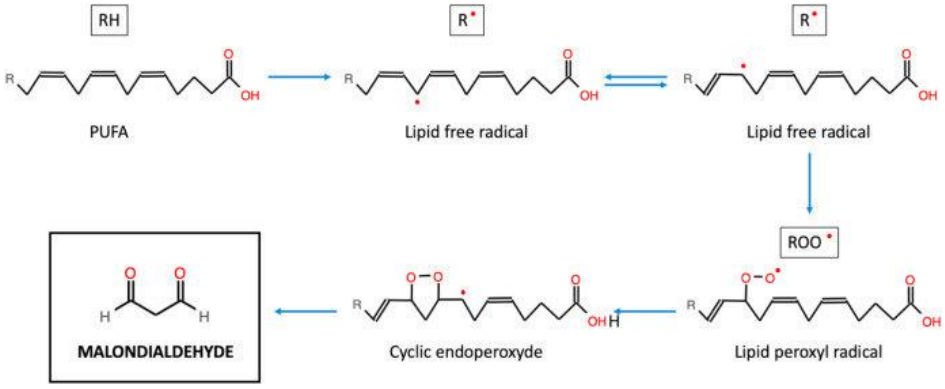

Figure 2.8 MDA formation through lipid peroxidation.

Catalase (CAT) catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen (O_2) and two molecules of water (H_2O). Catalase is a ubiquitous antioxidant enzyme present in most aerobic cells (Ighodaro and Akinloye, 2018) and catalyzes the reaction of hydrogen peroxide into water and oxygen. Overheating can inactivate catalase (Johansson and Borg, 1988). It is therefore essential to keep the enzyme cold during sample preparation and assaying. The enzyme is also very unstable at high dilution, thus should the samples be diluted immediately before the analysis (Herbert, 1955). In Figure 2.9 is the catalytic activity of CAT shown.

Catalytic activity:

$$2H_2O_2 \xrightarrow{CAT} O_2+2H_2O$$

Figure 2.9 Catalase Enzymatic Reaction.

Increased CAT activity indicates that there are higher levels of hydrogen peroxide (H_2O_2) in the organism, and the organism is exposed to higher levels of free radicals, which can lead to oxidative stress. The CAT activity is stable for several months at 0°C (Liu et al., 2013).

2.11 Disease in fish

Fish diseases frequently arise from multiple sources, predominantly including environmental issues, particularly substandard or swiftly changed water quality, nutritional deficiencies, and other husbandry variables such as the absence of quarantine before introducing new fish.

Symptoms may encompass anorexia, debilitation, lethargy, impaired buoyancy, deterioration in condition, conspicuous growths or visible pathogens, fin clamping, hemorrhagic lesions, erosions or ulcers, excessive mucus secretion, flashing behavior, abnormal body orientation or positioning within the water column, and other behavioral anomalies such as spinning, surface gasping, exophthalmia, and/or ascites.

2.11.1 Bacterial Diseases in Cichlids

Cichlids (Family Cichlidae) are susceptible to a variety of bacterial pathogens, which can result in severe economic losses and welfare concerns (Noga, 2010). Bacterial infections in cichlids are commonly associated with suboptimal environmental conditions, including poor water quality, nutritional deficiencies, and inadequate husbandry practices (Austin and Austin, 2016). Stress factors such as overcrowding, fluctuating water parameters, and the introduction of infected fish without quarantine can predispose cichlids to opportunistic bacterial infections (Roberts, 2012).

Clinical manifestations of bacterial diseases in cichlids are often nonspecific and include lethargy, anorexia, abnormal swimming behavior, exophthalmia, hemorrhages, ulcers, fin erosion, and ascites (Popma and Masser, 1999). Common bacterial pathogens isolated from diseased cichlids include *Aeromonas hydrophila*, *Pseudomonas* spp., *Vibrio* spp., *Flavobacterium columnare* (columnaris disease), and various *Streptococcu*s spp. (Zamri et al., 2013).

2.11.2 *Streptococcus agalactiae*

Among bacterial pathogens, *Streptococcus agalactiae* is recognized as a major etiological agent of streptococcosis in both food and ornamental fish, including cichlids (Evans et al., 2002). *S. agalactiae* is a Gram-positive, facultative anaerobic bacterium that poses a significant threat to aquaculture due to its pathogenicity and high mortality rates in affected populations (Amal and Zamri-Saad, 2011).

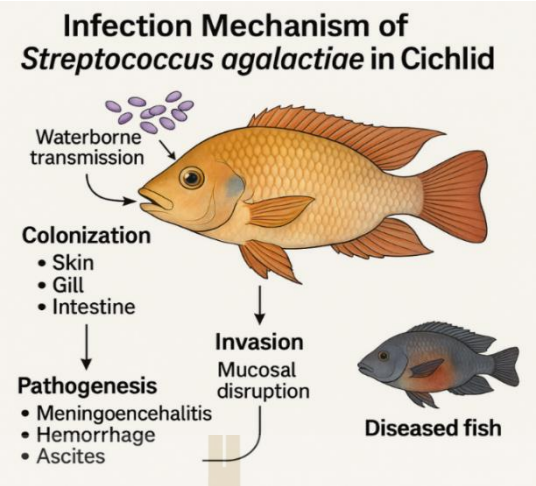

Figure 2.10 Schematic illustration of the fish immune system showing innate and adaptive immune responses.

In cichlids, *S. agalactiae* infections are characterized by clinical signs such as erratic swimming (spinning), lethargy, darkening of the body, exophthalmia, hemorrhages, and abdominal distension due to ascites (Sudheesh and Cain, 2017). Histopathological findings often reveal severe meningoencephalitis, systemic septicemia, and multifocal necrosis in various organs, including the brain, spleen, and kidney (Delannoy et al., 2013).

Transmission of *S. agalactiae* in aquaculture systems can occur through waterborne exposure, direct fish to fish contact, or via asymptomatic carriers (Pradeep et al., 2016). Environmental stressors such as elevated temperatures, poor water quality, and high stocking densities exacerbate the severity of outbreaks (Leal et al., 2020).

Several studies have documented outbreaks of *S. agalactiae* in tilapia (*Oreochromis* spp.) and other cichlids, reporting mortality rates exceeding 50% in severe cases (Zamri et al., 2014; Soto et al., 2012). The pathogen's zoonotic potential further underscores the need for effective prevention and control strategies (Mian et al., 2009).

2.11.3 Current Research and Control Measures

Recent research has focused on vaccine development, immunostimulants, and dietary interventions to mitigate the impact of *S. agalactiae* in aquaculture

(Kayansamruaj et al., 2020). Oral and injectable vaccines targeting *S. agalactiae* have shown promising results in enhancing specific immune responses and reducing mortality in cichlids (Kayansamruaj et al., 2018). Moreover, functional feed additives, such as Protein hydrolysis releases bioactive peptides with immune-reactive and antibacterial properties, which can stimulate non-specific immune responses in fish (Gisbert et al., 2021; Vijayaram et al., 2022). are being investigated for their potential to modulate immune responses and improve disease resistance.

Biosecurity practices, including quarantine of new stock, routine monitoring of water quality, and maintaining optimal husbandry conditions, remain critical components of integrated disease management (FAO, 2023).

CHAPTER III

MATERIALS AND METHODS

3.1 Ethics Approval

All experimental procedures were accepted by the Ethics Committee of Suranaree University of Technology, Animal Care and Use Committee (approval No. SUT-IACUC-0010/2023)

3.2 Centricon preparation duck blood protein hydrolysate

Duck blood protein hydrolysate (DBPH) was prepared following a previously reported method (Pérez-Gálvez et al., 2011) with some modifications. After that, DBPH was diluted with NaCl at a ratio of 2:1 and the DBPH mixture was boiled in a water bath at 95℃ for 15 minutes, followed by centrifugation at 12,500 rpm for 15 minutes at 4℃. The supernatant was transferred to a centrifugal filter tube with Centricon (10 kDa, Merck KGaA, Darmstadt, Germany) filter and centrifuged at 4000 × g for 50–60 minutes at 4℃. Using the filter, DBPH samples were separated by size while the debris was retained on the filter, as shown in Figure 3.1. The filtered solution was aliquoted and stored at –20℃ until use. Ultimately, the permeate fraction with a molecular weight below 10 kDa was collected and analyzed for its molecular weight via Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and size exclusion chromatography.

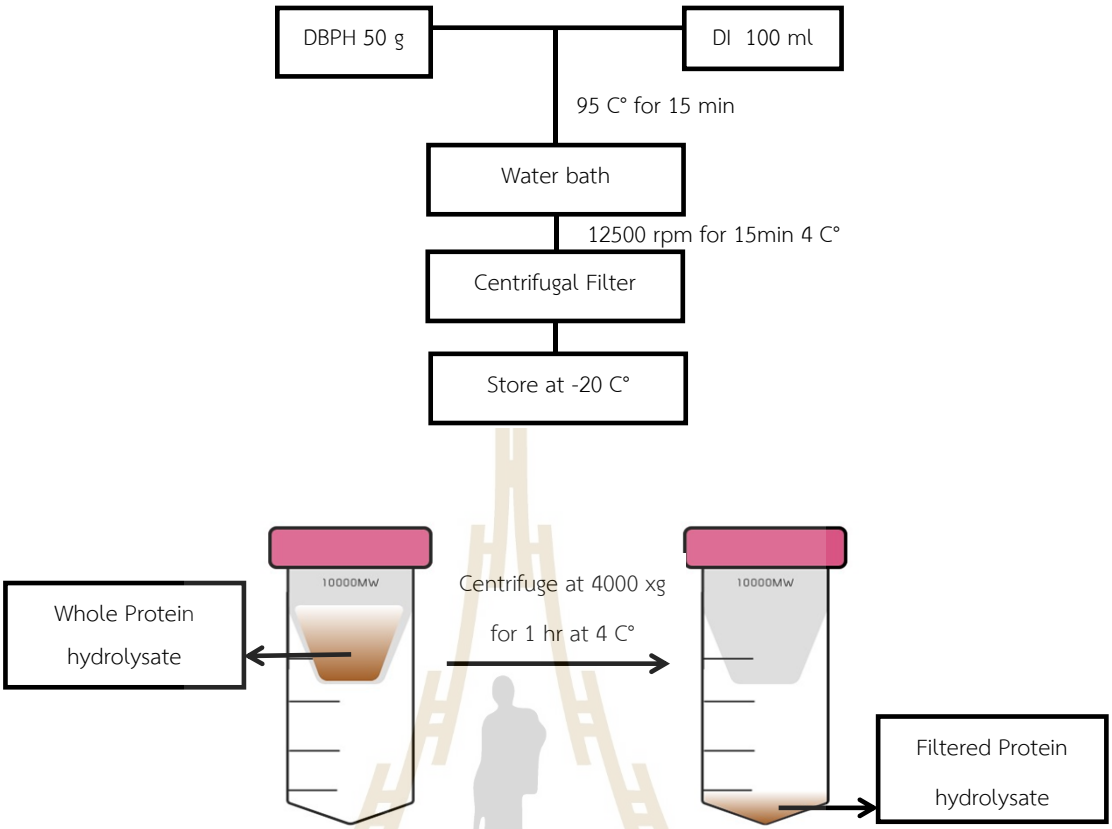

Figure 3.1 Filtration of Duck Blood Protein Hydrolysate.

3.3 Determination of Protein Molecular Weight and Protein Concentration

The DBPH samples was diluted with 20 µl of 2X Laemmli buffer at a 1:1 ratio and then the mixture was boiled in a heat block at 95℃ for 10 min. The sample was centrifuged at the maximal speed for 10 min at 25℃ and used to analyze the molecular weight of protein samples by SDS-PAGE technique.

The SDS-PAGE was prepared with the following chemicals.

15% separating gel	
40% Polyacrylamide	3.6 ml
1.5M Tris (pH8.8)	2.7 ml
10% SDS	100 µl
10% Ammonium persulfate	100 µl
TEMED	8 µl

ddH2O (DI)	3.2 ml
5% stacking gel (5%)	
40% Polyacrylamide	0.5 ml
1.5M Tris (pH6.8)	0.5 ml
10% SDS	40 µl
10% Ammonium persulfate	40 µl
TEMED (add it right before the gel is poured)	4 µl
ddH2O (DI)	3 ml

The DBPH samples was subjected to the gel as described above. Electrophoresis was performed at constant 50 V for 15 min for 5% stacking gel and 100 V for 50 min for resolving gel, respectively. Then, the gel was stained with staining solution (0.125% Coomassie brilliant blue R-250) on the shaker at room temperature overnight and destained twice in a solution containing 25% ethanol and 10% acetic acid on the shaker at room temperature for 2-3 hr or until the gel is clear. To visualize and analyze the images, we used the ChemiDoc MP Imaging SystemTM (Bio-Rad Laboratories, Hercules, CA, USA) determination of protein concentration.

The concentration of DBPH was determined using the Lowry protein assay with bovine serum albumin (BSA) as a standard protein. This method is based on the reaction of Cu^{+}, which is generated by the oxidation of peptide bonds from protein samples, with the Folin–Ciocalteu reagent. Briefly, the standard bovine serum was prepared at concentrations of 0, 0.1, 0.5, 1, 2, and 3 µg/ml to construct the standard curve. The Folin-Ciocalteu Reagent was diluted with ultrapure water at a ratio of 1:1 followed by transferring 10 µl of this diluent into 96 well plate. Then, 100 µl of copper sulfate solution was added to the same plate and incubated at room temperature for 30 m The protein concentration of the sample was measured by absorbance at 750 nm.

3.4 Molecular Weight Distribution Analysis

Molecular weight analysis is an essential parameter for evaluating peptide composition, estimating the extent of protein hydrolysis, and comparing the sizes of the peptides produced. In this study, the molecular weight distribution of gastrointestinal (GI) digests was analyzed

following the method described by Luasiri et al., 2024. A 100 µL aliquot of the peptide digest (10 mg/mL) was analyzed by chromatography using a Superdex Peptide 10/300 GL column (GE Healthcare, Piscataway, NJ, USA). The chromatography was performed with an AKTA Purifier (GE Healthcare, Piscataway, NJ, USA) using a mixture of 30% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) in deionized (DI) water. The system operated in isocratic mode at a flow rate of 0.7 mL/min, with a total elution volume of 30 mL. The peptide profile was determined by monitoring absorbance at 215 nm using UV detection. The study also utilized common substances such as cytochrome c, aprotinin, and synthetic peptides.

3.5 Diet Preparation

Preparation of DBPH and experimental media the filtered DBPH solution was aliquoted and stored at -20°C for further use. The protein concentration of DBPH was quantified using the Lowry protein assay using bovine serum albumin (BSA) as a standard. This colorimetric method is based on the reduction of Folin–Ciocalteu reagent with Cu^{+} ions, which are generated by the oxidation of peptide bonds within the protein sample. To generate a standard curve for calibration, BSA was prepared at concentrations of 0, 0.1, 0.5, 1, 2 and 3 µg/mL. The Folin–Ciocalteu reagent was diluted 1:1 with ultrapure water and 10 µL of this solution was pipetted into each well of a 96-well microplate. Then, 100 µL of copper sulfate solution was added to each well. The plates were incubated at room temperature for 30 min, the absorbance was measured at 750 nm using a microplate reader, and the protein concentration of the samples was calculated using a standard curve to control the same amount of protein for each preparation. Based on the quality and quantity of DBPH, five experimental formulas were formulated, Negative control: commercial diet mixed with 0.85% NaCl (saline), positive control: commercial diet supplemented with 0.1% Vitamin C, Treatment 1: commercial diet supplemented with 0.5% DBPH, Treatment 2: commercial diet supplemented with 1% DBPH and Treatment 3: commercial diet supplemented with 2% DBPH. The mixture of each diet was thoroughly homogenized with the respective supplement, then spread and allowed to dry for approximately 1 hour. After that, all diets were coated with squid oil at a ratio of 600 g of diet to 15 ml of squid oil (equivalent to 2.5% v/w) and then air-dried at room temperature for 2 hours. The prepared diets were then stored at 4°C used.

To ensure consistent nutrient content the approximate composition of each diet was therefore analyzed according to the official methods of the Association of Official Analytical Chemists (AOAC, 1990). All diets contained approximately 38% crude protein, 10% crude moisture, 4% crude fiber and 4% crude fat, with no significant differences between the treatment groups. During the experiment, fish were fed *ad libitum* twice daily along with the individual fish diets.

3.6 Experimental Design, Fish Culture and Sampling

This study was conducted at the Aquaculture Laboratory, Suranaree University of Technology, Nakhon Ratchasima Province, Thailand. All experimental procedures were approved by the Animal Care and Use Committee, Suranaree University of Technology (Approval No. SUT-IACUC-0010/2023). Before the feeding experiment, 40 healthy flowerhorn cichlids (initial weight: 3.34±0.67 g; initial length: 3.40±0.61 cm) were acclimated for 14 days in 40 separate glass tanks (23″ × 13″ × 12″) under the same controlled environment. Due to the aggressive nature of flowerhorn cichlids, they were housed separately to prevent injuries from aggressive behavior. Each tank was equipped with aeration, water filtration, and temperature control. During the acclimation, the fish were fed ad libitum twice daily using ready-to-eat food. After acclimation, the fish were randomly assigned to 5 dietary treatment groups using a completely randomized design with 8 replications per group (n = 8). The dietary treatment groups were as follows: Negative control group: commercial diet mixed with 0.85% saline (NaCl), positive control group: commercial diet supplemented with 0.1% vitamin C, experimental groups supplemented with 0.5%, 1% and 2% DBPH, respectively. The feeding experiment lasted for 30 days. During this period, all fish were fed the experimental diets sequentially twice a day until they felt full. At the end of the feeding experiment, the growth performance was assessed by recording the weight gain rate (WGR), final body length and survival rate (SR). The growth parameters were calculated using the following formula:

Weight gain rate (WGR, g) = final body weight – initial body weight

Survival rate (SR, %) = 100 × final number of fish / initial number of fish

After the feeding experiment, fish from each diet group were divided into two subgroups: pre-challenge group (n = 4) and post-challenge group (n = 4). In the pre-challenge group, blood samples were collected from fish in each treatment for analysis of serum humoral immune parameters and assessment of antioxidant enzyme activities. The DBPH group showed the best performance in terms of growth, humoral immune response and antioxidant activities (see Section 3 Results) were identified as the most suitable DBPH level group for further analysis.

For microbiota analysis, gut samples were collected from the negative control and the most suitable DBPH groups. In addition, liver and spleen samples were harvested from the negative control, positive control, and the most suitable DBPH groups for basic immune gene expression analysis.

3.7 Bacterial Challenge Test

Assessment of the immune-enhancing effectiveness of duck blood protein hydrolysate (DBPH) in flowerhorn cichlids. The bacterial challenge test utilized *Streptococcus agalactiae*, a pathogen recognized for inducing considerable morbidity and mortality in multiple fish species. After a 30-day feeding trial, fish from the negative control, positive control (supplemented with 0.1% vitamin C), and 2% DBPH-supplemented groups were selected for the challenge. Each fish was injected intraperitoneally with 0.2 ml of *S. agalactiae*, bacteria at a concentration of 1×10^8 CFU/mL. This dose is consistent with previous studies that have shown significant immune responses and mortality at similar bacterial doses in fish species. Abdel Rahman et al. (2023) 24 hours after infection, the fish were monitored for clinical signs of Streptococcus, including lethargy. The fish exhibited irregular swimming patterns and enlarged eyes. Tissue samples from the liver and spleen were collected for gene expression analysis to assess the activity of immune-related genes. In addition, serum samples were collected to assess the immune response of body fluids, including immunoglobulin M (IgM) levels, lysozyme activity, and complement C3 concentrations.

3.8 Immune Responses and Antioxidant Enzyme Activity

Immune Responses and Activity of Antioxidant Enzymes. Blood samples were obtained from the caudal vein of four fish in each treatment group following

anesthesia. The samples were permitted to remain at room temperature for two hours to enable serum separation and subsequently stored at -80℃ until further utilization.

3.9 Lysozyme Activity

Lysozyme activity was measured according to the method of Siwicki et al. Briefly, lysozyme at concentrations of 0, 2.5, 5, 10, 15, and 20 µg/ml in 6M PCB-NaCl (pH 6.0) was prepared as reference standards for the standard curve. After that, 10 µl of flowerhorn cichlid serum and each concentration of reference standard was added into wells of a 96-well flat bottom plate in triplicate. To determine serum lysozyme activity, a suspension of 190 µl of 0.3mg/ml dried Micrococcus lysodeikticus (ATCC 4698; Sigma-Aldrich, St. Louis, MO, USA) was quickly added into all wells of the plate. The reaction was carried out at 25℃, and the optical density (OD) was measured at 450 nm at the initial time (0 min), 30 min, and 60 min using a microplate spectrophotometer (BioTek™ EPOCH, Agilent Technologies, Santa Clara, CA, USA). The concentrations of lysozyme (µg/ml) were calculated from a standard curve of lysozyme concentrations.

3.10 Alternative Complement (ACH_{50}) Activity

ACH_{50} activity was determined following a previously described method [43]. Briefly, flowerhorn cichlid serum (50 µL) was two-fold serial diluted with EGTA-GVB buffer (gelatin veronal buffered saline, 10 mM ethyleneglycol-bis (beta-amino-ethyl ether) N-N'- tetraacetate) and then an equal volume of 5 × 107 cells/mL of GRBC suspension was added to the diluted plasma. After that, the mixture was incubated for 90 min at 25℃ and centrifuged at 3000× g for 10 min at 4℃. The optical density (OD) was measured at 415 nm using a microplate spectrophotometer (BioTek™ EPOCH). Flowerhorn fish diluted serum representing the volume of complement producing 50% hemolysis (ACH_{50}) of GRBCs was determined, and the number of ACH_{50} units/mL was calculated for each experimental group (in triplicate).

3.11 Activity of antioxidant enzymes

The activity of catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA) was measured using commercial kit (Abbkine Corporation, Georgia, USA) according to the manufacturer's recommended protocol.

3.12 Total Immunoglobulin (Ig)

The total immunoglobulin (Ig) concentration (mg/mL) in flowerhorn cichlid serum was determined using the total protein kit (Biuret method; Erba, Mannheim, Germany) according to the method of Siwicki et al. Briefly, flowerhorn cichlid serum (10 µl) was added in a 1.5 ml microcentrifuge tube, followed by the addition of an equal volume of 12% PEG solution (polyethylene glycol; Sigma-Aldrich, St. Louis, MO, USA). The mixing samples will incubate at 25°C for 30 min and centrifuged at 12,500 rpm at 4°C for 10 min. Following centrifugation, the mixture was separated into supernatant (non-Igs) and pellet (total Igs). After that, 4 µl of the supernatant, serum (total protein), and standard bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was added into a 96-well flat bottom plate in triplicate. The protein concentration was determined according to the manufacturer's recommended protocol, and the optical density (OD) was measured at 546 nm using a microplate spectrophotometer (BioTek™ EPOCH). After precipitation, total Igs was calculated by subtracting total protein from non-Igs. The concentrations of proteins were derived from the standard curve constructed with bovine serum albumin.

3.13 DNA extraction of bacterial 16S rRNA genes

The gut specimens were squeezed out with sterile instruments to collect the luminal content. The gut samples were cut laterally to remove the mucus layer of the fish gut by visual inspection according to the method of NucleoSpin® Tissue kit. A cover glass was used to separate the mucus layer from the gut samples. The luminal content and mucus layer were pooled and transferred to a sterile conical tube containing 6.5 mM dithiothreitol for mucus degradation. After incubation for 3 hours at 56°C, the lysate sample Vortex the samples. Add 200 µL Buffer B3, vortex vigorously and incubate at 70°C for 10 min. Vortex briefly, Adjust DNA binding conditions Add 210 µL ethanol (96–100 %) to the sample and vortex vigorously, Bind DNA For each sample, place one NucleoSpin® Tissue Column into a Collection Tube. Apply the sample to the column. Centrifuge for 1 min at 11,000 x g. Discard the flow-through and place the column back into the Collection Tube. And then Wash silica membrane Add 500 µL Buffer BW. Centrifuge for 1 min at 11,000 x g. 2nd wash Add 600 µL Buffer B5 to the column and centrifuge for 1 min at 11,000 x g. Dry silica membrane Centrifuge the column for 1 min at 11,000 x g. Elute, highly pure DNA Place the NucleoSpin®

Tissue Column into a 1.5 mL microcentrifuge tube (not provided) and add 100 µL prewarmed Buffer BE (70°C). Incubate at room temperature for 1 min. Centrifuge 1 min at 11,000 x g. The eluted DNA was stored at -80°C until use. To determine the quantity and quality of genomic DNA, 2 µL of each gDNA sample was analyzed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of the gDNA was assessed through agarose gel electrophoresis. Subsequently, three DNA libraries from the control group and another three from the DBPH group were amplified using the V3-V4 region of the bacterial 16S ribosomal RNA gene. The libraries were prepared and sequenced on Illumina Novaseq 6000, and data were then analyzed by bioinformatics (Biomarker Technologies BMKGENE; Münster, Germany).

To generate high-quality reads, the raw reads were filtered by trimmomatic v0.33, and the primer sequences were removed by cutadapt 1.9.1. Next, Dada2 in QIIME2 was performed to de-noise and remove chimeric sequences. Taxonomic annotation was conducted on operational taxonomic units (OTUs) using the SILVA reference database. The abundance of each species in the samples was calculated at the phylum, class, order, family, genus, and species levels using statistical analysis based on the composition of each sample. The abundance of data was generated by the R package for the characterization of microbial communities present in the samples. To measure species diversity within individual samples, alpha diversity analysis was assessed using various metrics, including the Chao1, Shannon, and Simpson indices. Conversely, beta diversity analysis was assessed to compare species diversity between different samples using principal component analysis (PCA) generated by the R package.

3.14 Challenge Trial

Preparation of *Streptococcus agalactiae*. The *S. agalactiae* virulent strain was isolated from the infected fish from an earthen culture pond in the northeast part of Thailand and maintained in the Laboratory of Biotechnology for Aquaculture, Suranaree University of Technology. A single colony was resuspended in tryptic soy broth (Merck KGaA, Darmstadt, Germany) and cultured at 37°C, 150 rpm for 16-18 h. The bacterial suspension was adjusted to a final concentration of $1x10^8$ CFU/mL with an optical density at 600 nm of 1.0.

3.15 Expression of DBPH on mRNA expression levels of inflammatory and antioxidant genes via qRT-PCR

3.15.1 Total RNA extraction

Experimental fish were dissected and liver, spleen, head kidney, and intestine were collected. Total RNA was extracted using TRIzol reagent (Gibco BRL, USA) according to the manufacturer's instructions. One ml of TRIzol reagent (Gibco BRL, USA) was added to a homogenized tube containing 100 mg of collected tissue. All samples were homogenized in a tissue homogenizing machine (MP automatic tissue extractor; Fast Prep®-24). After that, the homogenized sample was incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes, and 0.3 ml of chloroform per 1 ml TRIzol reagent was added to the sample. Sample tubes were shaken vigorously by hand for 15 sec and incubated at room temperature for 3 min. Then, the sample was centrifuged at 12,500 rpm, 4°C for 15 min. Following centrifugation, the mixture was separated into a lower red (phenol-chloroform phase), interphase, and a colorless upper aqueous phase. The aqueous phase was transferred to a new tube and RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol in an equal volume to the aqueous phase transferred. The sample was incubated on ice for 30 min and centrifuged at 12,000 rpm, 4°C for 30 min. The supernatant was removed, and 1 ml of 80% ethanol was added to the sample. After that, the sample was mixed with a vortex mixer and centrifuged at 8,000 rpm, 4°C for 5 min. The ethanol wash was removed, and the RNA pellet was air-dried for 5–10 min on ice. Total RNA was dissolved in DEPC. Total RNA samples were treated with DNase I (Fermentas, USA) to remove contaminating genomic DNA from RNA samples.

3.15.2 First strand cDNA synthesis

The first-strand cDNA was synthesized using the ImProm-II™ Reverse Transcription System kit (Promega Corporation, Madison, WI, USA). Oligo (dT) and 1 µg of total RNA from each tissue were incubated at 70°C for 5 min. Fifteen microliters of master mix consisting of 4 µl of 5X reaction buffer, 4.8 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 0.5 µl of RNase inhibitor, 1 µl of RNase transcriptase, and 3.7 µl of nuclease-free water were prepared on ice. The total volume of 20 µl was mixed well and then incubated at 25°C for 10 min, 42°C for 90 min, and 72°C for 15 min, respectively. The first-strand cDNAs were kept at -20°C until used.

3.15.3 Real-time PCR analysis Inflammatory and Antioxidant Gene Expression

To assess the influence of DBPH on mRNA expression levels of inflammatory and antioxidant genes following the feeding trial, liver and spleen tissues were obtained from each experiment (n = 4), both before and after the challenge. The total RNA, in 3.15.1 was then converted into complementary DNAs (cDNAs) in 3.15.2 Subsequently, partial fragments of these genes and the β-actin gene were amplified from the liver and spleen cDNA using specific primer sets, as illustrated in Table 3.1 The β-actin gene was used as an internal reference gene for normalization. The cleaned-up PCR products were inserted into a pGEM® T-Easy plasmid (Promega Corporation, Madison, WI, USA), and the plasmid copies were sequenced by Macrogen, Inc. (Seoul, Korea) to check that the standard plasmids for qRT-PCR analysis were correct. The sequence data for each gene are shown in Table 3.1 We used the CFX Opus Real-Time PCR System (Bio-Rad, Hercules, CA, USA) and THUNDERBIRD® SYBR®qPCR Master Mix (TOYOBO, Osaka, Japan) to check the mRNA levels of antioxidant genes three times, following our earlier research One microliter of first-strand cDNAs from First strand cDNA synthesis was analyzed by quantitative real-time PCR analysis using an the CFX Opus Real-Time PCR System machine (Bio-Rad, Hercuies, CA, USA). Ten microliters of the PCR mixture containing 1 µl first strand cDNA, 5 µl SYBR Green qPCR Master Mix (Toyobo), 2 µl dH2O, and 1 µl each specific primer was illustrated in Table 3.1. The PCR conditions was performed at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 55-63°C for 30 s. DNA melting curve analysis was used to verify the specificity of the primers. The melting curve analysis was used to confirm the specificity of the primers by evaluating the dissociation characteristics of double-stranded DNA during heating.

Table 3.1 Primers used for qRT-PCR analysis.

Primer name	5′ to 3′ Nucleotide Sequences	Product size (bp)	Annealing temperature (°C)	Sequence data	Product size (bp)
SODF	GGAGACCTGGGAAATGTGAC	171	55	**GGAGACCTGGGAAATGTGAC**TGCAGGAGCAGA	171
				TAATGTTGCCAAGATAGACATCACTGACAGTGTG	
				ATCAAGCTCACAGGTGCCGACTCCATCATTGGAA	
SODR	ACCAGTCTTCAGGCTCTCTT			GAACCATGGTGATCCATGAGAAGGCTGATGACCT	
				GGGTAAAGGAGGAGAT**GAAGAGAGCCTGAAGA**	
				CTGGT	
CATF	TCTCAACAGGAACCCAGTCA	174	55	**TCTCAACAGGAACCCAGTCA**ATTACTTTGCAG	174
				AGGTGGAGCAGCTGGCCTTCGACCCCAGCAACAT	
				GCCACCGGGCATTGAGCCCAGCCCTGACAAGATG	
CATR	ACAGGGATCTGCAGGTAGTT			CTGCAGGGTCGACTCTTCTCCTACCCAGACACAC	
				ATCGTCACCGGCTTGGGGCA**AACTACCTGCAGA**	
				TCCCTGT	
IL-1βF	GTGACCACTGGCAGAAAGAT	150	55	**GTGACCACTGGCAGAAAGATCTCGTCCTGTC**	150
				AGGAGACTTACAGCTGCAGGCCATCACTCTGAA	
				AGGAGGAAACTACCAACACAAAGTGAATTTTAAA	
IL-1βR	GACAGGACAACAGTCAGACC			ATGTCGCGGTACAACTCTTCCTCCGTCACTCCTG	
				GTGAT**GGTCTGACTGTTGTCCTGTC**	

Table 3.1 (Continue).

Primer name	5′ to 3′ Nucleotide Sequences	Product size (bp)	Annealing temperature (°C)	Sequence data	Product size (bp)
IL-6F	CCAACCAGCAGTGAGGAG	191	55	**CCAACCAGCAGTGAGGAG**CAGATGCTCAAGGT	191
				CAACAGTCCGTATGCTTTCCATAGAAAGATGAAA	
				GCGCACAACATCCTGAATCATCTCTTTGATTTCC	
IL-6R	GCTGCCATATTTCTCCTGGT			TCAAAGAAGTGAAAAGATCTATCTGTAGAATGGA	
				GATGAAG**ACCAGGAGAAATATGGCAGC**	
CCF	ACAGAGCCGATCTTGGGTTACTTG	229	55	**ACAGAGCCGATCTTGGGTTACTTG**GTCCAGAG	229
				AGCAAGGCGTCCATGTGTCAATGCAGTCATCTTT	
				CAGACACAGTCCGGTCTTTTCTGCATCAATGGGA	
CCR	TGAAGGAGAGGCGGTGGATGTTAT			GAGCTCCCTGGGTTCGTGCCACGATTGTTGCATT	
				CGAGAAAGCTAAAGCCCAGTCCACTACACCATCT	
				GTGGTCACTACATCTCCAGTCTCCCTTCTCTCCA	
				TC**ATAACATCCACCGCCTCTCCTTCA**	
CXCF	TGTCTGTGTCACCGTGTCAGGAAT	151	55	**TGTCTGTGTCACCGTGTCAGGAAT**CGTGTTGG	
				CCTGAAGTCGGAAATAAAGGACATTCAGATCTAC	
				CCAGCAACCATCTTCTGCAACAAAGTGGAGATTG	
CXCR	CCTTCAGCTCAGGGTTCAAGCAAT			TTGTCACCTTGAACAACAGCTATCGCT**ATTGCTT**	
				GAACCCTGAGCTGAAGG	

Table 3.1 (Continue).

Primer name	5′ to 3′ Nucleotide Sequences	Product size (bp)	Annealing temperature (°C)	Sequence data	Product size (bp)
β-actinF	ACAGGATGCAGAAGGAGATCACAG	155	55	**ACAGGATGCAGAAGGAGATCACAG**CCCTGGC CCCATCCACCATGAAGATCAAGATCATTGCCCCA CCTGAGCGTAAATACTCCGTCTGGATCGGAGGCT CCATCCTGGCCTCCCTGTCCACCTTCCAGCAG**AT** **GTGGATCAGCAAGCAGGAGTAC**	
β-actinR	GTACTCCTGCTTGCTGATCCACAT				

3.16 Statistical analysis

SPSS 25.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The results are reported as least squares mean values. The data was analyzed using a one-way analysis of variance, according to the complete randomized design (CRD) experimental plan to compare the differences between the means in each experimental group, using Tukey's multiple tests at the accepted significance level of $P < 0.05$. In addition, an independent sample T-test was conducted to evaluate the difference between the negative control group and the optimal percentage DBPH group for the microbiome analysis, as well as between the pre-and post-challenge group for gene expression analysis ($P < 0.05$).

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%).

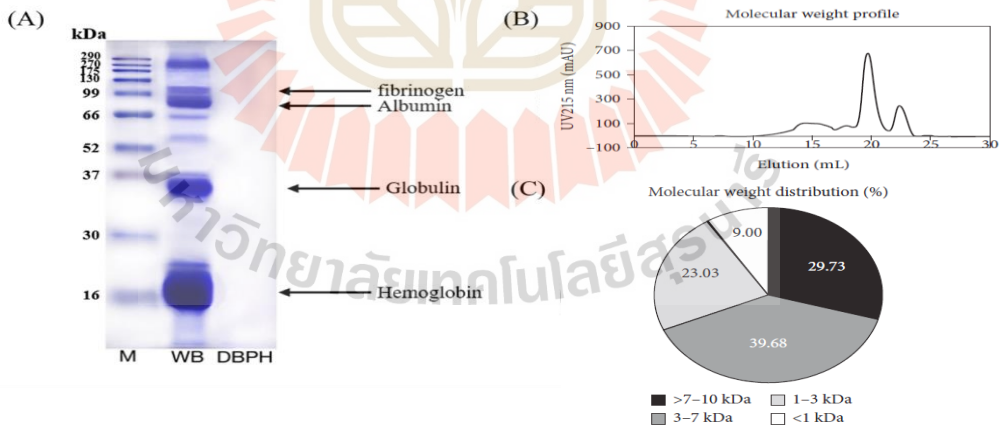

Figure 4.1 The SDS-PAGE profile (12.5% acrylamide) of samples containing 20 µg of protein (A). The samples include protein standard markers (M), whole duck blood (WB), and low molecular weight duck blood protein hydrolysate (DBPH). Additionally, size exclusion chromatography was used to obtain the molecular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

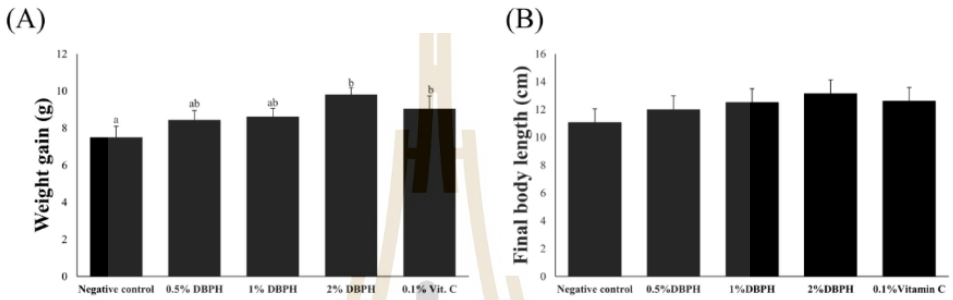

Figure 4.2 Weight gain (A) and Final body length (B) of flowerhorn fed with different experimental diets for 30 days. Means ± S.D. (n = 8) with different superscript letters is significantly different ($P < 0.05$).

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 ($P < 0.05$).

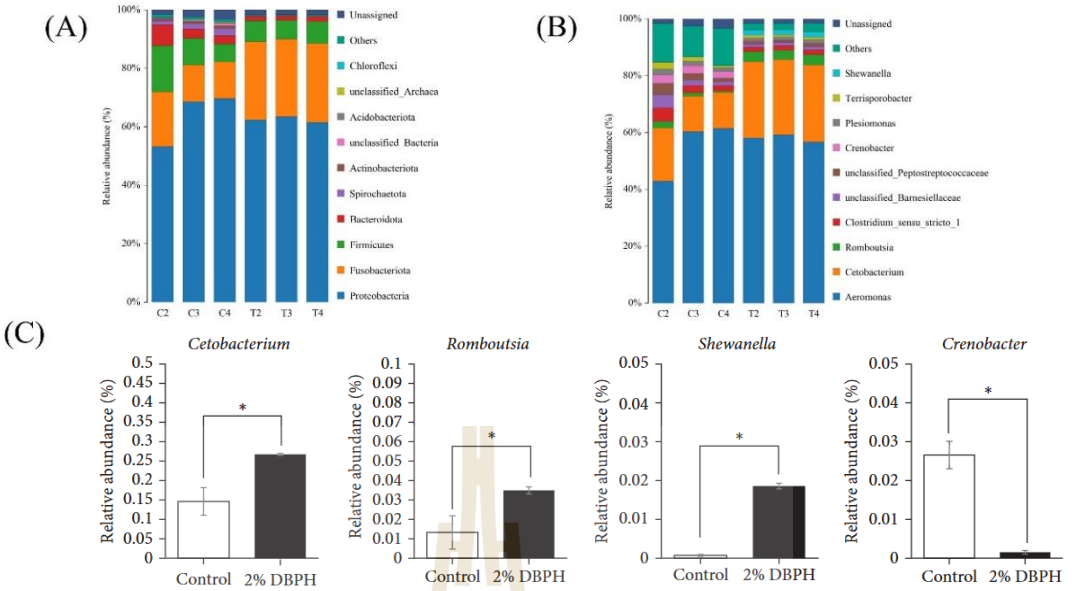

Figure 4.3 Intestinal microbiota composition of flowerhorn fish fed with the control diet and 2% DBPH for 30 days. Taxonomic distribution at phylum level (A), Taxonomic distribution at genus (B), and Abundance of significant intestinal bacteria communities at the genus level (C).

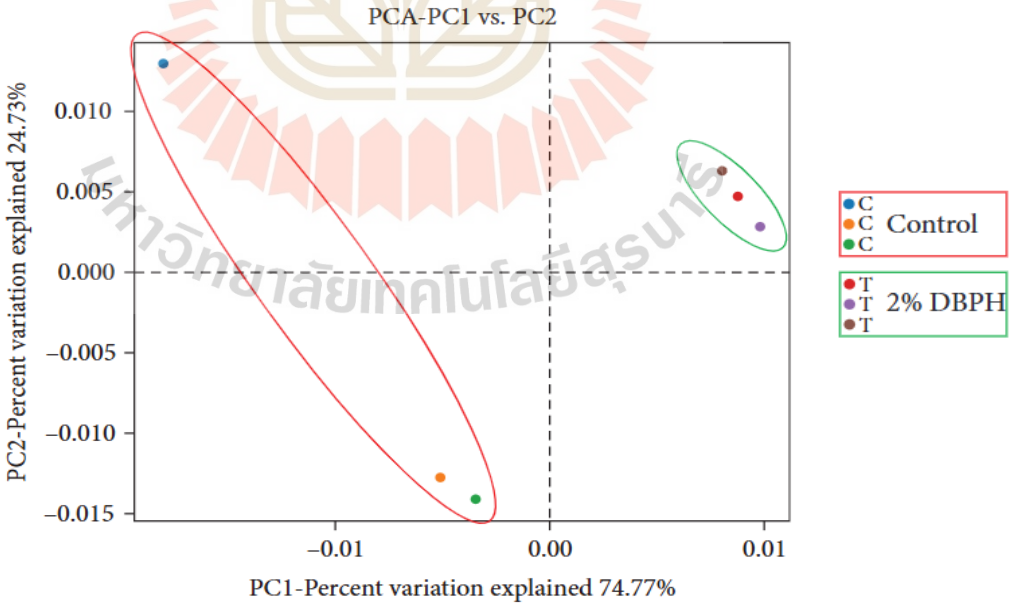

Figure 4.4 Principal component analysis (PCA) based on distances of intestinal bacteria communities of flowerhorn fish fed with the control diet and 2% DBPH for 30 days.

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)

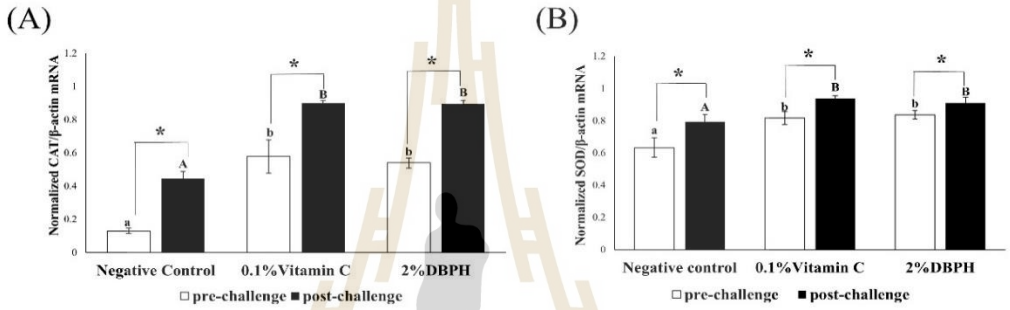

Figure 4.5 Quantitative real-time PCR analysis of CAT (A) and SOD (B) expression in the liver of flowerhorn fish after the 30-day feeding trial. Bars with asterisks indicate significant differences between pre- and post-challenge. Bars labeled with different lowercase letters denote significant differences 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).

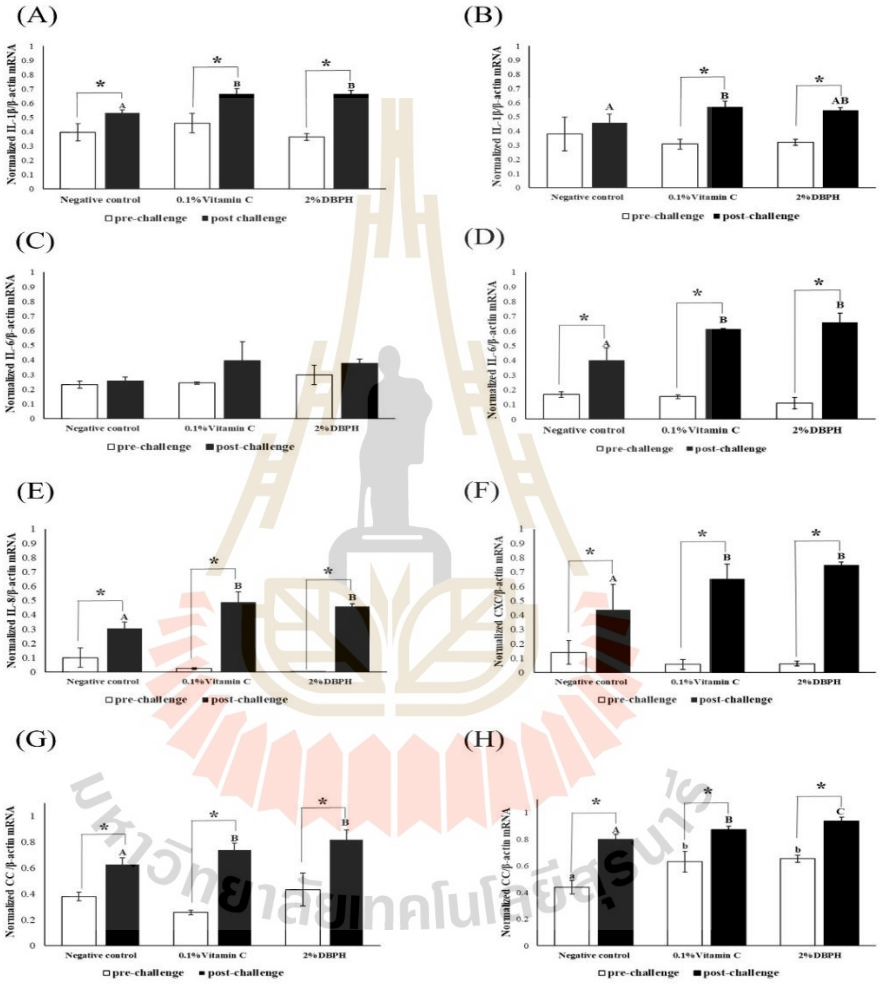

Figure 4.6 Quantitative real-time PCR analysis of IL-1β, IL-6, CC, and CXC chemokine expression in the liver (A, C, E, G) and IL-1β, IL-6, CC, and CXC chemokine expression in the spleen (B, D, F, H) of flowerhorn fish after the 30-day feeding trial. Bars with asterisks indicate significant differences 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 ($P < 0.05$).

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).

CHAPTER VI

CONCLUSION

This study provides insight into the health-promoting properties of low molecular weight duck blood protein hydrolysate (DBPH) in flowerhorn fish. Diets supplemented with 2% DBPH exhibited the highest growth, antioxidant activity, and humoral immune response enhancement under normal conditions. Additionally, DBPH could promote the abundance of the genera Cetobacterium and Romboutsia, which could serve as indicators of the overall health and well-being of the fish. Moreover, with a *Streptococcus agalactiae* challenge, fish fed with diets supplemented with DBPH exhibited an enhanced ability to modulate inflammatory genes as well as antioxidant gene expression (SOD, CAT). Overall, dietary supplementation with DBPH could improve the overall health of the flowerhorn fish by ameliorating humoral immune response, alleviating oxidative stress, and strengthening resistance against *S. agalactiae*.

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