CHAPTER II

LITERATURE REVIEW

2.1 Biology of Flowerhorn Cichlid

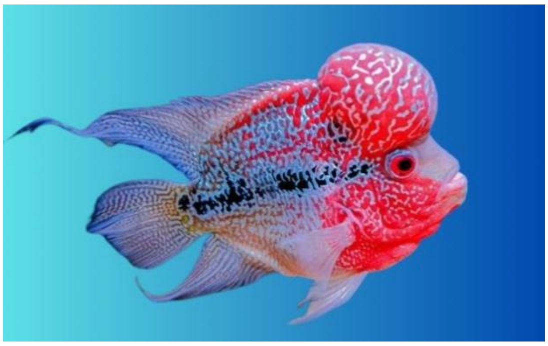

Figure 2.1 Flowerhorn fish Aquarium. (2024, August 20). Flowerhorn Cichlid Care Retrieved from https://www.blessingsaquarium.com/post/flowerhorn-cichlid-care.

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.

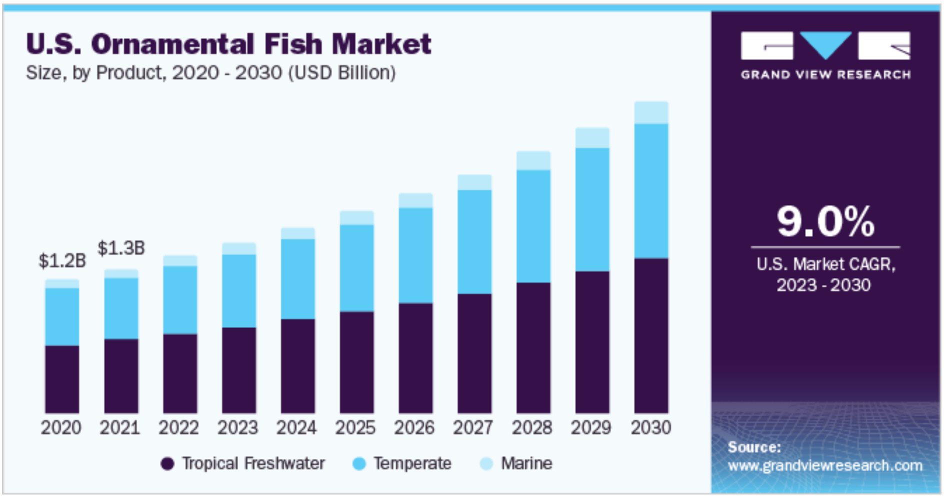

Figure 2.2 The Global Ornamental Fish Market. FQA (2021). The Global Ornamental Fish Market. Retrieved from https://www.grandviewresearch.com/industryanalysis/ornamental-fish-market.

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, Melanotaeniidae, 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).

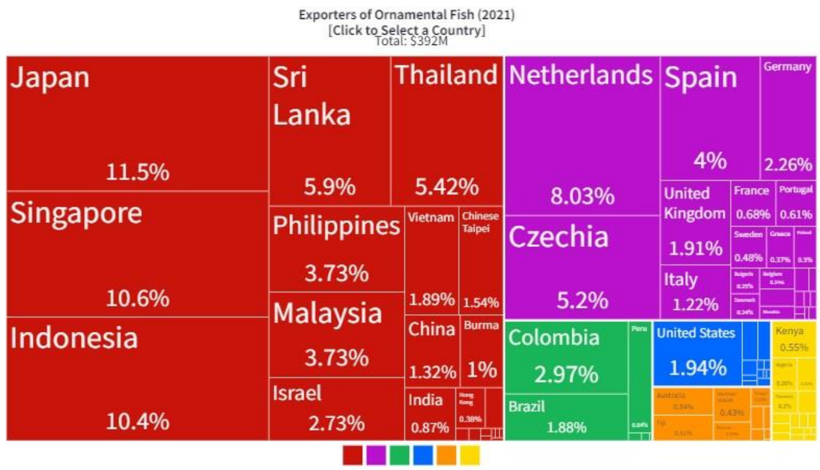

Figure 2.3 Exporters of Ornamental Fish. OEC. (2025, May 26). The Observatory of Economic Complexity. Retrieved from https://oec.world/en/profile/hs/freshwater-ornamental-fish.

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).

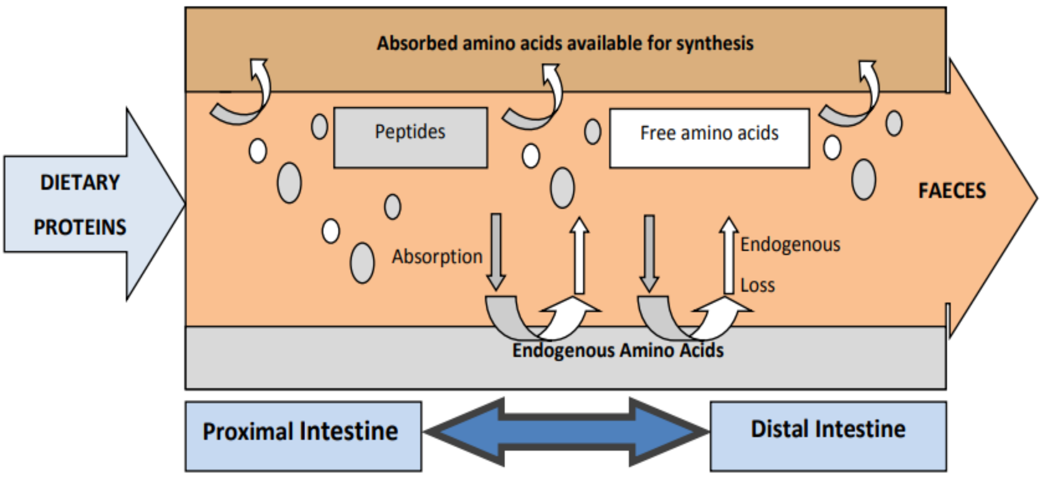

Figure 2.4 A conceptual diagram of digestion and absorption of dietary proteins in intestine of fish. (Chowdhury 2012).

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.

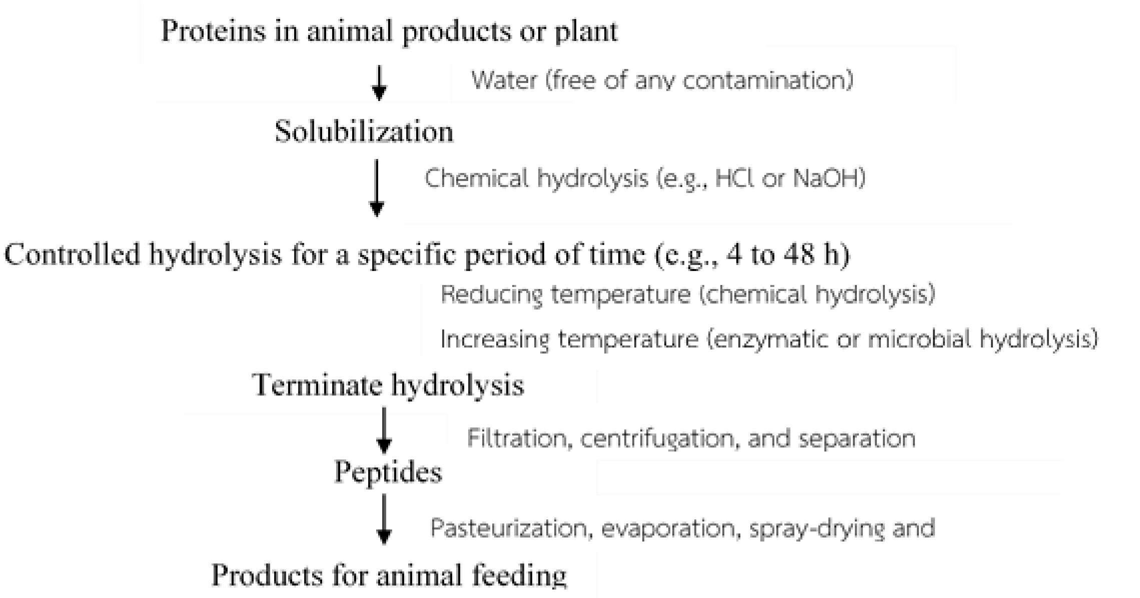

Figure 2.5 General procedures to produce peptides from animal and plant proteins. (Hou et al., 2017).

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	Alcalase	10% with PBM		(↑) Survival	Chaklader et al., 2020
	Carp hydrolysate Cyprinus carpio				(↔) Final body weight, specific growth rate,	
	Bluefin tuna Thunnus Maccoyii				feed conversion ratio and feed intake	
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.

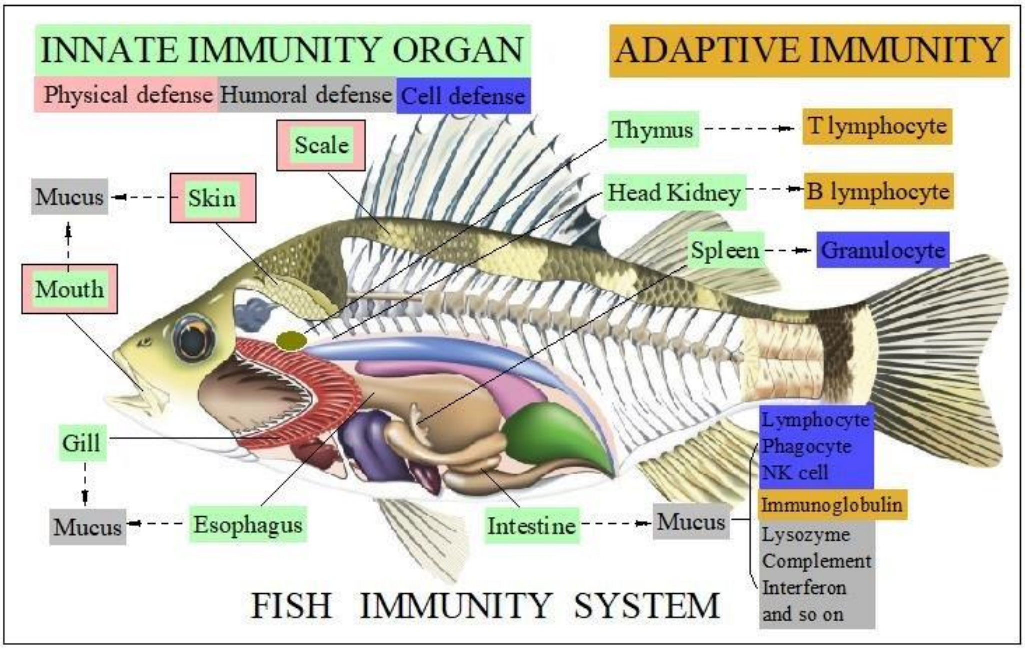

Figure 2.6 The innate immune system of fish. (Le et al., 2022).

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.

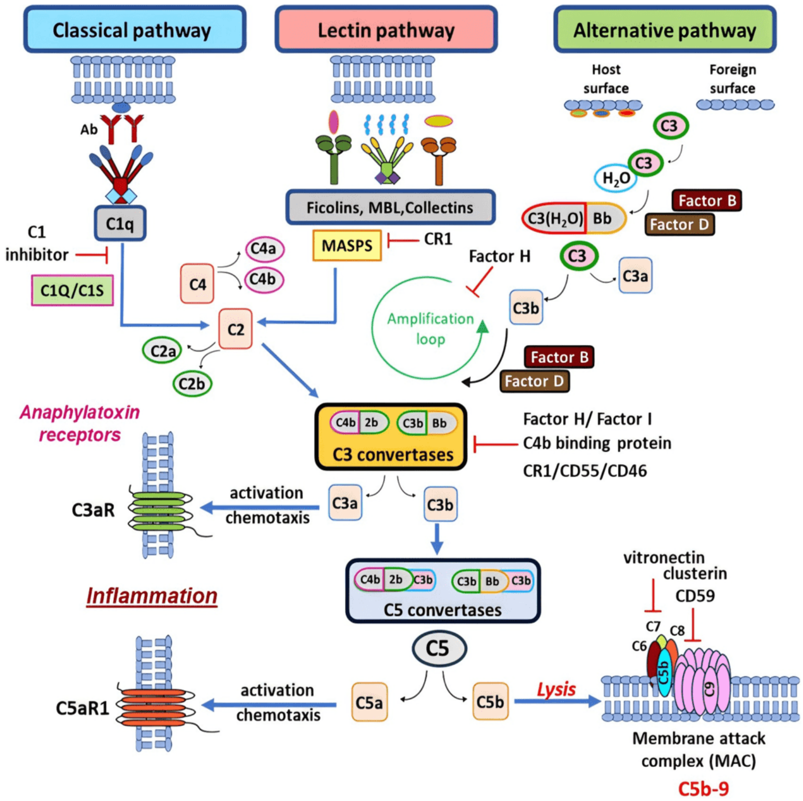

Figure 2.7 The complement cascade pathways (Detsika et al., 2024).

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.

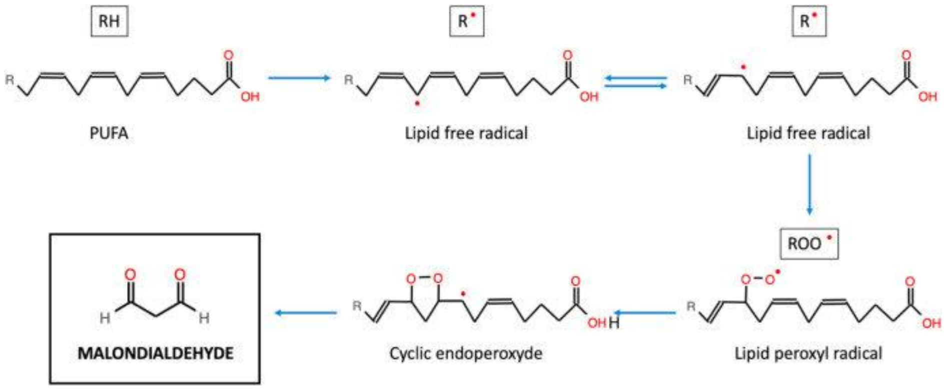

Figure 2.8 MDA formation through lipid peroxidation.

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).

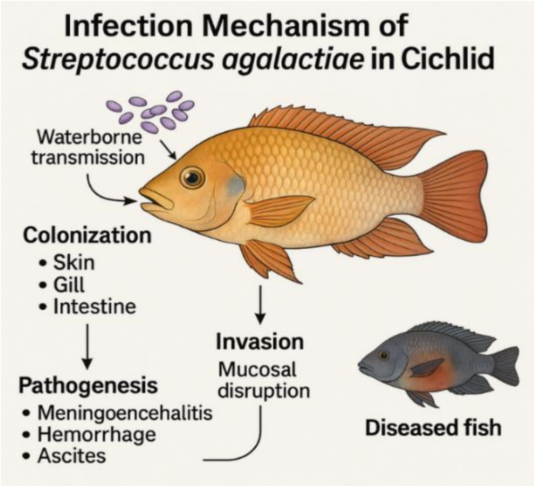

Figure 2.10 Schematic illustration of the fish immune system showing innate and adaptive immune responses.

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).