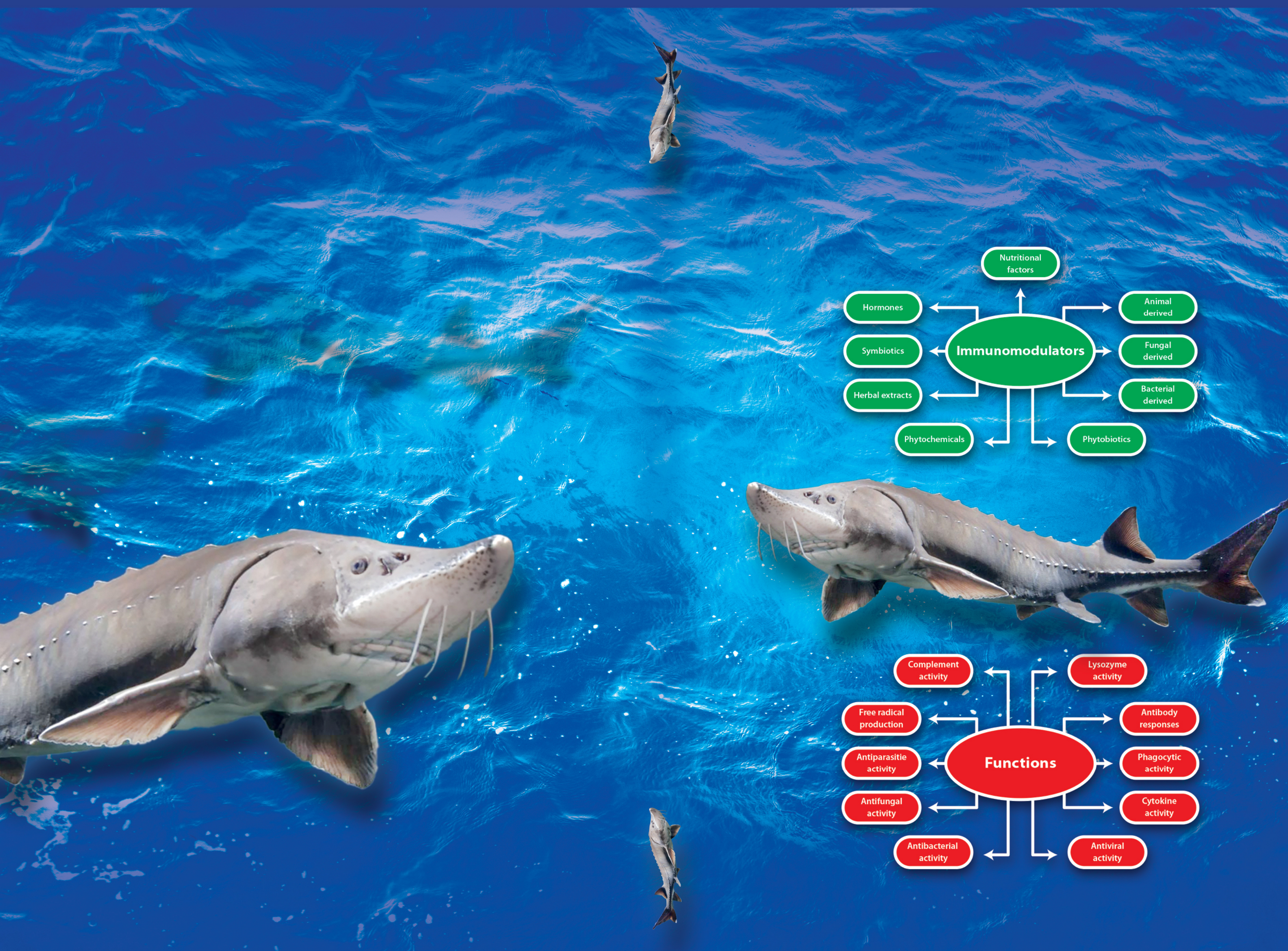
Immunomodulators in Aquaculture and Fish Health

Edited by
Preetham Elumalai
Mehdi Soltani
Sreeja Lakshmi



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Features:

- Reviews the pressing topic of reduction of antibiotic use in aquaculture
- Discusses herbal immunomodulators, nutrients, antioxidants, and pre- and pro-biotics
- Covers the topic of progressive immunomodulation using nanotechnology
- Discusses fish health management in the ever-growing aquaculture industry
- Includes natural and synthetic immunomodulators

The book is meant for researchers and industry experts in aquaculture, fisheries science, and veterinary medicine.

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Contents

Foreword

It is my great pleasure to write this foreword for a very timely book, "**Immunomodulators in Aquaculture and Fish Health,**" edited by my colleagues, Preetham Elumalai, Mehdi Soltani, and Sreeja Lakshmi.

As a believer in identifying opportunities and linking scientific evidence, innovation with improved sustainable aquatic production, together with my professional commitment in the aquaculture industry of over 40 years, I have found this volume of work to be a very extensive review. The wealth of information on the potential therapeutic and preventative roles of immunomodulators, in combating diseases in farmed aquatic species is truly insightful.

The importance of this book defines an era, where the performance of aquatic foods has been greatly recognized and concerted efforts have been initiated to enhance production and to bridge the ever-expanding supply-demand gap, for aquatic blue foods worldwide.

The burden of disease is high in aquatic production and currently estimated as $10 billion USD annually. With the decades of experience and lessons learned in aquatic animal health management, it is convinced that prevention is better than cure for aquatics and investing in prevention is more cost-effective than investing in therapy.

In this regard, I believe we should be aiming for more tools and procedures such as vaccines and vaccination and more research efforts should be supported, both at academic and commercial levels.

I congratulate the editors for this comprehensive volume and hope it will serve the purpose of increasing awareness of immunomodulators towards the implementation within aquatic animal health management.

Dr Rohana Subasinghe
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Aquaculture plays a vital role in global food security and economic development. It is the fastest-growing food production sector in the world and has been for some considerable time, with an average annual growth rate over the last 50 years of 8%. The latest FAO State of the World Fisheries and Aquaculture Report (2022) estimated global aquaculture production of aquatic animals at a record 87.5 million tonnes, with a value of USD 264.8 billion; this equates to 49% of total aquatic animal supply by volume and 65% by value. Approximately 2.5 million people are directly employed in the aquaculture sector around the world, with most of these in Asia, followed by Africa and Latin America. Women comprise 28% of these employment figures, slightly more than the average of 25% for the agriculture sector as a whole.

Infectious diseases represent a major constraint to the continued growth of global aquaculture, with estimated annual losses of at least USD 6 billion. The industry is particularly prone to disease outbreaks because of high stocking densities which increase pathogen transmission rates and reduce water quality, and also because of low genetic diversity in many breeding stocks, which may compromise the immune response to infection of cultured animals. Antimicrobials and antiparasitics are used therapeutically and prophylactically, but these treatments are often expensive and there is concern over the potential adverse effects of their widespread use, particularly the promotion of antibiotic resistance. Vaccination is another option for disease control, but vaccines often have limited efficacy, particularly for juvenile fish which do not have a fully developed immune response. There is therefore increasing interest in alternative approaches to disease control in aquaculture.

Immunomodulators are substances that affect the functioning of the immune system. A range of natural and synthetic products have been used or proposed, with varying degrees of scientific evaluation, to control infectious diseases in aquaculture. This book provides a very comprehensive and timely exploration of their efficacy and potential role in an aquatic animal health management system. The various sections of the book provide an overview of immunomodulators and their mode of action; the potential of immunomodulators to provide a more sustainable approach to disease control; the current use of immunomodulator products in aquaculture; and finally, their efficacy, limitations, and future prospects for the aquaculture industry. The book will be of great benefit to researchers in aquatic animal health, aquatic veterinarians, aquaculture managers, and all of us who wish to promote an economically viable and environmentally sustainable aquaculture industry.

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Editors

Preetham Elumalai, PhD, is an associate professor at the Department of Marine Biology, Cochin University of Science and Technology, Kochi, Kerala, India. He earned a master's degree from the University of Madras and a PhD in biochemistry and molecular immunology from the Institute for Immunology, University of Regensburg, Germany. His research practice includes bioassay-guided identification of novel marine compounds, unveiling fish lectins in innate immune defense, aquatic vaccine development, evaluation of cost-effective feed additives and nutrigenomics, and effects of environmental pollutants on marine ecosystems. He has been a partner in numerous EU-, Indian-, and UK-funded projects (e.g., IVVN, BactiVac). He has written more than 70 peer-reviewed articles and has two patents in his name apart from editing five books (Springer, CRC Press) and has presented his work at more than 60 national and international conferences. He has been awarded the prestigious INSA fellowship (2018); MASTS, Fellowship (2019); IVVN award, UK (2020); FRSB award (2021); and BactiVac award, UK (2022).

Mehdi Soltani, PhD, is a distinguished professor at the University of Tehran and an adjunct professor at Murdoch University, Australia. He earned a DVM from the University of Tehran and PhD in aquatic animal health from the University of Tasmania, Australia. Professor Soltani has an international reputation for research on aquatic animal health, with 290 published scientific papers, collaborations with researchers throughout the world, and editorship of scientific journals in fisheries and veterinary science. He chaired government advisory committees in fisheries and aquaculture and has also worked closely with the aquaculture industry. He developed and patented a number of fish vaccines, which are registered throughout the Middle East. He also taught numerous undergraduate courses and supervised many higher degree students. His research interests include vaccine development for fish pathogens; immunopathogenesis of infectious agents in fish/shellfish; and development of alternative therapies such as immunostimulants, probiotics, and phytobiotics for disease control in farmed fish and shellfish.

Sreeja Lakshmi, PhD, is a postdoctoral research scientist in collaboration with Moredun Research Institute (MRI), UK. She graduated from Calicut University and earned a PhD in biochemistry and functional genomics from the Institute for Molecular Biology, University of Regensburg, Germany. She has published research articles in peer-reviewed international journals and authored books and book chapters. She has been awarded prestigious research grants from the Bavarian Research Foundation (Bayerische Forschungsstiftung), Government of Bavaria, Germany; an HRD-Fellowship for Women Scientists from the Department of Health Research, Government of India; and a MASTS (Marine Alliance Science and Technology, Scotland) Award for Postdoctoral and Early Career Research Exchanges (PECRE). She has visited the University of Aberdeen, Scotland, and received an IVVN Fellowship grant from the International Veterinary Vaccinology Network (IVVN), UK.

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Section I

Immunomodulators: An overview

1

Immunomodulators: An Introduction

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1.1 Introduction

Before going into detail about immunomodulators, it's important to understand how immune systems work and how different variables can stimulate the immune system in different ways. Many substances are known to exist in our environment, and they are always capable of affecting the immune systems of living beings. For example, it has been shown that the immune system plays an important role in aquatic organisms subjected to various physical stress conditions (Mauro et al. 2021), noise pollution (Mauro et al. 2020), drug pollution (Mauro et al. 2022), or bacterial activity (Vizzini et al. 2021). Several biomarkers are also used to evaluate the health status of important animals in aquaculture (Mauro et al. 2022). In addition, aquatic organisms are also an excellent source of molecules with antimicrobial and antitumor activity (Mauro et al. 2022). In this chapter, we briefly introduce the function and role of immunomodulators, especially in the fish immune system. Fish have an immune system that is similar to that of higher vertebrates. As a result, every living entity must preserve its integrity and health status when challenged and must be able to recognise and distinguish between "self" (its molecules, cells, and tissues) and "non-self" (all other organisms or substances). The purpose of the immune system is to recognise the millions of non-self organisms that are potentially harmful to the self and to eliminate them or reduce their impact so that no damage occurs to the self (Takx-Köhlen 1992). This chapter does not explain self-immunity or self-tolerance phenomena that lead to suppression of the immune system and the spread of many autoimmune diseases in fish. We prefer to divide the immune system into two broad categories based on function, namely the innate immune system (non-specific immune system) as the first line of defence against pathogens (Carbone & Faggio 2016) and the adaptive immune system (specific or acquired immune system) (Marshall et al. 2018).

1.2 How Do the Immunostimulants Work in Fish?

Since their embryonic life stage, fish have relied on their innate immune system, and their survival depends on it. The skin is the principal non-specific defence in fish and plays a key role in protecting and preventing the entry of pathogens into the epithelium through the secretion of a mucus layer involved in the immunity system (Salinas et al. 2011). The cells and mediators involved will differ depending on the time, the trigger, the anatomical location (inflammation can affect any tissue), and the severity of the inflammation (Calder et al. 2013). Teleosts have a cellular defence system that includes macrophage-like phagocytic cells, neutrophils, and natural killer (NK) cells, as well as T and B lymphocytes, as well as various humoral defence components like complement (classical and alternative pathways), lysozyme, natural hemolysin, transferring factor, and C-reactive protein (Watts et al. 2001). Furthermore, teleosts and elasmobranchs are the most primitive groups that possess the major histocompatibility complex (MHC) and T-cell receptors, which are the primary components of the immune response against

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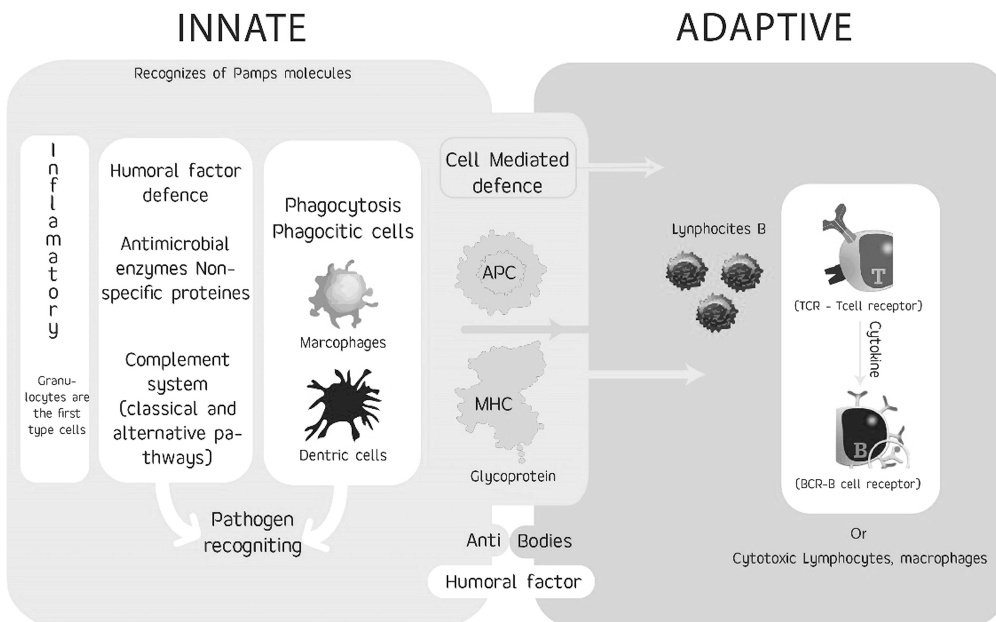

FIGURE 1.1 The immune system assumption in teleostfish.

The above figure represents types of immune system in fish. Innate immunity provides primary defence against pathogens by activating phagocytosis and antimicrobial, complement activation. On the other hand, adaptive immune system functions by stimulating specific lymphocytes.

pathogenic organisms (Zou & Secombes 2016). Sakai et al. (2021) investigated the role and function of cytokines in fish (interferon, interleukin 2, and macrophage activating factors), whereas among the lymphoid organs found in fish to mediate the responses have been the thymus, spleen, and kidney (Zapata 1996). The collaboration of fish innate and adaptive (memory) immune systems to eliminate intruders or activate defensive mechanisms is now well recognised; these two systems are classified into cell-mediated defence and humoral components (soluble substances). Figure 1.1 describes their main functions.

The innate system is made up of three parts: the tegument (skin and mucus), cellular components (granulocytes, monocytes, macrophages, and natural killer cells), and humoral components (granulocytes, monocytes, macrophages, and natural killer cells) (the complement system, antimicrobial enzyme system, and non-specific mediators such as interferon and interleukin). Furthermore, inflammation is thought to be an innate immune response driven by complex interactions between cellular and humoral components. Granulocytes are the first cells to arrive at the site of inflammation and are responsible for killing infections. Innate and adaptive immune systems normally work in concert, with innate responses serving as the host's first line of defence and enabling adaptive responses by antigen-specific T and B cells to produce antibodies in the presence of specific humoral components called histocompatibility molecules and glycoprotein receptors encoded by genes in the major histocompatibility complex (MHC).

1.3 Immunomodulators

The immunomodulators are all antigens (chemical or physical) that manage to vary the immune system's activities. When an agent depresses the immune system, also known as an immunosuppressant, they can cause a negative response. Immunosuppression is described by Dohms and Saif (1984) as "a state of transient or persistent dysfunction of the immune response resulting from insults to the immune system and increasing susceptibility to disease," and the qualifier "and frequently a suboptimal antibody response." Otherwise, if an immunomodulator can increase or promote activity, it is called an immunostimulant. Finally, another category is immune adjuvants, which hold the promise of being the actual modulators (De Paula Barbosa 2014) of the immune response, especially to enhance the vaccine's efficacy. An example in this regard is Freund's complete adjuvant, which is being used to enhance the potency of poor immunogenic substances (Tengjaroenkul & Yowarach 2011). Very often, the same agent can have both immunostimulant and immunosuppressive effects on an organism. Studies showed that the physiological response of fish to different doses of immune system stimulants could be different (Petit 2019). In other words, in some cases, excessive use of several immunostimulants simultaneously may cause immunosuppression in fish (Raa 1996).

1.4 Immune Suppressors

Immunosuppressive compounds are chemical, biochemical, and physical agents that suppress, decrease, or disrupt the immune system functions in fish. Studies show that all immunosuppressant compounds carry a severe risk of infection. Various types of immunosuppressive materials may change the immune response in fish when challenged by pathogens (Hidasi 2017). Most immunosuppressants may weaken the immune system by altering the gene expression of immune parameters. However, some immunosuppressive materials may decrease the absorption of vitamins and dietary supplements. Furthermore, immunosuppressive compounds reduce the ability of fish's innate and specific immune systems to respond to foreign objects. The interaction of immune suppressants with drugs may also reduce their effectiveness. The following part explains some immune suppression effects on the fish immune system.

1.4.1 Environmental Stressors

The functions of the immune system of fish depend on nutrition, environmental conditions, health, gender, and ontogeny. Temperature, dissolved oxygen, salinity, pH, and hardness ranges may also differ between fish species. Therefore, environmental fluctuations could alter the fish's physiological status and immunological response. For example, hyperosmotic stress could decrease the immune system response of *Scatophagusargus* to bacterial infection (Lu et al. 2022). In hostile biological conditions, fish may not be able to feed properly. As a result, they will not have enough energy to allocate to the immune system (Estensoro et al. 2012). Moreover, breeder malnutrition may lead to epigenetic changes in the offspring's immune and metabolic genes. Improper fish nutrition under stressful conditions can also lead to impaired immune priming by dendritic cells and monocytes and impair the function of effective memory T-cells. Acute and chronic stress are critical agents in suppressing the immune functions of fish (Guo et al. 2021).

Environmental stress can suppress the immune response in fish. As a result, fish that live in stressful environments may have a weaker immune system than fish that live in normal conditions. Guo et al. (2022) discovered that exposing Wuchang Bream (Megalobramaamblycephala) to ammonia nitrogen reduced immunoglobulin M (IgM), interleukin 1 (IL-1), and tumour necrosis factor (TNF-) levels while decreasing TLR mRNA expression. Following exposure to ammonia, there was a considerable drop in IgM and component C3 levels and lysozyme activity in the spleen and head kidney of Pelteobagrusvachellii (Qi et al. 2017). Moreover, those latter authors showed that high ammonia concentrations in the environment could disrupt the expression of immune-related genes in crucian carp (*Carassius auratus)* (Mazini et al. 2022). It was observed that the immune system's response would be decreased when fish were exposed to the stress of transporting between different farms. They discovered that an increase in corticosteroids was linked to immunological suppression in fish. Corticosteroids may affect the effectiveness of the immune system by altering mRNA expressions implicated in immunological parameters. Therefore, to better understand the impact of environmental stressors, each of the stressors has been discussed separately.

1.4.1.1 Agrochemicals

Agrochemicals include fertilisers, phytohormones, and pesticides (e.g., insecticides, pesticides, herbicides, and fungicides) used in the agriculture industry. Agrochemicals also include medications, disinfectants, hormones, and growth stimulants used in cattle, poultry, and aquaculture. Suppression of the immune system in fish exposed to agrochemicals can increase the susceptibility and vulnerability of fish to various pathogens (Banaee et al. 2019; Farag et al. 2021). Changes in intrinsic and specific immunological indices are perhaps the most important reason for suppressing the immune system of fish exposed to agrochemicals (Hassan et al. 2022). This section explains the reasons for the decay of the fish immune system after exposure to agrochemicals.

Due to their lipophilic nature, most pesticides easily cross biological barriers and enter the aquatic body. Pesticides can suppress the immune system by interacting with immune agents or causing oxidative damage in tissues involved in the immune system (Farag et al. 2021). Previous studies have shown that fish exposure to pesticides can cause changes in haematological parameters, including a decrease in leucocytes and an alteration in the differential count of white blood cells (Banaee et al. 2008). In fish treated with pesticides, decreased total immunoglobulin, C3, and C4 complement activities have also been reported (Hatami et al. 2019). A significant decrease was reported in lysozyme activity, respiratory burst activity, and total immunoglobulin levels in Nile tilapia (Oreochromis *niloticus*) exposed to cypermethrin (Abdel-Tawwab et al. 2020). Exposure to pesticides can also lead to changes in the gene expression of inflammatory cytokines such as TNF-, IL-1, and IL-6 (Acar et al. 2021; Wang et al. 2020).One study found that glyphosate exposure altered the levels of interferon- (IFN-) and IL-1 in the hematopoietic tissues of common carp (*Cyprinus carpio*).

1.4.1.2 Heavy Metals

Exposure to heavy metals at levels higher than the accepted dosages could suppress the immune response in fish. Also, the transmission of heavy metal contamination through the food chain can affect fish immune systems (Mohiseni et al. 2017). A significant decrease in immune functions of Vardar chub (*Squalius vardarensis,* Karaman) may be due to histopathological damage to the kidney and spleen (Jordanova et al. 2017). Bernier et al. (1995) demonstrated that bioaccumulation of heavy metals could cause suppression of the immune system, autoimmune diseases, increased susceptibility to pathogens, and inflammation reactions in fish. Banaee et al. (2019) demonstrated that alterations in humoral immune parameters in fish exposed to heavy metals indicated quenching of innate immune responses. Manganese reduced lysozyme activity and IgM levels while increasing the expression of tlr3, tnf-, il-1, and il-6 in juvenile yunlong groupers (*Epinephelus maureus* and *E. lanceolatus*) (Wang et al. 2022). Heavy metals were exposed to *Centrarcus lal.rax.* changes in bactericidal activity in the skin mucosa of gilthead seabream (*Sparus aurata*) after heavy metal exposure have also been reported (Guardiola et al. 2015). A significant decrease in HK-B cell proliferation, IgM level, and serum bactericide potential was observed in catfish exposed to arsenic (Ghosh et al. 2007). Heavy metal exposure in fish may affect leucocyte counts. Furthermore, changes in the granulocyte/granulocyte ratio suggest that heavy metals trigger the fish immune system. A significant increase in monocyte and neutrophil numbers and a significant decrease in lymphocyte numbers were reported in goldfish (*C. auratus*) after exposure to manganese (Aliko et al. 2018).

Genotoxicity may be linked to oxidative stress, DNA damage, and changes in mRNA expression. Indeed, Ghazy et al. (2017) found that the gene expression in the immune system changed in Nile tilapia that lived in waters contaminated with heavy metals. Heavy metals altered the expression of IL1, TNF-, IFN, Mx, Lyz, C3B, and CXCL-Clc in zebrafish (*D. rerio*) embryos (Cobbina et al. 2015). Moreover, significant changes in the mRNA transcription of immune-related genes were observed in the leucocytes of European sea bass exposed to cadmium, lead, and mercury (Morcillo et al. 2015).

1.4.2 Other Xenobiotics

Segner et al. (2021) showed that xenobiotics could bind to the aryl hydrocarbon receptor (AhR) as a vital transcription factor. Then, complex AHR and xenobiotics were set on sequence response elements and changed immune-related gene expression. Suppression of the immune system in aquatic animals exposed to xenobiotics may be due to energy shifts for detoxification. Decreased energy allocated to the immune system may reduce its performance.

1.4.3 Natural Toxins

Qiao et al. (2013) investigated the immunotoxicity of cyanobacteria in Crucian carp. The immunological fish response might be boosted by blood cyanobacteria, according to authors Rymuszka and Adaszek (2013), who assessed the effects of microcystin derived from cyanobacteria on carp leucocyte proliferation under *in vitro* conditions. They showed microcystin could increase apoptosis rates in white blood cells. Rymuszka and Sieroslawska (2018) showed that carp leucocytes' half-life decreased after exposure to nodularin, a cyanobacteria toxin. Contamination of food with aflatoxin toxins can reduce the potency of non-specific immune systems in fish (Bitsayah et al. 2018). In common carp fed with aflatoxins-contaminated feed, Bitsayah et al. (2018) found a substantial shift in complement C3, C4, and CH50, lysozyme activity, and total immunoglobulin content.

1.4.4 Anti-nutritional Agents

Some antinutritional compounds in the diet can suppress the immune system in aquatic animals. However, the immunodepressive effects of anti-nutritional compounds on the immune system depend on their bioavailability and their dosages in feed. By generating cytotoxicity, anti-nutritional substances may have an impact on immune function. Furthermore, by producing inflammation or modifying the quantity of inflammatory markers in the blood, these substances may impair immunological function. Inhibiting the absorption of micronutrients in the intestine by anti-nutritional compounds can also play a role in suppressing the aquatic immune system (Abdel-Tawwab et al. 2018). The immune system may be harmed by phytotoxins such as cyanogenic glycosides (Cho et al. 2013). Glycinin is a dietary allergen in soy, which can harm an animal's immune system (Sun et al. 2008). Gossypol, an anti-nutritional compound found in cottonseed, can cause apoptosis by activating caspase-3 (Sadahira et al. 2014).

1.4.5 Diseases

Increased inflammatory cytokines in chronic diseases may be a reason to suppress the immune system. Furthermore, toxins and enzymes secreted by the primary pathogen can reduce the immunity of fish against secondary pathogens (Ilgová et al. 2021). Simultaneous fish infection with two or more pathogens can weaken the immune system (Shameena et al. 2021). Ilgová et al. (2021) found that chronic pathogen infections could significantly delay the immune system response of fish. They showed that infection with monogeneans could increase the susceptibility of fish to secondary pathogens. Ilgová et al. (2017, 2020) demonstrated that infection with *Eudiplozoon nipponicum* reduced TNF-gene expression in common carp macrophages in vitro.

1.4.6 Sex Hormones

Hormone manipulation and changes in the steroid hormones may affect gene expression in a fish's immune response. Therefore, a decrease in the immune system capacity of adult fish may be related to changes in sex hormones. Szwejser et al. (2017) found that fish leucocytes have receptors and cytochrome P450 aromatase. As a result, oestrogen levels in the blood might affect the fish's immune system. Cabas et al. (2018) found a link between oestrogen levels and autoimmune illness and chronic inflammation in fish and discovered that fluctuations in sex steroids affected the immune systems of spotted snakeheads (*Channa punctatus*). They found that increased sex steroids could mitigate innate and cellular immune responses. Dietrich et al. (2021) showed that hormone therapy in common carp could change mRNA expression in hematopoietic tissues.

1.4.7 Drugs and Antibiotics

Antibiotics can suppress the fish immune system by disrupting the regulation of NF-B signalling and immunotoxic pathways (Qiu et al. 2020). Yang et al. 2020 indicated that antibiotics are toxic to fish at high dosages. They also showed that treatment with a high dose of antibiotics would suppress the fish's immune system. Increased mortality and an inflammatory response were observed in zebrafish treated with 260 ng of L-1 sulfamethoxazole (Zhou et al. 2016). Common carp exposed to sulfamethoxazole has reportedly seen a similar outcome (Iftikhar et al. 2022). Liu et al. (2020) found that exposure of zebrafish larvae to high doses of sulfamethoxazole could change the mRNA expression of cytokines such as IL-1, IFN-, IL-11, and TNF-. The genotoxicity effects of antibiotics on leucocytes could mitigate the efficiency of cellular immunity in fish (Grondel et al. 1985).

1.5 Immunostimulants

Immunostimulants have a crucial role in activating the non-specific defence mechanism in fish protection against pathogens, and they are valuable for controlling fish diseases. Previous studies have shown that numerous immunostimulants may be useful to fish cultures in aquafeed. Particularly for enhancing and improving the immunity and disease resistance in fish, increasing non-specific defence mechanisms immune stimulants may also be an effective strategy to increase fish performance. Although the use of immunostimulants in the aquaculture industry has been successful in some cases, research in this field is still ongoing (Jadhav et al. 2006).

Chemical agents, bacterial components such as probiotics (Abdel-Latif et al. 2022), polysaccharides (e.g., from plants) (Faggio et al. 2016, 2015), animal or vegetable extracts (plant-based) (Rashidian et al. 2021), feed additives and herbal extract (Elumalai, as pointed out above, immunostimulants) might mainly facilitate the function of phagocytic cells and increase their bactericidal activities (Abarike et al. 2019). Moreover, natural killer cells, complement, lysozyme, and other antibody responses may be stimulated by different types of immunostimulants. Immunological function and activation are associated with improved protection against infectious diseases in aquaculture due to their ability to serve as an alternative and supplement to vaccination. Moreover, they also have additional effects on growth performance and the survival rates of the fish under stress (Heo et al. 2001) (Figure 1.2).

1.5.1 Chemical Agents

Since immune system stimulants contain many chemical compounds that may affect the function of the aquatic immune system, they can be administered alone or in combination with vaccination. Some immunostimulants have been shown in studies to improve vaccination efficiency. Shahbazi and Bolhassani (2016) found that some biochemical compounds such as vitamin C and E, lactoferrin, interferon, growth hormone, prolactin, and recombinant cytokines can act as immunostimulants. The chemical agents could disrupt immunological structures and functions, making animals more susceptible to both infections and non-infectious agents. A wide range of substances target the immune system and prolonged exposure to these compounds can result in immunological dysfunction (Koller 2001). For example, levamisole is the most useful chemical agent used and is a

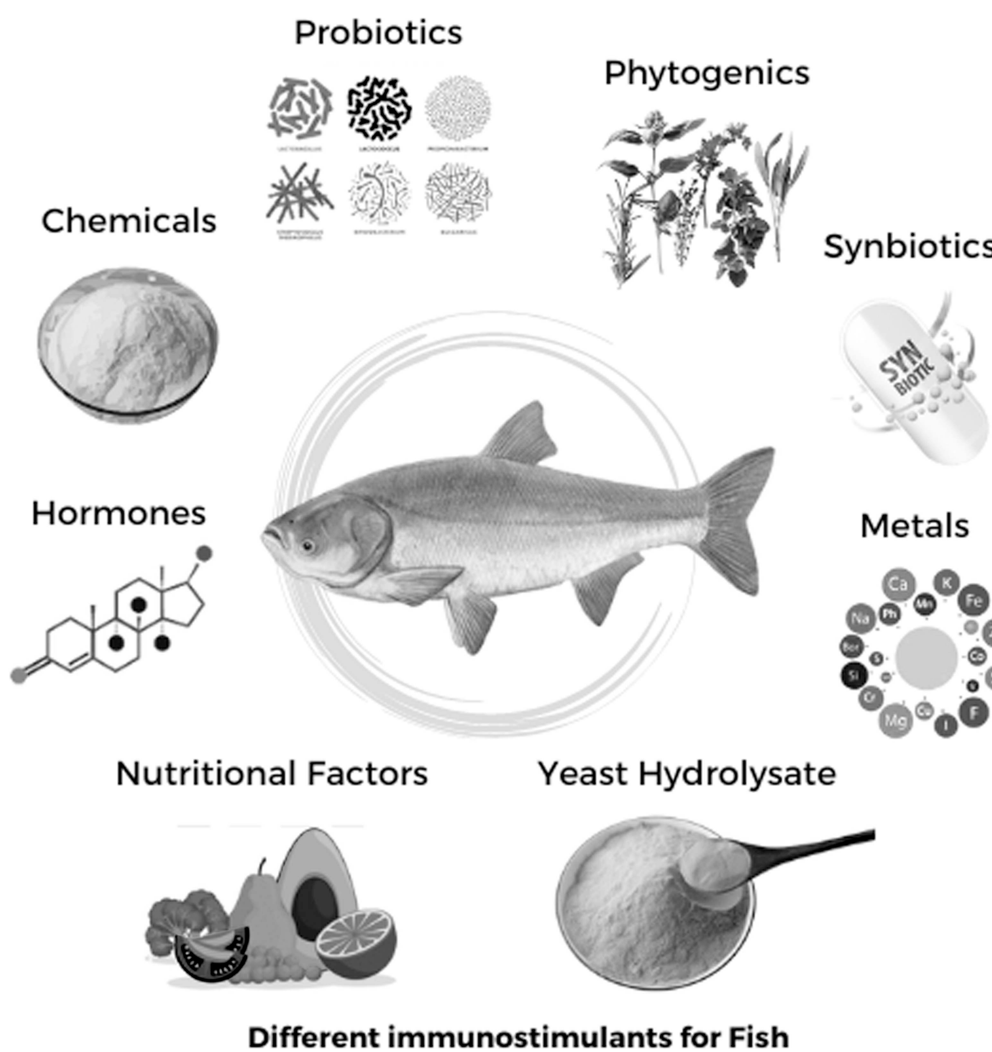

FIGURE 1.2 Different categories of immunostimulants for fish.

Various natural and synthetic agents that enhance immunological activity in fish are depicted in the figure. Bacterial agents, specifically prebiotics and probiotics are of high potential, along with immensely used phytochemicals, yeast derivatives, chemical agents and metals.

levo-isomer of tetramisole (Findlay & Munday 2000). It is a polysaccharide that can increase macrophage activity and provide resistance to specific harmful microorganisms. On the other hand, chitosan is a de-N-acetylated version of chitin. Both chitin and chitosan have the potential to be significant components in aquaculture. Chitosan treatment by injection or immersion was found to enhance brook trout (*Salvelinus fontinalis*) resistance to an *A. salmonicida* infection. Chitosan treatments had a substantial influence on the non-specific immunity and immunological response of both healthy and cortisol-treated *Labeo rohita* (Barman et al. 2013).

1.5.2 Biological Substances and Bacterial Derivates

The most common biological substances are bacterial derivatives, also known as killed pathogens, and their products. The use of immunostimulants such as probiotics and prebiotics has always been considered (Bachère 2003). Probiotics are a collection of non-pathogenic microorganisms often found in aquatic animal digestive systems. Oral administration of probiotics can change bacterial flora, boost the immune system, and stimulate growth performance (Villamil et al. 2002). Previous studies show that the administration of probiotics can significantly increase antibody production and non-specific immune parameters in fish (Abareethan & Amsath 2015). Therefore, it is essential to identify and isolate different strains of microorganisms to produce probiotics. Among the different probiotic strains commonly used in fish are *Bacillus,* e.g., *B. subtilis* and *B. licheniforms*; *Lactobacillus* sp. such as *L. delbrueckii subsp., L. bulgaricus, and L. acidophilus*; and *Bifidobacterium* sp. Numerous reports on the use of probiotics in aquatic environments have been published (Van Doan et al. 2020; Jahangiri & Esteban 2018; Chauhan & Singh 2019), and for more details, readers are referred to Chapter 10.

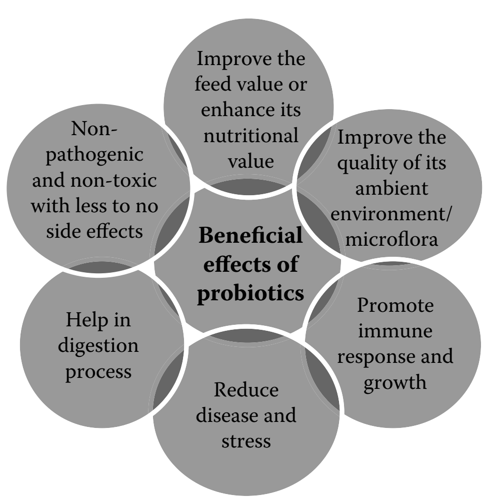

FIGURE 1.3 Benefits of probiotics.

Figure 1.3 illustrates the functions of probiotics as an immunostimulant in aquaculture. Probiotics promote immunity, growth, digestion, reduce stress and improve the feed value and quality of microflora in fish.

1.5.3 Lipopolysaccharide

Lipopolysaccharide (LPS) is the main component of the outer membrane of gram-negative bacteria such as *Salmonella typhimurium* and *Escherichia coli* (Miura & Mizushima 1968), and its preparations include O-antigens and endotoxins. The biological activity of LPS is a consequence of both hydrophobic domains known as lipid A (or endotoxin), a "core" oligosaccharide, and a distal polysaccharide (or O-antigen) (Neidhardt 1996). Moreover, LPS has been used as a potential immunostimulant. Toll-like receptor (TLR)-4 is mainly involved in the activation of the immune system by LPS through the specific recognition of its endotoxin (lipid A) moiety. LPS studies were made on fish both in vitro and in vivo, and they reported that LPS influences the growth and health status of fish. (Guttvik et al. 2002) showed that Atlantic salmon fry fed with LPS-coated feed (0.1% LPS) for 63 days had a reduced survival rate when challenged with a virulent strain of *A. salmonicida.* Furthermore, Paulsen et al. (2003) discovered that LPS stimulates plasma lysozyme activity originating from macrophages in various organs (e.g., blood polymorphonuclear and cells isolated from the head, kidney, and intestine) in their experiment on *Salmo salar*. In an in vitro experiment, Paulsen et al. (2001) in *Salmo salar* found that in head kidney macrophages grown in the presence of LPS, there was an increase in lysozyme production in the culture supernatants, which coincided with an accumulation of lysozyme gene transcript in stimulated cells.

1.5.4 Hormones and Cytokines

Hormones and cytokines are part of the neuroendocrine system. Their role as immunomodulators in the immune system has been studied in recent years. Acute stress may often be associated with fish life stages and have an impact on fish immunity and health (Figure 1.4). An example could be stress (resulting in potential advantages), thus involving short-term challenges resulting in immune activation or enhancing processes. Hormones generally can directly affect macrophages, lymphocytes, NK cells, and mitotic activity. Cortisol, growth hormone (GH), prolactin (PRL), reproductive hormones, melanin-concentrating hormone (MCH), and pro-opioid melanocortin (POMC)-derived peptides have all been shown to affect immune function in many fish species (Harris & Bird 2000). The growth hormone (GH, or somatropin) is a hormone from the family of prolactin and somatostatin; the main role of GH and insulin-like growth factor-I (IGF-I) is in the regulation of body size in growing animals. Previous studies showed that the administration of exogenous growth hormone (GH) improves many aspects of immune function, for example, cytotoxic (Sakai et al. 1996), phagocytic (Yada et al. 2006), haemolytic (Kim et al. 2013), and lysozyme activities (Harris et al. 2000) as non-specific defences, and immunoglobulin production as specific defences Yada (2007) observed that the activation of immune function during seawater adaptation was closely associated with increased plasma GH levels in some euryhaline fishes. Moreover, a previous study (Yada et al. 2006) showed that ghrelin (an important regulator of GH

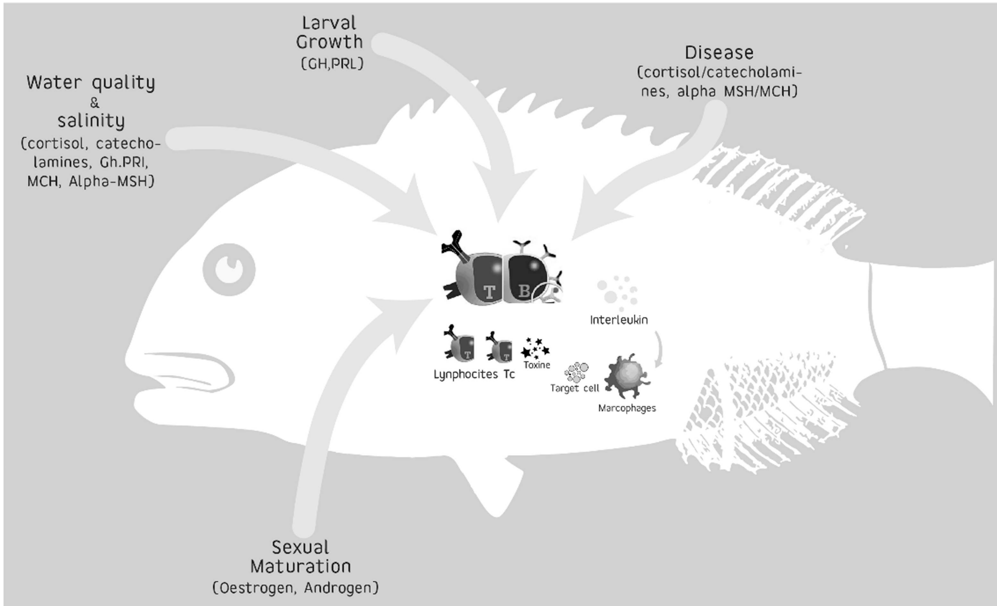

FIGURE 1.4 Interconnections between the general immune system and the endocrine system about environmental processes and fish life stages.

secretion) stimulates superoxide production associated with phagocytosis in trout leucocytes. While GH increased the mRNA levels of superoxide dismutase, which catalyses the dismutation of superoxide into oxygen and hydrogen peroxide, Also, Pontigo & Vargas-Chacoff (2021) found that GH may modulate the immune response in the SHK-1 cell line and leucocyte cultures of the head kidney in Atlantic salmon. Therefore, their work points out the independent action of GH on the immune system and the GH/IGF axis.

Cytokines are low-molecular-weight glycoproteins involved in regulating the immune system. These molecules are mainly secreted by cells of the innate and adaptive immune systems, but they play an important role in the innate immune response in fish. Kono et al. (1996) reported that IL-1 plays an essential role in fish immunity by activating lymphocytes and phagocytic cells and increasing resistance to *A. hydrophila* infection. Type I IFNs (homologs to human IFN- and IFN-) also have antiviral activity. Secombes & Belmonte (2016) discovered that type II IFN (IFN-) had bactericidal activity against intracellular parasitic bacteria. Studies to elucidate cytokines' functions in fish have recently begun. Still, more work is needed to select their appropriate functions, such as immunostimulants and vaccine adjuvants, to prevent infection in farmed fish. Sakai et al. (2021) developed a multiplex reverse transcription-polymerase chain reaction assay to investigate the immune response of fish when activated by an immunostimulant. Moreover, cytokines are also defined as biological response modifiers because of their ability to enable communication between different cell populations, in agreement with what was reported by Wilson et al. (2002).

Conforming to changes in environmental factors such as water quality, salinity and diseased conditions, the T-cell proliferation and cytokine expression also range, which is mediated through hormonal regulations. Hormones expressed during sexual maturation and larval growth enhance the expression of these immunological mediators.

1.5.5 Phytogenic Immunostimulants

(Plant extracts, herbals, garlic, ginger, triterpenic acid, polyphenols, olive oil, seaweeds)

Phytogenics are plant-derived natural products characterised by their richness in biologically active compounds that are mainly incorporated into the feed to enhance the innate immunity, health status, and growth performance of the animals. In farmed fish, phytogenics have been reported to contribute as antimicrobials, antioxidants, anti-inflammatory agents, immunostimulants, and sedatives. They work as promoters of growth and appetite stimulators, and they could influence the bile secretion and several enzymes associated with digestion (Chakraborty et al. 2011, Firmino et al. 2021, and Caipang et al. 2021). Botanicals, including herbs and spices, contain aromatic compounds and essential oils (extracted from parts of plants such as leaves, flowers, roots, and fruits), and many other medicinal plants come under phytogenics (Caipang et al. 2021). Plant extracts are active substances with desirable properties that are extracted from plant tissue for specific purposes, such as immunostimulant use. They have been known to have increased lysozyme activity, complement activity, phagocytic activity, an antibody response, elevated respiratory burst activity, and higher plasma protein (albumin and globulin) (Reverter et al. 2014; Harikrishnan R et al. 2011a). Saponin compounds, herbs, ginger, triterpenes from fungi and plants and seaweeds, etc. are some of the compounds and products which have an immunostimulatory effects on fish health and welfare and are discussed in detail in this book.

1.5.6 Nutritional and Dietary Factors

(Dietary amino acids, vitamin C, vitamin E, dietary nucleotides, organic acids, polysaccharides, probiotics, and food waste)

Nutrition plays a crucial role in maintaining the body's functioning and health. It provides all the essentials required by the

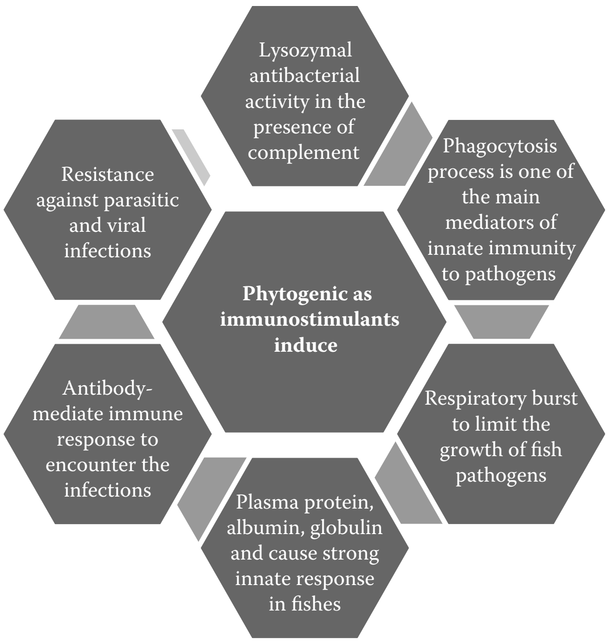

FIGURE 1.5 Activities of plant extract as an immunostimulant.

Figure 1.5 represents the role of plant extracts in enhancing immunity in fishes. Plant extract stimulate immunity by different cellular mechanism like phagocytosis, respiratory burst, antibody mediated responses, lysosymal antibacterial activity, and stimulate immunogenic plasma proteins.

body to maintain life, like metabolic energy and elements and compounds that act as co-factors for various physiological processes. When we talk about immunostimulants, dietary nutritional components include dietary amino acids (AA), vitamin C, vitamin E, dietary nucleotides, organic acids, polysaccharides, probiotics, food waste, etc. Dietary amino acids are essential acids that can improve the haemocyte count, phagocytic activity, respiratory burst in haemolymph, and lysozyme activity in cell-free haemolymph (CFH). Moreover, they can remarkably downregulate the malondialdehyde content (Luo et al. 2021). In addition to these, AAs are necessary for endogenous synthesis of protein and act as important energy substrates. These can also modulate the necessary metabolic pathways (Dawood et al. 2021) (Figure 1.6).

Vitamins are organic compounds necessary for animal growth and development. They are required in small quantities and must be provided with food, as they cannot be synthesised in the body. At present, vitamins C and E have gained popularity as immunostimulants (Kono et al. 1996). Vit E (tocopherols) are bioactive phenolic compounds, and a proper dose of this can (i) promote the differentiation and proliferation of lymphocytes and cytokines, (ii) enhance the production of antibodies and enhance complement activity when encountered with an antigen, and (iii) it can also improve phagocytosis activity and cytotoxicity. In channel catfish and turbot, VE enhances macrophage phagocytosis (Barman et al. 2013). Vitamin C (ascorbic acid) is involved as a cofactor in many bioactive processes, like neuromodulation, collagen synthesis, and cellular activities related to hormones, and the immune system.

Dietary nucleotides, chitin, and organic waste do also have compounds with immunostimulatory effects and are discussed in subsequent chapters. Bacterial and yeast cell walls mostly consist of glucans. When glucan was given to feed, it activated phagocytic cells in fish, and they also demonstrated an increase in the development rate of *Litopenaeus vannamei* juveniles, enhancing phagocytosis and the capacity of the cells to eliminate harmful pathogens. Additionally, they increase complement and lysozyme activity (Kono et al. 1996). Additionally, glucans improve the non-specific defence mechanisms of fish and shellfish and offer defence against bacterial infections (Barman et al. 2013). Prebiotic compounds called fungal polysaccharides are commonly regarded as a dietary component for controlling growth and health issues. Higher fungi are excellent providers of a variety of crucial natural compounds (Mohan et al. 2019). More details are discussed in subsequent chapters (Figure 1.7).

1.5.7 Trace Elements and Metals

Minerals that are less abundantly present in living tissues are referred to as trace elements (or trace metals). They are considered to be nutritionally essential, although if consumed at sufficiently high levels, they may prove toxic. Copper, chromium, iron, iodine, fluoride, manganese, molybdenum, selenium, and zinc are considered as essential trace elements. General functions of minerals include structural constituents of tissues, formation of the exoskeleton, osmotic pressure balance, muscle contractions, and nerve impulse transmission. They are

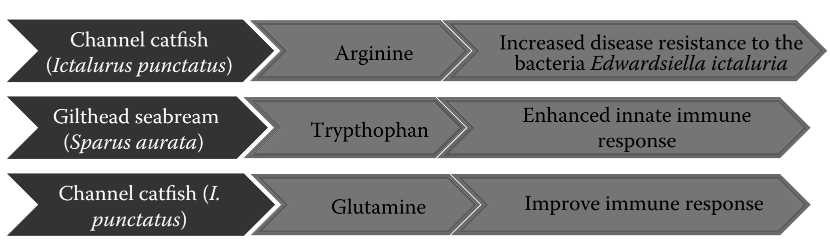

FIGURE 1.6 Effects of different amino acids on fish health.

Essential amino acids such as arginine and tryptophan and non essential aminoacid like glutamine have increased effect on fish immunity. Arginine, in channel catfish improve the resistance in fish against *Edwardsiella ictaluria.* Tryptophan in Gilthead seabream has a transient immune enhancement activity in the fish. Similarly, glutamine improve the immune response in channel catfish.

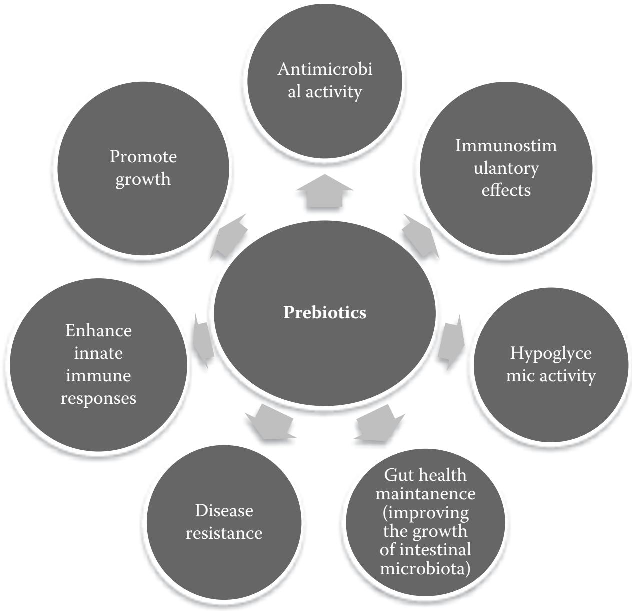

FIGURE 1.7 Functional properties of prebiotics.

Prebiotics have an enhancing effect on fish growth, antimicrobial activity, and disease resistance. They improve favourable intestinal microbiota and stimulate immune system in teleost.

also prime components for co-factors in metabolism, catalysts, enzymes, enzyme activators, hormones, pigments, and vitamins.

1.5.8 Synbiotics

Synbiotics are a combination of both prebiotics and probiotics and work as growth and immunity promoters. These have been used in aquaculture for over a decade, but the functional mechanism is still not very clear. Prebiotics, which are parts of synbiotics when hydrolysed to simpler mono- or disaccharides, show an exceptional increase in biomass and colonisation of probiotic bacteria on the surface of intestinal epithelial cells in the host. By releasing extracellular bacterial enzymes and bioactive substances from their metabolic activities, they also contribute to the growth of aquatic animals. These enzymes also improve the nutrient absorption capacity, which in turn help in effective utilisation of feed. Synbiotics stimulate the immune system's synthesis of nitric oxide, phagocytosis, and respiratory burst activity in fish.

1.5.9 Yeast Hydrolysate

Yeast is known for keeping the digestive system of an animal's body healthy and in balance; hence, it is widely used as a supplement in aquatic feeds. Yeast has a high protein and energy content, as well as high micronutrient content. Aside from being high in amino acids and proteins, yeast products also have immunomodulatory compounds like mannan oligosaccharides (MOS), chitin, glucans, and nucleic acids. Mannan oligosaccharides are well known to enhance the growth performance of rainbow trout (*Oncorhynchus mykiss*), *Aespeciallyes cerevisiae* is a well-known yeast from which glucan is extracted and purified, and an intraperitoneal injection of this improves both specific and non-specific immune responses in carp *(Cyprinus carpio) to* the bacterial challenge posed by *A. hydrophila.* Yeast hydrolysate is a hydrolysate of yeast cells obtained through various methods like acids, enzymes, or other hydrolysis. The extract may be obtained through autolysis, where the enzymes found in yeast itself are used to break down the protein, or through hydrolysis, where enzymes are added from external sources as depicted in Figure 1.8 (Gong et al. 2019).

1.6 How to Administer the Immunomodulators to Fish?

Overall, the immunostimulants could be administered through different routes. Although injection methods are the best strategy to enhance non-specific immune system responses, this method is costly and time-consuming. Furthermore, injection methods are only performed by experts. Therefore, it is applied in experiments where the fish are intended as brook stock in genetic studies. Another method is immersion, but its efficiency is less than that of injection; however, it requires crowding and an increase in the handling of fish stocks. Immune system stimulants may be prescribed as oral supplements. However, the immunostimulants' concentration in the diet depends on the size, ontogeny stages, and initial weight of the fish. This method consists of oral ingestion, produces a suitable non-specific immune response, and can be the most cost-effective method of administration. Top dressing can help you achieve these. The surface of the food is treated with the pure immunostimulant in this case. This is comparable to employing a layer of fish oil to top-dress antibiotic granules. This technique produces variable results depending on how well the immunostimulant adheres to the feed. At last, one method that is much more advanced is bio-encapsulation.

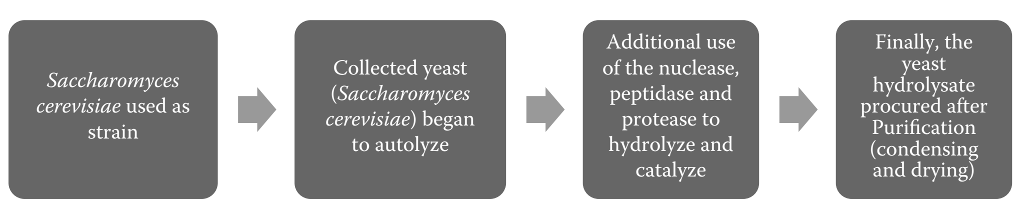

FIGURE 1.8 Extraction of hydrolysate.

Demonstrate the pathway for the extraction of hydrolysate from yeast to use as an immunostimulant for fishes. The yeast (*Saccharomyces cerevisiae*) are collected, autolyzed yeast is subjected to nuclease, peptidase, and protease to get the hydrolysed product, which is subsequently purified using condensing and drying.

1.7 Limitations of Immunostimulants

- Though showing extraordinary growth in the field of aquaculture, one of the important disadvantages of some immunostimulants is their high cost.
- The administration of the drug is important to consider as it depends on its efficacy. Immunostimulants show limited efficiency upon parental administration.
- Immunostimulants are not completely effective against all diseases.
- Overdoses of immunostimulants in feeds may cause immunosuppression.
- In some cases, aquatic animals may also fail to provide enhanced protection or an increase in immunity.
- Immunostimulants are successfully used in aquaculture against various infections and pathogens; however, the ability to improve innate resistance against many diseases (e.g., columnaris disease) has not been studied.

1.8 Factors Affecting the Efficiency of Immunostimulants

The effectiveness of an immunostimulant can depend on various factors, as follows:

- Solubility: Laminaran is an algal extract that can boost respiratory burst activity in leucocytes of the anterior kidney and activate macrophages in Atlantic salmon because it is more soluble than the fungal and yeast glucans. It has also been demonstrated to be a potential chemical for diet usage due to its greater solubility, so the solubility is considered an important aspect for immunostimulants.
- Duration of dose: Salmon that received M-glucan injections only took 2 days to produce their peak leucocyte responses. After 4–7 days of therapy with yeast beta-glucan, the respiratory burst activity increased. This demonstrated how immunostimulants might improve non-specific immunity with very brief dosages.
- Dosage: A high dose or overdose certainly does not seem to have an enhancing effect and can, in turn, inhibit the immune responses. At concentrations of 0.1–1 g/mL, the respiratory burst activity of glucans-treated macrophages increased. Whereas glucan had no effect at a concentration of 10 g/mL, it was inhibitory at a concentration of 50 g/mL. Very high vitamin E levels in feed, such as those of a >1,000–5,000 IE/kg diet, have an immunosuppressive effect. So, the dose per unit weight has a significant effect on efficacy.
- Time of administration: applying immunostimulants at the right time is very important in aquaculture. Mostly, application is needed before the outbreak of disease so that losses due to disease can be reduced.
- Method of Administration: The administration of immunostimulants through injection has been reported to be most effective against a range of pathogens. Vaccination seems to be impractical for small fish. Immersion is commonly used in intensive culture systems, despite the fact that it is less expensive than injection, produces a less non-specific immune response, and stresses the fish during handling. It is most effective during the acclimation of juveniles to ponds in field conditions. Oral ingestion is good for extensive aquaculture systems. It is cost-effective and enhances non-specific immune responses (Barman et al. 2013).

1.9 Evaluating the Efficacy of Immunostimulants

In vivo and in vitro methods can be used to assess the efficacy of an immunostimulant. The in-vivo method employs fish pathogens to assess the efficacy of immunostimulants, whereas the in-vitro method examines cellular and humoral immune mechanisms. In-vivo and in-vitro methods should be performed together to check the basic mechanisms for providing protection. In preliminary studies, in vitro methods are preferred. In vitro evaluation is based on lymphocyte proliferation, complement activation, total erythrocyte and leucocyte counts, chemokinesis, chemotaxis, lysozyme activity, and RBA phagocytosis. Other parameters include monitoring natural cytotoxic activity, macrophage-activating factor (MAF) levels, and C-reactive protein levels. However, these tests are too expensive to be conducted to check the efficacy of immunostimulants. A deep level of research is required to check the efficacy of various compounds for aquaculture species and their pathogens and to ultimately decrease the cost of the immunostimulants.

1.10 Timing of Administration

It is very important to use immunostimulants at the correct time and in the right concentration to boost the immune system. Anderson proposed in 1992 that it is best to use immunostimulants prior to the possibility of disease outbreaks in order to minimise disease-related loss. Furthermore, the effective dose and timing of exposure have a significant impact and are complicated by the culture system and feeding scenario. Studies in Atlantic salmon showed that the maximum non-specific disease resistance is attained only after the third week of injecting glucan at 10 mg/100 g, whereas the effects of low dosing at 1 mg/100 g last only for 1 week. Similarly, in African catfish, administration of glucan led to a maximum increase in the phagocytic cells at 7 days but not after 14 days. So, it is preferable to use them well in advance and at regular intervals (Barman et al. 2013).

1.11 Detection of Immunostimulants

Detection of immunostimulants in fish body is detected by using 'omic' technologies.

Methods of detection of immunostimulation are as follows and it is discussed in further chapters in detail:

- In vitro measurement
- In vivo measurement
- Phagocytic activity

1.12 Attributes of Immunostimulants

The most important attribute of an immunostimulant is that it directly influences the animal's health. It is biodegradable and biocompatible, and therefore safe for the environment. It enhances the immune system of animals, promoting good health, and is non-toxic to both fish and shellfish with no side effects observed. In aquaculture, it provides disease resistance to animals against a broad spectrum of pathogens and reduces mortality caused by opportunistic pathogens. It can also keep the host safe by providing enhanced immune stimulation to fight viral diseases. It increases the effectiveness of many antimicrobial substances, vaccines, and antibiotics. Moreover, it is cheap, easily available, and most importantly, an eco-friendly method for immune stimulation (Barman et al. 2013).

1.13 Vaccine

A vaccination is a biological treatment that increases immunity to a specific disease. Vaccine often comprises an agent that resembles a microorganism that causes a disease and is frequently created from weaker or dead versions of the pathogen. In order for the immune system to more quickly identify and eliminate any further interactions with this disease-causing microorganism, The agent prompts the body's immune system to identify the agent as foreign, eliminate it, and "remember" it so that the immune system will be better able to identify and eliminate any of these microorganisms that it comes into contact with in the future. "Prevention is better than cure" is the core tenet of a vaccination. The name "vaccine" originated from Edward Jenner's usage of the phrase "cow pox" in 1796 (Latin "variolvaccin," which was borrowed from the Latin "vaccn-us," from "vacca" cow). He was a pioneer in the use of cowpox vaccinations to stop the spread of smallpox.

FIGURE 1.9 Vaccination via injection.

Figure 1.9 represents vaccine administration through injection via intramuscular or intraperitoneal method to increase resistance against pathogens by stimulating immune system.

Fish immunisation started in 1942 when David C. B. Duff successfully immunised trout orally against the bacteria *Aeromonas salmonicida* (the first fish vaccine). He is known "Father of Fish Vaccination". The first commercially approved fish vaccination was a dead *Yersinia ruckeri* vaccine against enteric redmouth disease that was administered by immersion in 1976.

1.14 Immunostimulants vs Vaccine

Table 1.1 is the comparison of immunostimulants and vaccines (Dawood et al. 2021).

1.15 New Paradigm

1.15.1 Nutrigenomics

Nutrigenomics is a branch of science that integrates bioinformatics, nutrition, genomics, molecular biology, and epidemiology. It links the relationship between nutrients and cellular processes and shows how the dietary components alter the genetic makeup. Although the relationship between nutrition and the immune system is generally known, it is still unclear

TABLE 1.1

The Comparison of Immunostimulant with Vaccines

Immunostimulants (IS)	Vaccine
• More treatments are required as the prophylactic effect is short term	• One or two treatments are enough as prophylactic effect is long
• Efficacy of immunostimulants is good	• Efficacy of vaccine is excellent
• IS possess wide spectrum of activity	• Vaccine possess limited spectrum of activity
• Nontoxic with less side effects	• Nontoxic with less side effects
• No toxic residue accumulation	• No toxic residues accumulation
• Positive/no environmental impact	• No environmental impact
• Mainly enhance non-specific immune system before specific immune system matures.	• Enhance specific and nonspecific immune response
• Can be used at any stage of life cycle	• Cannot be used at any stage of life cycle
• Easy to supply to larvae of fish and shrimp	• Difficult to supply to larvae of fish and shrimp
• Cost-effective	• Costly

how nutrition, animal energy status, and immune function are linked together. The effects of diet on the immune system are becoming more transparent because of emerging omics technologies like transcriptomics (microarray and RNA-seq) and proteomics. Modules of genes can reveal changes in both local (intestinal) and systemic immune function by applying molecular pathway enrichment analysis. Using the omics, researchers can now investigate the effects of dietary manipulations such as fasting, feed additives, and protein replacement on gene expression, protein synthesis, and immune functions. It is a relatively new approach in aquaculture, but the scope it provides to understand the mechanism behind gene alteration through nutrition may lead to more intense research and the development of aquaculture (Samuel and Martin 2017).

1.15.2 Trained Innate Immunity

It is a concept that argues that not only can adaptive immunity provide immunity by memorising the pathogen, but an innate immune response can also recognise the pathogen and adapt to provide an immune response after exposure. It stimulates defence and increases nonspecific resistance to infection. One such example is the prophylactic effects of glucan injection in fish against *Vibrio salmonicida*. Though technological advancements and research have revealed the mechanism responsible for such immune responses to be effective programming of cells like monocytes, NK cells, macrophages, etc. through pattern recognition (MAP kinase dependent), we are still far from knowing the actual effectiveness, mechanism, and potential side effects it may cause. The approach is opening doors of application in various aquaculture fields like brood stocking, larval rearing, and first-feeding fish; however, assessments of this approach using modern tools like transcriptomics, epigenetics, proteomics, and metabolomics are needed (Zhangzuobing et al. 2019).

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Immunomodulators and Stress Oxidative

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Immunomodulators to Prevent Diseases and Minimize Antimicrobial Use

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Immunomodulation in Aquaculture Health Management

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Application of Immunostimulants for Aquaculture Health Management

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Herbal Immunomodulators for Aquaculture

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Prebiotics and Probiotics as Effective Immunomodulators in Aquaculture

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Immunomodulation in Fish Through Nutrients, Antioxidants and Hormones

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Progressive Immunomodulation Through Nanotechnology

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Efficacy and Limitations of Immunomodulators

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Current Status and Recent Advancements with Immunostimulants in Aquaculture

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