Sanjay Kumar Gupta
Sib Sankar Giri *Editors*

Biotechnological Advances in Aquaculture Health Management

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Dedicated to
All
COVID-19 Warriors
and Frontline Workers

Foreword

Aquaculture is one of the cheapest animal protein farming practices that has shown tremendous growth over the past few decades. Currently it contributes about 52 percentage of the total fish for human consumption globally (FAO 2020). Asian countries are major drivers that produce about 80 percentage of the total global production and contribute directly and indirectly to economic and food security. To accelerate the production in an ecologically safe and environmentally sound manner, responsible use of resources and quality inputs deserve due care and importance considering the sustainability of aquaculture. However, with the growing demand of the cheap quality protein, unsustainable intensification of the industry has been proliferated and getting popular in several parts of the globe, resulting in growing incidences of disease outbreak. With the further expansion of aquaculture, incidence of outbreaks of infectious disease is also expected to increase multi-fold and even at serious epizootological level, thus causing serious threat and limiting the growth of the industry. Biotechnological applications play a key role in aquaculture health management as it facilitates the rapid and reliable detection and identification of virulent pathogens. In the era of omics evolution, modern state-of-the-art technologies such as transcriptomics, proteomics, metabolomics, microbiomics, RNA interference and cell culture techniques would offer excellent application to decode the molecular mechanism of disease progression and in developing their control and management strategies.

This book has been compiled with chapters that encompass the fundamental facets connected with the biotechnological advances of disease management in aquaculture. Recent advances on the role of various dietary supplements, viz. probiotics, prebiotics, synbiotics, immunostimulants, etc., in health management have also been extensively covered, which would offer enormous benefits to global readers of all categories and also provide insights into the underlying mechanism of health promotion in aquaculture.

I sincerely would like to place a record of appreciation to the book editors and specialist contributors for their sincere efforts in the comprehensive collation of brilliant chapters on biotechnological advances in aquaculture health management. I would like to urge the reader to gain insights from the valuable compilation and to join the editors in this enthralling, exciting and gratifying attempt.

International Civil Service (FAO of UN), ICAR CIFE Dilip Kumar
Mumbai, India
28 May 2021

Foreword

Aquaculture is a multi-billion-dollar industry that is expanding rapidly to cater to the demands of livelihood and nutritional security of humankind globally. The sector has been confronting serious challenges which are sophisticated and multidimensional. Among these challenges, prevalence of infectious diseases takes the lion's share leading to huge economic losses. The annual loss of revenues incurred due to disease upsurges is estimated to the tune of six billion US dollars. The figure is projected to surpass 40% of the global production with emergent diseases in the shrimp sector. This poses a major threat towards the collapse of the aquaculture industry in several developing nations of Asia. Additionally, there are also upcoming issues of climate change and antimicrobial resistance that have aggravated the risks associated with this sector and need to be addressed judiciously on a priority basis. Hence, it is imperative to upgrade the application of technological advances in this sector under the umbrella of environmental sustainability and health promotion of the cultured species.

Understanding the potential of biotechnological tools such as transcriptomics, metabolomics, microbiomics, genome editing and RNA interference would not only prove to be the cornerstone in tackling the incidence of disease outbreak but also pave the way for the development of diagnostics and curative measures. The importance of disease prevention by boosting the inherent immunity of fish via

diet supplementation and vaccination, rather than disease management, would prove to be a paradigm shift in the enhancement of safety against infectious pathogens.

The biotechnological inventions can be applied towards refining the health strategies for qualitative and quantitative production from the aquaculture sector in a holistic manner. This book is a humble effort towards the compilation of the multifarious aspects of technological advances in aquaculture health management.

The book will be useful to readers who are exploring opportunities to make contributions in the field of aquaculture health management. The updated literature survey highlights the voids in the area that may be crucial to realize the potential of blue economy. I applaud the entire team of editors and contributors for putting their best efforts towards building a comprehensive compilation in the form of this book entitled *Biotechnological Advances in Aquaculture Health Management*. It should be helpful to both academicians (students, teachers, researchers) and aqua-preneurs for upgrading their skills and knowledge in the area of aquaculture health management driven by biotechnological advances.

Laboratory of Aquaculture and
Artemia Reference Center, Ghent University
Ghent, Belgium
28 May 2021

Peter Bossier

Preface

Globally, the aquaculture industry has witnessed phenomenal growth in the last two decades that has contributed to enhance the preference of protein-rich meat in the diet of the poorest of the poor. Aquaculture produce are recognized as one of the healthiest foods on the planet, as it holds great promise in transforming food systems, and eradication of hunger as well as malnutrition in numerous developing countries of the world. As per recent reports of FAO, the total fish production is expected to expand from 179 million tonnes in 2018 to 204 million tonnes in 2030. Out of which, aquaculture production is anticipated to touch 109 million tonnes in 2030, an escalation of 32% (26 million tonnes) over 2018 (FAO). With the increasing production, the global average annual fish consumption has also risen to the tune of 20.30 kg in 2017 (FAO). The global aquaculture market is projected to grow with a healthy growth rate of more than 7.1% during the estimated period of 2020–2027. Growing demand for quality fish products has promoted both horizontal and vertical expansion of aquaculture activities globally. However, the major obstacles in the sustainable growth of aquaculture are seriously challenged with the prevalence of disease outbreak caused by a diverse group of infectious pathogens and several non-infectious agents. Such detrimental circumstances often lead to deprived growth, compromised immune response and intense mortality and ultimately hamper the aquaculture production. Therefore, to rescue this sunrise sector, application of biotechnological interventions or tools could be a milestone step to ensure long-term economic and social benefits towards food security. The advent of high-throughput sequencing technology and omics approaches such as genomics, transcriptomics, microbiomics and metabolomics has enabled scientists/researchers not only to understand the complex biological processes and underlying molecular mechanism but also helped in precise and targeted invention of remedial measures to curb huge economic losses to farmers. The development of molecular diagnostics for the identification of diseases, prophylactic measures for control, management and analysis of biological resources are some of the prime concerns that have been detailed in this book. Prevention at the host's end is equally important in this aspect which paves the way to immunomodulation via dietary supplementation of prebiotic, probiotics, synbiotics and immunostimulants. The aquaculture sector needs innovative biotechnological approaches to overcome challenges such as water quality management, rapid disease diagnostic services, disease prevention and

management of outbreaks, supply of disease-free or high health broodstock and seed. Of late, biotechnological science is growing rapidly and has endowed us with several new tools and technology to create new horizons in aquaculture, especially health management. Some of the advanced biotechnological approaches such as vaccination, antimicrobial peptides, gene editing, metagenomics, RNA interference and cell culture techniques have shown promising results in managing the health of cultured aquatic organisms over different agro-climatic condition across the globe. Nanotechnology has emerged as an alternative approach with innovative materials and protocols to solve persisting issues in fish health management. Biofloc technology is becoming increasingly popular as an emerging avenue in aquatic animal healthcare and directed to maximize aquaculture productivity by using microbial biotechnology.

Apart from economic potential for the fisheries sector, health management through biotechnology also holds promise for sustainable management of aquaculture practices, which is crucial for the prevention of environmental degradation due to intensive farming within the aquaculture industry.

The editors have tried their best to make comprehensive coverage of the biotechnological advances in aquaculture health management. This book updates the subject matter, illustrations and problems to incorporate new concepts and issues related to biotechnological aspects of health management. The publication of this book has been possible through the enthusiastic support, assistance and cooperation of dedicated scientists/researchers of different institutions working in the areas of aquaculture and fisheries across the globe. The processing and editing of various chapters has taken a long time, and we express our sincere gratitude to all the contributors for bearing with us.

I wish this book would be of immense benefit to researchers, scientists, students, entrepreneurs, and even industry players working in the field of aquaculture, biotechnology, fish health management and fisheries.

Ranchi, Jharkhand, India — Sanjay Kumar Gupta
Seoul, South Korea — Sib Sankar Giri

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This book is an outcome of continued professional collaboration of both editors. We would like to express our sincere appreciation to all contributors who have contributed to this book in the form of chapters. Critical remarks and suggestions of two anonymous reviewers were very much helpful in picking up several relevant and innovative topics. Indeed, it is our utmost pleasure to acknowledge all the persons who have directly or indirectly helped us in completing this book.

We are immensely grateful to Dr. Dilip Kumar, Ex Director/VC of ICAR CIFE Mumbai, India, and Prof. Dr. ir. Peter Bossier, Director, Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium, for their encouragement, suggestions and support to the young researchers for their contributions in this book.

Unflinching support of family members, students, colleagues, and friends in this pandemic situation is a great source of motivation for us to complete this book.

We would also like to sincerely thank Springer Nature for accepting and publishing this work.

Finally, we would like to bow our heads before Almighty God, whose great power and eternal wisdom has embraced us with strength and audacity to complete this book.

28 May 2021

Sanjay Kumar Gupta
Sib Sankar Giri

"Once you replace negative thoughts with positive ones, you'll start having positive results."
—Willie Nelson

Contents

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Sib Sankar Giri, M.Sc., M.Phil., Ph.D. is a senior Research Associate in the Lab of Aquatic Biomedicine, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University (SNU), Seoul, South Korea. He received his Master of Philosophy and Doctor of Philosophy degrees in Biotechnology from Periyar Maniammai Institute of Science & Technology (Erstwhile Periyar Maniammai University), India, in 2008 and 2014, respectively. He has extensive research experience in aquaculture probiotics, fish immunostimulants, and microbial biotechnology. He was the recipient of INSPIRE fellowship from the Department of Science and Technology, Govt of India, for doctoral research. He has published more than hundred research articles in SCIE journals and wrote three book chapters in knowledge-based editions published by renowned international publishers. He co-authored a book titled *Life Within Life-A book on biodiversity*. He is an editorial

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Biotechnological Approaches in Fish Health Management

1

Md. Idrish Raja Khan and Tanmoy Gon Choudhury

Abstract

With the advancement in aquaculture practices and the unresponsible approach towards the aquatic environment, the fisheries sector is suffering from severe disease outbreaks, resulting in significant losses globally. Consequently, disease management approaches targeted towards the pathogen are not enough and indeed not a sustainable option. Over the years, an array of biotechnological approaches has shown promising response to improve the health of cultured organisms and accomplish the greater production while safeguarding the aquatic environment. Such biotechnological interventions include pathogen-free best management practices by avoiding possible use of chemical remedial agents and applying biocontrol approaches against pathogens of diverse origin. This chapter highlights various biotechnological approaches to biocontrol disease outbreaks in aquaculture, like the adoption of vaccines or immunostimulants, probiotic, prebiotic, symbiotic, paraprobiotics, phage therapy, antimicrobial peptides, gene therapy, RNA interference, etc. The biotechnological tools outlined in this chapter could be of utmost importance to achieve the better health management of cultured aquatic organism and the long-term sustainable development goal of aquaculture.

Keywords

Biotechnological approaches · Aquaculture · Vaccines · Probiotic · Phage therapy

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1.1 Introduction

Globally, aquaculture has emerged as one of the most promising agri-based commodities with a significant contribution to the global food basket (FAO 2020). It is a premium quality animal protein source, contributing to almost 17% of total animal protein and 7% of all protein sources (FAO 2018). These attainments are the collective legacy of involvement of scientific and intensified aquaculture practices. Growing demand for quality fish products has promoted both horizontal and vertical expansion of aquaculture activities globally. However, aquaculture production through modern approaches simultaneously made organisms vulnerable to an extensive range of diseases caused by a diverse group of pathogens (bacteria, virus, parasite and fungi) and several are non-infectious, such as soil and water quality-related disorders, malnutrition, etc. Such detrimental circumstances often lead to deprived growth, intense mortality and ultimately fettered aquaculture production, especially the aquatic organisms where the immune response is a primary concern owing to their inadequate immunocompetence.

Over the years, there had been remarkable advancement in biotechnology and its application in an array of fields, including its prospect in aquaculture, especially the health management of aquatic organisms. Biotechnological interventions offer a 'give and take management system' whereby biotechnological tools can be used for healthy aquaculture practices, from the production of seeds to table size fish production (Vijayan et al. 2013). Aquaculture sector needs innovative biotechnological approaches to overcome the challenges such as water quality management, rapid disease diagnostic services, disease combating and management outbreaks, supply of disease-free or high health broodstock and seed, etc. (Subasinghe et al. 2001; Mishra et al. 2020).

1.2 Diseases: A Major Obstacle in Aquaculture Production

Disease incidences are being recognized as a potential constraint for the growth of aquaculture. A wide variety of pathogenic microorganisms affects aquaculture and very often leads to catastrophic results. At present, the aquaculture industry is overwhelmed by the disease outbreak caused by numerous etiological agents. Therefore, there is an urgent necessity for adopting standard culture practices and minimizing the chances of any potential outbreak to sustain the growth of aquatic animal. With the shifting scenario in aquaculture, the farmers are emphasizing prophylactic measures rather than therapeutic approaches to prevent infections and to facilitate superior production. The prophylactic measures include chemotherapeutic application, balanced and fortified nutritional strategy with the introduction of various nutraceuticals or functional food, dietary immunostimulants, etc. However, infections are more frequently prevented and managed by intrusive chemotherapy these days, such as various drugs, especially antibiotics and other chemical remedial agents (Boutin et al. 2012). Because of its fast and effective response, farmers prefer the use of chemical therapeutics. However, the indiscriminate and irresponsible use

of antimicrobial compounds contributes to an increase in pathogen virulence by trans-horizontal chromosome transfer (Moriarty 1999). Additionally, the chemical substances also impose several other detrimental effects on the aquatic environment and simultaneously distress aquatic life through inhibition or decolonization of beneficial microbiota, biomagnification or bioaccumulation of various toxic compounds, etc. and ultimately rendering aquatic lives immunocompromised against an array of pathogens (Reda and Selim 2015).

1.3 Biotechnological Tools for Health Management in Aquaculture

Biotechnological interventions have a direct effect on key elements of fish health management, with direct knock-on effects over pathogens, and ultimately lead to lessening in the employment of chemical therapeutics, especially antibiotics. The application of various vaccines to prevent disease also significantly reduces the use of different chemical remedial agents (Engelstad 2005). Over the past decades, the biotechnological application has proven its significance in fish health management through the development of multimodal and multivalent vaccines along with various molecular diagnostic probes (Adams and Thompson 2006). In recent time, biotechnology has emerged, matured and arrived as a sustainable option, owing to beneficial and well-established field biotechnological interventions. These interventions are now more widely adopted to improve and sustain aquaculture activities. Here in this chapter, an effort has been made to summarize the biotechnological intervention can be employed for the health management purposes in aquaculture.

1.3.1 Vaccine

Currently, vaccines' administration is one of the important and useful prophylactic measures that can guard aquatic organisms from diseases caused by a diverse group of pathogens. Over the years, vaccination has gained considerable importance in the aquaculture industry, particularly in western countries, owing to its effectiveness in controlling different dreadful diseases. The effective vaccination strategy essentially involves several considerations before application, such as deciding as to which disease to vaccinate against, the vaccine type, vaccination method, the timing of vaccination, the use of re-vaccination, etc. (Toranzo et al. 2009). A vaccine is any biological preparation that can establish or improve immunity of the host upon administration and provide protection against a specific virulent pathogen. The recipient's immune system develops a primary response following exposure to the vaccine antigen either killed, attenuated, sub-unit or recombinant (Adams and Thompson 2006). An optimal vaccine must be able to induce innate immune mechanisms, sufficient antibody response and T-cell response(s), and concomitantly, a 'memory' is also developed which, upon secondary exposure of the same

antigen, shows vigorous response and eventually eliminates the pathogen over a quick span as compared to the very first exposure of the antigen (Mishra et al. 2020).

Over the years, vaccine application had established itself a proven and cost-effective approach to control an array of infectious diseases among cultured animals. The employment of vaccines eliminates the possibility of economic losses due to disease, reduces the need for chemical therapeutics, leaves no residues, and does not impact the emergence of drug resistance in pathogens (Subasinghe et al. 2001). In recent times, significant research and field trials have been made to develop effective fish vaccines against infectious viral and bacterial diseases. However, vaccines' commercial availability remains a major concern throughout the globe (Shefat 2018; Mishra et al. 2020).

Most of the commercial vaccines available in the market are inactivated (killed) vaccines. However, few reports suggest the failure of such vaccines, particularly against viruses, which lead the foundation for the development and adoption of live attenuated vaccines (Adams and Thompson 2006). However, the application of live vaccines also brings several concerns that the attenuated pathogen might back-mutate and revert to the virulent nature and may lead to catastrophic results (Thompson and Adams 2004). Licensing and government approval of such vaccines might be an obstacle for further implementation. Alternatively, preparation of sub-unit vaccines has also been adopted, where the specific disease-causing components of the virulent agents are isolated and then applied as vaccines (Adams and Thompson 2006). With the advancement in science, the vaccine development strategy has also taken a leap; currently, genetic approaches have been adopted to clone the virulent genes encoding specific antigens and then integrate them into bacterial DNA genome, i.e. recombinant vaccines (Leong et al. 1997; Table 1.1).

1.3.2 Immunostimulants

Immunostimulants are chemical substances responsible for the non-specific enhancement in innate immune responses (Mishra et al. 2020). Immunostimulants are promising nutritional supplements to aid in the resistance capability of organisms against various diseases and increase the survival of organisms by elevating the host's immune response irrespective of the pathogen. Immunostimulants protect organisms without any side effects and with no or minimal detrimental impact on the environment. The most accepted mode of action of immunostimulants is to facilitate the proper functioning of phagocytic cells to rouse the bactericidal and fungicidal activities (Barman et al. 2013). Immunostimulants are also well known for their establishment of recovery response in the immunosuppressive condition of organisms caused by any form of stress (Barman et al. 2013). A wide variety of compound has been found to have immunostimulatory nature, including synthetic chemicals (levamisole, FK-565, etc.), bacterial derivatives (β-glucan, LPS, EF203, etc.), polysaccharides (chitin, chitosan, schizophyllan, etc.), nutritional factors (vitamin C, vitamin E, etc.), along with several animal and plant products, hormones,

Table 1.1 Vaccines used in aquaculture

Type of vaccine	Antigen	Commercial name	Host organism	Mode of application	Remarks	Reference
Attenuated	*Aeromonas salmonicida*	Brivax II	Rainbow trout (*Oncorhynchus mykiss*)	IP injection	Cellular, humoural and mucosal immune response of host got elevated	Lawrence and Banes (2005)
	Edwardsiella ictaluri	NA (in vitro)	Channel catfish (*Ictalurus punctatus*)	IP injection		Liu et al. (2015)
Killed or inactivated	Infectious pancreatic necrosis virus (IPNV)	Alpha Ject® 1000	Salmon	IP injection	Amenable to autogenously	Biering et al. (2004)
	Spring viraemia of carp virus (SVCV)	Bioveta	Carps	IP injection	Easy administration adhesions associated with adjutants. Safe for use *V. Salmonicida*	Salgado-Miranda et al. (2013)
	A. salmonicida, *V. anguillarum*	MULTIVaC, Microtek	Salmonids	IP injection		Salgado-Miranda et al. (2013)
Vector-based vaccine	Infectious salmon anemia virus (ISAV)	NA (in vitro)	Atlantic salmon (*Salmo salar*)	IP injection	Apoptosis was detected in the infected cells, additionally further field level efficacy must need to be performed	Wolf et al. (2012)
	Infectious hematopoeitic necrosis virus (IHNV)	NA (in vitro)	Atlantic salmon (*S. salar*)	IP injection		Adams and Thompson (2006)
Recombinant protein	IPNV/VP2	Microtek	Salmon	IP injection	Economically viable and safe to host. Ability to produce sufficient quantities of the protective proteins. Safe and low-cost method	de Kinkelin (1994)
	Spring viraemia of carp virus (SVCV)	International Inc.	Carp	IP injection		Salgado-Miranda et al. (2013)
	Salmon rickettsiae		Salmonids			

(continued)

Table 1.1 (continued)

Type of vaccine	Antigen	Commercial name	Host organism	Mode of application	Remarks	Reference
		Pharos, S.A., Bayovac 3.1		IP injection		Adams and Thompson (2006)
DNA vaccine	IHNV	Aqua Health Ltd	Salmon	IM injection	Rouses cellular as well as humoral immunological indices in host. Possibility for construct a vector encoding several antigens	LaPatra et al. (2001)
	IPNV	Novartis	Rainbow trout (*O. mykiss*)	IM injection		Ballesteros et al. (2014)
	Pancreatic disease (PD)	NA (in vitro)	Rainbow trout (*O. mykiss*)	IM injection		Kurath (2008)
	Viral haemorrhagic septicemia virus (VHSV)	NA (in vitro)	Salmonids	IM injection		Meeusen et al. (2007)
	Nervous necrosis virus (NNV)	NA (in vitro)	European sea Bass (*Dicentrarchus labrax*)	Oral feeding		Valero et al. (2016)
Genetically attenuated pathogen	*A. salmonicida*	Brivax II	Rainbow trout (*O. mykiss*)	IP injection	Elevation in immunological response of host	Liu et al. (2015)
Synthetic peptide vaccine	Viral haemorrhagic septicaemia (VHS)	NA (in vitro)	Rainbow trout (*O. mykiss*)	IP injection	Possibility for construct a vector encoding several antigens	Coeurdacier et al. (2003)

Table 1.2 Commercially available immunostimulant preparations in aquaculture (Mishra et al. 2020)

Commercial name	Company	Contains	Method of application
Nutri-care	Nutricorp animal bio solutions	Extract of herbs, vitamins, enzymes, probiotics, amino acids and organic minerals	For salinity stress of below 30 ppt−10 g/kg of feed for 5 days, whereas above 30 ppt 15 g/kg of feed for 5 days
Lysozyme hydrochloride	Belovo, Belgium	Lysozyme isolated from hen's eggs	As a dietary supplement
Selenium yeast	Alko, Finland	Selenium yeast	As feed ingredient
Lactoferrin DMV	International, Netherland	Lactoferrin from bovine milk	As feed ingredient
Macroguard Biotec-	Mackzymal, Norway	β (1, 6) branched β (1, 3) glucan from yeast	With vaccines through injection through feed for 6−8 weeks continuously
DS 1999	International Aquaculture Biotechnologies, Ltd.	Bacterin	Direct incorporation in the culture medium
Levamisole	Janssen Pharmaceutica, Belgium	Tetrahydro-6-phenylimidazolthiazole hydrochloride	As a dietary supplement
Immustim	Immundyne, USA	β (1,6) branched β (1,3) glucan from yeast	Immersion for larvae/PLs and feed ingredient for grow-out shrimp

cytokines and others have been extensively studied and tested in aquaculture practices (Barman et al. 2013; Table 1.2).

1.3.3 Probiotics

Lilly and Stillwell (1965) coined the term 'probiotic' to designate unknown growth-promoting substances produced by ciliated protozoans. It is defined as a 'live microbial feed supplement having the ability to improve the microbial balance of host animal' (Fuller 1989). Probiotics confer health benefits to the host when they are functional in sufficient quantity. Dietary administration of probiotics helps to modify the beneficial microbial balance of the gastrointestinal tract and stimulates the growth, immune response and provides resistance against a broad range of virulent pathogens (Kesarcodi-Watson et al. 2008). Besides the health and growth-promoting potentials, probiotics have an extended application in aquaculture. Given the intricate relationship between an aquatic animal and its surrounding environment, the concept of probiotic application in aquaculture has been broadened to include improvement of water quality by directly applying probiotics into the water. This extended application has been suggested to describe probiotics as microbial

Table 1.3 Commercially available probiotic products applied in aquaculture (Mishra et al. 2020)

Name	Composition	Indications or result	Dose
Pro Marine	Probiotics fortified with vitamin C and calcium	Removes unwanted microorganism and accelerates encrustation	For boosting growth: 5 g/kg of feed twice daily
SUPERGUT	Contains the selective multi-strain of probiotics along with seaweed extract and molasses	Limit the activity of harmful pathogenic bacteria, maintain a healthy and balance gut microflora	10−20 mL/kg shrimp feed
PROBAC-G	*Lactobacillus*, *B. subtilis*, *Cerevisiae*, fungal diastase, vitamin B12, papain, pepsin	Improve feed convertion ratio (FCR) and resistances to disease due to favourable gut flora	500 g/L/tonne of feed
ALGUTPRO	Active/inactive forms of *Candida* spp., *Saccharomyces* spp. and growth enhancers	Single-cell protein and growth enhancer lead the proliferation of beneficial bacteria	For shrimp, 2 kg/tonne feed and for fish, 3 kg/tonne feed
VIBRION	Probiotic strains, alkaline protease and lipase, etc.	Efficiently reduces the harmful microbial load like *Vibrio* spp.	250−500 g/acre for 7−10 days
Environ	Probiotic mix	For eater and soil pollutant management	250−500 g/acre
Thiomax	Probiotics and micronutrients	Works in the bottom sludge layers	2−3 kg/acre
Spark-PS	Probiotics	Secretes various hydrolytic enzymes and digest complex organic substances	2−3 L/ha
GENTECH P.S.	Aqua probiotics	It helps to degradation of organic load	5 L/ha
AQUAGEN PRO	Probiotic strains	Enhances the ammonia-oxidizing activity	2−3 L/acre in 3−5 ft depth

'water additives' (Moriarty 1999). In the last two decades, the research and application of probiotics in aquaculture have expanded enormously. According to Khan et al. (2020) and Knipe et al. (2020), probiotic isolates belong to about 20 different bacterial genera. Most of the isolates were identified as members of either *Bacillus* spp. or *Lactobacillus* spp. The administration of probiotics in intensive and semi-intensive aquaculture practices has emerged as one of the most convenient and promising options to meet the nutritional requirements by producing premium quality animal protein with minimal application of chemicals. The most adopted probiotic candidate in the aquaculture system includes *Bacillus* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Streptomyces* spp., *Carnobacterium* spp. and yeast (Van Doan et al. 2019; Khan et al. 2021a, b; Table 1.3).

1.3.4 Prebiotics

Prebiotics can be defined as non-digestible food ingredients that are metabolized by specific health-promoting bacteria and selectively stimulating the growth and proliferation of gut inhabiting symbiotic bacteria (Ringø et al. 2010; Ganguly et al. 2012). Primarily, prebiotics are fragments of carbohydrate or complex oligosaccharides (3−10 sugar moieties) such as galactose, fructose or mannose. Additionally, short-chain fatty acids (SCFA) were also reported as prebiotic later as they also have a positive influence on colonic health (Ringø et al. 2010; Ganguly et al. 2012). Prebiotic of carbohydrate in origin can be categorized into monosaccharide, oligosaccharide or polysaccharide based on their molecular size or degree of polymerization (Ringø et al. 2010). Over the years, despite the potential benefits of prebiotic administration of several compounds such as fructooligosaccharides (*FOS*), short-chain fructooligosaccharides (scFOS) inulin, manno-oligossacharides (MOS), isomalto oligosaccharides (IMO), trans-galactooligossacharides (TOS), xylo-oligosaccharides (XOS), glucooligossacharide (GOS), soya bean oligosaccharides, lactosucrose, lactulose, etc. in health and growth performance of aquatic animals, the application of prebiotics in aquaculture is still under-explored and requires further investigation (Table 1.4).

1.3.5 Synbiotic

Gibson and Roberfroid coined the term 'prebiotics' and predicted that a combination strategy of probiotic and prebiotic can be adopted to achieve higher, which later termed 'synbiotic' (de Vrese and Schrezenmeir 2008). The supplementation of both probiotics and prebiotics leads to a positive influence on growth, immunomodulation, feed utilization, survival against infection, etc. (Cerezuela et al. 2011). Intestinal probiotic microbes selectively ferment dietary prebiotic to produce short-chain compounds from various prebiotic compounds such as lactose propionate, few lipids, etc. They are observed to have a beneficial role over uptake and bioavailability of nutrients (Breves et al. 2001; Bongers and van den Huevel 2003). Additionally, the synbiotics application has also shown increased fermentation and production of numerous SCFA, leading to reduced intestinal pH thereby eliminating the chances of any probable infection of certain pathogenic bacteria while triggering the augmentation of various endosymbionts such as *Bifidobacteria* and *Lactobacillus* (Breves et al. 2001; Bongers and van den Huevel 2003; Table 1.5).

1.3.6 Paraprobiotics

The term paraprobiotics was coined by Taverniti and Guglielmetti (2011) and defined it as non-viable beneficial microbial cells or cell fragments or cell extracts, which upon administration confer a health benefit in the host organism. The prefix

Table 1.4 Common prebiotic used in aquaculture

Prebiotic	Dose and length of administration	Fish	Results	Reference
Inulin	Oral feeding at 20 g/kg for 1 month	Turbot (*Psetta maxima*) larvae	Increased growth rate and proliferation of gut microbiota (*Bacillus* spp. and *Vibrio* spp.)	Mahious et al. (2006b)
	Oral feeding at 20 g/kg for 1 month	Siberian sturgeon (*Acipenser baerii*)	Increased growth rate	Mahious et al. (2006a)
	5 and 10 g/kg for 1 week	Gilthead seabream (*Sparus aurata* L.)	Significant inhibition in phagocytosis and respiratory burst in leucocytes	Cerezuela et al. (2008)
	Oral feeding of 0.8% for 114 days	Sharpsnout sea bream (*Diplodus puntazzo*)	No significant change in growth and digestibility	Piccolo et al. (2011)
FOS	Oral feeding at 20 g/kg for 1 month	Turbot larvae	Increased growth rate and proliferation of gut microbiota (*Bacillus* spp. and *Vibrio* spp.)	Mahious et al. (2006b)
	Oral feeding at 10 g/kg for 4 months	Atlantic salmon (*Salmo salar*)	No significant effect on feed intake, growth or digestibility	Grisdale Helland et al. (2008)
scFOS	0.8 or 1.2 g/kg for 8 weeks	Hybrid tilapia	Increased growth rate, feed intake, feed conversion, along with significant proliferation of *V. parahemolyticus*, *A. hydrophila*, *Lactobacillus* spp.	Hui-Yuan et al. (2007)
	1 g/kg for 56 days	Hybrid tilapia	Increased proliferation of gut endo-symbionts	Zhou et al. (2009)
	Fish meal (FM)-based diets enriched with 1% scFOS for 7 weeks	European sea bass (*Dicentrarchus labrax*)	No significant change in growth and survival	Guerreiro et al. (2015)
MOS	Oral feeding of 0.2% Artemia enriched by A1 DHA SelcoTM with MOS addition for 23–43 days	White sea bream (*Diplodus sargus*) larvae	No significant effect on growth, survival	Dimitroglou et al. (2011)

(continued)

Table 1.4 (continued)

Prebiotic	Dose and length of administration	Fish	Results	Reference
	FM-based diets enriched with 20 g MOS at 0.16% for 8 weeks	European sea bass (*D. labrax*)	Increased growth	Torrecillas et al. (2015)
	FM-based diets enriched with MOS at 0.21 g 0.05%, 0.1%, 0.2%, 0.3% and 0.4% till 75 days	European sea bass	Increased growth and disease resistance	Salem et al. (2016)

Table 1.5 Synbiotic used in aquaculture

Synbiotic combination	Aquatic organism	Effect	Reference
Enterococcus faecalis + MOS and plyhydroxybutyrate acid in different combinations for 12 weeks	Rainbow trout (*Oncorhynchus mykiss*)	Increased growth performance and immune response	Rodriguez Estrada et al. (2009)
B. clausii + MOS, FOS in different combinations for 56 days	Japanese flounder (*Paralichthys olivaceus*)	Increased growth performance and digestibility performance	Ye et al. (2011)
Biomin IMBO (*E. faecium* + FOS) in different combinations for 60 days	Rainbow trout (*O. mykiss*)	Increased growth performance and immune response	Mehrabi et al. (2012)
B. subtilis + FOS in different combinations for 10 weeks	Juvenile large yellow croaker, (*Larimichthys crocea)*	Increased growth performance and immune response	Ai et al. (2011)
B. subtilis + chitosan in different combinations for 8 weeks	Cobia, (*Rachycentron canadum*)	Increased growth performance and immune response	Geng et al. (2011)
L. plantarum 7–40 + galactooligosaccharide	Pacific white shrimp (*Litopenaeus vannamei*)	Increased growth performance and digestibility	Huynh et al. (2018)
B. lincheniformis + MOS	Pacific white shrimp (*L. vannamei*)	Elevate the dietary assimilation, intestinal SCFAs content and immune response of host to confer healthy growth	Chen et al. (2020b)

'*para*' is adopted from Greek word, which means 'atypical' or 'alongside of' (Choudhury and Kamilya 2019). Because of their crude nature, they are also called as 'ghost probiotic' (Choudhury and Kamilya 2019). Villamil et al. (2002) were the first who observed the immune augmentation potential of paraprobiotics,

Table 1.6 Paraprobiotic application in aquaculture

Paraprobiotic	Organism under study	Mode of administration	Post-administration responses	Reference
L. plantarum	Giant fresh water prawn (*Macrobrachium rosenbergii*)	In vivo	Significant rise in immune response and diseases resistance	Dash et al. (2015)
B. pumilus SE5	Orange-spotted grouper (*Epinephelus coioides*)	In vivo	Significant rise in immune response	Yan et al. (2016)
Pseudomonas aeruginosa VSG2	*Labeo rohita*	In vitro	Significant expression of immune genes and immune cell activity	Giri et al. (2016)
L. plantarum	Pacific white shrimp (*L. vannamei*)	In vivo	Elevation in growth, immune response and tolerance towards salinity stress	Zheng et al. (2017)
B. amyloliquefaciens FPTB16	*Catla catla*	In vivo	Significant rise in immune responses	Singh et al. (2017)
Lactobacillus plantarum	Whiteleg shrimp (*Penaeus vannamei*)	In vivo	Positively modulated the gastrointestinal microbiota	Zheng et al. (2020)

Lactococcus lactis in turbot. This study leads the foundation for future research, which revealed that paraprobiotic preparations may play a vital role in mediating growth and immune responses, resistance from various diseases, etc. (Choudhury and Kamilya 2019). Several researchers have studied the capability of paraprobiotic in elevating immune responses in different in vitro models. These studies advocate for the potency to augment immunity levels significantly through enhanced myeloperoxidase content, nitric oxide production, respiratory burst activity, phagocytic activity, proliferative of leukocytes in fish head-kidney, etc. (Villamil et al. 2002; Salinas et al. 2006; Román et al. 2012; Kamilya et al. 2015; Table 1.6).

1.3.7 Bacteriophage

Bacteriophages are the viruses that kill or lyse the bacterial host cell and release new progenies (Al-Sum and Al-Dhabi 2014). Employment of precise killing capability of phages to control virulent bacterial pathogens is called phage therapy or phagotherapy. After the infection on bacterial host, phages utilize the bacterial host's bio-machinery for all kinds of metabolic support to survive (Al-Sum and

Al-Dhabi 2014). As the natural environment is replete with loads of bacterial host, the occurrence of phages is natural. It can flourish in soil up to 10^{7-8} virions/g and in water approximately 10^7 virions/mL either in a fresh or saline environment (Ninawe et al. 2020; Park et al. 2020). The life cycle of bacteriophages can be categorized into two stages; the first is lytic (virulent) and the second is temperate. For the phage therapy, lytic phages are preferred because of their bacteriolytic activity (Choudhury et al. 2017). Although bacteriophages were discovered way back at the beginning of the nineteenth century, the focus of research on their therapeutic potential against bacterial diseases was limited because of the poor understanding of phage life cycle and phage-bacteria interactions (Almeida et al. 2009). However, the emergence of multidrug-resistant bacteria has substantially encouraged researchers to explore the potential of phage therapy. The strain-specific lytic capability of phages can be employed as bioagents against a wide range of bacterial pathogens. Owing to the specificity of phages to their host, the probability of disturbing natural microflora of aquatic environment or host inhabiting beneficial bacteria will be null, which is very unlikely with the administration of common broad-spectrum antibiotics (Fortuna et al. 2008). Nowadays, work associated with phage therapy against bacterial pathogens in aquaculture has been accepted worldwide and encourages researchers to explore the application and efficacy of phage therapy in different circumstances under various cultural conditions (Table 1.7).

1.3.8 Quorum Sensing Inhibition or Quorum Quenching

Quorum sensing is a bacterial cell-to-cell mechanism of communication, in which they produce, release and detect small signaling molecules to coordinate the expression of specific genes or proteins (Defoirdt et al. 2004). The mechanism was first reported in a bacterium *V. fischeri* (Nealson et al. 1970). Later on, similar systems were also found to be present in many other bacteria, where they produce acylated homoserine lactones (AHLs), autoinducer-2 (AI-2), cholerae *quorum-sensing* autoinducer-1 (CAI-1), etc. as communicating molecules (Nealson et al. 1970; Defoirdt et al. 2004). Bacterial phenotypic characteristics that are coordinated by the quorum sensing system include antibiotic production, nodulation, conjugation, biocorrosion, bioluminescence, swarming, sporulation and most importantly expression of virulence factors (toxins, lytic enzymes, siderophores, adhesion molecules, etc.) and biofilm formation (De Kievit and Iglewski 2000; De Windt et al. 2003; Defoirdt et al. 2004). Quorum sensing probably increases the probability of pathogen infection to their host successfully by expression and coordination of these virulent factors to overwhelm the host's immune system making the organism immunocompromised (Donabedian 2003). Over the years, it has been reported that disruption of bacterial communication through probiotic application, chemical administration, gene mutation, etc. can significantly reduce the expression of virulence factor and may provide novel avenues for disease control in aquaculture (Swift et al. 1999) (Table 1.8).

Table 1.7 Phage-based commercial products for phage therapy in aquaculture

Name of the company/ institute	Bacterial host	Product description	Reference
Intralytix	*Escherichia coli*, *Vibrio tubiashii* and *V. coralliitycis*	Phage cocktail to control infections in oyster	Intralytix Inc. (2018)
Fixed Phage Ltd.	*V. parahaemolyticus*	aquaPHIX™ is a phage-based solution that binds with feed pallets applied directly in the aquaculture system	Mattey (2020)
ICAR-CIBA	*Vibrio* spp.	LUMI^{PHAGE} for biocontrol of luminescent bacterial disease (LBD) in shrimp larvae	ICAR-CIBA (2017)
Phage Biotech Ltd	*V. harveyi*	Phage therapy to treat *Vibrio* infections in shrimp	Phage Biotech (2017)
ACD Pharma	*Yersinia ruckeri*	CUSTUS$^{®}{}_{YRS}$ is phage-based solutions against Yersiniosis, or enteric red mouth disease (ERM) of Atlantic salmon	ACD Pharma (2017)
Proteon pharmaceutical	*Pseudomonas* spp. and *Aeromonas* spp.	BAFADOR® is a phage-cocktail applied via immersion	Grzelak (2017)
Mangalore Biotech Laboratory	*Vibrio* spp.	LUMI-NIL MBL is a phage formulation to control luminous vibriosis in shrimp	Mangalore Biotech Laboratory (2019)

1.3.9 RNA Interference (RNAi)

RNAi is a post-transcriptional gene silencing that is evolutionally conserved, where dsRNAs are introduced into a cell to cause sequence-specific degradation of homologous mRNAs (Almeida and Allshire 2005). This method is a defence strategy adopted by fungus, plants and invertebrates to counter the introduction of unwanted nucleic acids (viruses, etc.). However, the technique has been reported in several other eukaryotic organisms revealing that the mechanism corresponds to control the endogenous gene expression (Voinnet 2002). In the RNAi machinery, the post-transcriptional activity to degrade the cytoplasmic RNAs in a sequence-specific manner can be adopted as key to advance towards antiviral strategy in aquaculture, especially in shellfish culture practices (Molnár et al. 2005). Recent advancements in the application of RNAi-based technology promise as a potential molecular tool for silencing particular genes involved in expression of virulence factor or validation of potential drug targets in various organisms (Golding et al. 2006; Hong-Geller and Micheva-Viteva 2010). In this perspective, RNAi-based silencing or molecular scissoring could help in protection from viral infection (Table 1.9).

Table 1.8 Effect of quorum quenching on the virulence of pathogens

Pathogens	Quorum quenching method	Virulence capacity	Host	Challenge method	Bacterial density	Reference
V. harveyi	luxS, luxO, luxP gene mutation	Abolished	Brine shrimp Artemia	Bath challenge	10^4 CFU/mL	Defoirdt et al. (2005)
A. hydrophila	ahyR gene mutation	Reduced	Epithelioma papillosum of carp (*Cyprinus carpio*) cells	Coculture 30 min	MOI 1	Bi et al. (2007)
			Swordtail (*Xiphophophorus helleri*)	Intraperitoneal injection with 0.1 mL bacterial suspensions	$10^{4–10}$ CFU/mL	
A. hydrophila	ahyI gene mutation	Reduced	Epithelioma papillosum cyprini (EPC) cells	Coculture 30 min	MOI 1	Chu et al. (2011)
			Gold fish (*Carassius auratus gibelio*)	Intraperitoneal injection with 0.1 mL bacterial suspensions	$10^{4–10}$ CFU/mL	
V. parahaemolyticus	opaR gene mutation	Increased	Chinese hamster ovary cells	Coculture 5 h	1.5×10^6 CFU/mL, MOI 15	Gode-Potratz and McCarter (2011)
V. alginolyticus	luxT gene mutation	Slightly reduced	Zebra fish	Intramuscular injection	10^4–10^7 CFU per fish	Liu et al. (2012)
V. harveyi HAH	Probiotic application, *Cobetia* sp.	Reduced	*P. vannamei*	In vitro and in vivo tests	Immersion @ 10^4–10^6 CFU/mL	Yu et al. (2019)
A. hydrophila	Probiotic application, *B. licheniformis* T-1	Reduced	Zebra fish	Injected intraperitoneally	2.6×10^8 CFU/mL	Chen et al. (2020a)

Table 1.9 Applications of RNAi against viral infection in shrimps

Target species	Target gene	Delivery method	Pathogen	RNAi inducer	Reference
Giant tiger prawn (*P. monodon*)	YRP65	Transfection	Yellow head virus (YHV)	In vitro transcribed dsRNA	Ongvarrasopone et al. (2011)
	vp15 and vp28	Injection	White spot syndrome virus (WSSV)	siRNA	Ho et al. (2011)
	ns1and vp	Injection	Densovirus (DNV)	Bacterially expressed dsRNA	Sellars et al. (2011)
	PmRab7	Injection	Laem-Singh virus (LSNV)	Bacterially expressed dsRNA	Sudhakaran et al. (2011)
	GAV, b-actin	Oral	Gill-associated virus (GAV)	Bacterially expressed dsRNA	Posiri et al. (2011)
Pacific white shrimp (*L. vannamei*)	Rab7	Injection	Taura syndrome virus (TSV)	Bacterially expressed dsRNA	Mejía-Ruíz et al. 2011
	rr2 and dnapol, ORF	Injection	WSSV-TSV	In vitro transcribed dsRNA	Hirono et al. (2011)

1.3.10 Antimicrobial Peptides (AMPs)

AMPs are a group of conserved oligopeptides (>5−100 amino acids) that play a key role in innate immunity and are known for exhibiting broad-spectrum antimicrobial responses (Paria et al. 2018). They are cationic, which can precisely target bacterial cell membranes because of their negative charge and cause the destruction of the organism by disruption of the cellular membrane and impaired cellular functioning (Reddy et al. 2004). Owing to their broad-spectrum antimicrobial capacity, these molecules may serve as an alternative biodegradable antimicrobial agent in aquaculture without any concerns towards the development of drug resistance (Lai and Gallo 2009; Table 1.10).

1.3.11 Gene Therapy

Gene therapy is a genome editing technology for precise and targeted genetic modifications, enabling knocking out or adding specific gene or DNA fragments by engineered nucleases (Li et al. 2019). There are four major ways of editing through programmed nucleases: zinc finger nucleases (ZFNs), mega-nucleases, clustered regularly interspaced short palindromic repeat-associated nuclease Cas9 (CRISPR-Cas9) and transcription activator-like effector nucleases (TALENs; Li et al. 2019). These nucleases precisely recognize specific sites in the genome and

Table 1.10 Antimicrobial peptide application in aquaculture

Protein/AMPs	Administration method	Pathogen	Infected organism	Results	Reference
AMP (CEME, cecropin-melitin)	Intraperitoneal injection	*V. anguillarum*	Coho salmon (*Oncorhynchus kisutch*)	Increased survival percentage	Jia et al. (2000)
AMP (synthetic hepcidine)	Intraperitoneal injection	*V. anguillarum*	European bass (*D. labrax*)	Significant reduction in the mortality	Alvarez et al. (2016)
AMP (FSB-AMP)	Oral	*V. parahaemolyticus*	Pacific white shrimp (*L. vannamei*)	Significant reduction in the mortality	Cheng et al. (2017)
AMP (Scy-hepc)	Oral	*A. hydrophila*	Sea bream (*Sparus macrocephalus*) and hybrid grouper	Increased survival percentage	He et al. (2018)
AMP (synthetic hepcidines)	Intraperitoneal injection	*Edwarsiella tarda*	Mudskipper (*Boleophthalmus pectinirostris*)	Significant increase in survival	Chen et al. (2018)
AMP (hepcidine)	Oral and intraperitoneal injection	*Flavobacterium columnare*	Grass carp (*Ctenopharyngodon idellus*)	Significantly increased survival	Wei et al. (2018)

generate DNA double-strand breaks (DSBs). Production of DSBs will lead to activation of natural DNA repair mechanism, non-homologous end joining (NHEJ) which will cause direct rejoining and ultimately lead to insertions or deletions of gene (Cox et al. 2015). As a result of this mutation, the gene may loss of function; this particular approach offers a novel perspective in disease management in aquaculture as we can design the specific nucleases and precisely achieve predictable substitution, insertion or deletion of target genes of our interest (Osakabe and Osakabe 2014).

Aquaculture industry is suffering severe loss every year caused by diseases of diverse origin in nature. Several strategies either prophylactic or therapeutic approach were adopted to resolve the issue; however, the problem remains the same. On the other side, biotechnological science is growing rapidly and has endowed us with several new tools and technology that availed capability to create new horizons in aquaculture, especially health management. Some of the advance biotechnological approaches such as vaccination, antimicrobial peptides, gene editing, etc. have shown promising results in managing the health of cultured organisms of both finfish and shellfish organisms over different agro-climatic condition all over the globe. However, there is a lot of scope in the optimization and commercialization of these methodologies at different culture conditions in a farmer-friendly manner.

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RNA Interference and Its Potential Applications in Aquatic Animal Health Management

2

Vikash Kumar, Suvra Roy, Bijay Kumar Behera, and Basanta Kumar Das

Abstract

In the past two decades, with the discovery of RNA interference (RNAi) or post-transcriptional gene silencing (PTGS), the modern molecular biology field has been boosted with its immense applications. This rapid and powerful silencing method is useful in studying the gene function as well as in therapeutic applications for disease treatment. The RNAi or PTGS is a biological process of mRNA degradation induced by complementary double-stranded (ds) small interfering RNA (siRNA) sequences that mediate suppression of target protein-coding gene expression and provide resistance to both exogenous and endogenous microbial nucleic acids. This sequence-specific natural gene silencing mechanism has revolutionized experimental biology. RNAi technology has important practical applications in aquatic animal health, including functional genomics, therapeutic intervention, and other areas. Here in this chapter, we introduced the RNAi or PTGS mechanisms and the current understanding of gene silencing in aquatic animals in both fish and shellfish and will propose key areas of aquaculture fields where gene silencing could be applied.

Keywords

RNA interference · Post-transcriptional gene silencing · Disease resistance · Aquatic animal health management

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2.1 Introduction

RNA interference (RNAi) or post-transcriptional gene silencing (PTGS) is a naturally occurring biological regulatory mechanism first described in *Caenorhabditis* sp. model (Fire et al. 1998). Following this ground-breaking discovery, molecular biology has advanced tremendously for both in vitro and in vivo studies. RNAi mechanism has been described in all eukaryotic species ranging from unicellular organism to complex species from both animal and plant kingdoms (Ghildiyal and Zamore 2009). RNAi is essentially an endogenous cellular mechanism utilized by the host to prevent the transcription of foreign genetic material and inhibits the accumulation of microbial pathogens. RNAi is triggered by gene-specific double-stranded RNA (dsRNA), leading to post-transcriptional silencing of specific genes by inhibiting the messenger RNA (mRNA) expression or translational in a cell or organism (Fabian et al. 2010; Fire et al. 1998; Meister and Tuschl 2004; Schuster et al. 2019). The basic concept of RNAi-mediated silencing pathways is the association of double-stranded (ds) small RNAs of ~21–22 base pairs nucleotides, having characteristic 2 nt 3′ overhangs, with a Argonaute protein superfamily to form RNA-protein core complex termed the RNA-induced silencing complex (RISC) (Elkayam et al. 2012; Kim and Rossi 2008; Nguyen et al. 2016; Schirle et al. 2016). In eukaryotes, three classes of small RNAs exist: (1) small interfering RNAs (siRNAs), (2) microRNAs (miRNAs) and (3) PIWI-interacting RNAs (piRNAs) (Ketting 2011). Interestingly, these small RNAs guide Argonaute proteins onto target RNAs molecules via Watson-Crick base pairing that results in silencing in gene. The siRNAs, miRNAs or piRNAs pathways follow the basic principle of post-transcriptional gene silencing; however, the general mechanism for effector functions and small RNA biogenesis is relatively different. For instance, the piRNA biogenesis is dicer independent, whereas siRNAs and miRNAs depend on RNase-III dicer enzymes for processing of double-stranded RNA (dsRNA) precursors into small RNAs (Carthew and Sontheimer 2009).

Aquaculture, farming of aquatic animals and plants, continues to dominate the food-producing sector in the world (Kumar 2020; Kumar et al. 2021; Roy 2020; Roy et al. 2019, 2020). However, due to the global demand increase, the pressure for intensification and expansion of aquaculture systems has rendered aquaculture business fragile and created ideal ground for disease outbreaks, having a devastating impact on socio-economic development worldwide (Kumar et al. 2016, 2019a, b, 2020a, b, 2021; Tran et al. 2020). Moreover, in recent years, the application of RNAi technique has become a potential tool to investigate the functionality of gene of interest and suppress the infection or replication of many pathogens that cause severe economic losses in aquafarming. Therefore, there is an urgent need to thoroughly understand the cost-effective RNAi technology to manage heath of aquatic animals and avoid severe mortality and production losses in aquaculture.

2.2 Mechanism of RNAi Technology

The genetic studies on two model organisms, e.g. *Drosophila* sp. and *Caenorhabditis* sp., have helped in the understanding of mechanism underlying RNAi (Reshi et al. 2014). However, the RNAi mechanism was first recognized in plants, when post-transcriptional gene silencing using small specific RNA oligonucleotides of 20−25 bases was studied. The RNAi-mediated gene silencing involves mRNA expression and translational repression by non-specific binding (imperfect) of small RNAs to the 3' UTR region of target mRNA molecule. Further studies using biochemical and molecular genetics reveal that RNAi occurs via a two-stage process involving an initiation stage and an effector stage (Fig. 2.1) (Hammond et al. 2001).

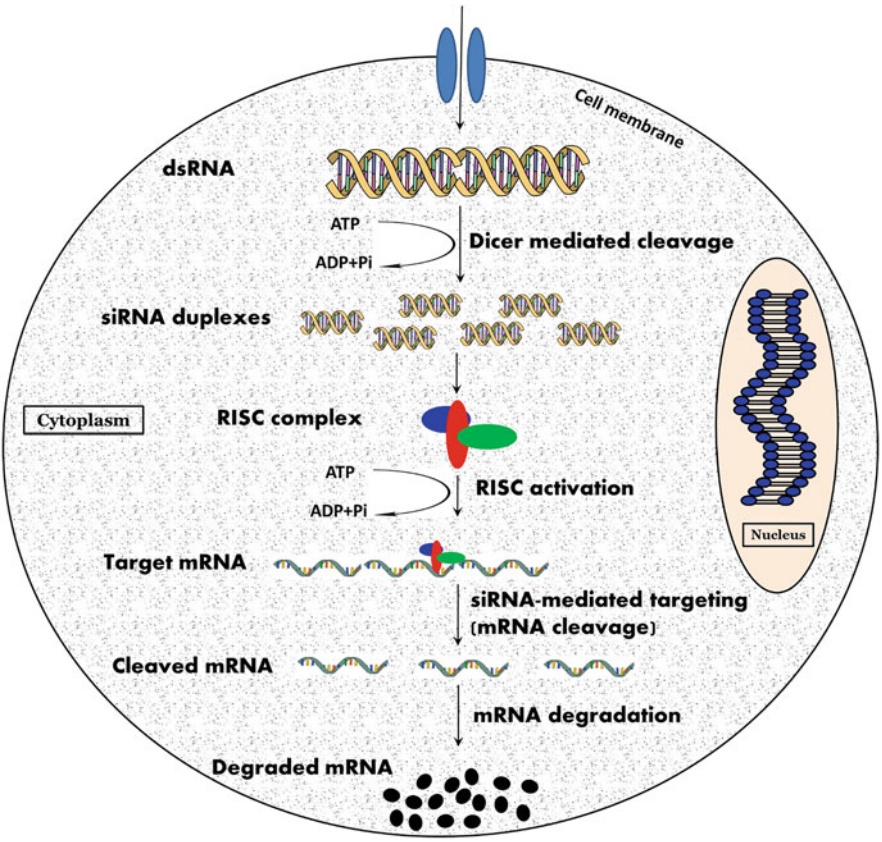

Fig. 2.1 Schematic representation of RNAi molecular mechanism in aquatic animals

2.2.1 Initiation Stage

The initiation stage was reported to take place inside cell cytoplasm. Briefly, a protein molecule known as dicer (ribonuclease III) specifically recognizes the dsRNA sequences and generates 21−23 nucleotides in length small interfering RNAs (siRNAs) molecules (Fig. 2.1) (Reshi et al. 2014).

2.2.2 Effector Stage

The generated double-stranded small interfering RNAs were unwound into single-stranded molecules by a protein complex (containing dicer, Argonaute proteins, etc.) known as RNA-induced silencing complex (RISC). Among two strands, the sense strand is released, while antisense strand is retained by RISC. The antisense strand further guides RISC onto the complementary mRNA molecules, resulting in degradation of target mRNA (Fig. 2.1) (Lingel and Izaurralde 2004). The endonucleases bind to the mRNA region homologous to the siRNA and carry out degradation, resulting in inhibition of expression or translation of target molecules (Zamore et al. 2000).

2.3 RNAi Technology in Aquatic Animal Health Managements

Aquatic animals including both fish and shellfish are susceptible to a variety of pathogens, posing a constant threat to the aquaculture production and severe ecological and economic losses. However, the development of RNAi technology, as a promising gene knockdown tool, has shown promising results in both health and disease management of aquatic animals (Kumar et al. 2018). Fishes were the first vertebrate in which RNAi knockdown of gene expression using long dsRNA was performed. The comprehensive RNAi study was first done in embryos of rainbow trout (*Oncorhynchus* sp.), carrying a green fluorescent protein (GFP) expressing the vector. The siRNAs were able to reduce the number of strongly fluorescent embryos by 60% compared to the negative control embryos, thus providing the first evidence of an effective siRNA-mediated gene silencing in fish (Boonanuntanasarn et al. 2003). The major proteins involved in the RNAi pathway, including dicer and Argonaute, have been identified in fish and shrimp species, e.g. *Danio rerio* (zebrafish), *Gobiocypris rarus* (rare minnow), *Penaeus monodon* (giant tiger prawn), *Litopenaeus vannamei* (whiteleg shrimp), *Marsupenaeus japonicus* (kuruma shrimp) and *Fenneropenaeus chinensis* (Chinese white shrimp), confirming the existence of RNAi machinery in aquatic animals (Table 2.1) (Nguyen et al. 2016; Saksmerprome et al. 2009).

There are reports which highlight that RNAi has become promising tool to study the function of genes in a cell or animals. Additionally, the RNAi-based sequence-specific dsRNA has shown to be effective against severe aquatic animal pathogens. For instance, genes from shrimp pathogens such as yellow head virus (YHV), white

Table 2.1 Major core RNAi genes identified in aquatic animals

Species	Genes	Tissue	References
D. rerio	Dicer	Fertilized eggs	Andrews et al. (2014)
	Dicer 1	Retina	Akhtar et al. (2015)
	Argonaute 2	Fertilized eggs	Kretov et al. (2020)
G. rarus	Argonaute 2	Gill, heart, intestine, kidney, liver, muscle and spleen	Su et al. (2009)
L. vannamei	Dicer 1	Fertilized eggs	Yao et al. (2010)
	Dicer 2	Gill, hepatopancreas, heart, intestine, stomach, nerve, pyloric caecum and epidermis	Chen et al. (2011)
	Argonaute 1	Gill	Labreuche et al. (2010)
	Argonaute 2	Gill	
P. monodon	Dicer 1	Lymphoid organ and haemolymph	Su et al. (2008)
	Dicer 2	Gill, haemolymph and haemocyte but not in tissue muscle	Li et al. (2013)
	Argonaute 1	Lymphoid organs, muscle, gill, hepatopancreas, ovary and nerve cord tissue	Dechklar et al. (2008)
	Argonaute 2	Lymph tissue, gill, haemocytes, eyestalk, heart, ovary, epidermis, stomach, brain, muscle, hepatopancreas, intestines and nerve	Yang et al. (2014)
	Argonaute 3	Haemocytes, lymphoid organ, ovary, heart, hepatopancreas, nerve cord, brain gill, thoracic ganglia, and eyestalks	Phetrungnapha et al. (2013)
	TRBP[a]	Ovaries and lymphatic organs	Yang et al. (2013)
F. chinensis	TRBP[a]	Haemocytes	Wang et al. (2012)
M. japonicus	TRBP[a]	Haemocytes	

[a]*TRBP* transactivation response RNA-binding protein

spot syndrome virus (WSSV), and Taura syndrome virus (TSV) have been targeted by dsRNA, resulting in enhanced survival of shrimp species (Tirasophon et al. 2007; Westenberg et al. 2005; Yodmuang et al. 2006). The detailed application of RNAi technology in aquatic animals is summarized below.

2.3.1 Functional Genomics

RNAi technique can be applied to study the physiological status of different aquatic species. For instance, RNAi technique is being used to determine the process of moulting, reproductive functions (gonad-stimulating hormone and gonad-inhibiting

hormone) and growth by identifying the genes regulating the function of this processes (De Santis et al. 2011).

Additionally, there are several RNAi-related studies on aquatic model organisms, e.g. *D. rerio* (zebrafish) and *Artemia franciscana* (brine shrimp), which help in understanding of animal physiology. Zebrafish, a cyprinid freshwater fish, share very high physiological and genetic similarities with higher vertebrate, including the digestive tract, brain, vasculature, musculature and innate immune system. For instance, zebrafish has almost 70% of functional similarities with human genes responsible for disease, whereas the brine shrimp, an aquatic invertebrate highly osmotolerant, characteristically small and branchiopod crustacean that can be grown in gnotobiotic conditions (germ-free environment, allowing host-associated microbial communities control), serves as exceptional model organism to study the host-pathogen interactions in commercially important shrimps and other crustacean's species. RNAi studies on these model organism have unravel the mechanisms of stress resistance, controlling the process of cell division, by identifying the genes involved in the differentiation, development and reproductive processes (Kumar et al. 2018; Nguyen et al. 2016).

2.3.2 Sex Control

The RNAi technique to control the sex of aquatic animals has remarkably high practical application in aquaculture sector. For instance, an important shellfish species, *Macrobrachium rosenbergii* (giant freshwater prawn), have a very high economic value; however, due to higher growth rate and bigger in size, only male population is preferred for culture. In this prawn species, a highly expressed androgenic gland specific gene, namely, insulin-like AG (Mr-IAG), plays important role in male sex differentiation (Ventura and Sagi 2012). Interestingly, injecting the male prawn juvenile, at an early developmental stage, with Mr-IAG-specific dsRNA, resulted in in vivo silencing of Mr-IAG gene and functional and full sex reversal of male population into neo-females. Subsequently, if the neo-female population were crossed with untreated males, the resulted progenies will be all male population (Ventura et al. 2009). The above experiment demonstrates that RNAi technology is useful in maintaining all male population in prawn species without changing the genome structure, and hence it could become a promising strategy to regulate the sex in other aquatic animals, without creating transgenic animal species.

2.3.3 Disease Management

The fish and shrimp species are constantly exposed to pathogenic microbes in aquatic environment due to which disease outbreak caused by bacteria, viruses and parasites is often observed in aquaculture system. Moreover, with the development of RNAi technology, the understanding on immune mechanism and role of genes potentially involved in tolerance against pathogenic microbial infection has

significantly increased. This has created a positive impact on developing health management protocols for inhibiting the proliferation of pathogenic microbes and improved host survival. However, still RNAi technique is mostly done in smaller scale, and application for commercial disease management is very limited in aquaculture. The RNAi technology used for antimicrobial strategies in aquaculture is summarized below.

2.3.3.1 Antibacterial

The RNAi technique helps in understanding the functional role of genes and underlying immune mechanism, imparting tolerance in aquatic species against pathogenic bacterial infection. In aquatic species, using RNAi, it was shown that prophenoloxidase, p38 mitogen-activated protein and crustin play important role in the multiplication of pathogenic bacteria. For instance, RNA-mediated knockdown of prophenoloxidase inactive precursor resulted in significantly increased bacterial load in shrimp species (Fagutao et al. 2009). Similarly, knockdown of p38 mitogen-activated protein kinases leads to significant lower expression of antimicrobial peptide genes (PEN4, crustin and ALF2) and higher mortality of shrimp species against pathogenic bacterial pathogens (*Vibrio alginolyticus* and *Staphylococcus aureus*) (Yan et al. 2013). In another study, the role of *M. japonicus* crustin (MjCRS) was determined by RNAi technique, and results showed that MjCRS is an important antibacterial defence peptide and silencing results in upregulation of pathogenic bacteria numbers and decreased survival of shrimp species (Hipolito et al. 2014).

2.3.3.2 Antiviral

RNAi technique has been widely used as a powerful tool to identify the genes that participate in viral replication and protect the aquatic animals from viral infection (Wang et al. 2013). Through sensing the viral nucleic acid, RNAi-mediated immunity is triggered to inhibit the replication of the RNA and DNA viruses and knocking down the virus-specific genes or downregulating host genes that are related with viral replication mechanisms. In aquatic animals, siRNA- and miRNA-mediated RNAi antiviral immunity is being reported (Ongvarrasopone et al. 2011).

In siRNA-mediated RNAi silencing, the exogenous microbial dsRNA sequences were cleaved by dicer protein into a siRNA duplex. The siRNA duplex generally possesses 3′ OH, 5′ phosphate (PO_4) and 3′ dinucleotide overhangs molecules. Afterwards, the siRNA duplex combines with Argonaute protein and forms a precursor RNAi-induced silencing complex (pre-RISC). In complex, one passenger strand from duplex is cleaved by Argonaute protein. Later, the Argonaute protein and guide strand in RISC target the complementary strand of mRNAs, resulting in inhibition of translation (Saksmerprome et al. 2013). In shrimp, siRNA-mediated post-transcriptional silencing is reported as important antiviral immune mechanism that leads to inhibition of viral proliferation and infection by degradation of viral mRNA. For example, the viruses after entry and replication inside host produce dsRNA molecules, which initiate an siRNA-mediated antiviral mechanism. The viral dsRNA is processed by dicer2 protein, which cleaved the strand and produces

siRNAs molecules. Afterwards, the siRNAs bind with Argonaute protein and form RISC, resulting in degradation and inhibition of viral replication. Interestingly, it has been found that siRNA contains a 2nd−7th seed region that is responsible for initial recognition of target molecule and a 12th−17th nt supplementary region that helps in binding to the target molecule (Gong and Zhang 2021).

Moreover, miRNAs molecule is derived from the spliced short introns or endogenous noncoding RNA transcripts that are folded into incomplete stem-loop structures. In miRNA-mediated RNAi silencing, the primary miRNAs (pri-miRNAs) are transcribed by the RNA polymerase from genome and later cleaved into approximately 70 nucleotide precursors, called pre-miRNAs, by Drosha protein. Simultaneously, the pre-miRNAs bind with exportin 5 and exported to cell cytoplasm, where pre-miRNAs are processed by Dicer protein into miRNA duplexes. The miRNA duplex binds with Argonaute protein and forms pre-RISC. Later, after the passenger strand is removed from duplex, the RICS containing Argonaute protein and guide strand targets the complementary strand resulting in post-transcriptional gene silencing of target mRNA (Gong et al. 2018). Interestingly, the miRNA 2nd−7th bases are the key domain that recognizes target mRNA through Watson-Crick base pairing, which is generally known as seed sequence. In aquatic animals, miRNAs are reported as a key regulator for variety of biofunctions, e.g. cell development, metabolism, differentiation, proliferation, immunity and apoptosis (He et al. 2015). There are also growing evidence that miRNA plays critical role in antiviral immunity in aquatic organisms by targeting multiple genes through individual miRNA or one gene by several miRNAs. It has been also demonstrated that miRNA promotes apoptosis and cellular phagocytosis by targeting the specific genes, resulting in suppression of viral proliferation and infection.

2.3.3.3 Antiparasitic

An important application of RNAi, apart from being antibacterial and antiviral tool, is its considerable potential for the development of parasitic control technologies. Being very sequence-specific in action, it may be possible to develop species-specific dsRNA for parasites. In mammals, RNAi has been used to suppress gene expression in the intra-mammalian life stages (adults and schistosomula) of *Schistosoma mansoni*, which affects more than 200 million people worldwide and is responsible for 300,000 deaths annually (Da'dara and Skelly 2015).

In aquaculture, the first evidence of gene silencing mediated by dsRNA in a fish parasite was reported in 2007 (Ohashi et al. 2007). Since then, the RNAi technology has been mostly applied to study gene function of various parasites responsible for disease outbreaks in aquaculture. Despite being considered a suitable and capable tool for functional genomics, very little research has been done on its use in the manipulation of the gene function of fish parasites. RNAi techniques have been successfully used to study the microsporidian parasite (*Heterosporis saurida*) of lizardfish (*Saurida undosquamis*) and *Myxobolus cerebralis* (causative agent of whirling disease infection) in the salmonid fishes. The study highlights that RNAi could be a promising tool to enlighten better about the gene functions in parasites for

targeted drug delivery, silencing gene expression and reducing the role of vectors to transmit disease by parasites.

2.4 Delivery Strategies

For successful RNAi-induced silencing, suitable and specific systems are required for delivering RNAi molecules to their target host cell. Depending on the species and organ, different dsRNA delivery strategies can be used, such as electroporation, microinjection, oral pathway, lipid nanoparticles, polymer-based systems and protein-based systems. However, numerous extra- and intracellular biological barriers to RNAi delivery exist in an organism necessitating the smart designing of delivery strategies (Attasart et al. 2010; Timmons and Fire 1998; Tseng et al. 2000). A variety of delivery strategies have been developed for the successful delivery of siRNA molecules, both in vivo and in vitro conditions. Some of the delivery strategies used in aquatic species are discussed below.

2.4.1 Electroporation

Electroporation is another microbiology technique, which utilizes electrical field in order to increase the permeability of the cell membrane, allowing introduction of chemicals, drugs or DNA molecules into the cell. The first experiment on electroporation delivery method to introduce foreign DNA was conducted on *P. monodon* in 1999 (Tseng et al. 2000). Afterwards, several works have been done to validate the method of electroporation-based introduction of foreign DNA using *Artemia* model system (Arenal et al. 2000). Electroporation technique was also employed to deliver siRNA molecule into embryos of model shrimp (*Artemia sinica*) to knock down the As-sumo1 gene. These studies support the electroporation technique to deliver nucleic acid into embryos of fish and shellfish at earlier life stages. Additionally, electroporation can be performed with significantly large numbers of zygotes or embryos at the same time.

2.4.2 Microinjection

Microinjection, utilizing glass micropipette to inject a liquid substance, is a direct method to introduce DNA into either cytoplasm or nucleus. The microinjection method has also been used widely to introduce nucleic acids, including dsRNA, into fishes and shellfish, at different developmental stages. Studies on aquatic animals, e.g. *Daphnia magna*, *Macrobrachium rosenbergii*, *L. vannamei*, *P. monodon* and *A. franciscana*, have reported that microinjection of siRNA resulting in significant induced post-transcriptional silencing in host animals (Chimwai et al. 2016; Han et al. 2019; Kumar et al. 2018).

2.4.3 Oral Delivery

Oral delivery of RNAi therapeutic molecule, either naked or conjugated with a polymer or in the form of bacteria that contain the specific dsRNA/siRNA or in the form feed, has been successfully applied to many aquaculture species (Sarathi et al. 2008). Antiviral activity of RNAi molecule incorporated in nanoparticulate feed has been reported in shrimp species, targeting the WSSV vp28 gene. The study demonstrated that gene knockdown by RNAi incorporated feed results in significant increased protection of shrimp species against viral challenge (Ufaz et al. 2018). In another study, oral delivery of RNAi molecule against WSSV infection in *P. monodon* leads to reduced percentages in cumulative mortality and delayed average time of death (Thammasorn et al. 2015).

2.4.4 Vector-Based Delivery

Vectors are tools commonly used by molecular biologists to deliver genetic material into the cells. This process can be performed in both in vivo (living organism) or in vitro (cell culture) condition. Vector-based delivery methods include those derived from viruses and plasmids. Viral vectors are recognized as efficient delivery systems for RNAi technology (Qayoom and Mushtaq 2020). However, induction of strong immune response and the risk of vector integration with host genome are often associated this method. These delivery methods are still in laboratory use and require further work and validation before they can be used in commercial aquaculture.

2.5 RNA Interference (RNAi) Procedure in Crustaceans: A Case Study from *A. franciscana* Model System

2.5.1 Culture of *A. franciscana* for Microinjection

At first, the *Artemia* cysts were hydrated in distilled water for 1 h. Later, the cysts were decapsulated using sodium hydroxide (NaOH) and sodium hypochlorite NaOCl for 2 min; afterwards, the reaction was stopped by adding sodium thiosulphate ($Na_2S_2O_3$). The decapsulated cysts were washed and suspended in filtered 35 g/L autoclaved seawater (FASW) and incubated for 28 h at 28 °C for hatching with constant illumination of approximately 27μE/m^2 s. The developmental stage instar II (mouth is opened to ingest particles) hatched larvae were collected and daily fed with green algae (*Tetraselmis suecica*) until they grow to adults in a 28 °C controlled temperature room with approximately 27μE/m^2 s constant illumination.

It is important to mention that approximately after 2 weeks of *Artemia* culture, the females are manually separated and kept in a separate tank from male population to avoid the mating and fertilization.

2.5.2 Preparation of *A. franciscana* hsp70 and gfp dsRNA

The dsRNA specific to the *A. franciscana hsp70* gene transcript is amplified using gene-specific primers, and each primer included with T7 promoter (5′ TAATACGACTCACTATAGGG 3′) at their 5′ ends (forward primer, 5′ GATGCAGGTGCCATTGC 3′ and reverse primer, 5′ AGCTCCTCAAAACGGGC 3′). In addition, a green fluorescence protein (gfp) fragment was also amplified to prepare dsRNA of *gfp*, which could serve as a negative control. Here also T7 promoter was included at their 5′ ends (forward 5′ *AGA*GCGCTTCTCGTTGGGG 3′ and reverse 5′ *AGA*CCTGAAGTTCATCTGC 3′). After PCR, the purified product was incubated overnight at 37 °C with T7 enzyme mix, reaction buffer and four ribonucleotides (ATP, CTP, GTP and UTP). Afterwards, the dsRNA was incubated with RNAse or DNase I for 1 h at 37 °C for 1 h for nuclease digestion to remove any ssRNA and DNA template that did not anneal.

The dsRNA of *hsp70* and *gfp* was quantified using nanodrop spectrophotometer and further subjected to agarose gel electrophoresis (1.5%) in order to check the efficiency and integrity of duplex formation.

2.5.3 Microinjection of Artemia Females with dsRNA

The *hsp70* and *gfp* dsRNA were mixed separately in 1:10 ratio (v/v) with phenol red (0.5%) in Dulbecco's phosphate-buffered saline (DPBS). The diluted *hsp70* or *gfp* dsRNA (250 nL of solution containing approximately 80 ng dsRNA) was injected to egg sacs of adult *Artemia* female with a FemtoJet® microinjector using Femtotips II microinjection capillary tips while viewing under stereomicroscope.

The females were injected with each *hsp70* and *gfp* dsRNA. Injected females were kept for 2 h, and animals which remained healthy, retained dye and could swim properly were used in the further experiments. The injected females were transferred to 6-well plate (1 female/well) containing sea water (35 ppt). In each well, a healthy adult male was transferred, and the pairs were fed daily with green algae, *Tetraselmis suecica*. The plates were maintained in a 28 °C controlled temperature room with approximately 27μE/m^2 s constant. After 5 days, larvae were collected from each mating pairs, injected with dsRNA *hsp70* and *gfp*, for further analysis.

2.5.4 Validation of RNAi Microinjection Efficiency

2.5.4.1 Survival of *A. franciscana* Larvae

The *Artemia* larvae collected from single female injected with either *hsp70* or *gfp* dsRNA were transferred to sterile falcon tubes (50 mL) containing 30 mL seawater. Subsequently, the larvae were challenged at 10^7 cells/mL with pathogenic bacterial pathogen strain. The survival of *Artemia* larvae was recorded after 48 h of pathogen addition.

2.5.4.2 Detection of *hsp70* mRNA in Brine Shrimp Larvae

The larvae from injected *Artemia* females with dsRNA either *hsp70* or *gfp* were collected in sterile falcon tubes (50 mL) containing 30 mL seawater. Subsequently, the larvae were washed with distilled water and resuspended in eppendorf tubes (1.5 mL) with RLT buffer. Later, the larvae were homogenized, and according to the manufacturer's protocols, the total RNA was isolated using RNA extraction kit. Equal amounts of larvae total RNA (determined by nanodrop spectrophotometer) were used for synthesis of cDNA, using first-strand synthesis qRT-PCR kit. Subsequently, cDNA product (5μL) was used for *hsp70* PCR amplification using set of forward and reverse primers. The PCR products were transferred in 1.5% agarose gel (w/v), stained with nucleic acid gel stain (GelRed™) and visualized by imaging system (ChemiDoc MP).

2.5.4.3 Detection of Hsp70 Protein in Brine Shrimp Larvae

As mentioned before in detection of hsp70 mRNA protocol, the larvae from dsRNA injected females were collected in sterile eppendorf tubes (1.5 mL) and homogenized in equal volume of loading buffer (5μL). Later the samples were vortexed, heated at 95 °C for 5 min, and 15μL of each sample were transferred in SDS-PAGE gel (10%). A positive control, i.e. HeLa (heat shocked) cells were loaded in one well. Later, the gels were then stained with Coomassie Biosafe to visualize the quality of samples. Subsequently, the gels are transferred to polyvinylidene fluoride membrane (PVDF) for antibody probing. At first, the membrane was incubated with blocking buffer at room temperature for 60 min and then with primary antibody (monoclonal anti-Hsp70 antibody). Later, the membrane was incubated with secondary antibody (anti-mouse IgG). Finally, western ECL substrates (chemiluminescence reagent) were added to membrane for 5 min, and the bands were visualized in imaging system (ChemiDoc MP).

2.6 Conclusion and Future Perspective

RNAi has undergone an information explosion in the last two decades, and it is now established that as much as 5% of the human genome is dedicated to encoding and producing, the >1000 microRNAs (miRNAs) regulate at least 30% of our genes. It is now understood that RNAi is a promising tool that controls the vital processes of a biological organism, such as cell growth, tissue differentiation, heterochromatin formation and cell proliferation. RNAi can successfully result in the knockdown of single or multiple genes, providing a quick and convenient method to analyse the gene function. More specifically, small interfering RNAs (siRNA) of ~20−22 bp dsRNA molecules having a characteristic 2 nt 3′-overhangs allow recognition and subsequent binding of RNAi machinery, ultimately leading to a homology-dependent degradation of the target indigenous mRNA. The possibilities of dsRNA molecules production are evolving from in vitro to in vivo synthesis with lower cost, allowing more practical applications in broader scale. Additionally, the newly developed dsRNA delivery techniques allow manipulations at the cellular and

tissue level especially, and studies have shown initial success in dsRNA delivery simultaneously on many individuals. RNAi can now be applied to control the sex of a population and has shown possibility for widespread application, enhancing the animal production and prevention of microbial diseases in aquatic animals. In conclusion, RNAi technology promises great potential for use in both research and applied sciences and could become a core component in aquatic animal health management.

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3 Biotechnological Advances in the Development of Outer Membrane Protein-Based Vaccines for Use in Aquaculture

Biswajit Maiti, Anirban Chakraborty, and Indrani Karunasagar

Abstract

Aquaculture is one of the fastest-growing food-producing sectors globally, and the pressure on the system to have maximum productivity in a limited time with very high stocking densities requires high feed inputs. This is contradictory to the natural culture systems, and thus it is often hampered by various disease problems with bacterial cause most often being a consequence of the above culture practice. Therefore, a strategy to prevent diseases through better management practices would be most appropriate. Towards this, vaccination offers itself an acceptable strategy that the farmer can easily adopt for successful aquaculture without any environmental effects. However, the availability and selection of suitable vaccine candidates are relentlessly researched upon for having a vaccine that offers greatest protection to the commonly encountered pathogens. Though outer membrane proteins (OMPs) of bacteria play an important role in virulence, deciphering them as vaccine candidates offer promise as these molecules directly interact with host cells due to exposed epitopes and can induce an effective immune response. They are not only crucial for host immune response but serve as targets for drug therapy. In recent years, many studies have demonstrated the efficacy of OMP-based vaccines and recommended them as potentially important molecules for vaccine development that would provide for long-lasting and improved immunity. A comprehensive review of biotechnological advances

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on the use of OMPs as the potential new-generation vaccine candidates for fish is presented.

Keywords

Outer membrane proteins · Vaccination · Fish · Aquaculture · Biotechnology

3.1 Introduction

Food and nutritional security are being addressed globally through aquaculture as it is an important source of protein and can have different species cultured in cold and warm waters. Being one of the fastest-growing food industries, it has social relevance as it has supported the livelihood of artisanal fisher folk and is the primary income source for many around the world, including several developed and developing countries. Asia contributes significantly to aquaculture production in the world. Finfish is the most commonly cultured group and contributes to 66% of total fish aquaculture production (FAO 2018). The intensive culture practices with high feed input contribute to the deterioration of the culture environment as the naturally occurring useful microbes are not enough to carry out the various biogeochemical cycles towards bioremediation. A consequent increase in sulphides, ammonia, and other pollutants stress the animals and result in them becoming susceptible to opportunistic pathogens in that particular culture pond. All of these factors result in infectious diseases and their spread within and to neighbouring culture ponds and become a major setback for successful aqua-farming.

Aquaculture also faces many other problems like overexploitation of fish, urbanization, and untreated industrial waste to freshwater. The most important is the economic losses due to infectious diseases and the consequent effect on livelihood, causing adverse effects on the entire world's aquaculture practices. There are many different types of infectious agents for the morbidity and severe mortality observed in cultured freshwater fish (Subasinghe et al. 2001). They could be viruses, bacteria, parasites, or fungal agents. Bacterial diseases are the major cause of concern because various opportunistic and few primary pathogenic bacteria are responsible for disease outbreaks. They are also the most common agents of morbidity and mortality among natural populations of fish, but such diseases in the wild often go unnoticed. In aquaculture systems, representatives of many bacterial taxa are naturally associated with aquatic animals and are autochthonous to the aquatic environment. They are known to cause diseases in fish that are stressed or weakened due to sudden environmental changes (Novoslavskij et al. 2016). It is very important to develop good husbandry techniques to minimize the disease problem and make aquaculture successful. Treatment is resorted to during any disease outbreaks, with the antimicrobials being administered orally or by immersion or parenteral administration. It is to be expected that there would be loss of appetite during an illness, and therefore the fish is unlikely to take in the antibiotic orally (Assefa and Abunna 2018). Treatments become ineffective if it does not reach the target site. The cost of

the antimicrobials, together with the environmental hazard associated with the accumulation of the antimicrobials and/or their metabolites, causes serious economic loss to the farmers and damage to the environment with the buildup of residues and antimicrobial resistance in the native organisms of the aquatic environment.

The best method for disease control, both economically and ethically, would be prevented through appropriate vaccination. Gaps in knowledge and delivery technology prevent the cost-effective use of many vaccines for aquaculture (Dadar et al. 2017). Vaccines against bacterial diseases have successfully reduced antibiotic use in aquaculture and have been identified as crucial factors in the Atlantic salmon industry's success in Norway. However, it is necessary to select the immunogenic protein molecules as a potential vaccine candidate. Outer membrane proteins (OMPs) from fish pathogens have been identified and utilized as promising candidates for vaccine development. It stimulates both specific and innate immune response molecules and can control the disease burden in aquaculture (Maiti et al. 2020).

3.2 The Use of Vaccines for Controlling Bacterial Fish Diseases

Good husbandry techniques contribute to a great extent in minimizing the occurrence of diseases in aquaculture during disease outbreaks. The conventional approach is to administer therapeutics either orally or through immersion techniques. In some cases, even injection method is also followed. However, during a diseased condition in fish, there is an alteration in eating habits and other physiological conditions, which negatively influences the success of the conventional vaccination approaches. Thus, a prophylactic approach where vaccination of the cultured species is done using immunogenic antigens can be a good alternative to protect animals against infectious agents. This strategy has shown promising results in higher animals, including humans, in protecting against a number of bacterial and viral agents. Fish vaccination is very popular in countries like Norway, North America, and the USA. Over the years, several bacterial and viral vaccines have been successfully developed and commercialized either in mono- or multivalent forms (Bostock 2002; Evelyn 2002). The control of bacterial disease outbreaks through antibiotic treatment is generally not recommended. Besides its harmful effects on the environment, prolonged use of antibiotics can lead to the emergence of antimicrobial resistance that may get transferred to fish or human pathogens and other innocuous bacteria (Karunasagar et al. 1994). Therefore, the use of vaccines is a better way to protect cultured fishes from bacterial diseases in aquaculture.

Although significant advances have been made in the field of fish vaccine development, the number of commercially available vaccines that are truly effective is very few. This is mainly due to antigenic variation among serotypes and their acceptability. In most aquaculture practices, inactivated bacterial vaccines are usually the preferred choices. They are produced by inactivating the disease-causing microorganism with chemical (formalin) or heat. But this type of inactivated vaccine, referred to as bacterin, is not highly satisfactory when used for some specific

outbreak with a specific antigenic type as energy is wasted by the fish immune system in producing antibodies to many antigenic molecules, protective or otherwise, at the same time. Therefore, it is important to search for conserved protective antigens that could be targeted for developing effective vaccines. The availability of the whole genome sequence information of several bacterial species has led to a new approach, called reverse vaccinology, based on screening potential vaccine candidates followed by proteomic analysis of the molecules (Rappuoli et al. 2011). In the case of a subunit vaccine, a single antigen is usually expected to induce immunity to a single pathogen (monovalent) or multi-organisms (polyvalent). The development of vaccines using biotechnological approaches can be potent, safer, better characterized with specificity and long-term immunity without the need for boosters, being the supreme factors.

The estimation of protection efficacy or vaccine efficacy is a well-defined term generally used to understand the relationship between the mortalities resulted from a disease outbreak or challenge of any specific pathogen in vaccinated and control groups. Results are commonly expressed by the term relative percent survival (RPS) based on the formula derived by Amend (1981) or by estimating the percentage of survival using the Kaplan Meyer's survival analysis (post-challenge survival proportion).

$$\mathrm{RPS} = [1 - (\%\text{mortality in the vaccinated group}/\%\text{mortality in the control group})] \times 100$$

3.3 Advantages of Outer Membrane Proteins (OMPs)-Based Vaccines

The outer membrane (OM) of Gram-negative bacteria comprises a group of integral proteins, commonly referred to as OMPs. These proteins are essential for maintaining the integrity and function in the selective permeability of the membrane. About 2−3% of the genes encode OMPs and cover about 50% of the total OM mass. These OMPs are considered as potential vaccine candidates since they are: (1) exceedingly immunogenic due to their exposed epitopes on the cell surface, presenting themselves as loops (usually designated as L1, L2, and so on) (Fig. 3.1) broadly conserved among different serovars and also sometimes shared among other Gram-negative bacteria. These OMPs can be present as monomers, homo-dimers, and/or homo-timers in the outer layer of the membrane. Due to their location in the outermost layer of the bacterial cell wall and the exposed epitopes on the cell surface, they are ideal candidates with a very high degree of immunogenicity (Maiti et al. 2020). Further, studies on the sequence analysis of OMPs demonstrated that they are relatively conserved and show sequence similarity among related species, which could offer cross-protect across different fish pathogens.

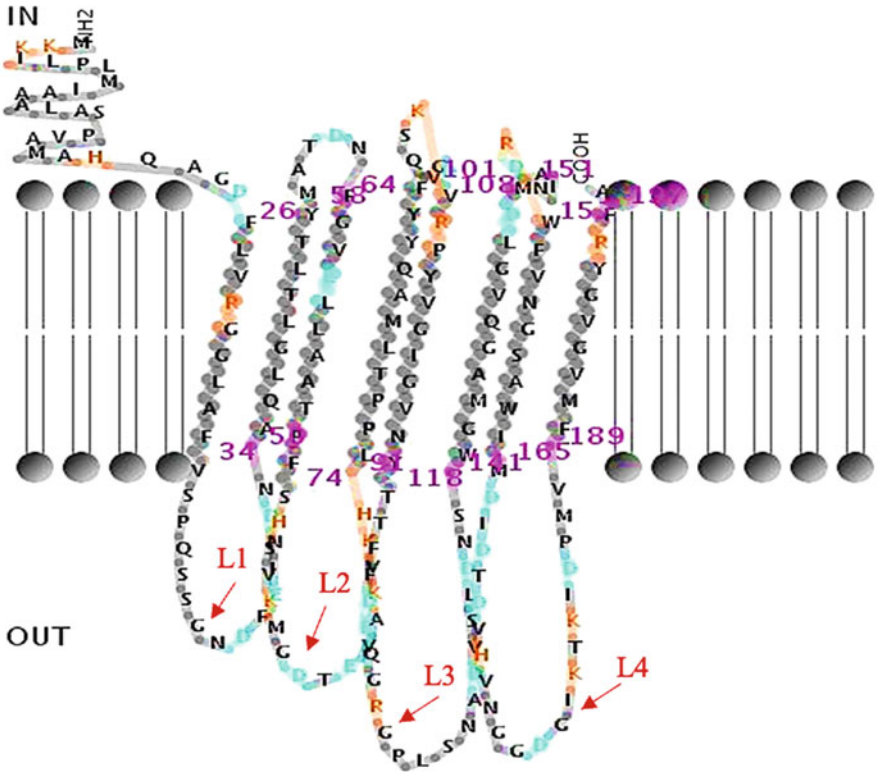

Fig. 3.1 Predicted 2D structure of OmpW of *A. hydrophila* displays four loops (L1 to L4) which are exposed to the outside of the membrane (Bagos et al. 2004)

3.4 Selection of Potential OMPs Through In Silico Biology

It is necessary to screen and select the proper molecule(s) as a vaccine candidate as it should be present in high volume. For the above reasons, these vaccines were proposed and developed using reverse vaccinology, a term initially used by Dr. R. Rappuoli (Rappuoli 2000). The bioinformatic tools allow us to directly access nucleotide and protein sequences, which are crucial in genomics and proteomics. Using these tools, one can study the sequences of OMPs to know its feasibility as a vaccine candidate. Indeed, various molecules have been screened using in silico analysis, and the selected molecules were targeted to develop recombinant proteins as subunit vaccines (Baliga et al. 2018). Over the years, several studies were conducted to identify potential OMPs-based vaccines against bacterial fish pathogens using in silico tools (Mahendran et al. 2016; Baliga et al. 2018; Ormsby et al. 2019; Maiti et al. 2020). Further, most of these studies have proposed the use of OMPs as potential vaccine candidates. Using genetic information from whole-

genome sequence (genome mining) followed by the application of recombinant DNA, technology has opened a new field of vaccine development, and modern vaccines are important examples of success in this new field. The whole genomic sequence can be screened for potential vaccine molecules (OMPs), predicting the immunogenicity and antigen profiling of those proteins. Various in silico tools can be used to study and map specific epitopes for the selection of suitable vaccine candidates. For instance, Signal P 3.0 server can be used to determine signal peptide in OMPs, while EMBOSS-GUI server can locate the antigenic sites present in the protein. Similarly, PRED-TMBB can be used for the prediction of the two-dimensional (2D) structure of the target protein.

3.5 OMPs as Potential Vaccine Candidates for Bacterial Pathogens of Fish

Traditional fish vaccines are whole-cell live-attenuated or inactivated/killed bacteria using chemicals or heat. Many of the commercialized vaccines are of this type. Although traditional vaccines are usually safe and effective, there are few disadvantages. Application of whole-cell vaccine, especially live vaccines, can result in immunocompromised situations in fish, making them susceptible to potentially fatal secondary infections. Another issue is the use of adjuvants and mineral oils in antigen formulation since these substances offer increased immunity by allowing a slow release of antigen and the resultant prolonged response by the cells. However, the adverse effects of adjuvants such as decreased growth rates, chronic peritonitis, adhesions, granulomas, and pigmentation in the peritoneal cavity in the host are also reported (Afonso et al. 2005; Oda et al. 2006). In recent years, modern vaccines like subunit vaccines and DNA vaccines are being evaluated for commercial use, and vaccination trial studies have shown promising results.

The OMPs can be used as subunit vaccines, which usually contain one or more pure or semi-pure antigens instead of the whole cell. One of the major advantages of subunit vaccines is that they induce specific immunity against a particular pathogen's virulence factor and also helps in maintaining the overall immune system. Extracted total crude OMPs from the cell as well as purified OMPs have been used to evaluate the immunogenicity and protection efficacy of fish against specific pathogens. In the past decade, specific recombinant OMPs were tested as subunit vaccines for various fish species. Moreover, unlike toxins and most of the enzymes, the OMPs do not induce toxic effects on the host. Evidence obtained through the work carried out by several groups reveals that total OMPs and/or any particular OMP is useful as potent immunogenic molecules and provides significant protection to fish when challenged with pathogenic bacteria.

3.5.1 Extracted Total OMPs as Vaccine Candidates

The antigen preparation and the methods of delivery influence the protective efficacy of any vaccine candidates. Selected immunogenic OMP can be formulated with oil (adjuvant) or phosphate-buffered saline. So far, trial studies have relied on injection as the preferred method of delivering OMPs (antigen) because injection induces high immunogenicity.

Many researchers have used crude OMPs from bacteria to evaluate the immunogenicity and protective efficacy in different fishes. In a study by Maji et al. (2006), a crude OMP extracted from *A. hydrophila* that contained two polypeptides of 23 kDa and 57 kDa, respectively, was shown to be highly immunogenic in pathogen-challenged (*A. hydrophila*) goldfish. Similarly, Viji et al. (2013) reported the efficacy of extracted total OMPs in protecting goldfish against *A. hydrophila* infection. Immunized rohu (*L. rohita*) showed good immunogenic responses and strong protection against edwardsiellosis when they were vaccinated with OMPs of *E. tarda* (Kumar et al. 2014). No vaccinated fish were claimed to have died, whereas all the fish died in the control group, indicating 100% protection even after 168 days post-challenge. The study also identified two specific OMP proteins, i.e., Omp85, a conserved OMP, and GroEL protein, using MALDI-TOF mass spectrometry. Brudal et al. (2015) used a zebrafish model for a vaccination study using isolated OMPs. Vaccinated zebrafish could survive when challenged with a high dose of *Francisella noatunensis* without causing any major adverse effects. In another study, crude OMPs were extracted from *Stenotrophomonas maltophilia* and channel catfish vaccination (*Ictalurus punctatus*). Vaccinated catfish could induce immune system and offered high RPS (RPS up to 73.33) in the challenge study (Wang et al. 2016).

3.5.2 Recombinant OMPs as Vaccine Candidates

Recombinant vaccine is prepared by utilizing recombinant techniques to produce high amounts of antigen (Fig. 3.2). The vaccine can be administrated to the host by injection, orally, or by immersion. The advantage of recombinant vaccine is that there is hardly any chance of adverse effects on the host. Moreover, each batch of vaccine has the same potency and is more stable during long-term storage. This technique also allows the use of processed bacterial antigens or cleaved epitopes, which stimulate T-suppressor cells. In conventional vaccines, the availability of large quantities of specific protein is a limitation. The extraction and purification of a specific protein from bacteria is quite cumbersome, and the yield is less. Therefore, genetic manipulation of a specific gene coding for a particular protein becomes very useful since a large protein requirement is made possible.

It has been observed that specific OMPs are better immunogens, and they improve immunogenicity and protection efficacy compared to total crude OMP extracts. Bader et al. (2004) extracted total OMP from *E. ictaluri* and studied its immunogenic responses in channel catfish. The authors reported a weaker immunogenic response when catfish (~16 g body weight) was vaccinated with a lower dose

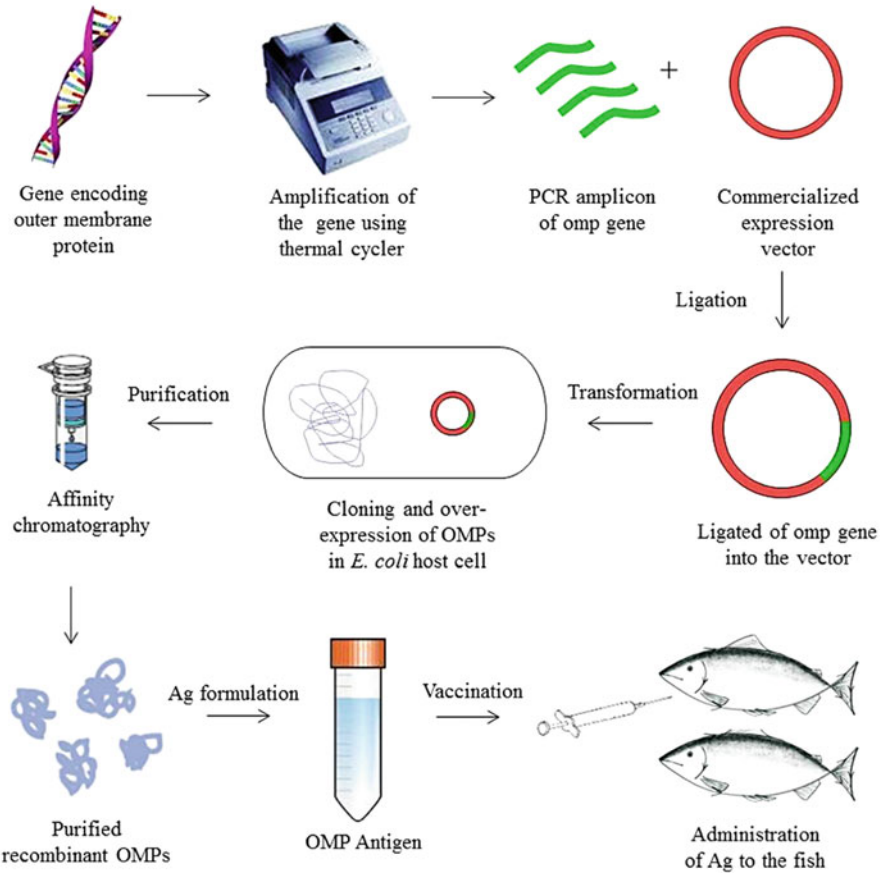

Fig. 3.2 Flow chat diagram of fish vaccination using recombinant OMPs

(3.13 μg or 6.25 μg) of OMP. However, with higher dose (12.5 μg), the protective efficacy was comparatively better (RPS = 67.5). The various OMPs that have been characterized as potential vaccine candidates for fish are shown in Table 3.1. For instance, a recombinant OmpTS of *A. hydrophila* was found to be immunologic and provided significant protective efficacy (RPS = 57) to the Indian major carp, rohu (Khushiramani et al. (2007). Similarly, a 20 kDa OMP protein (OmpW), which is highly conserved among the *Aeromonas* sp., was shown to be highly immunogenic against *A. hydrophila* (Maiti et al. 2009). The same group further evaluated the protective efficacy of this OMP (OmpW) in combination with a 43 kDa adhesin protein (Aha1) of *A. hydrophila* in common carp (*Cyprinus carpio*) and observed significant protection to carp against experimental *A. hydrophila* challenge with RPS of 52 and 71, respectively (Maiti et al. 2012). In another study, Khushiramani et al. (2012) showed that a recombinant Omp48 protein induced significant protective immune response in rohu against more than one fish pathogen, viz, *A. hydrophila*

Table 3.1 Recombinant OMP-based vaccine trials conducted against bacterial fish pathogens

Targeted OMPs	Used against (bacterial infection)	Fish used in trail experiment	References
Total OMPs	*A. hydrophila*	Goldfish	Viji et al. (2013)
Total OMPs	*E. tarda*	Rohu	Kumar et al. (2014)
Total OMPs	*A. hydrophila*	Goldfish	Maji et al. (2006)
Total OMPs	*A. hydrophila*	Rohu	Behera et al. (2010)
Total OMPs	*A. hydrophila*	Rohu	Rauta and Nayak (2015)
Total OMPs	*A. hydrophila*	Rohu	Behera and Swain (2013)
Total OMPs	*Francisella noatunensis*	Zebrafish	Brudal et al. (2015
Total OMPs	*Stenotrophomonas maltophilia*	Channel catfish	Wang et al. (2016)
OmpTS	*A. hydrophila*	Rohu	Khushiramani et al. (2007)
OmpK	*V. anguillarum*	Rohu	Hamod et al. (2012)
OmpR	*A. hydrophila*	Rohu	Dash et al. (2014)
Omp48	*A. hydrophila* and *E. tarda*	Rohu	Khushiramani et al. (2012)
OmpA	*E. tarda*	Common carp	Maiti et al. (2011)
OmpW	*A. hydrophila*	Rohu	Dubey et al. (2016a)
OmpA	*E. tarda*	Fringed-lipped peninsula carp	Dubey et al. (2016b)
Omp38	*V. anguillarum*	Asian sea bass	Kumar et al. (2008)
Omp38	*V. anguillarum*	Asian sea bass	Kumar et al. (2007)
OmpU	*Vibrio harveyi*	Orange-spotted grouper	Nguyen et al. (2018)
OmpA	*Edwardsiella anguillarum*	Japanese eels	LiHua et al. (2019)
OmpU	*Vibrio vulnificus*	Japanese eel	Le et al. (2018)
OmpN	*Edwardsiella ictaluri*	Channel catfish	Yang et al. (2016)
OmpII	*A. hydrophila* and *Edwardsiella anguillarum*	European eels	He et al. (2020)
FrpA	*Photobacterium damselae*	Sole	Valderrama et al. (2019)

(continued)

Table 3.1 (continued)

Targeted OMPs	Used against (bacterial infection)	Fish used in trail experiment	References
OmpA	*Vibrio ichthyoenteri*	Flounder	Tang et al. (2017)
TolC	*Vibrio harveyi*	Hybrid grouper	Zhu et al. (2019)
OmpF	*Aeromonas hydrophila*	Rohu	Yadav et al. (2018)
OmpC	*Vibrio anguillarum* and *Edwardsiella tarda*	Flounder	Xing et al. (2018)
OmpF and OmpK	*A. hydrophila*	European eel	Zhang et al. (2019)
Aha1 and OmpW	*A. hydrophila*	Common carp	Maiti et al. (2009, 2012)
OmpA, OmpC, OmpK, and OmpW	*A. salmonicida*	Rainbow trout	Diao et al. (2020)
OmpA1, Tdr, and TbpA	*A. hydrophila*	Channel catfish	Abdelhamed et al. (2017)
OmpV, OmpU, OmpT, Omp1, and Omp2	*Vibrio ichthyoenteri*	Flounder	Tang et al. (2019)
Multiple OMPs	*Flavobacterium columnare*	Grass carp	Luo et al. (2016)
Multiple OMPs	*Vibrio parahaemolyticus*	Zebrafish	Peng et al. (2016)

and *E. tarda* (RPS of 69 and 60, respectively), suggesting that the Omp48 could even be useful against multiple infections. The OmpA, one of the highly conserved proteins of *E. tarda*, is also present in other Enterobacteriaceae family members such as *E. coli*, *E. aerogenes*, *K. pneumoniae*, *V. alginolyticus*, *Proteus* sp., *Salmonella* sp., *Citrobacter* sp., etc. In a challenge study with pathogenic *E. tarda*, Maiti et al. (2011) reported a high immunogenic response (RPS = 54.3) in common carps (*C. carpio*) that were vaccinated with purified recombinant OmpA. Similarly, Hamod et al. (2012) observed that adult rohu vaccinated with adjuvant-coupled recombinant OmpK had significant survivability (RPS = 67.8) when challenged with a LD_{50} dose of *Vibrio anguillarum*. In yet another study, a recombinant form of OmpR, a member of the two-component regulatory system of *A. hydrophila*, was formulated with mineral oil, and modified adjuvants were used for the vaccination of rohu (*L. rohita*) juveniles (Dash et al. 2014). The study showed high immune responses and good protective efficacy upon challenge with a pathogenic dose of *A. hydrophila*. Interestingly, a significantly decreased level of mortalities was observed at 140 days after immunization compared to 56 days upon challenge. This suggested that the use of mineral oil or adjuvants allowed the slow release of antigens that ensured long-lasting protection against the pathogens. Luo et al. (2016) determined the immunogenicity and protective efficacy of various recombinant OMPs in grass carp and reported RPS up to 72 when challenged with

Flavobacterium columnare. In recent studies, the recombinant OMP-FrpA of *Photobacterium damselae* showed significant immune responses in sole and offered a protective effect (RPS of 73) against photobacteriosis (Valderrama et al. 2019). He et al. (2020) identified the OmpII of *A. hydrophila* and used it for the vaccination trial of European eels. The results showed that the recombinant OmpII protein could provide cross-protection against multiple infections, including *A. hydrophila* (RPS = 83.33), *E. anguillarum* (RPS = 55.56), and *V. vulnificus* (RPS = 33.33).

Multiple OMPs have also been compared and evaluated for their protective efficacy in fish against pathogens. Five recombinant OMPs were compared for vaccination study against *Vibrio ichthyoenteri* infection in flounder. The OmpT offered a significantly higher RPS of 76.9 than other OMPs (Tang et al. 2019). Diao et al. (2020) compared four recombinant OMPs, namely, OmpA, OmpC, OmpK, and OmpW, to determine the potential vaccine candidate for rainbow trout. The OmpC offered the highest RPS of 81.6, followed by OmpA (71.1), OmpK (55.3), and OmpW (42.1).

3.5.3 OMPs-Based DNA Vaccine

Another emerging strategy in vaccination is the use of a DNA vaccine where a vector (carrier) like plasmid coding for a protective antigen allows the host machinery to transcribe and translate the selected gene (Liu 2003). DNA vaccine can be stable and can protect the host against target pathogens for more than one generation. Additionally, these vaccines possess some distinct advantages over traditional whole-cell vaccines, such as the stimulation of B- and T-cell responses, lack of other virulence factors, and limited side effects. However, DNA vaccines also have limitations. The most vital one is the possibility of integrating plasmid DNA with the host genome, which can further transfer to other aquatic animals and humans (Rogan and Babiuk 2005). Other concerns include the cost of preparation and the method of administration.

A porin gene, encoding 38-kDa major OMP (*Omp38*), of *V. anguillarum* was targeted by Kumar et al. (2007) to construct a DNA vaccine. Juveniles of Asian sea bass (*Lates calcarifer*) were vaccinated with this DNA vaccine through intramuscular injection. Upon a challenge with LD_{50}, *V. anguillarum*, the vaccinated sea bass exhibited significant protection compared to the control group. Further, to make the vaccine delivery more practical and simpler, the same group used the oral route of DNA vaccine delivery through chitosan nanoparticles. The results showed moderate protection (RPS = 46) in vaccinated Asian sea bass (*L. calcarifer*) and when artificially infected with *V. anguillarum* (Kumar et al. 2008). In another study, Liu et al. (2017) constructed and evaluated a DNA vaccine using *ompC* of *E. tarda*. The constructed vaccine was able to induce an innate immune system and was found to be protective against *E. tarda* infection. DNA vaccine encoding *ompK* could offer good RPS (RPS 50) in flounder against *V. anguillarum* (Xu et al. 2019). The *ompK*-based DNA vaccine could further increase the immune response ($CD4\text{-}1^+$ and $PCD4\text{-}2^+$ T lymphocyte) in flounder (*Paralichthys olivaceus*) (Xing et al. 2020).

3.6 Biodegradable Nanoparticles in Fish Vaccine Delivery

To make the vaccination process successful, not only is it important that the vaccine candidate is effective, but the delivery of the vaccine needs to be easy and eco-friendly. In the majority of the studies conducted in fish by the various investigators, injection of OMP-based vaccine proved to be the most effective form of administration, providing the much-needed response and protection. However, in an aquaculture system, the most practical approach of vaccine delivery is the oral route. But there are several obstacles associated with successful oral vaccination in fish. For example, the antigen (Ag) is usually destroyed due to protease activity present in the intestinal tract, and the Ag may not enter the gut mucosa to initiate the immune system. Other problems associated with oral delivery include the rejection of vaccines by animals, degradation of the vaccine in environment, etc. Perhaps, due to these problems, only a handful (five) of oral vaccines is available commercially, both for humans and other animals (Embregts and Forlenza 2016). Therefore, scientists are constantly looking at various alternative methods of Ag delivery that are not only effective but also least toxic to fish. One way vaccine degradation can be avoided is by encapsulation with biodegradable polymers such as nanoliposomes, calcium phosphate, carbon nanotubes, poly lactic-co-glycolic acid (PLGA), chitosan, etc. Indeed, researchers have attempted this method, and a number of studies have been reported. To enlist a few, Behera et al. (2010) observed long-lasting immune repose (both innate and adaptive immune) in Indian major carp, rohu juveniles with PLGA microparticle-encapsulated OMPs of *A. hydrophila*. Further, the combination showed protection against *A. hydrophila* infection (Behera and Swain 2013). Rauta and Nayak (2015) also used biodegradable PLA/PLGA nanoparticles for the delivery of OMP vaccines to rohu and reported significantly higher percentage survival against *A. hydrophila* infection in the groups PLA/PLGA encapsulated groups compared to control groups. Dubey et al. (2016a, b) conducted vaccination trials through oral delivery of recombinant OMPs as vaccine candidates. Two recombinant OMPs, namely, OmpW and OmpA, were encapsulated in PLGA and chitosan nanoparticles, respectively, and orally vaccinated among fish. These studies reported superior protection (significant differences in PCSPs between the vaccinated and control groups) in vaccinated fish, i.e., OmpW-vaccinated Rohu (*L. rohita*), and OmpA-vaccinated fringed-lipped peninsula carp (*L. fimbriatus*) after the challenge with lethal dose of *A. hydrophila* and *E. tarda*, respectively. Zhu et al. (2020) used single-walled carbon nanotubes-based for targeted delivery of nanovaccines against fish viral disease. Two recent reviews have summarized the developments in nanoparticles-based vaccine delivery systems quite comprehensively (Vinay et al. 2018; Maiti et al. 2020).

3.7 Conclusion

Bacteria are naturally present in the aquatic environment of fish farms and, when stressed, cause infectious disease outbreaks that bring about colossal loss to the aquaculture industries. The indiscriminate use of antibiotics in aquaculture leads to the development of antibiotic resistance and the spread of the resistance to other bacteria in the environment, including the pathogens and other innocuous bacteria participating in the mineralization process in the environment. Further, the residues of some antibiotics with zero tolerance in aquatic animals are a consequence of their use, and this majorly results in rejection of export of fish and fisheries products. Therefore, it is pertinent to develop appropriate preventive measures for successful aquaculture. OMPs are highly immunogenic molecules. Hence, vaccination using immunogenic molecules like OMPs can be useful alternative to prevent diseases. Although vaccination is crucial in fish health management, maintaining proper environmental conditions (physicochemical parameters), hygiene and sanitation, biosecurity, and optimum nutrition of cultured farms is equally important to prevent the occurrence of infectious diseases. Over the years, several improvisations have been made in the process of vaccine development. Biotechnology has played a pivotal role in developing vaccines that are effective, safe, and easy to deliver. Vaccination of fish against potential pathogens using safe and protective immunogen along with other health management techniques including good animal husbandry practices could be the best way to control bacterial diseases in aquaculture.

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Application of Carbon Nanotubes in the Advancement of Fish Vaccine

4

Sib Sankar Giri and Se Chang Park

Abstract

Various ways are adopted to control diseases in aquaculture. The development of efficient drug delivery system is of fundamental importance to improve effects of drug. In this regard, the unique properties of nanoparticles attracted extensive research on the applications of nanoparticles in aquaculture. Recently, nanodelivery system has been developed to improve the administration and efficacy of vaccines. Nanoparticles can be easily turned up to have specific chemical and physical characteristics. Likewise, carbon nanotubes (CNTs) are new alternative and efficient tool for transporting and translocating therapeutic molecules. As the functionalized CNTs are not immunogenic and have low toxicity, highly biocompatible, they hold tremendous potential in nanomedicine and nanobiotechnology. CNT-based drug delivery is promising for higher efficacy with lower side effects in achieving the higher effectiveness of drugs. CNTs are being utilized delivery vehicles for vaccines to protect farmed fish against disease-causing pathogens. This book chapter sheds the light on CNTs as a potential novel tool as vaccine carrier against various bacterial and viral diseases in fish. The importance of CNTs to enhance sustainable aquaculture has also been highlighted in this chapter.

Keywords

Nanoparticles · Carbon nanotubes · Fish · Pathogens · Diseases resistance

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4.1 Introduction

World human population is expected to reach 9.6 billion by 2050, and feeding the growing population will be a critical challenge. Global food security can be achieved through increased food production, reduced food waste, and increased food nutritional quality. Globally, fish accounted for about 17% of animal protein consumed by the global population (FAO 2018). Between 1961 and 2016, the average annual increase in global food fish consumption (3.2%) was higher than population growth (1.6%) and that of meat from all terrestrial animals combined (2.8%) (FAO 2018). Global fish production peaked at about 171 million tons in 2016, with aquaculture representing 47% of the total (FAO 2018). The rapid growth in aquaculture system has been driven by a variety of factors, including pre-existing aquaculture practices, population and economic growth, and expanding export opportunities. In intensive aquaculture, diseases that occur among wild population manifests in confined farm environment. It is known that the stresses associated with farming operations favor the transmission of pathogens (Alderman 1988). Now, aquaculture sector is under uncertainty, and the progress is not commensurate with challenges facing the aquaculture industry. Diseases such as white spot syndrome virus (WSSV), yellow-head virus, and early mortality virus have been causing severe loss to aquaculture worldwide. Several recent reports have indicated that bacterial pathogens (54.9%), viruses (22.6%), parasitic agents (19.4%), and mycotic agents (3.1%) are responsible for the disease outbreaks in fish cultures (Dhar et al. 2014). To prevent and treat infections in aquaculture, prophylactic and therapeutic use of antimicrobials and chemical disinfectants is a common practice. However, they provide temporary solution while adversely affecting the environment and nontarget organisms, resulting in uncertainty over the sustainability of aquaculture. The overuse of antimicrobials in aquaculture has led to the development of resistant bacteria. Stimulated by the overuse of antimicrobials, bacterial mutation, recombination, and horizontal gene transfer have resulted in the emergence of new antimicrobial-resistant bacteria and eventually disseminate antimicrobial resistance genes into animal and human populations. Therefore, there is an increasing demand for new technology to provide safe and healthier aquatic products, free of chemical residues. Among various alternatives, effective vaccines are the major tools to resolve disease outbreaks in aquaculture. Accordingly, a number of successful vaccines have been developed for aquaculture.

The first scientific report on fish vaccination was the use of inactivated orally administered *Aeromonas salmonicida* vaccine. Compared with human vaccine history, fish vaccine development has a very short history starting in the 1970s with the first licensed fish vaccine made commercially available in 1976 (Evelyn 1997). The range of bacterial infections for which vaccines are commercially available now comprises classical vibriosis (*Vibrio* (*Listonella*) *anguillarum*), cold-water vibriosis (*Aliivibrio* (*Vibrio*) *salmonicida*), *Vibrio ordalii*, furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*), yersiniosis (*Yersinia ruckeri*), pasteurellosis (*Photobacterium damselae* subsp. *piscicida*), edwardsiellosis (*Edwardsiella ictaluri*), winter ulcer (*Moritella viscosa*), and streptococcosis/lactococcosis

(*Streptococcus iniae/Lactococcus garvieae*) (Ringø et al. 2014). To limit the impact of the infectious diseases in aquaculture, a continuous effort to improve vaccine strategies for fish is required. One of the vaccine strategies that have been tested recently is DNA vaccination. DNA vaccination has been experimentally tested in various fish species, mainly against viral pathogens (Gomez-Casado et al. 2011). DNA vaccines that direct neutralizing antibodies towards G-protein, an essential component for viral cell attachment, have shown good protective effects against rhabdovirus, infectious hematopoietic necrosis virus (IHNV), and viral hemorrhagic septicemia virus (VHSV) in rainbow trout. However, DNA vaccines that are based on other rhabdoviral proteins do not provide the same levels of protection (Corbeil et al. 1999). The immunogenicity of vaccines can be enhanced by using adjuvants and efficient delivery system (Vinay et al. 2018). Traditional adjuvants such as mineral oil have been routinely used for vaccine delivery; among the most common are Freund's complete/incomplete adjuvant and more recently Montanide (Ji et al. 2015). However, vaccines containing mineral oil as adjuvant cause serious side effects, such as granulomas, adhesion, pigmentation, poor feeding, and growth retardation (Ringø et al. 2014). Further, vaccination methods (oral, immersion, and injection) have certain disadvantages. For example, immersion vaccination requires a large quantity of vaccine, and it is difficult to measure the dose of antigen received. Furthermore, in-feed delivery of drugs and vaccines, especially peptides, DNA and RNA, are degraded by the gastric digestion or are denatured before they can be absorbed in the intestine (Florence et al. 1995). Hence, it is necessary to develop novel adjuvants and delivery systems that are safe and potent for aquaculture species.

With nanotechnology, it is possible to synthesize nanometer-sized particles that can be utilized for a broad spectrum of applications in aquaculture (Fig. 4.1). Nano-encapsulated or nano-coated drug delivery systems allow the drugs, vaccines, adjuvants, and enzymes, to be protected in the stomach, passed on to the intestine and retained there for a longer time for better absorption and assimilation (Aklakur et al. 2016). Using nanotechnology, several vaccines nano-carriers with a variety of compositions, sizes, and surface properties have been developed (Peek et al. 2008). Several of these have been designed and investigated for their utility in the delivery of antigens to immune cells to promote a protective immune response. Nanocarriers facilitate targeted and/or sustained release of antigens or adjuvants to antigen-presenting cells (Gregory et al. 2013). Particles smaller than 10μm are readily taken up by phagocytic cells, such as macrophages and dendritic cells (DC). This property has been used to improve the cellular uptake of antigens, thereby increasing the efficiency of antigen recognition and presentation (Oyewumi et al. 2010). Solid nanocarriers protect protein-based antigen vaccines from degradation and facilitate entry into the gut-associated lymphoid tissue and mucosa-associated lymphoid tissues, which makes them suitable for vaccine delivery via oral or mucosal routes (Kim et al. 2014).

Nanomaterials and nano-formulations based on natural polymers and matrices are promising tools in the efforts to address the challenges of the fishing industry and aquaculture. Carbon nanotubes (CNTs) are allotropes of carbon, made of graphite

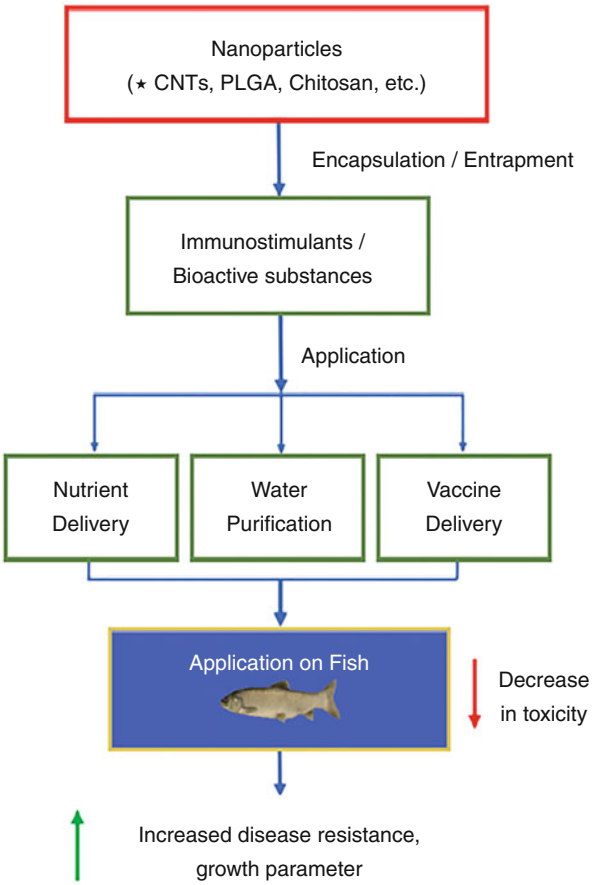

Fig. 4.1 Application of nanotechnology in fish aquaculture. (After modification from Shah and Mraz 2020)

and constructed in cylindrical tubes with nanometer in diameter and several millimeters in length. CNTs have been described at the atomic level for the first time in 1991 by Iijima (Battigelli et al. 2014). They can be classified as single-walled carbon nanotubes (SWCNTs), and multiwalled carbon nanotubes (MWCNTs), depending on the number of graphene shells coaxially arranged to form the tubes. Their particular physiochemical properties, especially their ability cross biological barriers promoted their study in nanomedicine (Battigelli et al. 2014). Moreover, CNTs have been successfully applied in pharmacy and medicine due to their high surface area that is capable of adsorbing or conjugating with a wide variety of therapeutic and diagnostic agents (drugs, genes, vaccines, antibodies, biosensors, etc.). In this review, we provide an overview of recent advances in the area of nanovaccinology, but we limit our review only to carbon nanoparticles. Carbon nanotubes (CNTs), which are relatively inert, nontoxic, and non-immunogenic by them, are applied as scaffolds for vaccine development owing to their unique properties. Single-walled CNTs (SWCNTs) are mainly exploited as potential

vaccine delivery vehicles because of their desirable chemical and physical characteristics (Scheinberg et al. 2013). Recent advances in carbon-based nanodelivery systems have been discussed in detail. The interaction of nanoparticles with the biosystem is also discussed to provide an overview of nanoparticle processing, as well as its clearance from the body. We have concluded with remarks about the future prospects of carbon nanovaccinology in aquaculture.

4.2 Method of Vaccine Administration

Mainly three routes of vaccination are used in aquaculture: orally, immersion, injection via intramuscular or intraperitoneal route.

The oral vaccination offers the most attractive approach of immunization of fish for a number of reasons: ease of administration of antigens, less stressful, and it is applicable to small- and large-sized fish (Giri et al. 2021). Vaccines are produced either by top coating the feed with antigen or mixing antigen into the feed. In oral vaccination, it's difficult to determine the exact amount of vaccine consumed by each fish. Also, depending on whether fish are gastric or agastric, the intact antigen has to pass through the digestive system to reach the second segment of hindgut where antigens are absorbed. However, several researchers have continued to develop novel methods of protecting efficacy of antigen during the delivery beside its protective nature. The use of nanodelivery system has been explored as an alternative strategy to address said problems and to enhance the vaccine efficacy.

Vaccination by injection route delivers a minute and known amount of antigen directly to the fish. Vaccines delivered by this way likely to be most effective and provide protection for long duration (Mitchell 1995). Injection vaccines are often multivalent, containing either combination of bacterins or different bacterins and killed virus or viral proteins. Injection vaccination is important for salmon and trout during the production cycle to harvest (Plant and LaPatra 2011). However, this process has several limitations like handling, injecting, labor intensive and costly, and not practical for small fish (weight under 20 g). Nakanishi et al. (2002) reported a new vaccine administration method (i.e., multiple puncture/immersion) for juvenile rainbow trout against beta-hemolytic *Streptococcus*, and they found that the method was equal in effectiveness to intraperitoneal injection. However, further studies on the longevity of protection, applicability with other vaccines, etc. have not been reported further.

Immunization by immersion method can be regarded as the simplest method of vaccination. It can be achieved by one of the following ways: direct immersion (DI), hypersonic infiltration (HI), and spray. However, compared to DI, HI improved the immune responses of *Cyprinus carpio* to *Aeromonas salmonicida* bacterin (Huising et al. 2003). Bath immunization does not create handling stress, but it requires large quantity of vaccine (Nakanishi and Ototake 1997). Commonly, fish are immersion vaccinated with a concentrated solution for short period of time. Ultrasound, a method use for vaccine delivery, generates a sound wave of approximately 20 kHz that enhances the permeability of cells (Plant and LaPatra 2011).

4.3 The Use of Carbon Nanotube Against Viral Diseases in Aquaculture

CNTs are stable and capable of carrying multiple antigens. The application of CNTs in fish vaccine delivery system is quiet recent, and they have been used against both viral and bacterial diseases in aquaculture (Table 4.1).

Hemorrhagic disease caused by grass carp reovirus (GCRV) results in tremendous loss of grass carp (*Ctenopharyngodon idella*) industry. Zhu et al. (2014) used functionalized SWCNTs as carrier to manufacture SWCNTs-VP7 subunit vaccine with chemical modification. Major capsid protein (MCP) of GCRV was used developing VP7 subunit vaccine. They immunized grass carps by VP7/SWCNTs-VP7 subunit vaccine against GCRV by intramuscular injection (i.m.) and bath immunization. Immunization with SWCNTs-VP7 resulted in better immune responses than the VP7 subunit vaccine alone. The use of SWCNTs as carrier reduced the immunization doses/concentrations (about five to eight times). A relative percentage survival greater than 95% was observed in smaller-size fish (0.2 g) with SWCNTs-VP7 bath immunization. Immune responses like serum respiratory burst activity (RBA), complement activity, lysozyme activity, superoxide dismutase (SOD) activity, alkaline phosphatase (ALP) activity, immune-related genes, and antibody levels were significantly enhanced in fish immunized with vaccine (Zhu et al. 2014). In another study, those authors vaccinated (i.m.) grass carp with VP7 of grass carp reovirus as (a) naked pcDNA-vp7, (b) SWCNTs-pcDNA-vp7, (c) empty plasmid vector, or phosphate-buffered saline. The ability of the different immune treatments to induce transgene expression was analyzed at 28 days post-vaccination. They detected higher levels of transcription and expression of the VP7 gene in muscle tissues of grass carp in SWCNTs-pcDNA-VP7 treatment groups. Further, the serum RBA, SOD activity, complement, lysozyme level, immune-related genes, antibody levels, and RPS were significantly enhanced in fish immunized with SWCNTs-pcDNA-vp7 vaccine (Zhu et al. 2015). Therefore, SWCNTs could be a promising carrier for plasmid DNA vaccine.

The DNA vaccine has made it challenging to avoid the problem of easily metabolized and degraded in vivo. To reduce the lethal hemorrhagic disease caused by GCRV, a novel SWCNTs-pEGFP-VP5 DNA vaccine and ammonium-functionalized SWCNTs by a chemical modification method were prepared (Wang et al. 2015). After i.m injection (1, 2.5, and 5μg) and bath administration (1, 10, and 20 mg/L), the ability of the different immune treatments to induce transgene expression was analyzed at 28 days postimmunization. Higher levels of transcription and expression of VP5 gene were detected in muscle tissues of grass carp in SWCNTs-pEGFP-vp5 (Wang et al. 2015). The antibody levels, immune-related genes, and RPS were significantly enhanced in fish immunized with SWCNTs-pEGFP-VP5 vaccine. A RPS of nearly 100 was observed in bath immunization group at dose 20 mg/L and in injection group at a dose of 5μg. Those results indicated that ammonium-functionalized SWCNTs could provide extensive application prospect to aquatic vaccine.

Table 4.1 Application of SWCNTs as vaccine carrier in fish

Name of vaccine	Fish species	Pathogen	Route of vaccination	RPS/ survival percent	Reference
SWCNTs-aerA	Grass carp	*Aeromonas hydrophila*	Bath immersion	80.6, 84.9	Gong et al. (2015)
SWCNTs-aerA	Grass carp	*A. hydrophila*	i.m.	79.6	Gong et al. (2015)
SWCNTs-pEGFP-aerA	Grass carp	*A. hydrophila*	i.m.	83.7	Liu et al. (2016)
SWCNTs-A0KIY3	Zebrafish	*A. hydrophila*	Bath immersion	86.11	Guo et al. (2018)
SWCNTs-A0KIY3	Zebrafish	*A. hydrophila*	i.p.	94.44	Guo et al. (2018)
SWCNTs-BL	Grass carp	*A. hydrophila*	i.p.	85	Zhang et al. (2020b)
SWCNTs-VP7	Grass carp	GCRV	i.m.	95	Zhu et al. (2014)
SWCNTs-pcDNA-vp7	Grass carp	GCRV	i.m.	100	Zhu et al. (2015)
SWCNTs-pEGFP-vp5	Grass carp	GCRV	i.m.	100	Wang et al. (2015)
SWCNTs-G	*Micropterus salmoides*	MSRV	Immersion	70.1	Guo et al. (2020)
SWCNTs-pcDNA-ORF149 koi		KHV	i.m.	81.9	Hu et al. (2020)
SWCNTs-pcDNA-ORF149	*Cyprinus carpio*	CyHV-3	Immersion	56	Hu et al. (2021)
SWCNTs-MCP	*Micropterus salmoides*	LBUSV	Immersion	80.1	Jia et al. (2020a)
SWCNTs-pcDNA-MCP	*Micropterus salmoides*	LBUSV	Immersion	61.11	Jia et al. (2020b)
SWCNTs-MCP	Grouper	TGIV	Immersion	89.47	Liu et al. (2020)
SWCNTs-MCP	Grouper	NNV	Immersion	70.76	Liu et al. (2021)
SWCNTs-M-VP4-3	Grass carp	GCRV	Immersion	88.33	Qiu et al. (2021)
SWCNTs-VP4	Grass carp	GCRV	Bath treatment	58.3	Qiu et al. (2020)
SWCNTs-VP4-3	Grass carp	GCRV	Bath treatment	76.7	Qiu et al. (2020)
SWCNTs-M-VP7	Grass carp	GCRV	Immersion	96	Zhu et al. (2020)
SWCNTs-MG	*C. carpio*	SVC	Bath treatment	63.5	Zhang et al. (2020a)

(continued)

Table 4.1 (continued)

Name of vaccine	Fish species	Pathogen	Route of vaccination	RPS/ survival percent	Reference
SWCNTs-pcDNA-M	*C. carpio*	SVCV	i.m.	51.3	Zhang et al. (2018)
SWCNTs-pEGFP-G	*C. carpio*	SVCV	i.m.	57.5	Zhang et al. (2017)
SWCNTs-pEGFP-M	*C. carpio*	SVCV	Immersion	46.3	Zhang et al. (2019)
SWCNTs-pcDNA-MCP	*Siniperca chuatsi*	ISKNV	Immersion	82.4	Zhao et al. (2020a)
SWCNTs-M-MCP	*Siniperca chuatsi*	ISKNV	Immersion	86.7	Zhao et al. (2020b)
SWCNTs-Mor	*Siniperca chuatsi*	ISKNV	Bath treatment	77.33	Zhao et al. (2021)
SWCNTs-pcDNA-vp4-3	Grass carp	GCRV	i.m.	74	Zheng et al. (2021)

GCRV grass carp reovirus, *ISKNV* infectious spleen and kidney necrosis virus, *KHV* koi herpesvirus, *SVC* spring viremia of carp, *SVCV* spring viremia of carp virus, *SWCNTs* single-walled carbon nanotubes, *NNV* nervous necrosis virus, *TGIV* iridovirus of Taiwan, *LBUSV* largemouth bass ulcerative syndrome virus, *CyHV3* cyprinid herpesvirus 3, *MSRV Micropterus salmoides* rhabdovirus, *MCP* major capsid protein, *i.p.* intraperitoneal

The protective effect on grass carp against GCRV-II infection was assessed using CNT as carrier (Qiu et al. 2020). The VP4 gene of GCRV-II was divided into four segments and, respectively, expressed in *Escherichia coli*. The VP4 and VP4-3 epitope were conjugated with SWCNTs to vaccinate grass carp. Immunization with SWCNTs-VP4 provided better immune responses in grass carp than naked VP4 subunit vaccine. Besides, the immune-related parameters were higher in SWCNTs-VP4-3 treatment groups than SWCNTs-VP4 groups. The fish survival rate in SWCNTs-VP4-3 was 76.7% during 10 days postinfection. These results indicated that SWCNTs-VP4-3 can be a plausible candidate for preventing and controlling GCRV-II among grass carp. In another study, those authors optimized the immunization program of SWCNTs-vaccine (SWCNTs-M-VP4-3) against GCRV to determining the best immunization program for grass carp (Qiu et al. 2021). They optimized different parameters affecting the vaccination such as immunization time, antigen concentration, and fish density during immersion vaccination of grass carp. The highest relative percent survival (88.33%) was observed 12 h of immunization time, 10 mg/L of antigen concentration, and 15 fish per liter of fish density (Qiu et al. 2021). Those results indicated that the administration protocol which induced highest immune response of the host had the highest vaccine effectiveness against the disease. Further, Zheng et al. (2021) constructed the DNA

vaccine using SWCNTs as delivery vector (SWCNTs-pcDNA-vp4-3) and immunized (i.m.) grass carp with various doses against GCRV-II. The highest serum antibody level was recorded in SWCNTs-pcDNA-vp4-3 immune group. The RPS in SWCNTs-pcDNA-vp4-3 immune group (5μg per fish) was 74%.

Spring viremia of carp (SVC) is viral disease that is responsible for significant morality in several carp species. *Rhabdovirus carpio*, a bullet-shaped RNA virus, is the causative agent of SVC. SVC infection has been reported in various carp species including *Cyprinus carpio*, *Carassius auratus*, *Ctenopharyngodon idella*, *Aristichthys nobilis*, *Hypophthalmichthys molitrix*, *Tinca tinca*, *Silurus glanis*, and *Oncorhynchus mykiss*. Zhang et al. (2017) used SWCNTs as a candidate DNA vaccine carrier in *C. carpio* juveniles and immunized via bath (1, 5, 10, 20, 40 mg/L) or injection (1, 4, 8, 12, 20μg). Art 22 days post-vaccination, higher levels of transcription, and expression of G gene were detected in muscle, spleen, and kidney tissues in SWCNTs-pEGFP-G treatment groups. The complement activity, SOD activity, ALP activity, and antibody levels were significantly enhanced in nano-vaccine-immunized group. The RPS reached to 57.5% in SWCNTs-pEGFP-G group than that of naked pEGFP-G (40.0%) at the highest vaccine dose (40 mg/L). However, fish in injection group could reach the similar RPS at a dose of 12μg (Zhang et al. 2017). Their study suggested that ammonium-functionalized SWCNTs are the promising carrier for DNA vaccine in large-scale vaccination of fish by bath administration approach. In another study, a SWCNTs-DNA vaccine encoding matrix protein of SVCV (SWCNTs-pcDNA-M) was injected i.m. at a dose of 10μg conferred up to 51.3% protection to fish against i.p. challenge with SVCV (Zhang et al. 2018). Further, SWCNTs as a promising vehicle enhanced about 17.5% of the immune protective effect in SWCNTs-pcDNA-M vaccinated common carp compared with fish injected with naked pcDNA-M DNA vaccine (Zhang et al. 2018). Moreover, vaccination with SWCNTs-pcDNA-M increased serum antibody production, none specific immunity parameters and expression of immune-related genes in fish (Zhang et al. 2018). Further, immersion vaccination with SWCNTs-pEGFP-M expressed the antigen proteins in fish kidney and spleen against SVC (Zhang et al. 2019). The stronger and longer duration immune responses were observed in fish vaccinated with SWCNTs-pEGFP-M in comparison with those vaccinated with pEGFP-M alone, and the use of SWCNTs increased the immune protective effect of naked DNA vaccine by 23.8% (Zhang et al. 2019).

Koi herpesvirus (KHV), otherwise known as cyprinid herpesvirus 3 (CyHV-3), is listed as a notifiable disease to the International Office of Epizootics. KHV has spread too many parts of the world due to global fish trading (Boutier et al. 2019). KHV ORF149 gene has been proved encoding one of the main immunogenic proteins for KHV (Hu et al. 2020). Those researchers coupled a plasmid expression vector for ORF149 to SWCNTs for an anti-KHV vaccine. The SWCNT vaccine conferred an 81.9% protection against intraperitoneal challenge with KHV, and SWCNTs as delivery vehicle enhanced 33.9% protective effects than that of naked DNA vaccine. The serum antibody production, enzyme activities, and immune-related gene expression were higher SWCNT-DNA vaccine group (Hu et al. 2020). Those researchers further developed an immersion DNA vaccine system

based on SWCNTs. To evaluate its efficacy against KHV, juvenile koi fish was vaccinated via immersion with SWCNTs-p149 (Hu et al. 2021). A stronger and prolonged immune response (serum antibody production, enzyme activities, and immune-related genes expression) was detected in fish vaccinated with high concentration SWCNTs-p149. Those studies demonstrate that SWCNTs as a promising carrier for DNA vaccine against KHV might be used to vaccinate large-scale juvenile koi fish by bath administration as well as intramuscular vaccination approach.

Rhabdovirus is highly virulent pathogens of aquatic organisms with a wide range of hosts. There are several successful DNA vaccines containing the viral G protein have been reported against fish rhabdovirus. A kind of live vaccine for *Micropterus salmoides* rhabdovirus (MSRV) exhibited 100% RPS via i.p. vaccination (Lijuan et al. 2018). Guo et al. (2020) reported a kind of immersion SWCNTs-loaded subunit vaccine which composited by glycoprotein (G) of MSRV and evaluated its protective effect on largemouth bass *M. salmoides*. A stronger immune response including serum antibody levels, enzyme activities, complement C3 content, and immune-related genes (*IgM*, *TGF-β*, *IL-1β*, *IL-8*, *TNF-α*, *CD4*) expression were induced obviously with SWCNTs-G vaccination. After bath immunization with SWCNTs-G (40 mg/L) for 28 days largemouth bass (*Micropterus salmoides*) exhibited RPS of 70.1% against MSRV challenge (Guo et al. 2020). In another study, those authors group applied SWCNTs-based vaccine against largemouth bass ulcerative syndrome virus (LBUSV) (Jia et al. 2020a). For this, SWCNTs containing MCP of LBUSV (SWCNTs-MCP) were evaluated for their protective effect on largemouth bass by immersion immunization. An elevation in serum antibody levels, enzyme activities, complement C3 content, and immune-related genes (IgM, TGF-β, IL-1β, IL-8, TNF-α, and CD4) expression was recorded in the SWCNTs-MCP-immunized groups compared with the pure MCP group (Jia et al. 2020a). The use of 40 mg/L SWCNTs-MCP resulted in highest RPS 80.56% in fish. Therefore, SWCNTs-based subunit vaccine can be used as a new immunization method against various viral diseases in fish. In a recent study, those researchers demonstrated that SWCNTs-DNA vaccine could be effective against the LBUSV infection (Jia et al. 2020b). They demonstrated strong increase in serum antibody levels, enzyme activities, and immune-related genes (IL-6, IL-8, IFN-γ, IgM, and TNF-α) expression in the SWCNTs-pcDNA-MCP-immunized groups. The RPS for SWCNTs-pcDNA-MCP group (40 mg/L) was 61.11%. Thus, SWCNTs-DNA vaccine may be new tool against LBUSV.

Iridovirus of Taiwan (TGIV) is threatening the grouper culture for more than two decades. Liu et al. (2020) constructed a prokaryotic expression vector of TGIV's MCP to acquire the vaccine, and SWCNTs were used as vaccine carrier. Juvenile pearl gentian grouper was bath vaccinated with various concentrations (5, 10, 20 mg/L) for 6 h and challenged with TGIV after 28 days. SWCNT-MCP vaccine induced a higher level of antibody in fish. The activities of related enzymes (acid phosphatase, ALP, SOD) and the expression of immune-related genes (Mx1, IgM, TNFα, lysozyme, CC chemokine 1, IL1-β, IL-8) increased significantly in SWCNTs vaccinated group (Liu et al. 2020). Interestingly, immunization with SWCNTs vaccine (20 mg/

L) exhibited RPS of 89.47%. In another study, those authors produced recombinant MCP of nervous necrosis virus (NNV) to develop a SWCNT-based vaccine against NNV (Liu et al. 2021). Bath immunization with SWCNT-MCP vaccine significantly increased the immune responses in fish. Data of challenge test revealed that the vaccines carried by SWCNTs (20 mg/L) provided RPS of 70.76%, while 33.33% was provided by the vaccine without SWCNTs. Those researches showed the immersion subunit vaccine loaded by SWCNTs can protect pearl gentian grouper from NNV as well as TGIV.

Infectious spleen and kidney necrosis virus (ISKNV) belongs to the genus *Megalocytivirus* from the family Iridoviridae. *Megalocytiviruses* threaten the aquaculture industry and causing severe economic losses in China, Japan, and Southeast Asia (Guo et al. 2012). Zhao et al. (2020a) bath immunized juvenile mandarin fish with SWCNTs-pcDNA-MCP or pcDNA-MCP. The higher ($p < 0.05$) immune response (immune-related genes expression, serum antibody production, enzyme activities, and C3 content) was recorded in SWCNTs-pcDNA-MCP group. After 14-day challenge, the RPS in SWCNTs-pcDNA-MCP-immunized group was 82.4%, but RPS in naked pcDNA-MCP-immunized group was 54.2%. In another study, those researchers optimized the efficacy of SWCNTs-based immersion subunit vaccine (SWCNTs-M-MCP) against ISKNV in mandarin fish (Zhao et al. 2020b). They vaccinated mandarin fish via bath immersion and then designed an orthogonal experiment to optimize different parameters affecting vaccination such as immune duration of bath immunization, immune dose, and fish density when immunized. The highest RPS (86.7%) was recorded in the group with 8 h of immune duration, 20 mg/L of immune dose, and 8 fish per liter of fish density (Zhao et al. 2020b). Importantly, these results provide a helpful reference for the effective use of vaccine to control ISKNV in fish farming.

Due to the presence of several impenetrable barriers, there is limited success in the therapeutic use of antiviral drug in several fish species. Recently, Zhao et al. (2021) selected moroxydine hydrochloride (Mor) for the treatment of ISKNV using SWCNTs as drug carrier. They challenged mandarin using live ISKNV via bath immersion route for 6 h, and then fish were bath treated with various doses of Mor-SWCNTs. The use of SWCNTs significantly reduced the Mor dosage to kill ISKNV. After 4 h treatment, the Mor concentration was 103.48μg/g in 40 mg/L Mor group, and 182.35μg/g in 40 mg/L Mor-SWCNTs groups. After 11 days, cumulative mortality (11.51%) and infection rate (3.81%) in 40 mg/L Mor-SWCNTs group were significantly reduced. Further, enzyme activities, complement C3 content, and expression of immune-related genes were significantly higher Mor-SWCNTs, and aforementioned activities reached the highest level at 3 days posttreatment (Zhao et al. 2021). Those results revealed that drug delivery with functionalized SWCNTs as drug carrier has potential application value to control fish viral diseases in aquaculture.

Zhang et al. (2020b) constructed a targeted SWCNTs-mannosylated antigen-based vaccine deliver system (SWCNTs-MG) that can recognize the signature receptor (mannose) of antigen-presenting cells (APCs), and feasibility of SWCNTs-MG was studied against SVC in *Cyprinus carpio* model for rhabdovirus

prevention. Within 6 h of immersion vaccination, SWCNTs-MG was detected in internal immune-related tissues (gill, muscle, and intestine). Moreover, mannose modification could facilitate the binding and cellular uptake of nanovaccine by APCs and further enhance host-protective immune responses against SVC infection (Zhang et al. 2020a). Same research group constructed a SWCNT-M-VP7 vaccine to target APCs (Zhu et al. 2020). The targeting ability of CNTs-M-VP7 was confirmed in vitro and in vivo. The immersion CNTs-M-VP7 vaccine crossed into the grass carp body through mucosal tissues and then to immune-related tissues. CNTs-M-VP7 significantly induced the maturation and presenting process of APCs, which then triggered robust immune responses (Zhu et al. 2020). Those studies could be a research template for the application of targeted nanovaccine system in aquatic animals.

4.4 The Use of CNTs Against Bacterial Diseases in Aquaculture

Aeromonas hydrophila is the most common etiological agent of motile Aeromonas septicemia (MAS). Epidemic of disease caused by *A. hydrophila* outbreaks is responsible for huge economic loss in aquaculture industry. Gong et al. (2015) applied functionalized SWCNTs applied as a delivery vehicle for recombinant *A. hydrophila* vaccine administration via bath or injection in juvenile grass carp. The SWCNT-aerA vaccine augmented the production of specific antibodies, stimulated the induction of immune-related genes, and induced higher level of survival rate. Further, a similar RPS (approximately 80%) was found in both a 40 mg/L bath immunization group and a 20μg injection group (Gong et al. 2015). Further, SWCNTs as a delivery vehicle for DNA vaccine were studied in juvenile grass carp (Liu et al. 2016). SWCNTs-DNA vaccine induced a better protective immunity in fish against *A. hydrophila*.

In iron homeostatis, iron-related proteins play important roles. Iron-related proteins may be potential vaccine candidates against *A. hydrophila* infection in fish. For that, Guo et al. (2018) overexpressed four iron-related recombinant proteins (P55870, A0KGK5, A0KPP0, and A0KIY3) from *A. hydrophila* and encapsulated with SWCNTs. Zebrafish were immunized with SWCNTs-encapsulated proteins via both intraperitoneal injection and bath immunization. Encapsulation with SWCNTs evoked an immune response in zebrafish. The immunization with SWCNTs-protein provided higher RPS (94.44%) against *A. hydrophila* i.p. challenge. Further, bath immunization with 40 mg/L SWCNTs-encapsulated protein resulted into RPS of 86.11% against pathogen challenge (Guo et al. 2018). Those results indicated that zebrafish vaccinated with SWCNT-P55870 and SWCNT-A0KGK5 could be promising vaccine candidates against pathogenic *A. hydrophila* infection.

In another study, Zhang et al. (2020b) sonicated *A. hydrophila* using ultrasonic cell crusher to obtain bacterial lysate (BL). A chemical modification method was used to link BL and functionalized SWCNTs to prepare SWCNTs-BL vaccine. Grass carps were vaccinated with BL or SWCNTs-BL via immersion (5, 10 mg/L) or injection (5, 10μg/fish). After 28 days, fish were challenged with live

A. hydrophila. Vaccination with SWCNTs-BL increased the serum antibody titer, enzymatic activity, and expression of some immune-related genes (especially IgM and TNF-α). The immunizing dose had great influence on RPS, and the RPS in injection group was higher than that of immersion group. Moreover, the immunoprotective effects of SWCNTs-BL were better than BL. Those results indicate that SWCNTs could be used as vaccine carrier to improve the effectiveness of vaccine.

4.5 Biocompatibility and Toxicity of CNTs

Nanomaterials designed for biological uses must be assessed for cytotoxicity and biocompatibility. Cytotoxicity is their effects on cell functions and viability. Biocompatibility involves comprehensive in vitro and in vivo evaluation of nanomaterials (Kyriakides et al. 2021). There is very limited data on the toxicity of SWCNTs to aquatic animals. In a semi-static system, rainbow trout was exposed to various concentrations of SWCNTs for up to 10 days (Smith et al. 2007). SWCNT exposure caused a dose-dependent rise in ventilation rate, gill pathologies (edema, altered mucocytes, hyperplasia), and mucus secretion with SWCNT precipitation on the gill mucus. No major hematological or blood disturbances were observed in terms of red and white blood cell counts, hematocrits, whole blood hemoglobin, and plasma $Na^{(+)}$ or $K^{(+)}$ (Smith et al. 2007). However, dietary supplementation of SWCNTs (500 mg/kg) for 6 weeks did not cause obvious toxicity in juvenile rainbow trout (Fraser et al. 2011). Further, the acute toxicity test using *Oryzias latipes* and *Daphnia magna* did not show any mortality/immobilizing effects up to the concentration of 100 mg/L SWCNTs, indicating its nonhazard category in the Globally Harmonised System (GHS) classification (Sohn et al. 2015). In a study, rainbow trout was intravenously injected with unfunctionalized SWCNTs, and after 96 h, tissues samples were collected for histological and biochemical studies (Boyle et al. 2018). Histological examination indicated the presence of carbon nanomaterials in kidney. There were no significant changes in hematology, or ionoloregulatory disturbance in blood plasma. Moreover, higher lipid peroxidation was detected only in kidney and spleen of fish injected with SWCNTs (Boyle et al. 2018). Those studies revealed the minimal environmental relevant toxicity of CNTs in aquatic species.

Functionalized CNTs have been reported to activate immune-related pathways in monocytes suggesting that such carbon-based nanomaterials may function as immunostimulatory agents (Pescatori et al. 2013). Recently, several researches reported the intracellular and extracellular degradation of biodegradation of CNTs (Bhattacharya et al. 2016). Activated neutrophils and eosinophils are found to release granule contents (e.g., myeloperoxidase (MPO), eosinophil peroxidase (EPO)) which enables extracellular destruction of microbes, and this pathway may be associated with the enzymatic degradation of SWCNTs (Andón et al. 2013).

4.6 Conclusion

Today, there are several materials that can be used to produce antigen delivery system for fish immunization, providing many alternatives to cope with the development of vaccines against the relevant pathogens and ensure a good health of fish. Different kinds of nanotechnology-based research have been carried out to strengthen the important milestones in aquaculture. The rational vaccine development highly relies on a mechanistic understanding of the immune system. The knowledge gained via application of nanotechnology to immunological studies will translate into new strategies for the development of vaccine. Here, we have outlined few summary points.

- Only few studies have been done on the use of CNTs as adjuvant for vaccine delivery in aquaculture species. However, how CNTs generally interact with fish tissues in vitro and in vivo has to be investigated more, but it has been studied in detail in animal models. One of the major challenges is to define the most promising immunotherapeutic application of CNTs. CNTs should show clear benefits in terms of overall therapeutic efficacy, safety, and cost-effectiveness (Fadel and Fahmy 2014). Future studies are necessary to explore in detail the mechanisms of clearance and degradation of CNTs in the host. However, biodegradation of CNTs in a living system is a complex process. In addition to the immunological effects of functionalization of CNTs on cells, the long-term persistence of CNTs in living systems needs to be explored further.
- The cost of highly purified CNTs remains expensive. For example, the prices of high purity MWCNTs (> 98%) and SWCNTs (> 95%) in September 2017 were 5 and 200 USD/g, respectively (Song et al. 2018). Therefore, the cost of nanoparticles should be kept within the reach of fish farmers for large-scale aquaculture usage. The future research must emphasize on reaping the benefits of technology keeping cost constraints and requirements in mind. Therefore, the development of synthesis techniques that can lower the cost of CNT is vital. In this regard, major focus may be shifted on the usage of NPs from natural products.
- CNTs and other nanoparticles provide many advantages like better release kinetics, stability, and targeted delivery over conventional adjuvant. Some of the problems of nanotechnology intervention in aquaculture are yet to be addressed. These problems include scalability, possible environmental impacts, and cost-effectiveness (Luis et al. 2019). It is worthy to mention that natural bioactive substances (e.g., herbal substances) received much attention in aquaculture. Synthesis of efficient vehicles for encapsulation of those natural bioactive substances may be of great interest in aquaculture.
- Another important point to be taken into consideration is that the use of carbon nanoparticles for the improvement of aquaculture is still marginal. Most of the research discoveries are converting for commercial exploitation. The use of carbon-based nanoparticles can ameliorate the negative effects of metal-nanoparticles.

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Biotechnological Interventions in Developing Vaccines Against Aeromonas Infection in Aquaculture

5

Sukanta Kumar Nayak, Jyoti Prakash Dash, and Pranabkanti Dutta

Abstract

Vaccination is one of the most effective means for disease prevention and control in any living system, and aquatic organisms are no exception. In aquaculture, the sustainability is at stake due to emergence and reemergence of infectious diseases. Among several pathogens *Aeromonas* species such as *A. hydrophila*, *A. salmonicida*, *A. sorbia*, *A. bestiarum* and *A. veronii* are the major fish pathogens that cause severe economic loss in many fish species ranging from freshwater to marine water. Over the years, several attempts have been made to develop vaccines against different *Aeromonas* species that includes the use of inactivated form, live attenuated, its toxins, or surface antigens, outer membrane proteins, lipopolysaccharide-based vaccines against different *Aeromonas* species. However, the modern biotechnological tools together with reverse vaccinology approaches have opened a new era in the field of aquatic vaccinology to formulate, develop and deliver different types of recombinant vaccines which can induce effective immunoresponses in diversified fish species to fight against *Aeromoniasis*.

Keywords

Aeromoniasis · *Aeromonas* species · Ghost vaccine · Subunit vaccine · Reverse vaccinology · Recombinant vaccine · Vaccine

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5.1 Introduction

Infectious diseases remain a major threat for the development and sustainability of aquaculture practices throughout the globe. Aquaculture industry is experiencing serious setbacks due to emergence/reemergence of diseases caused by bacteria, virus, fungi, parasites, etc. Despite multiple approaches to innovative therapy, diseases remain a major concern and constraint for this sector. Since the availability of first commercially licensed fish vaccines against enteric red mouth, in 1976, followed by a vibriosis vaccine, significant advances in terms of research and development have been made to develop fish vaccines. The development of vaccine and vaccination strategy as a fish health management approach is still emerging. While several commercial vaccines against different diseases are already available, many experimental vaccines against certain other diseases of economic importance are currently being persuaded in many countries. In past few decades, efforts have been made to develop vaccines against different bacterial diseases like aeromoniasis, edwardsiellosis, pseudomoniasis, vibriosis, nocardiosis, columnaris, etc. Among different bacterial pathogens, numerous efforts are being made to develop vaccines against important bacterial pathogens belonging to various *Aeromonas* species which are associated with many diseases in a wide range of fish species throughout the world. This chapter basically focuses on various efforts in general and biotechnological interventions in particular made in developing a suitable vaccine against *Aeromonas* species in general and *A. hydrophila* in particular.

5.2 Characteristics Features of *Aeromonas* Species

The genus *Aeromonas* consists of ubiquitous, facultative anaerobic, Gram-negative γ-proteobacteria autochthonous to aquatic environments (Janda and Abbott 2010). The genus *Aeromonas* belongs to Aeromonadaceae family of class Gammaproteobacteria and order Aeromonadales. It consists of 32 species and is constituted by facultative anaerobic, Gram-negative, rod-shaped and non-spore-forming bacteria. Among different species of *Aeromonas*, *A. hydrophila*, *A. salmonicida*, *A. sorbia*, *A. bestiarum* and *A. veronii* are the major fish pathogens that cause severe economic loss. All these bacteria are unique and vary greatly with respect to their distinct biochemical properties (Table 5.1). Serologically, great variations are also reported among several *Aeromonas* species. For example, the antigenic diversity among *A. hydrophila* is mainly attributed to the H and O somatic antigens. There are at least 12 O-antigen groups and 9 H-antigen groups that are further divided into several additional serotypes in *A. hydrophila*.

Table 5.1 Biochemical characteristics of certain important *Aeromonas* species associated with disease conditions of fish

Parameters	*A. hydrophila*	*A. salmonicida*	*A. caviae*	*A. sobria*	*A. bestiarum*
Gram reaction	−ve	−ve	−ve	−ve	−ve
Morphology	Rod shaped, single polar flagella,	Coccoid rod shaped, single polar flagella	Rod shaped	Rod shaped	Rod shaped
Motility	+ve	−ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	+ve	+ve
Methyl red	−ve	+ve	−ve	−ve	−ve
Voges−Proskauer	+ve	−ve	+ve	−ve	+ve
Indole production	+ve	−ve	+ve	+ve	+ve
Citrate utilization	+ve	−ve	+ve	+ve	−ve
Oxidative/ fermentative reaction	+/+	+/+	+/+	+/+	+/+
Hydrogen sulphide production	−ve	−ve	+ve	+ve	+ve
Gas from glucose	+ve	+ve	−ve	+ve	+ve
Nitrate reduction	+ve	+ve	−ve	+ve	+ve
KCN	+ve	−ve	+ve	−ve	+ve
Urease	−ve	−ve	+ve	−ve	−ve
Lysine decarboxylase	+ve	−ve	−ve	+ve	+ve
Ornithine decarboxylase	−ve	−ve	−ve	−ve	−ve
Starch hydrolysis	+ve	+ve	+ve	+ve	+ve
DNAse hydrolysis	+ve	−ve	+ve	−ve	+ve
Gelatin	+ve	+ve	+ve	+ve	+ve
ONPG	+ve	−ve	+ve	+ve	+ve
Tween 80	+ve	+ve	+ve	+ve	+ve
Fermentation of sugars					
Glucose	+ve	+ve	+ve	+ve	+ve
Lactose	+ve	+ve	−ve	−ve	−ve
Sucrose	+ve	−ve	+ve	+ve	+ve
Sorbitol	−ve	−ve	−ve	−ve	−ve
Mannitol	+ve	+ve	+ve	+ve	+ve
Inositol	−ve	−ve	−ve	−ve	−ve
Mannose	+ve	−ve	−ve	+ve	+ve
Fructose	+ve	+ve	+ve	+ve	+ve
Maltose	+ve	−ve	+ve	+ve	+ve
Raffinose	+ve	−ve	+ve	−ve	−ve
Cellobiose	−ve	−ve	−ve	−ve	−ve

5.3 Vaccines Against *Aeromonas* Species

Aeromonas species are mainly the leading cause of morbidity and mortality of diversified fish species. With the emergence of antibiotics resistivity, less effective drugs and chemotherapeutic agents, vaccine and suitable vaccination strategy against *Aeromonas* species is becoming inevitable to prevent severe economic losses in aquaculture industry. Although experimental vaccines against many *Aeromonas* species exhibited very promising results, till date except *A. salmonicida* vaccine for salmonids only, no commercial vaccines against any other *Aeromonas* species are available**.** There are several constraints in the development of vaccine against *Aeromonas* species. For example, in case of *A. hydrophila* issues like selection of suitable antigen(s), existence of a wide range of serotypes, pathotypes and variants, production cost, convenience of administration, ideal carrier/adjuvant, safety and ultimately potency/effectiveness need thorough scientific validations of lab to land studies.

Aeromonas species possess several virulence factors which are responsible individually and/or combined for the pathogenicity in different fish species (Lowry et al. 2014). The heterogeneity among the pathogenic strains with multifactorial virulent mechanisms such as lipopolysaccharide (LPS), outer membrane poteins (OMPs), extracellular products (ECPs) and ECPs factors like haemolysins, aerolysin, cytotoxin, enterotoxin proteases siderophores, surface array proteins and acetylcholinesterase S-layer, adhesions, etc., are possibly the major obstacle in selecting potent strain(s) for vaccine development. Despite, several attempts have been made to develop and evaluate different forms of conventional as well as recombinant vaccine(s) against different *Aeromonas* species.

5.4 *Aeromonas hydrophila* Vaccines

Aeromonas hydrophila is a prevalent and opportunistic pathogen of aquatic organisms. Most of the cultured and wild freshwater fish species like American eel (*A. rostrata*), Asian sea bass *(Lates calcarifer*), ayu (*Plecoglossus altivelis*), brown trout (*Salmo trutta*), blue tilapia, (*Oreochromis aureus*), blunt snout bream (*Megalobrama amblycephala*), chinook salmon (*Oncorhynchus tshawytscha*), carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), European eel (*A. anguilla*), eel (*A. japonica*), goldfish (*Carassius auratus*), golden shiner (*Notemigonus crysoleucas*), Indian major carps {rohu, *Labeo rohita*; catla, *Catla catla*; mrigal, *Cirrhinus mrigala*}, rainbow trout (*Oncorhynchus mykiss*), snakehead fish (*Ophicephalus striatus*), spotted murrel (*Channa punctatus*), snake head fish (*Channa striata*), pacu (*Piaractus mesopotamicus*), Stinging catfish (*Clarias batrachus*), Nile tilapia (*Tilapia nilotica*), etc., are susceptible to *A. hydrophila* infection (Hsu et al. 1981; Karunasagar et al. 1989; Ogara et al. 1998; Sahoo et al. 1998; Shome et al. 1999; Nielsen et al. 2001; Das et al. 2005, 2011; Mohanty et al. 2008; Pridgeon and Klesius 2011; Silva and Mourino 2012; Mu et al. 2013; Kumar et al. 2016). It's involvement in disease conditions/complications like haemorrhagic septicemia, red mouth disease, epizootic ulcerative syndrome, red sores, necrosis,

ulceration and haemorrhagic septicaemia, dropsy, fin rot, tail rot, etc., has been demonstrated in many wild as well as cultured fish species throughout the world. Over the years, numerous attempts have been made to use inactivated, live, attenuated, biofilm-based, ECPs, OMPs, LPS of *A. hydrophila* along with several recombinant forms as vaccine candidate in fish (Shome and Shome 1999; Swain et al. 2002; Chandran et al. 2002; Kalita et al. 2006; Kamilya et al. 2006; Behera et al. 2010, 2011; Wu et al. 2012; Saikia and Kamilya 2012; Bharadwaj et al. 2013; Sen et al. 2014; Kalita et al. 2018; Kumar et al. 2018; Zhao et al. 2020).

5.4.1 Conventional *A. hydrophila* Vaccines

Several conventional types of formulations involving whole cells of *A. hydrophila* along with its components like S-layer, OMPs, LPS, etc,. have been evaluated as vaccine candidate. The whole cell vaccination is a novel and widely used strategy to control diseases since the naïve conformation of the antigen is maintained in such formulations, which in turn help to induce strong immune responses in host. In aquaculture, several experimental vaccines based on whole cell such as inactivated, killed, live-attenuated, free cell/biofilm-based against *A. hydrophila* have been developed (reviewed in detail by Nayak 2020; Mzula et al. 2019). However, great degree of variations in their efficacy has been recorded by researchers depending upon the type of vaccine preparations.

Similarly, several extracellular-based compounds involved in virulence (aerolysin, hemolysin, lipases, metalloproteases, glycerophospholipid-cholesterol acyltransferase, etc.), digestion (amylases and chitinases) and host cell attachment (S layer, flagella) of *A. hydrophila* were also evaluated by researchers as an effective vaccine candidate against *Aeromoniasis*. Literatures indicate that *A. hydrophila* LPS, the outer membrane of the Gram-negative bacteria, can stimulate immunity in many fish species (Selvaraj et al. 2004; Nayak et al. 2007; Swain et al. 2008). Likewise, the S-layer protein, a major virulent factor mostly consisting up of a single protein in the outermost cell envelope component of *A. hydrophila*, is also found to be a potent vaccine candidate. Although limited studies have been made to develop S-layer-based *A. hydrophila* vaccine in fish, reports showed that different strains of *A. hydrophila* share antigenic similarities of S layer proteins and induce protective immunity in fish (Munn et al. 1982; Trust et al. 1983; Dooley et al. 1986; Dooley and Trust 1988; Phipps and Kay 1998).

On the other hand, recently OMPs-based vaccine has attracted a lot as vaccine candidate in controlling *A. hydrophila* infections in different fish species (Yadav et al. 2017, 2018; Dash et al. 2017; Dubey et al. 2016; Divya et al. 2015; Rauta and Nayak 2015; Dash et al. 2014a, b; Khushiramani et al. 2012; Thangaviji et al. 2012, 2013; Maiti et al. 2012; Khushiramani et al. 2007a, b). By virtue of their close proximity to host cells, OMPs are often associated with virulence. OMPs are highly conserved among the Gram –ve bacteria with strong ability to induce host immune responses. Recently, OMPs are also targeted as recombinant vaccines to deliver into fish by appropriate carrier molecules.

5.4.2 Recombinant *A. hydrophila* Vaccines

Developing an effective vaccine against *A. hydrophila* is very crucial and with the recent advances made in biotechnology, bioinformatics, microbiology, immunology, and molecular biology, currently it is possible to develop a potent vaccine against this bacterium to make an efficacious vaccine to control this pathogen. Recombinant DNA technology, reverse vaccinology technology with innovative tools and new delivery techniques have enabled significant progress in any vaccine development against *A. hydrophila*.

DNA vaccination concept is based on the expression plasmid carrying particular gene codes for a selected antigenic protein which is expressed within the host to elicit immune response. A DNA vaccine typically includes the transfection of a specific antigen coding DNA sequence onto the cells of an immunized species. While several DNA vaccines are available for veterinary uses, literatures tend to increase in this aspect in aquaculture perspective. In case of fisheries, DNA vaccine can be a promising tool for solving various aspects of microbial diseases including *Aeromoniasis*. Vaccination of DNA vaccine against *A. hydrophila* supplemented with functionalized single-walled carbon nanotubes upon bath immunization into grass carp (*Ctenopharyngodon idella*) lead to significant enhancement of the expression of immune-related genes (IFN-I, TNF-α, CRP, IL-8, IgM, MHC I and CD8α) in the intestine, kidney and spleen (Liu et al. 2016). Similarly, DNA vaccine containing apolipoprotein A1 of *A. hydrophila* can also induce immune response in fish like channel catfish (Pridgeon and Klesius 2013).

Additionally, DNA vaccines can be constructed with multiple genes of different pathogens. DNA vaccine expressing two antigenic outer membrane protein/peptides of *A. hydrophila* has been reported to induce immune responses in fish (Rauta et al. 2017). Though DNA vaccine has a lot of advantages, its use is limited for *Aeromonas* species in general and against *A. hydrophila* in particular. One of the reasons could be the production of carbohydrate and highly glycosylated proteins of the bacteria. Producing a plasmid DNA encoding, these genes are often not feasible as that of non-glycosylated proteins.

Like DNA vaccine, recombinant protein/peptide vaccine with identified immunogenic protein(s) from the pathogen is used as antigens for vaccine formulation. The important benefit of recombinant protein-based vaccines is their more effectiveness because they contain the specific antigenic protein and not the whole pathogens. These vaccines are prepared by taking the specific immunogenic regions of a pathogen and further inserted into the expression host which expresses the protein on a large scale, and the protein is purified as vaccine. In fish like *L. rohita*, rOMPR was demonstrated to upregulate the expression of immune-related genes such as complement component 3, chemokine, tumour necrosis factor-α, interleukin 1β, toll-like receptor 22 and manganese superoxide dismutase (Dash et al. 2014a, b). Similarly, significant increase in immune parameters like ceruloplasmin level, myeloperoxidase, anti-protease activities along with significant upregulation of IgM, IL1β, lysozymes C and G, NKEF-B, C3, CXCa and TNF-α in the fish like *L. rohita* confirmed the vaccine potential of rOMPC *of A. hydrophila* (Yadav et al.

2021). Furthermore, recombinant 46 kD maltoporin has been reported to retain its natural immunogenicity and cross-protection in European eel (*A. anguilla*) against multiple *A. hydrophila* serotypes (Feng et al. 2017).

Besides, a number of components can be used as potent candidate for subunit vaccine. These can be outer membrane proteins, outer membrane lipoprotein, capsid protein in case of viruses, etc. Recombinant OMPs of *A. hydrophila* as subunit vaccines are now preferred as vaccine candidate due to their profoundly immunogenic nature. The GAPDH protein based subunit vaccine of *A. hydrophila* LSA34 in turbot (*Scophthalmus maximus*) (Guan et al. 2011), OMP-G from *A. hydrophila* and *A. sorbia* in European eel (*A. anguilla*) (Guan et al. 2011), flagellar protein FlgK in channel catfish (Yeh and Klesius 2011); OMP48 from *A. hydrophila* in rohu (*Labeo rohita*) (Khushiramani et al. 2012) are few examples of such types of vaccines.

Recombinant proteins as well as OMPs from *A. hydrophila* have already been used as subunit vaccine. Although these subunit vaccines have several desirable qualities, in many cases, their ability to stimulate a potent immune response can be weaker than killed or live whole cell preparations. Therefore, subunit vaccines of *A. hydrophila* often depend on effective adjuvant to elicit the appropriate immunity and may require multiple booster immunizations to produce long-term protective immunity.

5.4.3 Recombinant Live-Attenuated *A. hydrophila* Vaccines

Live vaccines are believed to be better in terms of inducing protective immunity against pathogens. This is because of their ability not only to replicate but also to secrete/produce immunogens which in turn can stimulate a protective immune response. Certain researchers used to screen live-attenuated *A. hydrophila* strain, from the pathogenic *A. hydrophila* strain cultured on rifampicin containing medium (Jiang et al. 2016). Similarly, an exoenzyme mutant of *A. hydrophila* strain J-1 was generated through transposon insertion mutagenesis by Liu and Bi (2007). Immunisation of transposon Tn*916*-generated mutant of *A. hydrophila* J-1 defective in certain exoproducts as a live-attenuated vaccine (@10^7 CFU) protected better with relative percent survival of more than 60% against challenge with wild J-1 strain in swordtail fish (*Xiphophorus hellerii* Heckel). Likewise, the *A. hydrophila aroA* gene encoding 5-enolpyruvylshikimate 3-phosphate synthase live vaccine also significantly protected against the wild-type strain of *A. hydrophila* (Moral et al. 1998).

Even certain mutant strains of *A. hydrophila* have been reported to cross-protect the host against heterologous pathogens. Auxotrophic live-attenuated vaccine *aroA* mutant of *A. hydrophila* has been demonstrated to cross-protect the Hooke and DK30 strains of *A. salmonicida* with relative percent survival of more than 60% (Vivas et al. 2004). This correlated moderately with the activation of the humoral and cellular specific immune responses of vaccinated trout, which showed cross-reactivity against antigens shared by the two bacterial species, i.e. *A. hydrophila* and *A. salmonicida* (Vivas et al. 2004).

5.4.4 Bacterial Ghost Vaccines

Bacterial ghosts (BGs) represent a potential new concept in vaccines. BGs are intact bacterial cell envelopes, most commonly Gram-negative bacteria that are emptied of their content by gentle biological or chemical poring methods. Recently, the genetic inactivation of Gram-negative bacteria which produced bacterial ghosts has been reported as a promising new approach in non-living vaccine technology (Szostak et al. 1996; Jalava et al. 2002). Bacterial ghosts are produced by the controlled expression of PhiX174 lysis gene E. E-mediated lysis of bacteria results in the formation of empty bacterial cell envelopes, which have the same cell surface composition as their living counterparts. In addition, they display all surface components in a natural nondenatured form and are able to induce a strong mucosal immune response (Jalava et al. 2003; Mayr et al. 2005a, b).

Earlier researchers have succeeded in preparing such platforms for different pathogens in different fish species, for example, *Edwardsiella tarda* ghosts in tilapia and olive flounder (Kwon et al. 2005, 2007), *Vibrio parahaemolyticus* ghosts protect zebrafish (Ji et al. 2020), *Vibrio mimicus* ghosts in grass carps (Cao et al. 2018), *Pseudomonas aeruginosa* ghosts in Nile tilapia (Ghazy et al. 2020), *Aeromonas veronii* in koi (Jiang et al. 2019); *Citrobacter freundii* ghost in crucian carp (Pan et al. 2021) and *Flavobacterium columnare* ghosts in grass carp (Zhu et al. 2012). All these studies have demonstrated strong immune responses in fish as well as better survival on challenge.

The BGs-based vaccine using *A. hydrophila* was developed by researchers by cloning the lysis E gene in the bacteriophage PhiX174 followed by ligation into the prokaryotic expression vector pBV220. The lysis plasmid pBV220-Lysis E was constructed and then transformed to *A. hydrophila* strain to prepare *A. hydrophila* ghosts. Oral immunization with *A. hydrophila* ghosts can elicit systemic and mucosal adaptive immune responses and has higher potential to induce protective adaptive immunity than normal vaccine. The *A. hydrophila* ghosts were found to improve the antibody in serum with the relative percent survival of 76.8% as compared to 58.9% in formalin-killed vaccine in carp (*Carassius auratus gibelio*) (Tu et al. 2010).

5.5 *Aeromonas salmonicida* Vaccines

A. salmonicida, the oldest known fish pathogen, is the etiological agent of furunculosis throughout the world. This pathogen, currently endemic in most of the aquaculture-producing countries (both in freshwater and marine waters), causes huge economic losses to the salmonids and various other fish species. This pathogen can affect several fish species such as Atlantic cod (*Gadus morhua*), Arctic charr (*Salvelinus alpinus*), Atlantic halibut (*Hippoglossus hippoglossus*), Atlantic wolffish (*Anarhichas lupus*), goldfish (*Carassius auratus*), carp (*Cyprinus carpio*), Japanese flounder (*Paralichthys olivaceus*), lumpsucker or lumpfish (*Cyclopterus lumpus*), etc (Fuller et al. 1977; Humphrey and Ashburner 1993; Ford 1994; Cipriano et al.

1994; Hänninen et al. 1995; Dalsgaard and Madsen 2000; Fernandez-Alvarez et al. 2016).

It causes severe septicemia and acute mortality in salmonids and other susceptible fish species. The subacute or chronic form of the disease is characterized by the presence of lesions resembling boils, i.e. furuncles, in the musculature. Furunculosis caused by infection with *A. salmonicida* subsp. *salmonicida* is a well-known threat to aquaculture for more than a century. The disease probably exerts a significant impact on global aquaculture practices, making it a target for international research, particularly in the field of vaccination. As per *Bergey's Manual of Systematic Bacteriology*, there are five subspecies of *A. salmonicida*: *achromogenes*, *masoucida*, *smithia*, *pectinolytica* and *salmonicida* (Martin-Carnahan and Joseph 2005) but for convenience *A. salmonicida* subsp. *salmonicida* has been categorized into "typical" and "atypical" which can infect non-salmonid as well as salmonids are the phenotypically deviating isolates (Wiklund and Dalsgaard 1998).

Vaccines are available against atypical furunculosis of salmonids, but their efficacy is dependent on the characteristics of the infective strain. Although the vaccine against furunculosis for salmon can cross-protect against atypical *A. salmonicida* in certain fish, commercial vaccines for non-salmonid fish are yet to be available.

The focus of research regarding the control of *A. salmonicida* pathogen shifted towards vaccination during the early 1990s, resulting in the development of oil-adjuvanted vaccines. Since the introduction of successful oil-adjuvanted vaccines against this disease, a number of studies have been conducted to cross-check its protective and possible adverse consequences of this vaccine. Furthermore, various whole cells, LPS, A-layer and OMPs-based vaccines against *A. salmonicida* have induced protective immunity against furunculosis (Erdal and Reitan 1992).

The whole-cell bacterin-based *A. salmonicida* vaccines have been reported to provide inadequate protection against infection. The antibody responses correlate with antigen dose and in vivo protection for oil-adjuvanted, experimental furunculosis (*A. salmonicida* subsp. *salmonicida*) vaccines in Atlantic salmon (*Salmo salar*). Recently, Lim and Hong (2020) also developed a formalin-killed whole cell vaccine from highly pathogenic *A. salmonicida* which can exhibit protection with relative survival rate of 81.8% and 82.9% at 8 weeks and 16 weeks post-vaccination in fish, respectively.

Midtyng et al. (1996) found that intraperitoneal administration of adjuvanted polyvalent vaccine can induce anti-furunculosis immunity in fish like Atlantic salmon and is apparently necessary for an effective immunoprophylaxis of salmonid fish against furunculosis. Furthermore, immersion and injection vaccination with an avirulent strain of *A. salmonicida* were also found to be effective against furunculosis in salmonids (Cipriano and Starliper 1982). Likewise, Villumsen and Raida (2013) successfully used bath method to vaccinate the experimental *A. salmonicida* bacterin. Several researchers have also explored both commercial and an experimental auto-vaccine to verify the vaccine efficacy in rainbow trout, *O. mykiss*; turbot, *S. maximus*; and Atlantic halibut, *H. hippoglossus* (Gudmundsdottir et al. 2003; Santos et al. 2005).

Similarly, OMPs of *A. salmonicida* are also proved to be good antigen against furunculosis. In fish, immunogenic iron-regulated outer membrane proteins (IROMPs) of *A. salmonicida* are also explored as vaccine candidates (Hirst and Ellis 1994). Recently, improvisation in terms of recombinant vaccine against this pathogen has also been made. Diao et al. (2020) prepared recombinant OMPs, namely, OmpA, OmpC, OmpK and OmpW of *A. salmonicida* subsp. *masoucida* and demonstrated that among 4 OMPs, rOmpC significantly induced strong humoral immune response with production of sIg^+ lymphocytes and antibodies with significant upregulation in the expression of genes like MHC-II, TCR, CD4, CD8, IL-8 and IgM leading to highest relative percentage survival of 81.6% in rainbow trout (*O. mykiss*). Similarly, immunization of recombinant adenovirus harbouring the highly immunogenic Vapa gene of typical *A. salmonicida* was found to not only enhance antibody-mediated adaptive immune response with an increase in specific antibodies along with IgM and IgT but also lead to more survival (40%) in rainbow (*O. mykiss*) (Ling et al. 2019).

Besides, researchers have also evaluated live-attenuated vaccine like aromatic-dependent mutant of *A. salmonicida*, the aroA::Kar mutant by introducing it into the chromosome of virulent *A. salmonicida* 644Rb and 640V2 by allele replacement using a suicide plasmid delivery system (Vaughan et al. 1993). Earlier, Vivas et al. (2004) used an auxotrophic aroA mutant of *A. hydrophila* as a live-attenuated vaccine and demonstrated significant rise in specific and non-specific immune responses along with protection against *A. salmonicida* within rainbow trout (*O. mykiss*).

5.6 Vaccines Against Other *Aeromonas* Species

5.6.1 *Aeromonas veronii* Vaccines

Aeromonas veronii is capable of infecting both fish and mammals, including humans. It's a conditional pathogen causing high mortality in many freshwater fish species leading to significant economic losses to the aquaculture industry. Several fish species that are found to be susceptible to this pathogen include goldfish, Nile tilapia (*O. niloticus*), Pacific red snapper (*Lutjanu speru*), etc. (Hickman-Brenner et al. 1987; Abolghait et al. 2013; Martha and Carlos 2015; Jagoda et al. 2017; Yang and Zhang 2019).

Several conventional approaches made earlier with extracellular products from an inactivated *A. veronii* demonstrated immunogenicity in fish. The formalin-killed cells and extracellular product alone and/or in combination with Freund's adjuvant showed that the ECPs can produce and there was no lecithinase, urease or gelatinase activity. This study indicates that the ECPs of *A. veronii* can effectively enhance the ability of koi fish to resist bacterial invasion with protease, lipase, amylase and haemolyase activity (Song et al. 2018).

Currently, recombinant bacterial strains containing various antigenic components are directly delivered into host which cannot only survive throughout the intestinal

tract but also stimulate immunity in host. Flagellins, OMPs, are used as antigenic component to prepare recombinant bacteria to protect the fish against *A. veronii* infection. The recombinant *Lactobacillus casei* expressing flagellin A of *A. veronii*, Lc-pPG-1-FlaA (surface-displayed) and Lc-pPG-2-FlaA (secretory) has been demonstrated not only to survive and colonize in the intestinal mucosa when fed orally but also activated the innate immune system to trigger the cell immune response and inflammatory response with significantly high serum IgM, ACP, AKP, SOD and LYZ activity and significantly upregulated the expression of IL-10, IL-8, IL-1β, TNF-α and IFN-γ in the tissue of common carp, *C. carpio* (Kong et al. 2019). Besides, both the recombinant lead to higher survival {Lc-pPG-1-FlaA (70%) and Lc-pPG-2-FlaA (50%)} after being challenged by *A. veronii*.

Likewise in a similar approach, the ability of recombinant *L. casei* expressing OMPAI as oral vaccine against *A. veronii* infection has also been demonstrated. Zhang et al. (2018) generated two recombinant *L. casei* (surface-displayed or secretory) by cloning a 1022 bp gene fragment of the 42 kDa OMPAI antigen of *A. veronii* into pPG-1 (surface-displayed) and pPG-2 (secretory) and upon oral immunization with recombinant *L. casei* expressing OMPAI confers protection against *A. veronii* challenge in common carp, *C. carpio*. It was demonstrated that common carp fed with the recombinant vaccine candidate stimulated high serum or skin mucus specific antibody titres and induced a higher lysozyme, ACP, SOD activity and significantly upregulated the expression of IL-10, IL-β, IFN-γ, TNF-α genes. Further, higher survival rates after being challenged with *A. veronii* indicated the beneficial effects of Lc-pPG1-OMPAI and Lc-pPG2-OMPAI on immune response and disease resistance of common carp against *A. veronii* infection.

Currently, virulence gene deletion is becoming a mainstream direction for preparing a live-attenuated vaccine, which is notable for weak virulence, inexpensive production, immune convenience and strong immunity induction (Yang et al. 2013). Zhang et al. (2020) explored to design an effective live-attenuated vaccine of *A. veronii*. They have demonstrated that the live-attenuated vaccine is suitable for the development of a safe and effective vaccine against *A. veronii* infection in loach loach (*Misgurnus anguillicaudatus*) aquaculture. A very safe dose of 1×10^7 CFU/mL live vaccine can lead to an increase in enzyme activity parameters (SOD, LZM, ACP and AKP) in the skin mucus and serum along with specific IgM antibodies and cytokine IL-1β contents in the serum and increased cytokine (IL-15, pIgR, IL-1β and TNF-α) expression in the liver and spleen with high relative percent survival.

Besides, attempts have also been made to design *A. veronii* ghosts vaccine by PhiX174 gene *E*-mediated inactivation (Xu et al. 2014; Jiang et al. 2019). Immunization of *A. veronii* ghosts vaccine leads to significant enhancement of myeloperoxidase, respiratory burst, lysozyme and antibody in fish like koi (*C. carprio koi*) (Jiang et al. 2019). Furthermore, they have observed high relative percent survival of *A. veronii* ghosts-immunized fish (73.92%) as compared to 43.48% in formalin-killed immunized fish upon challenge with the parent strain *A. veronii*.

5.6.2 *Aeromonas sorbia* Vaccines

Aeromonas sorbia has been reported to be associated with diseases of fish (Wahli et al. 2005; Chen et al. 2013). However few studies have been made to develop vaccine against this pathogen. Earlier studies, though limited, showed that conserved OMP-G (OMP fragment of 747 bp), among *A. sobria* and *A. hydrophila* strains, can induce better immunogenicity with significant increase in antibodies and higher protection in fish like eels (*A. anguilla*) (Guan et al. 2011). Similarly, another recombinant ompTS amplified from two *A. sorbia* strains, one *A. hydrophila* strain and one *A. caviae* strain, has been reported to be more effective in providing protection in fish (Khushiramani et al. 2007a, b).

5.6.3 *Aeromonas bestiarum* Vaccines

Aeromonas bestiarum is one of the causal agents of motile *Aeromonas septicemia* in fish. There are scientific evidences supporting the association of A. *bestiarum* in disease conditions of fish. Like *A. sorbia*, limited vaccination studies against this bacterium have been done. Earlier, the formalin killed inactivated form of this bacterium has been demonstrated to elevate various non-specific immune responses leading to higher survival in fish like *C. carpio* (Kozinska 2004).

5.7 Application of Reverse Vaccinology to Develop Vaccine Against *Aeromonas* Species

Reverse vaccinology has changed the concepts and approaches for vaccine candidate selection and design in several systems, and aquaculture is no exception. With the ever-increasing information on microbial genomics, this technology has become a promising in silico approach to develop next-generation vaccine design (Fig. 5.1). The use of functional genomics approaches, such as DNA microarrays, proteomics, and comparative genome analysis to identify potential vaccine candidates based on in silico prediction, is the hallmark feature of this technology. It involves the development of vaccine through prediction of accurate antigens, independent of their abundance and immunogenicity. Such vaccines are highly specific which includes pathogen genome sequence analysis, prediction of unique/novel antigens and recombinant protein expression followed by immunogenicity assessment in host. Unlike conventional method, which requires a substantial period in identifying the ideal antigenic component(s) of a pathogen through culture-based assays, this technology has become a powerful tool for predicting the unique and ideal candidate vaccine antigen(s) through bioinformatic tools. Several programmes that are used in reverse vaccinology include *NERVE* for epitope prediction, *Vaxign* programme and *RANKPEP* for peptide bonding predictions, *SPAAN*, *MAAP*, *Protegen*, *BLAST*, *SPLIT*, *DAS*, *CELLO*, etc.

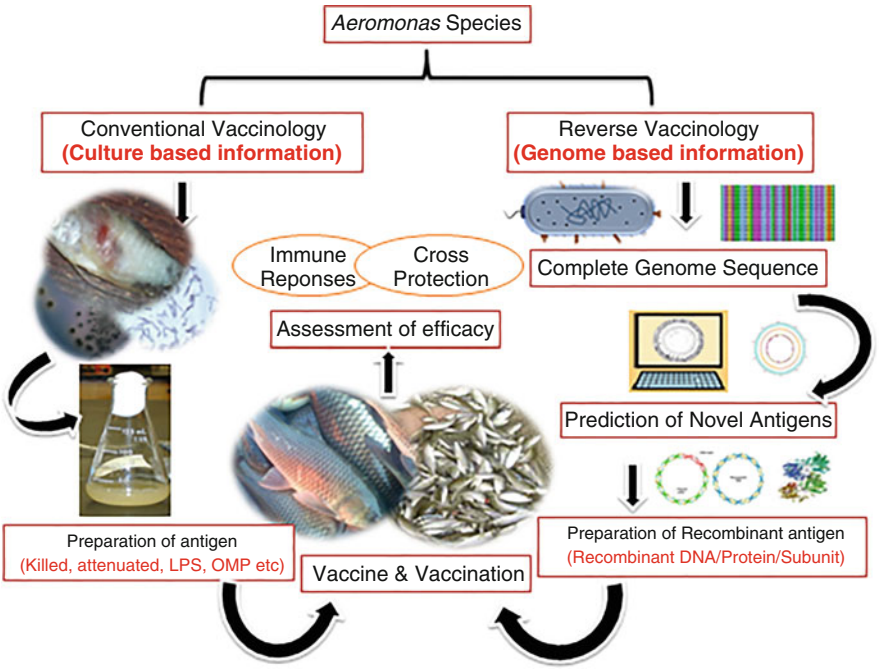

Fig. 5.1 Different types of approaches made to develop vaccine against different *Aeromonas* species in fish

A number of vaccines have been developed after the mid-1990s using the reverse vaccinology approach. Several vaccines against different aquatic pathogens like *E. tarda*, *Flavobacterium columnare* (Mahendran et al. 2016), *Photobacterium damselae* subsp. *piscicida* (Andreoni et al. 2013) are designed using this technology. Using this approach, different experimental vaccines, including plasmid DNA vaccines, recombinant live vector vaccines, subunit vaccines, etc., against various *Aeromonas* species have already been designed and evaluated in fishes.

Reverse vaccinology is adopted to design unique subunit vaccine(s) against various pathogens by using specific targeted epitopes identified from total OMPs. The antigenic diversity among *A. hydrophila* strains limits the vaccine development process, but the OMPs which are conserved across serotypes are potential and efficacious vaccine candidates. Recently, Bhattacharya et al. (2020) identified novel 9mer peptide epitopes (VGFDGSQYG and LAGKTTNES) in the OMPs sequences of *A. hydrophila* that can interact with both the T-cells and B-cells with maximum possible numbers of MHC alleles. Earlier, Singh et al. (2011) cloned the thermostable hemolysin gene of a number of strains of *A. hydrophila* to develop thermostable vaccines against *A. hydrophila*. Nonetheless, this technology helped to design a potent fish vaccine against *A. hydrophila* involving iron-regulated secreted proteins (Wang et al. 2019).

The availability of the complete sequence information of several *Aeromonas* species makes it possible to carry out the in silico analysis of its genome for vaccine development. However, mere recognizing potential epitopes may not be adequate to develop vaccine and may need additional in vivo and in vitro validations to ascertain their efficiency. Furthermore, though such approach has several advantages which in turn can save a lot of time and money, it is mostly appropriate against protein-based candidates. Also at times, proper computational support is also deficient in this technology and needs more upgradation.

5.8 Biotechnological Challenges to Develop *Aeromonas* Vaccines

Traditional vaccine development programme includes the selection of antigenic strain/component(s) based on culture-based assessment. However, modern biotechnology uses genetic engineering along with other technologies for the development and production of vaccines. With the availability of complete genome sequence for innumerous pathogens including *Aeromonas* species, technology to introduce individual/multiple gene(s) into suitable vector, novel delivery systems, development of adjuvant/potent carriers and most importantly significant progress in science immunology, it is now possible to develop an effective vaccine. While the potency of a vaccine against *Aeromonas* species is critically correlated to vaccine quality and efficacy, their efficacy is regulated by several interrelated factors such as type of disease induced by the pathogen, used strain/component(s) as antigen, host, vaccination schedule, idiosyncratic response of a particular fish species against a specific vaccine and several other factors, such as age, stages of growth, environmental conditions or genetic predisposition, if any. The most important impediment to the development of an ideal vaccine against *Aeromonas* species is to find the effective strain(s) and/or ideal component(s) for vaccination. Nonetheless, the success of a vaccine against *Aeromonas* species is strongly regulated by route of administration, dose and nature/type of antigen, adjuvant/carrier in fish. Besides, antigenic relatedness among the *Aeromonas* species and strains of a particular species from a wide range of sources will help to develop potent vaccine(s). In this regard, modern technologies will be helpful in determining the unique epitopes to develop monovalent/multivalent vaccines.

Further, currently with the successful intervention of modern technology, several antigen/carrier-antigen complex to prevent the degradation in the gastrointestinal transit during oral vaccination is also developed in fish. Nowadays, several biodegradable polymeric microparticles and nanoparticles-based carrier have been explored to deliver antigenic components of *Aeromonas* and other species (Behera and Swain 2011, 2012, 2013a, b). In this regard, most of the recent biodegradable carrier-based vaccines involving various components of *Aeromonas* showed considerable stability of the antigen along with effective induction of protective immunity after oral supplementation in fish. Different types of adjuvant/carriers are used in *A. hydrophila* vaccine especially ECPs, OMPs, S-layer proteins and LPS-based

formulations. These antigens are mostly poor antigens and are often degraded if supplemented through oral route. Natural polysaccharides like chitosan in bulk and/or nanoforms, liposome-based, microspheres-based polylactic-co-glycolic acid (PLGA), alginate, etc., for delivery of such antigens are demonstrated to induce better immune response upon vaccination against different *Aeromonas* infection. These carriers are safe, non-toxic, biocompatible in nature and possess the ability of control and sustained release of antigens and also protect proteolytic and acidic digestion of the antigen in the gastrointestinal tract of host. A functionalized single-walled carbon nanotubes vehicle to deliver the recombinant protein aerA of *A. hydrophila* has been demonstrated to augment the production of specific antibodies, to upregulate the immune-related genes and also to induce high survival rate compared to free aerA subunit vaccine irrespective of route (bath or intramuscular injection) of vaccination (Gong et al. 2015).

Finally, the safety aspects of the vaccines in general and recombinant forms in particular are an issue. Although vaccines are mostly safe, sometimes such vaccines could be risky. Therefore, complete information about the vaccines as well as their possible side effects needs proper scientific valediction and surveillance.

5.9 Conclusion

Vaccination probably is the best approach to prevent and control infectious diseases in any animals, and fish is no exception. In aquaculture, reduction of mortality and morbidity due to *Aeromonas* species which is involved in a broad spectrum of diseases in a wide range of host is the crucial factor for its sustainability. Experimental vaccines against various *Aeromonas* species can significantly reduce and control the aeromoniasis in fish. However, vaccine either conventional or recombinant form is yet to be commercially available, except against furunculosis. With the advent of biotechnological interventions and techniques like reverse vaccinology, more novel mono-/multivalent vaccine(s)-based DNA/subunit/peptide vaccine with ideal carrier/adjuvant against different *Aeromonas* species/strains with improved quality and efficacy, ability to induce strong specific long-term immunity without booster dose requirement, free of adverse reactions, cost-effective and cheap can be developed in the near future to protect fish against *Aeromonas* infection.

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Gene Editing Technology for Fish Health Management

6

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Abstract

Optimal fish health in recent times is clearly demarcated by sustainability in the upscaled aquacultural practices. Towards this, the application of selective genetic breeding techniques in farmed aquatic species generates tremendous potential for substantial production in terms of both the economically viable traits-growth and development. Acquiring such favourable changes in the undesired gene functions leads to greater genetic gain within organisms. So far, the methods of gene manipulation have focussed on the aspects of direct gene knockdown or gene elimination. However, recent advancements make use of efficient and targeted gene editing at random locus/loci within the genome. To date, a number of genes for a variety of traits have been employed for carrying out precise gene editing in different fish species. The major traits include those involved in reproduction, growth, development, pigmentation, disease resistance, etc. mainly among model fish species like zebrafish, medaka, etc. as well as economically relevant species such as Atlantic salmon, tilapia, rainbow trout, etc. Moreover, it has been significantly noted that among the different methods followed for incorporating the required genetic change, CRISPR Cas9 technique has successfully superseded the initial technologies in ensuring improved precision in editing at much broadened target sites, thus lowering the off-target rates while bringing about better gene activation or inhibition.

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Gene · CRISPR Cas 9 · Aquaculture · Traits · TALEN technique

6.1 Introduction

Sustainability in aquaculture is greatly hindered by the intensification of production. Thus, ensuring reliability in substantial upscaling necessitates the application of modern technologies to reduce the associated risks and minimize the losses. Much lately, the development of genetic improvement programmes has turned the table for paving a way to sustainable aquaculture at par (Gratacap et al. 2019). Although production in aquaculture is still underpinned by selective genetic breeding techniques for economically viable traits, there is still much scope for the progress in reproductive biology of farmed aquatic species to achieve greater genetic gain (Houston 2017). The resultant genetic variation may either arise from wild populations or from random de novo mutations. So far, the methods used to induce such genetic changes in organisms have focussed on the manipulation of undesired gene functions by elimination of the gene under study. However, recent methods aim at efficient and facile gene editing rather than elimination.

Gene editing refers to the specific and targeted modification at any random locus/loci within the genome. It aims at identification of gene of interest and its cloning after precise and efficient manipulation at the desired locus (Ye et al. 2015; Sun 2017). Genetic manipulations through genome editing have been employed within the aquaculture industry way back since the 1990s. Fishes, being the largest group of vertebrates, bestow unique opportunities for active application of the newly emerged gene editing technologies, owing to their biological and technical advantages over other vertebrate species. The group is endowed with the highest biodiversity that can serve as a source of abundant information and insights into the evolutionary history of developing gene functions in vertebrates as a whole. They have hence been increasingly popular towards performing functional studies, disease modelling, research and promotion of aquaculture through targeted gene manipulation. The major traits that have been dealt in detail among important fish species include reproductive aspects, growth, development, pigmentation, disease resistance, etc. The influence in them has largely been seen on model fishes like zebrafish, medaka, etc. as well as economically relevant species like Atlantic salmon, tilapia, rainbow trout, etc. (Yáñez et al. 2014).

During its initial years, genome editing has been tremendously applied together with transgenesis in animals, to allow precision breeding. However, contrary to transgenics, genome editing technologies overcome the concerns of public safety to genetically modified organisms (GMOs) by avoiding the introduction of any foreign non-host DNA with the transgene, into the targeted native genome. Rather, these techniques focus on the principle of *gene knockout* instead of *gene knockdown* utilized in the older methods. Gradual advances in the field further incorporated *gene knock-in* as a gene editing tool for the insertion of DNA fragments at specific

sites. Thus, in turn, the merger of genome editing with transgenics has enabled the advancement of fish breeding to its post-genomic era (Sun and Zhu 2019).

This chapter outlines the various methods employed for gene editing in fishes, with key emphasis on the reproductive aspect in impacting the incorporated genetic change. It also portrays how the initial technologies were superseded by the advent of CRISPR/Cas9 system. Further, technical developments, innovations and novel extensions of this system have ensured improved precision in editing within much broadened target sites to lower the off-target rates. This has in turn led to better gene activation or inhibition.

6.2 Types of GE Technology

A number of methods have been implemented in fishes to facilitate targeted genome editing. These have been discussed in detail as follows:

6.2.1 Gene Targeting/Knockout

This method is typically referred to as the first generation of genome editing method and is a dominant reverse genetic approach. It was first established in mouse models (Thomas and Capecchi 1987; Doetschman et al. 1987; Thompson et al. 1988) and is less common among non-rodent animals including fishes. However, gene knockdown using antisense morpholino oligomers (MOs) (Bedell et al. 2011) as a part of gene knockout, has been applied in prominent fish species like zebrafish (Bill et al. 2009) and medaka (Aller et al. 2013). Though this method proved to be convenient and efficient, it suffered from the problems of low specificity and high toxicity, besides being inheritable (Eisen and Smith 2008).

6.2.2 RNAi-Induced Gene Silencing

This is another variant of gene knockdown in organisms. Although it has not been well established in fish species, there are a few successful reports (Zhao et al. 2001; De Rienzo et al. 2012; Shinya et al. 2013).

6.2.3 TILLING (Targeting-Induced Local Lesions IN Genomes)

This approach is generally used as a screening method to identify either single individuals or large mutant libraries, with desired mutation incorporated within the gene of interest, from entire population treated with mutagens (Till et al. 2007). However, since it largely involves DNA sequencing, it is an expensive method beyond the reach of many laboratories (Huang et al. 2012).

6.2.4 Reverse Gene Editing Technologies

These are novel technologies that enable reverse genetic study of gene functions. There are the following methods included within this set of technologies. Overall, these methods make use of an engineered nuclease for specific cleavage at the target site. These methods have brought about gene editing in more than 40 fish species such as zebrafish, medaka, etc. with great efficiency (Zhu and Ge 2018).

6.2.4.1 Zinc Finger Nucleases (ZFNs)

Zinc finger nuclease (ZFN) represents itself as an ideal tool for incorporating mutations at targeted loci within organisms (Gupta and Musunuru 2014). This method involves the use of an artificial restriction endonuclease (FokI) equipped with a zinc finger DNA-binding domain (Urnov et al. 2010). It was first discovered in the year 1996 by Kim et al. (1996). The key feature of this approach, i.e. the ZFN, comprises of 3−6 zinc finger motifs, each recognizing 3 nucleotides within the DNA sequence, specifically targeting a total of 9−18 bp (Carlson et al. 2012). After recognition, dimerization of two ZFNs directs the FokI enzyme for cleavage at the target locus (Durai et al. 2005). However, constraints in the design and construction of typical zinc motifs limit their use owing to their low efficiency (Urnov et al. 2010) (Table 6.1).

6.2.4.2 TALEN

This is a more advanced approach to gene editing established in 2011 (Miller et al. 2011). This method is more or less similar to ZFNs in facilitating cleavage through dimerization via the FokI enzyme. However, contrary to ZFNs, TALENs recognize single nucleotide within the DNA sequence through tandem array of repeat modules named Repeat Variable Diresidue (RVD) (Moscou and Bogdanove 2009). This recognition favours specific cleavage between the TALEN sites (Christian et al. 2010). TALENs generally have an upper hand over ZFNs in being highly specific, efficient, inexpensive and easier for construction of plasmids encoding TALE proteins. The various functional studies conducted on different fish species using TALEN technique have been detailed in Table 6.2.

6.2.4.3 CRISPR/Cas9

This method is regarded as the most advanced and dominant technique in genome editing, so far. It was first discovered as a gene editing complex, effecting adaptive immune system against bacteriophage infection in *Streptococcus pyogenes* (Fridovich-Keil 2019), by Jennifer Doudna, Emmanuelle Charpentier and his colleagues in the year 2012. Further, it was refined by Feng Zhang and his colleagues. Unlike ZFNs and TALENs, CRISPR uses Cas9 nuclease to effect random but targeted incision in the host DNA, guided by the gRNA (guide RNA). The gDNA is a chimera of the targeting CRISPR RNA (crRNA) and the transactivating crRNA (tracrRNA). The gRNA specifies the target DNA sequence for Cas9 by means of its seed region which is located at the 3′ end of the spacer sequence present in the crRNA, 10–12 base pairs adjacent to the protospacer

Table 6.1 Functional studies conducted for fish health management using genome editing ZFN technique

Target fish species	Trait	Encoded protein	Target genes	Results	References
Channel catfish (*Ictalurus punctatus*)	Reproduction	Luteinizing hormone-β	*lhb*	Sterility in mutant founders; no homozygous mutants	Qin et al. (2016)
Rainbow trout (*Oncorhynchus mykiss*)	Sex determination	Sexually dimorphic on the Y-chromosome	*sdy*	Mosaic F0 mutants; heterozygous F1 mutants with ovarian structure resulting from male-to-female sex reversal; edited gene transmission	Yano et al. (2014)
Medaka (*Oryzias latipes*)	Sex differentiation	Gonadal somatic cell derived factor	*gsdf*	Homozygous mutant females with decreased dmrt1 expression	Zhang et al. (2016)
Zebrafish (*Danio rerio*)	Growth and development	No tail protein	*btl*	Defective tails in F0	Doyon et al. (2008)
Channel catfish (*Ictalurus punctatus*)	Sterility	Luteinizing hormone-β	*lhb*	Controlled escape	Qin et al. (2016)
Yellow catfish (*Pylodictis olivaris*)	Growth	Myostatin a	*mstna*	No significant phenotype in muscle growth in either F0 or F1 generation	Dong et al. (2011)

adjacent motifs (PAMs) (Doudna and Charpentier 2014). The seed region is complementary to the target DNA sequence in the host. Thus, this entire assembly of components of CRISPR-Cas9 system enables it to eliminate the requirement of laboursome protein engineering steps, followed in other methods, in editing the target DNA. The Cas9 nuclease simply requires a target-specific gRNA for each targeted DNA. Therefore, this method allows for cost-effective, convenient and efficient large-scale genome modification in species. In addition, it also allows for biallelic disruption of alleles (Jao et al. 2013) as well as simultaneous delivery of multiple gRNA for multiplex gene targeting or multi-gene knockout. Besides, it can

Table 6.2 Functional studies conducted using genome editing TALEN technique

Target fish species	Trait	Target genes	Encoded protein	Results	References
Zebrafish (*Danio rerio*)	Reproduction	*gnrh3*	Gonadotropin-releasing hormone	Fertile mutants (both sexes) with unaffected sexual maturation and gametogenesis	Spicer et al. (2016)
Medaka (*Oryzias latipes*)	Reproduction	*gnrh1*	Gonadotropin-releasing hormone	Infertile females due to anovulation	Takahashi et al. (2016)
Zebrafish (*Danio rerio*)	Reproduction	*kiss1/2*, *kissr1/2*	Kisspeptin(s), kisspeptin(s) receptor	Fertile and normal mutants (both sexes)	Tang et al. (2015)
Zebrafish (*Danio rerio*)	Reproduction	*fshb*, *lhb*	Follicle stimulating hormone-β, luteinizing hormone-β	Delayed onset of puberty and subsequent follicle activation in female fshb mutants; unaffected follicular growth in lhb mutants; infertile female mutants probably due to failed oocyte maturation and ovulation	Chu et al. (2014, 2015), Zhang et al. (2015b)
Medaka (*Oryzias latipes*)	Reproduction	*fshb*, *lhb*	Follicle-stimulating hormone-β, luteinizing hormone-β	Infertile female mutants; lhb mutants were infertile due to anovulation while fshb mutants showed inhibited folliculogenesis at PV stage	Takahashi et al. (2016)
Zebrafish (*Danio rerio*)	Reproduction	*prl*	Prolactin	Fertile mutants with inability to survive beyond 16 dpf in freshwater; no phenotype in brackish water	Shu et al. (2016)
Medaka (*Oryzias latipes*)	Sex determination	*dmy*	DM-domain on Y chromosome	Fertile female mutants; sex reversal of males into females	Luo et al. (2015)
Tilapia (*Oreochromis niloticus*)	Sex differentiation	*dmrt1*	Double sex and mab-3 related transcription factor 1	Testicular regression with spermatogonial degeneration or loss of germ cells in F0 mutants	Li et al. (2013, 2014)

Zebrafish (*Danio rerio*)	Sex differentiation	*dmrt1*	Double sex and mab-3 related transcription factor 1	Infertile males with testis dysgenesis; male-to-female sex reversal	Webster et al. (2017)
Zebrafish (*Danio rerio*)	Spermatogenesis	*fshb*, *lhb*, *fshr*	Follicle stimulating hormone-β, luteinizing hormone-β, follicle stimulating hormone receptor	Delayed spermatogenesis at puberty in fshb but not in lhb mutants	Zhang et al. (2015a, b)
Zebrafish (*Danio rerio*)	Spermatogenesis	*cyp17a1*	Cytochrome P450	All male mutants with decreased androgen levels	Zhai et al. (2017)
Tilapia (*Oreochromis niloticus*)	Spermatogenesis	*rspo1*	R-spondin 1	Delay in spermatogenesis	Wu et al. (2016b)
Tilapia (*Oreochromis niloticus*)	Spermatogenesis	*β-cat1/2*	β-catenin1/2	Retardation of ovarian differentiation and masculinization in F0 XX females; upregulation of dmrt1, cyp11b2, sox9 and serum 11-KT level	Wu et al. (2016a)
Medaka (*Oryzias latipes*)	Spermatogenesis	*dmc1*	Disrupted meiotic cDNA	Infertile mutant males with increased apoptosis of spermatocytes and production of malfunctional sperms with abnormal morphology such as multiple tails or heads	Chen et al. (2016)
Zebrafish (*Danio rerio*)	Folliculogenesis	*fshr*, *lhcgr*	Follicle-stimulating hormone receptor, luteinizing hormone cognate receptor	Female-to-male sex reversal at different times of development; no visible phenotype and complete arrest of folliculogenesis at early PG stage	Zhang et al. (2015a)
Medaka (*Oryzias latipes*)	Folliculogenesis	*cyp19a1a*	Aromatase	All-male fshr mutants due to sex reversal induced by reduced expression of aromatase encoded by the mutated gene	Murozumi et al. (2014)

(continued)

Table 6.2 (continued)

Target fish species	Trait	Target genes	Encoded protein	Results	References
Tilapia (*Oreochromis niloticus*)	Folliculogenesis	*foxl2a*	Forkhead box transcription factor L2a	Mosaic F0 mutants due to female-to-male sex reversal	Li et al. (2013)
Medaka (*Oryzias latipes*)	Folliculogenesis	*foxl3*	Forkhead box transcription factor L3	Production of sperm within the female ovary; unaffected cyp19a1a expression	Nishimura et al. (2015)
Zebrafish (*Danio rerio*)	Folliculogenesis	*bmp15*	Bone morphogenetic protein 15	An arrest of folliculogenesis at PV stage or stage II, partly due to reduced cyp19a1a expression	Dranow et al. (2016)
Zebrafish (*Danio rerio*)	Folliculogenesis	*pgr*	Nuclear progestin receptor	Infertile female mutants	Zhu et al. (2015), Tang et al. (2016)
Cavefish (*Phreatichthys andruzzii*)	Pigmentation	*oca2*	Oculocutaneous albinism 2	Reduced pigmentation	Ma et al. (2015)
Zebrafish (*Danio rerio*)	Development	*sp7/ Osterix*	Non-annotated transcription factor	Affected ossification and/or bone formation	Niu et al. (2017)
Zebrafish (*Danio rerio*)	Development	*stat3*	Signal transducer and activator of transcription	Spine development and immune function phenotypes in F0 mosaic mutants	Xiong et al. (2017)
Zebrafish (*Danio rerio*)	Disease model	*mpl*	Myeloproliferative leukaemia protein	Reduced number of thrombocytes and high chance of bleeding useful in understanding of human CAMT	Lin et al. (2017)
Zebrafish (*Danio rerio*)	Disease model	*rb1*	Retinoblastoma 1	Mosaic mutants with tumour development mostly in the brain at early stage of 3.5 months	Solin et al. (2015)

Yellow catfish (*Pylodictis olivaris*)	Growth	*mstnb*	Myostatin b	No significant muscle growth within generations	Dong et al. (2014)
Zebrafish (*Danio rerio*)	Disease resistance	*vhl*	von Hippel–Lindau protein	Enhancement of antiviral responses	Du et al. (2015)

Dpf days post fertilization, *PV* pre-vitellogenic, *CAMT* congenital amegakaryocytic thrombocytopenia

conduct precise editing by generating double-strand breaks that trigger DNA repair mechanisms, further leading to loss of indel mutations or knock-in of an exogenous fragment (Durai et al. 2005; Gratz et al. 2014).

The only drawback this technique suffers from is the potential off-targeting effects (Cho et al. 2014; Schaefer et al. 2017) which usually arise when the seed region shares false homology with the gRNA due to potential mismatches. These off-target effects are, however, minimized by using truncated gRNAs (Fu et al. 2014) or a mutant of Cas9 nuclease named Cas9 nickase that creates a single-strand break in DNA, along with 2 gRNAs that cleave at different sites of the target DNA (Ran et al. 2013). Besides, delivery of Cas9 ribonucleoprotein (RNP) complex, instead of DNA plasmids, into cells is crucial for mitigating the induced off-target effects.

CRISPR-Cas9 method has been particularly applied in the following:

- Model fishes, including zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*)
- Farmed fishes, such as yellow catfish (*Pelteobagrus fulvidraco*), tilapia (*Oreochromis niloticus*), common carp (*Cyprinus carpio*), Atlantic salmon (*Salmo salar* L.), channel catfish (*Ictalurus punctatus*), rice field eel (*Monopterus albus*) and Chinese tongue sole (*Cynoglossus semilaevis*) (Zhu and Ge 2018)
  Parallel to this, this method has also been useful for non-model species with long generation time mainly to detect the mutant phenotypes in the founder generation.

Recently, this method has been successfully applied in major species such as Atlantic salmon, rainbow trout, carps, namely, rohu, grass carp and common carp; catfish (channel and southern catfish); Nile tilapia and gilthead sea bream for different traits of interest such as pigmentation, growth, sterility, immunity, germ cell and muscle development, omega-3 metabolism and disease resistance (clearly summarized in Table 6.3). Herein, the CRISPR/Cas9 protocols involved either delivery through injection in vivo or via cell lines. The in vivo mutations are generally induced in newly fertilized eggs (generally at one-cell stage of development). It has been noted that some traits such as reproduction in tilapia (*Oreochromis niloticus*) and growth in *Ictalarus punctatus* exhibited germline transmission. Few other traits, viz., muscle development in common carp (*Cyprinus carpio*) and immunity in rohu carp (*Labeo rohita*), showed homology-directed repair and in vitro mutations, respectively. Further, a number of functional studies have been carried out using the above forms of GE technology in applied fields of fish health management as discussed below.

6.3 Functional Studies in fish health management

6.3.1 Functional studies in Reproduction

Functional studies in vertebrates have always been limited to the delineation of conserved function of genes based on their expression patterns, mainly due to the lack of approaches available for altering the gene function. Further among fishes,

Table 6.3 Summary of the functional studies conducted for fish health management using genome editing CRISPR Cas9 technique

Target fish species	Trait	Target genes	Encoded protein	Results	References
Channel catfish (*Ictalurus punctatus*)	Growth	*mstn*	Myostatin	More muscle cells in fry; increased mean body weight by 29.7%	Khalil et al. (2017)
Red sea bream (*Pagrus major*)	Growth	*mstn*	Myostatin	16% increase in skeletal muscle tissue; short body length and presence of small centrum	Kishimoto et al. (2018)
Nile tilapia (*Oreochromis niloticus*)	Sex determination	*nanos2, nanos3, dmrt1, foxl2*	Nanos homolog 1, Nanos homolog 2, double sex and mab-3-related transcription factor 1, Forkhead box transcription factor L2	Sex reversal and loss of cyp19a1a in gonads of nanos 3 mutated females (XX); no significant sex differentiation in somatic cells of nanos 2 mutated males (XY); disruption of germ cell development in dmrt 1 mutated cells; transmission of foxl2 and dmrt1 genes in F1	Dai et al. (2015), Ye et al. (2015), Gao et al. (2016)
Zebrafish (*Danio rerio*)	Somatic growth	*socs1a, mstnb*	Suppressor of cytokine signalling 1, myostatin b	Accelerated growth due to knockout of negative regulators of GHR; regulation of JAK-STAT pathway; generation of hyper-muscular phenotypes by regulation of receptors controlling myogenic proliferation	Dai et al. (2015), Ye et al. (2015), Gao et al. (2016)
Tilapia (*Oreochromis niloticus*)	Sex determination	*amhy*	Y-linked anti-Muellerian hormone	Male-to-female sex reversal	Li et al. (2015)

(continued)

Table 6.3 (continued)

Target fish species	Trait	Target genes	Encoded protein	Results	References
Chinese tongue sole (*Cynoglossus semilaevis*)	Sex differentiation	*dmrt1*	Double sex and mab-3-related transcription factor 1	Increased and decreased expression of female (foxl2 and cyp19a1a) and male related genes (sox9a and amh), respectively; development of ovary-like testis in females as well as disrupted spermatogenesis in males	Cui et al. (2017)
Tilapia (*Oreochromis niloticus*)	Sex determination	*gsdf*	Gonadal somatic cell derived factor	F0 mosaic XY fish with ovotestis due to partial/complete male-to-female sex reversal; homozygous female mutants with significantly increased estradiol (E2) levels; sex reversal in F2 males as well	Jiang et al. (2016)
Nile tilapia (*Oreochromis niloticus*)	Steroidogenesis	*sf-1, nr5a1*	Steroidogenic factor-1, nuclear receptor 5a1	Increased expression of foxl2 and cyp19a1a and decreased expression of dmrt1, amh and cyp11b2 in mutant XY males of F0 generation with testis dysgenesis	Xie et al. (2016)
Tilapia (*Oreochromis niloticus*)	Folliculogenesis	*cyp19a1a*	Cytochrome P450 19a1a	Induced female-to-male sex reversal in mosaic F0 mutants; increased expression of dmrt1 and cyp11b2 and decreased oestrogen production	Li et al. (2013)
Southern catfish (*Silurus meridionalis*)	Reproduction	*cyp26a1*	Cytochrome P450 26a1	Initiation of germ cell meiosis in the testis	Li et al. (2016)

Tilapia (*Oreochromis niloticus*)	Folliculogenesis	*sf-1*	Steroidogenic factor-1	Female-to-male sex reversal in some F0 XX females (due to gonadal dysgenesis) and most heterozygous F1 XX females; fertile males	Xie et al. (2016)
Zebrafish (*Danio rerio*)	Folliculogenesis	*nERs*, *namely*, *esr1*, *esr2a*, *esr2b*	Nuclear oestrogen receptors, namely, oestrogen receptor 1, oestrogen receptor 2 alpha, oestrogen receptor 2 beta	No significant phenotype in any single mutant; arrest of folliculogenesis at PV stage in females, through female to male sex reversal	Lu et al. (2017)
Zebrafish (*Danio rerio*)	Pigmentation	*Tyr*, *slc45a2/alb*	Tyrosinase, solute carrier 45 a2	Varying degrees of pigment loss in F0 larvae	Irion et al. (2014)
Atlantic salmon (*Salmo salar*)	Pigmentation	*tyr and slc45a2*	Tyrosinase, solute carrier 45 a2	Varying degrees of pigment loss in F0 mosaic founders	Edvardsen et al. (2014)
Northeast Chinese lamprey (*Lethenteron morii*)	Pigmentation	*slc24a5*	Solute carrier 45 a2	Loss of pigmentation in the skin and retina in F0 founders	Zu et al. (2016)
Tilapia (*Oreochromis niloticus*)	Spermatogenesis	*dmrt6*	Double sex and mab-3-related transcription factor 1	Delayed 11-KT production and spermatogenesis	Zhang et al. (2014)
Zebrafish (*Danio rerio*)	Survival and growth	*akt2*	Protein kinase B	Significant phenotypes in F0 mosaic mutants	Zhang et al. (2017a)
Zebrafish (*Danio rerio*)	Cranial vasculature development	*gspt1l*	G1 to S phase transition 1	Significant phenotypes in F0 mosaic mutants	Wang et al. (2017)
Rohu (*Labeo rohita*)	Immunity	*tlr22*	Toll-like receptor 22	Enhanced innate immunity	Chakrapani et al. (2016)

(continued)

Table 6.3 (continued)

Target fish species	Trait	Target genes	Encoded protein	Results	References
Atlantic killifish (*Fundulus heteroclitus*)	Model for monitoring AHPs	*ahr2a and ahr2b*	Aryl hydrocarbon receptor 2	Single and double homozygous mutants useful in monitoring AHPs in marine environments	Aluru et al. (2015)
Zebrafish (*Danio rerio*)	Disease model	*stxbp1a, stxbp1b*	Syntaxin-binding protein 1	Mutants displayed typical symptoms of human epilepsies for studying disease mechanisms and developing therapy	Grone et al. (2016)
Zebrafish (*Danio rerio*)	Disease model	*atp6v1h*	ATPase H+ transporting V1 subunit H	Exhibited phenotypes similar to human osteoporosis	Zhang et al. (2017b)
Common carp (*Cyprinus carpio*)	Inhibitor of skeletal muscle growth	*mstnba*	Myostatin	Increased muscles in F0 founders	Zhong et al. (2016)
Zebrafish (*Danio rerio*)	Stress tolerance	*fih*	Factor inhibiting HIF	Increased hypoxia tolerance	Cai et al. (2018)
Tilapia (*Oreochromis niloticus*)	Sex differentiation	amhy amhrII	Y-linked anti-Mullerian hormone, anti-Mullerian hormone receptor type 2	Male to female sex reversal in F0, F1 and F2 males	Li et al. (2015)
Tilapia (*Oreochromis niloticus*)	Sex differentiation	cyp26a1 aldh1a2	Cytochrome P450 26A1, aldehyde dehydrogenase 1 A2	Advanced and delayed meiosis in F0 males and females, respectively	Feng et al. (2015)

GHR growth hormone receptor, *HIF* hypoxia-inducible factor, *JAK-STAT* Janus kinases-signal transducer and activator of transcription proteins, *E2* estradiol, *PV* pre-vitellogenic, *AHPs* aryl hydrocarbon pollutants

major functional studies have been performed with the reproductive axis of adult fishes using recent reverse genetic editing tools. Previously, the lack of such tools had resulted in generation of no productive data on the functional importance of particularly gonadotropin-releasing hormone (GnRH) in fish reproduction. The GnRH has a major extensive role in the neuroendocrine regulation of secretion of gonadotropins by the hypothalamic-pituitary-gonadal (HPG) axis in chiefly effecting reproduction in fishes.

CRISPR-Cas9 technology has been largely employed in a non-target organism Nile tilapia (*Oreochromis niloticus*), known for its stable XX/XY sex determination system, for further understanding of the genetic basis of sex determination. The technique brought about the targeted disruption of endogenous genes, namely, nanos2, nanos3, dmrt1 and foxl2, to produce loss-of-function mutants with an efficiency up to 95%. The study concluded the successful generation of rapid and heritable mutations in the selected genes in comparison to those previously produced and reported by TALENs.

6.3.2 Functional Studies in Growth

Growth-associated traits in several groups of fishes have been incorporated by carrying out gene editing in the myostatin gene, as evident in channel catfish and common carp. The myogenic proliferation has also been negatively regulated in some species. Besides, somatic growth has been achieved via the transfer of growth hormone (GH) (Ye et al. 2015) or by the identification of regulators that negatively control the GH receptor via the JAK-STAT pathway (Dai et al. 2015). Further, tilapia and yellow catfish were also shown to exhibit sexual dimorphism in growth performance through the breeding of mono-sex or sex-controlled strains (Mei and Gui 2015). Herein, knockout of dnd gene, known to encode germplasm components in Atlantic salmon and zebrafish, was performed for differential editing of genes (Wargelius et al. 2016).

6.3.3 Functional Studies in Sterility

CRISPR/Cas9 has been applied in the production of sterile animals in aquaculture. This becomes desirable to minimize the rates of introgressive hybridization and repeated backcrossing with the wild stock or parent generation and also to avoid the negative impacts of early maturation in young ones. Consequently, this method has been recently used to induce sterility in Atlantic salmon and catfish.

Sterility in salmon species is envisaged as a process to prevent disease transmission between farmed and wild populations as well as the escape of fishes in open sea cages that tends to affect the gene pool. These problems were till now tackled through triploidization which involved the creation of infertile triploid species. However, the resultant species exhibited intolerance to suboptimal rearing conditions and susceptibility to deformities. Thus, their production was replaced

with the inclusion of CRISPR Cas9 technique to generate dead end (dnd) knockdown sterile counterparts. These candidates overcame the possibility of negative pleiotropic effects such as reduction in growth and flesh quality and higher susceptibility to diseases, associated with early maturation of the farmed species. This was attributed to subjection to targeted mutagenesis to inhibit the formation of germ cells by the dnd gene. Further extension to the development of knock-in mutations is desirable for an inducible on-off system for sterility as already developed for medaka and zebrafish.

6.3.4 Functional Studies in Immunity and Disease Resistance

The establishment of immunity and disease resistance as target traits for aquaculture emerged with the development of carp models using CRISPR-Cas9 genome editing for studying fundamental immunology. In this context, the targeted disruption of TLR22 gene resulted in the elucidation of the host response to pathogenic invasions. In addition, improved cell lines were also developed for the production of vaccines by knocking out key components of the interferon pathway.

Disease resistance traits have basically been attempted in fishes mainly using molecular genetic markers. The resultant marker-assisted selection and QTL studies led to the identification of loci of large effect that correlates with the actual quantitative polygenic trait variation.

6.3.5 Functional Studies in Development

Developmental genes in fishes have been targeted using the novel genome editing tools mainly to outperform MO- or RNAi-mediated gene knockdown for performing reverse genetic functional studies.

6.3.6 Functional Studies in Pigmentation

Pigmentation refers to the production of melanin in melanocytes (Cichorek et al. 2013). The genes involving pigmentation have been performed in a range of fishes, namely, zebrafish, Atlantic salmon, lamprey and cavefish. The choice of these genes is attributed to their ease in phenotype analysis using different gene editing protocols. Few genes that have been analysed in this relation include the golden gene (slc24a5) and tyrosinase (tyr).

6.3.7 Functional Studies in Metabolism

Functional studies in metabolism generally take into account the editing of genes to improve the flesh quality. Among the various traits responsible for defining the

quality of flesh, omega-3 metabolism plays a crucial role. Others include the combination of traits like appearance, taste, smell, firmness, juiciness, process characteristics and lack of intermuscular bones.

6.4 Applications

The practicality of genome editing lies in its varied applications in aquaculture. This potential relates to the following:

1. Delivery to large-sized externally fertilized embryos via microinjection.
2. Regulation of background genetic effects to assure successful editing in sibling species by comparison between large nuclear families of ample sample size.
3. Feasibility of extensive "phenotyping" for assessing the resistance to disease at earlier stages by means of disease challenge models. For example, the variation at a major quantitative trait locus (QTL) in Atlantic salmon was studied as a part of disease resistance to infectious pancreatic necrosis virus (IPNV).
4. Selective breeding programmes for the creation, identification and dissemination of improved germplasm containing favourable alleles for a particular trait.

6.5 Future Aspects

Genome editing approaches can be expanded in the future to accommodate more systematic studies that could possibly delineate the phenomena, processes as well as pathways involved in the understanding of key economical traits such as sex differentiation, body growth, intermuscular bone development, etc. Similarly, desirable characteristics could be designed for farmed fishes like teleosts to enhance their reproductive behaviour. Moreover, sterility technology in aquaculturable species may result in the reduction of escapes in interbreeding while ensuring the progress of the edited genes to the wild stock. Also, the approach offers to address global crisis in aquaculture posed by diseases and environmental stressors. Further advances for the designing of effective species-specific target guide RNAs in accordance with the available species-specific reference genome are warranted.

The future of genome editing in aquaculture also demands the integration of editing technologies with the commercial breeding programmes by careful management of genetic diversity, mass delivery of CRISPR Cas9 complex to entire broodstock population and targeting of multiple causative alleles for a trait or multiple traits at a given time. This would hence ensure the designing of farmed fishes exhibiting a combination of diverse beneficial traits.

Most importantly, since genome editing is accompanied by genome compensation which involves the masking of the edited phenotypes, large-scale screening is warranted in the future to aid in the in-depth understanding of sequence variations and functional dynamics in a gene and their relationships.

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Applications of Fish Cell Cultures

7

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Abstract

Cell culture serves as a reliable and proficient tool in diverse research fields such as virology, physiology, toxicology, immunology, oncology, genetics, and pharmacology. These systems can be employed for pathogen detection, confirmation, and characterization especially of viruses. It is also applicable in the case of intracellular bacteria, myxosporean or microsporean parasites as well. Fish cell cultures have gained more popularity in recent years and have prominent roles in viral disease diagnosis. Since treatment options are limited for many viral diseases, early disease diagnosis and proactive management measures are key for successful fish health management. The ability to propagate fish viruses in vitro using cell cultures is imperative in advancing research on viruses and to facilitate disease management strategies such as vaccines and antiviral agents. Moreover, potential host range of pathogens via susceptibility to cell cultures, virus-host cell interactions, and virus localization studies using cell cultures provide a better understanding of the viral pathogenesis. Availability of suitable fish cell cultures for propagation of viruses and disease diagnosis is very limited, which is a major concern in this area. The wide array of applications exemplifies the versatility, cost-effectiveness, and high potential of fish cell cultures in various research fields. The recent swift growth observed in research employing cell cultures is definitely an outcome of the progress in this sector and also due to increasing ethical demands for reduction and replacement of animal use in research. In the near future, innovations in 3D cell culture and CRISPER-Cas9

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genome editing will further enhance the research prospects of fish cell culture systems.

Keywords

Cell culture · Primary · Established · Intensive aquaculture · Fish health management

7.1 Introduction

Cell culture refers to the in vitro culture of cells in a controlled environment (Carrel and Burrows 1911; Lynn 2009; Sykes and Rankin 2014). Since cells in culture mimic the host animal in vivo, these systems can either replace or reduce the number of animals used in research; thereby taking into account the three "Rs"—replacement, reduction, and refinement (Russell and Burch 1959; Balls et al. 1983; Vertrees et al. 2008). In addition to being employed in various life sciences fields, cell cultures are utilized for the mass production of viruses, vaccines, hormones, clotting factors, antibodies, enzymes, and growth factors (Capstick et al. 1962; Beale 1981; Bibila et al. 1994; Segner 1998; Merten 2006; Lovitt et al. 2014; Maguire 2016; Zhang et al. 2017; Verma et al. 2020).

Preference for cell cultures in research is mainly attributed to the convenience in using these systems. Also, cells can be genetically manipulated, quantified, and characterized using molecular techniques and can be cryopreserved and revived for future applications (Mazur 1984; Thangaraj et al. 2018). In addition to reducing the number of animal studies and their associated costs, this approach also alleviates many of the ethical concerns associated with animal use in research. This is corroborated by recent market trends suggesting the animal activist groups, environmental agencies, and cosmetic industry are assertive in reducing animal testing and finding alternate in vitro models, and toward this effort, place restrictions on the sale of products where the ingredients were tested on animals.

The two distinct types of cell cultures in use are primary and established cultures. In primary cultures, cells are isolated directly from the host tissue. These non-transformed and non-immortalized cells are biologically and physiologically closer to the host and thereby serve as an appropriate in vivo model (Uysal et al. 2018). When compared to primary cells, established cell lines are cancerous and might have lost normal host physiological properties (Luginbuhl and Black 1961; Chacon et al. 1997; Ulrich and Pour 2001; Alge et al. 2006; Pan et al. 2009; Kaur and Dufour 2012; Uysal et al. 2018).

However, established cell lines are more commonly used in research due to ease of handling, immortal nature, and indefinite culture possibilities. Serial passaging is known to cause genotypic and phenotypic variations in cell lines. Cells that do not represent the original host tissue could result in false-negative or false-positive findings. For these reasons, established cell cultures are less preferred as a biologically relevant option.

Progress in cell culture technology and increasing ethical pressures for reduction and replacement of animals in research are accelerating the rapid growth in this field (Allen et al. 2005). Study of biochemical pathways, host-pathogen interactions, and pathogen-mediated cellular responses can be facilitated by cell cultures functioning as model systems (Verma et al. 2020). Besides, cell cultures are used to evaluate several bacterial and fungal toxins and for screening and discovery of antiviral agents (Quiot et al. 1985; Fryer and Lannan 1996; McIntosh et al. 1997; Ku et al. 2009; Marecki et al. 2019).

The diverse applications of cell cultures include providing excellent model systems for studying the normal physiology and biochemistry of cells, the effects of drugs and toxic compounds on cells, and aberrations of cell functions in cancerous cells. It is also used in drug screening and development and large-scale manufacturing of biological compounds. The major advantage of using cell cultures for any of these applications is the consistency and reproducibility of results obtained from using a batch of homogenous cells.

Cell culture has proven to be an effective biological alternative to the use of animals in research and demonstrates great promise in advancing biomedical research, particularly in the fields of virology, drug discovery, cancer biology, and regenerative medicine. This review will discuss some of the key applications, contemporary research progress, and future potentials of cell cultures in diverse research fields.

7.2 Fish Cell Cultures

Fish cell cultures have proven to be a successful alternative to the use of animals in research (Kelly et al. 1978; Nicholson 1989; Sommerset et al. 2005). In addition to satisfying the social and ethical concerns regarding animal use for research purposes, fish cell cultures offer multiple advantages such as easy dosing of drugs, reduced waste production, and reproducible and rapid test results in a cost-efficient manner (Wolf and Ahne 1982; Babich et al. 1991; Bols and Lee 1991; Arango et al. 2013; Verma et al. 2020). Also, fish cells can be cultured in a wide range of incubation temperatures and osmolarity conditions and are easily adapted to bicarbonate buffered media all with minimal requirement of standardized equipment (Leibovitz 1963; Wolf and Quimby 1976a, b; Lannan 1994; Pandey 2013). Similarities in blood plasma constituents and physiological functions offer similar culture methodology of terrestrial mammals.

Typically, fish cell cultures have been applied to cell biology studies where the specific tissue or organ giving rise to the cell line was not relevant. This has often been the case for studies in virology, radiation biology, thermal biology, and toxicology (Hightower and Renfro 1988; Nicholson 1989; Babich and Borenfreund 1991). Comparatively, development of mammalian cell lines with specific functions has led to significant understanding of mammalian biology at the cellular level (McKeehan et al. 1990). Similarly, fish cell lines would be invaluable in advancing basic knowledge in endocrinology, physiology, and immunology in addition to

providing practical information that could be used to enhance the health, growth, and reproduction of fish (Bols and Lee 1991).

The popularity of fish cell lines in various research fields is due to their ease of generation and maintenance. Since fish species represent 48% of invertebrates, it can provide plenty of resources for developing cell models to facilitate research (Altman and Dittmer 1972). However, the absence of functional in vitro models is still a huge concern in fish research field. These systems are urgently needed to support disease diagnosis, vaccine development, and antiviral agent studies. Though there are more than 30,000 species of fish, the fish cell culture field remains largely unexplored. Only around 300 established fish cell lines of freshwater and marine origin are currently available to researchers on a global scale. Considering the large diversity of fish, there is untapped potential for the development of cell cultures from various fish species and tissues which would allow for the study of species-specific as well as tissue-specific responses of cells toward different etiologic agents. A list of fish cell lines available from ATCC (American Type Culture Collection) and ECACC (The European Collection of Authenticated Cell Cultures) including the species of fish and tissue of origin is provided (Table 7.1).

7.3 Applications of Fish Cell Cultures in Diverse Research Fields

Since the development of the very first fish cell line from rainbow trout gonad (RTG2), several fish cell lines have been developed from different fish species, later to be employed in diverse research fields including virology, immunology, toxicology, endocrinology, biomedical research, disease control, biotechnology, and radiation biology (Wolf and Quimby 1962; Officer 1964; Wolf 1988; Hightower and Renfro 1988; Babich and Borenfreund 1991; Bols and Lee 1991; Villena 2003; Schirmer 2006; Ryan et al. 2008, 2009). The potential utility of cell cultures in genetic engineering studies offers good prospects for studying functional genomics related to various fish diseases (Collet et al. 2018).

Virus disease outbreaks in farmed fish and related mass mortalities stimulated the development of fish cell lines. Clem et al. (1961) initiated the first marine fish cell line from the fins of blue striped grunt, *Haemulon flavolineatum* (GF1). By 2010, around 300 fish cell lines were established (Lakra et al. 2011). The first established cell line from channel catfish (*Ictalurus punctatus*) was an ovary cell line that has been in use for about four decades in the diagnosis of catfish viruses (Bowser and Plumb 1980). Leukocyte cell lines, including monocyte-like cell lines (Vallejo et al. 1991) and B cell lines (Miller et al. 1994), have been developed and used to demonstrate immune functions in catfish. Compared with the ovary and leukocyte cell lines, fibroblast cells can be sampled at embryonic stages and provide material for rapid and early genetic screening. The CCf (channel catfish fin) cell line has been used in genetic studies including replication banding in which cultured cells provided control of timing for blockage and release of DNA synthesis and convenience for subsequent removal of chemicals and rinsing (Zhang et al. 1998). The

Table 7.1 List of commercially available established fish cell lines from the cell repositories, American Type Culture Collection (ATCC), and European Collection of Authenticated Cell Cultures (ECACC). Species of origin, family, tissue of origin, and morphology of commercially available cell lines are provided (Driever and Rangini 1993; Ghosh and Collodi 1994; Paw and Zon 1999). This is not an exhaustive list

Cell line	Species of origin	Family	Tissue of origin	Morphology/ cell type
ZF4 (ATCC® CRL-2050™)	*Danio rerio* (zebra fish)	*Cyprinidae*	Embryo	Fibroblast
ZEM2S (ATCC® CRL-2147™)	*Danio rerio* (zebra fish)	*Cyprinidae*	Embryo	Fibroblast
AB.9 (ATCC® CRL-2298™)	*Danio rerio* (zebrafish)	*Cyprinidae*	Caudal fin	Fibroblast
SJD.1 (ATCC® CRL-2296™)	*Danio rerio* (zebrafish)	*Cyprinidae*	Caudal fin	Fibroblast
RTgill-W1 (ATCC® CRL-2523™)	*Oncorhynchus mykiss* (rainbow trout)	*Salmonidae*	Gill	Epithelial
RTH-149 (ATCC® CRL-1710)	*Oncorhynchus mykiss* (rainbow trout)	*Salmonidae*	Liver hepatoma	Epithelial
PLHC-1 (ATCC® CRL-2406)	*Poeciliopsis lucida* (live bearer)	*Poeciliidae*	Liver hepatoma	Epithelial
G1B (ATCC® CRL-2536™)	*Clarias batrachus* (walking catfish)	*Clariidae*	Gill	Pleomorphic
RTG-2 (ATCC® CCL-55™)	*Oncorhynchus mykiss* (rainbow trout)	*Salmonidae*	Gonad	Fibroblast
BF-2 (ATCC® CCL-91™)	*Lepomis macrochirus* (bluegill)	*Centrarchidae*	Caudal trunk	Fibroblast
FHM (ATCC® CCL-42™)	*Pimephales promelas* (fathead minnow)	*Cyprinidae*	connective tissue and muscle	Epithelial
G14D (ATCC® CRL-2760)	*Ictalurus punctatus* (channel catfish)	*Ictaluridae*	Peripheral blood	T lymphocyte
3B11 (ATCC®CRL-2757)	*Ictalurus punctatus* (channel catfish)	*Ictaluridae*	Peripheral blood	B lymphoblast
28S.3 (ATCC® CRL-2758)	*Ictalurus punctatus* (channel catfish)	*Ictaluridae*	Peripheral blood	T lymphoblast
42TA (ATCC® RL-2759)	*Ictalurus punctatus* (channel catfish)	*Ictaluridae*	Peripheral blood	macrophage
1G8 (CRL-2756)	*Ictalurus punctatus* (channel catfish)	*Ictaluridae*	Peripheral blood	B lymphoblast

(continued)

Table 7.1 (continued)

Cell line	Species of origin	Family	Tissue of origin	Morphology/ cell type
EPC [*Epithelioma Papulosum Cyprini*] (ATCC® CRL-2872™)	*Pimephales promelas* (fathead minnow)	*Cyprinidae*	Skin	Epithelial
BB (ATCC® CCL-59)	*Ictalurus nebulosus (Brown bullhead)*	*Ictaluridae*	Muscle; Connective tissue	Fibroblast
SOB-15 (ATCC® CRL-2301)	*Oncorhynchus mykiss* (rainbow trout)	*Salmonidae*	Liver; Epithelium	Epithelial
WBE (ATCC® CRL-2773)	*Morone chrysops (white bass)*	*Moronidae*	Embryo	Epithelial
OmB (ATCC®CRL-3481)	*Oreochromis mossambicus* (Tilapia)	*Cichlidae*	Brain	Fibroblast like
RTG-P1 (ATCC®CRL-2829)	*Oncorhynchus mykiss* (rainbow trout)	*Salmonidae*	Gonad	Fibroblast
ASK (CRL-2747)	*Salmon salar* (Atlantic salmon)	*Salmonidae*	Kidney	Epithelial
CCO (ECACC 95060212)	*Ictalurus punctatus* (channel catfish)	*Ictaluridae*	Ovary	Fibroblast
CSE-119 (ECACC95122019)	*Oncorhynchus kisutch* (Coho salmon)	*Salmonidae*	Unknown	Fibroblast
E11 (ECACC 01110916)	*Channa striatus* (Snakehead fish)	*Channidae*	Whole fry tissue	Fibroblast
CHH-1 (ECACC 92110412)	*Oncorhynchus tshawytscha* (Chinook salmon)	*Salmonidae*	Heart	Not available
SSN-1 (ECACC96082808)	*Channa striatus* (striped snakehead)	*Channidae*	Fry	Fibroblast
CHSE/F (ECACC00021714)	*Lepomis macrochirus* (bluegill)	*Centrarchidae*	Embryo	Fibroblast-like
KF1 (ECACC 10072801)	*Cyprinus rubrofuscus)* (Koi carp)	*Cyprinidae*	Fin	Fibroblast
CCB (ECACC 10072802)	*Cyprinus carpio carpio* (Common carp)	*Cyprinidae*	Brain	Squamous
SSE-5 (ECACC 95122021)	*Oncorynchus nerka* (Sockeye Salmon)	*Salmonidae*	Embryo	Epithelial-like
CHSE-214 (ECACC 91041114)	*Oncorhynchus tshawytscha* (Chinook salmon)	*Salmonidae*	Embryo	–

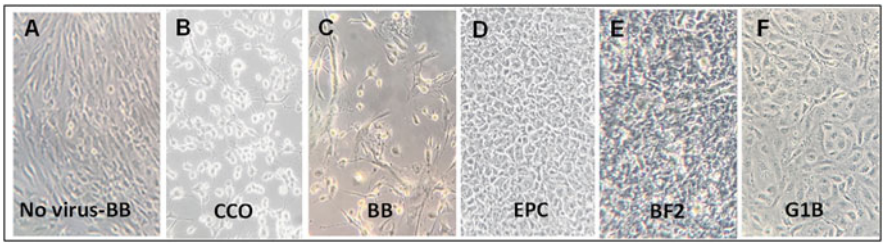

Fig. 7.1 The blue catfish alloherpesvirus (BCAHV) was inoculated onto various fish cell lines from different families to determine the potential host range and host-specificity of the virus. The CCO and BB from family Ictaluridae (**b-c**), EPC from Cyprinidae (**d**), BF2 from Centrarchidae (**e**), and G1B from Clariidae (**f**) were used. Panel (**a**) indicates control BB cells without any BCAHV. Virus replication and exhibition of CPE were restricted to cell lines from family Ictaluridae (CCO and BB) indicating the host preference of BCAHV. The CPEs primarily involved rounding of cells, syncytia (cell clumping) formation, and dissociation from culture surface (Dharan et al. 2021. Accepted (JWAS 10.1111/jwas.1285010.1111/jwas.12850))

CCf cell line was used to prepare chromosomes for study of sister chromatid exchange (Zhang et al. 1998). Another use of this cell line included providing a model for in vitro expression of transgenes for channel catfish.

Cell lines are used as research tools in virology to diagnose viral infections by observing characteristic cytopathic effects (CPEs), to isolate and propagate the viruses, and to develop attenuated or inactivated vaccines (Fig. 7.1). Cell culture-based assays also aid in the screening and discovery of novel antiviral agents (Fig. 7.2; Marecki et al. 2019). Researchers prefer fish cell cultures over that of mammalian origin, since most of the cell cultures from fish consist of normal cells which retain parental characteristics as opposed to the tumorigenic origin of mammalian cell lines that deviate from parental characteristics.

The fish cell technology still needs a lot of improvements, and its future will see diverse applications in molecular biology especially in gene editing, production of recombinant proteins, and regenerative therapies. Fish cell cultures are increasingly being applied to studies of fish, which, as the largest and most diverse group of vertebrates, are important model systems in embryology, neurobiology, endocrinology, and environmental biology. So far, fish cell lines have been an underutilized research resource, and this situation will hopefully change in near future.

7.3.1 Fish Cell Cultures in Virology

Being obligate intracellular parasites, viruses require host cell machinery for replication and propagation. Fish cell cultures can function as an effective replacement for animals in research and are more commonly used in the field of virology (Kelly et al. 1978; Nicholson 1989; Ott 2004; Sommerset et al. 2005). Cell cultures are considered “the gold standard” due to their diverse roles in virus disease diagnosis such as the detection, identification, propagation, isolation, confirmation, and

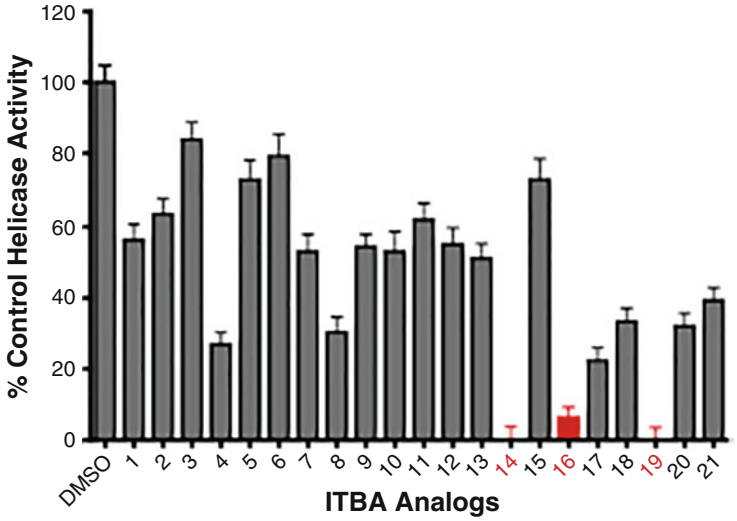

Fig. 7.2 A series of indole thio-barbituric acid (ITBA) analogs were screened to identify three analogs (red) that efficiently inhibited hepatitis C virus helicase. The IC_{50} values for hepatitis C virus helicase activity were found to be between 21 and 24 μM (Marecki et al. 2019)

characterization of viruses (Hsiung 1984; Leland and Ginocchio 2007; Jabbour and Snyder 2014).

Since it is not always practical to obtain viruses from infected animals for research purposes including purification, crystallization, cellular localization, genome sequencing, $TCID_{50}$ determination for animal infectivity studies, etc., cell cultures are considered as a reliable source (Wolf and Darlington 1971; Bowser and Plumb 1980). The OIE (Office International des Epizooties) protocols require the use of cell cultures in viral disease diagnosis and confirmation of viruses in addition to the molecular identification assays. While molecular identification only confirms the presence of the virus nucleic acids or proteins, the viability of virus can only be confirmed by growing in host cells.

Propagation of viruses in cell cultures for subsequent studies has started since 1913 (Stinehardt et al. 1913). Fish cell cultures are a reliable and efficient tool to study emergent and emerging fish viruses and to develop effective management strategies. The ability to isolate fish viruses in vitro using cell cultures is imperative in advancing research on viral diseases. Several viruses (catfish herpesviruses, tilapia lake virus, Largemouth bass virus, Koi herpesvirus, Golden shiner virus) severely impact aquaculture (Fijan 1968; Wolf and Quimby 1969; Plumb 1986; Hightower and Renfro 1988; Nicholson 1989; Waltzek et al. 2009; Davison et al. 2009; Hanson et al. 2011; Hellberg et al. 2016). The viral etiology of the fish pandemics caused by infectious pancreatic necrosis (IPN) and infectious hematopoietic necrosis (IHN) viruses were studied in detail only after the development of appropriate fish cell lines (Kelly et al. 1978; Sommerset et al. 2005). Detailed information on the infectious

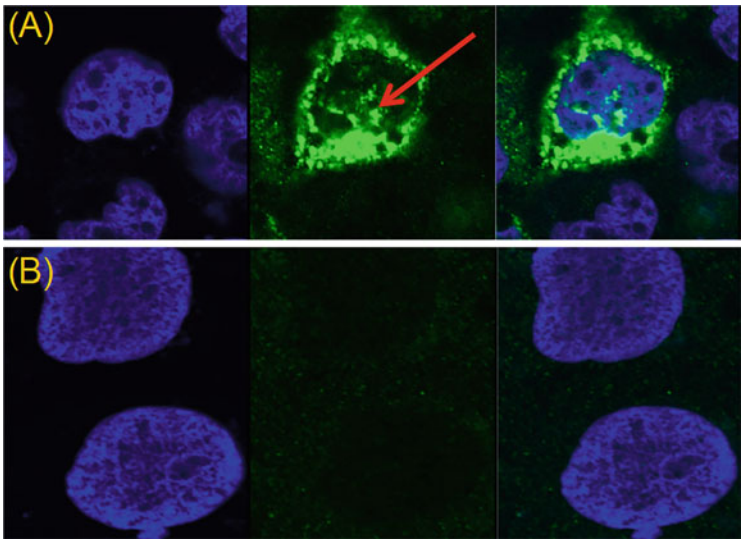

Fig. 7.3 Immunofluorescence (IF) assay to observe localization of virus enzymes in hepatoma cells. Panel 1 shows nucleus stained with DAPI (blue); panel 2 shows viral helicase detected using GFP (green) conjugated anti-mouse secondary antibody; and panel 3 is a merged view of panel 1 and 2. (**a**) Cells with virus indicating perinuclear localization and translocation of viral enzymes to the nucleus (red arrow) (**b**) cells without virus showing reduced/no staining (Aarattuthodi et al., unpublished observations)

agents will facilitate better management strategies for intensive fish production systems to enhance production efficiency and improve animal health and thereby impact the profitability of the aquaculture industry. In the case of emerging viruses, where information is limited, it is vital to understand these pathogens and preferably develop control strategies. For the fish viruses, the infectious cycle, mode of infection, pathogenicity, potential host range, and viral replication inhibition strategies need to be determined. These are critical to establish comprehensive management strategies including vaccine development and identification of effective antiviral agents. In addition, virus-host cell interactions and virus localization studies will provide a better understanding of the viral pathogenesis.

Cellular localization studies identify the position of viruses either in nucleolus or cytoplasm or both in the host cell. Cell cultures are also increasingly employed to determine cellular translocation and localization of viral proteins during acute and chronic infections and also in the screening and discovery of antiviral agents (Marecki et al. 2019). Immunohistochemistry and immunofluorescence assays were used to examine liver biopsies from patients infected by the hepatitis C virus (HCV) to determine the localization of the HCV-NS3 protein in these cells (Figs. 7.3 and 7.4) (Aarattuthodi et al. unpublished observations). The viral hemorrhagic septicemia virus (VHSV) was detected in fathead minnow (FHM) cell line by using RNA probes targeting viral transcripts from a fragment of nucleoprotein

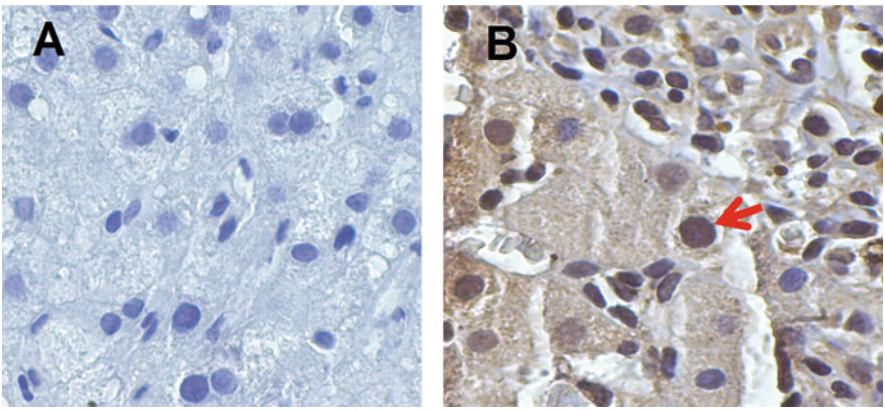

Fig. 7.4 Immunohistochemistry (IHC) to observe the cellular localization of virus. (**a**) Negative control showing liver biopsy section. (**b**) Positive staining (red arrow) for virus helicase was observed throughout the cytoplasm. Positively stained nuclei were also observed (Aarattuthodi et al., unpublished observations)

(N) and glycoprotein (G) genes. The RNA probes were able to detect viral mRNAs in formalin-fixed VHSV-infected FHM cells at different time points post inoculation.

Fulfillment of River's postulates is required to identify new and emerging viruses as the cause of idiopathic fish losses associated with suspected viral infections (Rivers 1937). This includes virus propagation in host cells and proof of filterability, which is normally achieved by inoculating virus in cell cultures and observing specific cytopathic effects (CPEs) on cell cultures. For crystallization purposes, virus in large scale using fish cell cultures can be produced that in turn would provide optimal binding sites for antiviral agents and other inhibitors.

Cell culture is widely used for the propagation of viruses as it is convenient and economic. Some of the finfish cell lines were used to study the viruses isolated from mollusks and crustaceans. For example, Akoya virus infecting pearl oysters could be cultured in eel kidney (EK-1) and EPC cell lines (Miyazaki et al. 1999). Bluegill fry (BF2) were used to propagate a reo-like virus isolated from the juvenile American oyster, *Crassostrea virginica* (Meyers 1979). The molluscan virus was able to produce CPEs within 48−72 h post inoculation in BF2 cell cultures.

Virus isolation relies on the availability of permissible cell cultures. Host-specific fish viruses such as golden ide herpesvirus, angelfish herpesvirus, and rainbow smelt herpesvirus are only visualized through electron microscopy, and a detailed virus characterization for such viruses could be delayed due to lack of specific cell cultures for their propagation (Hanson et al. 2011). Host and tissue specificity of viruses necessitate the development of cell cultures from appropriate hosts and from different tissues to represent diverse cell types (epithelial cells, fibroblast cells, etc.). For example, cyprinid herpesviruses (koi herpesvirus, goldfish hematopoietic necrosis virus, and carp pox virus), salmonid herpesvirus, acipenserid herpesvirus, and

walleye herpesvirus are highly host-specific, and most of them are refractory to nonspecific cell cultures (Hedrick et al. 2000; Hanson et al. 2011). Species-specific cell cultures are relevant to study the evolving and infectious fish viruses that affect the aquaculture industry to establish efficient and comprehensive pathogen-targeted management strategies. However, availability of suitable cell cultures for diagnosis and propagation of fish viruses is very limited. Scarcity of host-specific cell lines forces researchers to rely on general fish cell lines that might not be conducive for the growth and replication of uncharacterized viruses. The unavailability of suitable fish cell cultures hinders investigations on newly emerging unknown viruses (Bang 1960; Baron et al. 1996; Pandey 2013).

7.3.2 Development of Vaccines

Infectious pathogens especially viruses cause substantial production and economic losses in intensive aquaculture (Aarattuthodiyil and Dharan 2019). Lack of effective therapeutic options and practical vaccines exacerbate the situation. Since some of these disease outbreaks have the potential to jeopardize farm operations and or entirely wipe out production facilities, preventative strategies are increasingly important.

Vaccination-based preventive strategies are reported to be more effective in reducing the viral outbreaks in aquaculture (Hedrick and McDowell 1987; Dixon 1988; Hayman and Lobb 1993; Nusbaum et al. 2002; Ronen et al. 2003; Wise et al. 2015). Vaccination helps to induce high-level neutralizing antibodies in fish. In tilapia, channel catfish, Atlantic salmon, and sea bream increased antibody titers are observed in immunized fish (Mor and Avtalion 1990; Olsen et al. 1997; Hanif et al. 2005).

Since viruses are obligate intracellular parasites, cell cultures allow their propagation for the subsequent development of attenuated or inactivated vaccines (Wolf 1988; Hetrick and Hedrick 1993; Hanif et al. 2005; Sommerset et al. 2005; Ma et al. 2019). Attenuated viral vaccines can be developed by repeatedly passing the wild-type virus through appropriate fish cell cultures, which has proven to weaken the virus (Noga and Hartmann 1981). The long processing time required with egg-vaccine production (9 months) is a main factor that stimulated the use of cell cultures for vaccine production. Attenuation of the virus using cell culture systems avoid undesired recombination, complementation, and reversion to a pathogenic virus as evidenced in previously published studies.

In the wake of emergent and emerging fish viruses that severely impact economically relevant fish species, it is critical to establish proactive management strategies such as vaccines that are far more effective in controlling these pathogens and will be crucial for successful hatchery and farm operations. Cell cultures allow for large-scale production of vaccines. The most widely used koi herpesvirus vaccine was developed through repeated passaging of wild-type koi herpes virus in koi fin cell culture (Ronen et al. 2003). Noga and Hartmann (1981) developed three different cell lines from kidney, gill, and gonad tissues of adult walking catfish, *Clarias*

batrachus. A live-attenuated channel catfish virus (CCV) vaccine was produced by serial passaging of wild-type CCV in *Clarias batrachus* kidney cell line and was found to exhibit cross protection against CCV infection in catfish fingerlings. Catfish immunized against CCV using attenuated vaccines are protected from virus exposure (Hedrick and McDowell 1987; Arnizaut and Hanson 2011). The exposed fish developed CCV neutralizing antibodies that can persist for over 2 years after exposure (Bowser and Munson 1986).

Similarly, a nonpathogenic, attenuated cyprinid herpesvirus (CyHV3) was isolated by serial transfer (20 passages) of the virus in Koi fin (KF-2) cells (Ronen et al. 2003; Perelberg et al. 2005). The CyHV3 vaccine protected 100% of the immunized fish in challenges with wild-type CyHV3. Protective immunity was evident from the elevation of CyHV3-specific antibody titer. The attenuated CyHV3 vaccine is widely used as a preventive measure in koi and carp farms in Israel. Back passages of the virus extracted from vaccinated fish into naïve fish indicated no reversion of the attenuated virus to the pathogenic phenotype (Dishon 2009) as in the case of gene 50 deleted CCV vaccine and the attenuated V60 CCV vaccine (Noga and Hartmann 1981).

The use of fish cell cultures in the development and large-scale production of bacterial vaccines also seem promising. *Edwardsiella ictaluri* causes enteric septicemia of catfish (ESC), which is an economically devastating bacterial disease in the catfish industry. An orally delivered, live-attenuated *E. ictaluri* vaccine is developed and is found to be efficient against several field isolates (Wise et al. 2015; Aarattuthodiyil et al. 2020). Since *E. ictaluri* is an intracellular bacterium, vaccine development could be attempted using susceptible cell cultures, which would provide a consistent source of the vaccine. Similar approach could be utilized for other infectious bacteria and parasites.

7.3.2.1 Screening and Identification of Antiviral Agents

Screening of several antiviral agents (Indo-thiol Barbiturates, ITBA) identified three ITBA analogs that inhibit hepatitis C virus (HCV) enzymatic activities (Marecki et al. 2019) without killing human hepatoma cells (Fig. 7.2). These potential agents could aid in the treatment of HCV infection. Similarly, Hao et al. (2021) reported the efficiency of acyclovir, a guanosine derivative commonly used to cure herpes infection in human, against channel catfish virus infection in CCO cells. Acyclovir was capable to suppress viral DNA synthesis and replication and also to prevent CCV-induced cytopathic effects in CCO cell culture. Acyclovir was also found to exert effective antiviral activity against cyprinid herpesvirus-3 (CyHV-3) infection in common carp brain (CCB) and koi fin cells (KF-1) (Troszok et al. 2018).

Anti-CyHV3 activity of exopolysaccharides isolated from the algae *Arthrospira platensis* was evidenced by the inhibition of KHV replication in CCB cell lines even after 22 days post incubation (Reichert et al. 2017). Similarly, Balmer et al. (2017) studied the efficiency of a broad-spectrum antiviral compound LJ001 (a lipophilic thiazolidine derivative) against infectious hematopoietic necrosis virus (IHNV) using in vitro culture of EPC cell lines. The antiviral compound hinders viral entry by inhibiting virus-host cell membrane fusion. The EPC cell cultures were employed

to test the efficacy of JL122 (an analogue of LJ001) against three different fish rhabdoviruses (IHNV, VHSV, and SVCV) (Balmer et al. 2018). JL122 was able to prevent viral infection by inhibiting membrane fusion of enveloped viruses with their host cell and their subsequent entry.

An antiviral state in CHSE-214 against IPNV was induced by transfection with Polyinosinic polycytidylic acid (poly I:C). Poly I:C were able to produce interferons which can convert the cells into antiviral stage by expressing special proteins responsible for the inhibition of viral particles (Jensen et al. 2002). Similarly, Peng et al. (2010) developed a rapid immunofluorescent assay to detect potential antiviral agents. Using the method, the threshold dosage of poly I:C to induce interferon production in zebrafish cell line (ZF4) to make the cells into an antiviral stage against infectious pancreatic necrosis virus (IPNV) was determined. Fluorescent quantum dots (QDs) were hybridized with IPNV particles to serve as imaging nanoprobes. Huang and Han (2010) reported the development of a cell culture-based platform using grouper fin (GF-1) cell line for screening antiviral compounds against betanodavirus. The potential of various micromolecules to inhibit betanodavirus-induced CPEs were analyzed using MTT assays. Anti-nodaviral property of dasyscyphin C extracted from leaves of *Eclipta prostrata* was tested using in vitro culture of SIGE (Sahul Indian Grouper Eye) cell line. The sensitivity of grouper nervous necrosis virus (GNNV) to dasyscyphin was evidenced by the inhibition of virus propagation in SIGE cells (Krishnan et al. 2010). Regulation of immune relevant genes by antimicrobial peptides was studied using trout macrophage cell line RTS 11(Chiou et al. 2006). A study was done on human herpes virus to find out the interfering RNA (siRNAs) that target the specific mechanism of herpes virus posttranscriptional regulation by ORF57 and Epstein-Barr virus (EBV) SM proteins (Verma et al. 2013).

7.3.3 Toxicology Studies

Cell cultures are extensively used either alone or combined with animal trials to evaluate the effects of new drugs and chemicals and to determine the maximum permissible dosage. Especially important are liver- and kidney-derived cell cultures. The relative sensitivity of cell cultures from different fish species to drugs or contaminants at the cellular or molecular level can be studied. Fish cell cultures can be a suitable alternative for the use of animal tests to establish the potential toxicity of compounds with the benefit of avoiding high costs and variability of results. Cell culture tests are rapid, allow efficient screening of novel compounds, and sometimes can allow the identification of metabolic targets of inhibition. Cell culture tests can also be designed to evaluate various effects such as reduced growth rate, breakdown of membrane permeability, and tissue specificity of the toxicants.

The use of fish cell lines as in vitro model for environmental toxicology studies especially cytotoxicity analysis is considerable (Rachlin and Perlmutter 1968; Babich et al. 1991; Babich and Borenfreund 1991; Castaño et al. 1996; Segner 1998; Fent 2001). Fish cell lines being the most significant representative for the

aquatic environment can be used as an experimental model to study the genotoxicity of various chemicals, their mode of action (MoA), and other cellular damages. Additionally, the progress and mechanisms of aquatic toxicity could be studied using fish cell lines (Segner 1998). Fish cell lines are used as an alternative to in vivo test, revealing the mechanism of toxicity pathways, and for the screening of potential hazardous material (Rehberger et al. 2018). *Poeciliopsis lucida* hepatoma cell line (PLHC-1) and a subclone (PLHC-1/dox) were used to test the ability of 33 commonly used human drugs to moderate the activity of xenobiotic efflux transporter proteins in fish (Caminada et al. 2008). Fish cell lines derived from *Etroplus suratensis*, *Lates calcarifer*, and *Catla catla* were used to evaluate the cytotoxicity of chromium (Taju et al. 2017). The cytotoxicity of chemicals such as alkylbenzenes, phthalate diesters, pesticides (acting directly on cells), and benzopyrene (metabolism-mediated) were studied using a hepatoma cell line PLHC-1, derived from *Poeciliopsis lucida* (Babich et al. 1991).

Heavy metals, fungal toxins, and other organic and inorganic toxicants are capable of modulating an immune response in fish (Zeeman and Brindley 1981). Fish cell lines are widely used as research tools for the ranking and risk assessment of engineered nanomaterials for their cytotoxicity. Bermejo-Nogales et al. (2017) used *Poeciliopsis lucida* liver (PLHC-1) cell line and RTG-2 cell line to conduct an in vitro assay to assess the risk associated with nanomaterials derived from cerium, silicon, zinc, and carbon compounds revealing the suitability of cell lines as the best alternative to in vivo tests. The genotoxic potential of nanomaterials (NMs) was assessed with RTG-2 cells following exposure to nano-silver and nano-titanium (Klingelfus et al. 2019). Metabolism and DNA-binding mechanism of aflatoxins were tested using primary cultures of hepatocytes derived from *Salmo gairdneri* (Loveland et al. 1987).

Similarly, comet assays using fish cell lines represent a sensitive and reliable test for the detection of cellular DNA damages induced by toxic materials and complex chemicals in aquatic organisms (Leme 2019). The RTG-2, RTgill-W1, and RTL-W1 cell lines from rainbow trout (*Oncorhynchus mykiss*) and ZFL and ZF4 from zebrafish (*Danio rerio*) are most commonly used for comet assays (Zegura and Filipic 2019) to assess DNA damages arising from environmental genotoxicants, indicating their potential as environmental biomarkers (Kienzler et al. 2013). Another approach is the "neutral red dye uptake method" by which the cell viability is determined (Borenfreund and Puerner 1985). These methods help to determine the toxicological index IC_{50} (half maximal inhibitory concentration) and are useful in ranking the elements in terms of their toxicity. Environmental risk assessment procedures like toxicity studies are carried out using fish cell lines (Castano et al. 2003). Fish-derived cell lines are being used to determine the mechanism of toxicity, relative toxicity, and evaluating the toxicity of environmental samples. Furthermore, as in vivo tests often focus on apical endpoints which do not allow to discriminate the underlying mode of action, the in vitro assays could add diagnostic information regarding the mode of action of the test compound.

The in vitro effect of lindane (organochlorine insecticide) on cell phagocytosis against *Yersinia ruckerii* was tested using macrophages derived from kidney and

spleen of *C. carpio* (Cossarini-Dunier 1987). Similarly, the effect of lindane on the replication of spring viremia of carp virus was tested in EPC cell lines (Cossarini-Dunier and Hattenberger 1988). Also, the effect of polycyclic aromatic hydrocarbons in inducing cytochrome P4501A enzyme activity was determined using rainbow trout liver cell line RTL-W1 and primary hepatocytes (Behrens et al. 2001). EK cell line derived from kidney of European eel, *Anguilla anguilla*, was found to upregulate interferons after stimulation with polyinosinic polycytidylic acid, poly (I:C) suggesting their potential for use in aquatic toxicological studies (Chen et al. 2019).

The growing concern over possible toxicological consequences of environmental hormonally active substances has caused the development of methods for reliable and specific detection and characterization of endocrine active chemicals. Induction of vitellogenin (VTG) in fish is used as a biomarker for exposure to estrogen-active substances (Sumpter and Jobling 1995) and has been utilized for chemical screening and environmental monitoring (Purdom et al. 1994; Knudsen et al. 1997; Thorpe et al. 2009; Burki et al. 2006). Since VTG induction can be measured in fish hepatocytes in vitro, the use of VTG induction response in isolated fish liver cells has been suggested as in vitro screen for identifying estrogenic-active substances (e.g., Pelissero et al. 1993; Smeets et al. 1999; Segner and Braunbeck 2003). Compared to other in vitro screening assays, the hepatocyte VTG assay has the potential to detect the effects of estrogenic metabolites, since the hepatocytes in vitro remain metabolically competent, and to detect antiestrogenic effects. During recent years, the presence of endocrine-disrupting compounds (EDCs), which may adversely affect the hormone systems and thereby the homeostasis, development, and reproduction of exposed organisms, has attracted much attention. Aquatic wildlife appears to be particularly at risk by EDCs since watercourses are a sink for a variety of EDCs, including industrial chemicals such as bisphenol A (BPA), several pesticides with suspected endocrine activity, or estrogens excreted by humans, e.g., the natural estrogen (Ternes et al. 2002; Nakada et al. 2004; Rutishauser et al. 2004).

The use of biomarkers in ecotoxicology studies is getting more importance nowadays. Biomarkers can indicate the exposure to toxicants and possible impacts of exposure. Cell lines can be used to identify new biomarkers and to study their basic mechanism for the action of ecotoxicants like dioxin, environmental estrogens, etc. Fish cell cultures are also found sensitive to several bacterial or fungal toxins cultures (Ku et al. 2009). The extracellular products of *Aeromonas* sp. and *Vibrio anguillarum* were found to be toxic to SICH cells, a cell line derived from heart tissues of *Catla catla* (Ahmed et al. 2009). The EPC cell line derived from carp was found to be a suitable substrate for the study of intracellular antigen production and the expression of putative virulence factors produced by *Renibacterium salmoninarum*, an intracellular bacteria infecting salmonid (McIntosh et al. 1997). Rainbow trout cell lines such as RGB, RGG, and RGH are susceptible to extracellular products of *Photobacterium damselae* spp. *piscicida* (Ku et al. 2009).

7.3.4 Model Systems

The very first application of cell cultures as they were developed in the early 1900s was to study the behavior of cells in response to normal and induced stress. Since then, cell cultures have been increasingly used as model systems to study basic cell biology, cellular communications, signaling pathways, apoptosis, interactions between cells and pathogenic agents, effects of drugs, the process and triggers for aging, metabolic effects of nutritional elements, mutagenesis, and carcinogenesis. Fish cell cultures act as appropriate research models to study physiological responses without hindrance from environmental disturbances to which animals are sensitive. Another advantage of using cell culture for any of these applications is the consistency and reproducibility of results from using a batch of clonal cells. Reporter gene assays that identify genes expressed at a particular cellular event are an important part of life sciences research. Fish pigment cell lines are used to examine the formation and translocation of organelles and to study the growth, shape, and differentiation of cells (Matsumoto et al. 1983).

Cell cultures are used to study the functions of various immune cells, cytokines, lymphoid cells, as well as antitumor drug permeability and efficacy analysis, cell senescence studies, and cytokine expression profiling. Cell-based high-throughput screening is routinely supplemented with sophisticated optical assays to investigate the cellular response to stimuli such as small molecules, siRNAs, or antibodies. The complex interactions underlying disease outbreaks, host-pathogen interactions, and pathogenesis can be better revealed using cell culture methods. Consequently, models utilizing cultured cells are critical for the identification of specific molecules and/or mechanisms used in initial pathogen-host cell interactions. The macrophage cells from tilapia gill were used to study the attachment of different pathogens during infection (Saggers and Gould 1989).

Cell culture environment is selective and defined which can be easily manipulated by introducing new genetic materials into the cell and can be used to study the expression (knockdown or overexpress genes of interest) of new genes/proteins and their effect on the health of the cell. Additionally, cell cycle regulation, differentiation processes, growth stimulators, specialized cell functions, cell-cell, and cell-matrix interactions are also studied using cells in culture.

The zebrafish (*Danio rerio*) has become a widely used vertebrate model for bacterial, fungal, viral, and protozoan infections. Due to its genetic tractability, ease of manipulation, and optical transparency during early life stages, it is a particularly useful model to address questions regarding the cellular microbiology of host-microbe interactions. Howe et al. (2013) sequenced the zebrafish genome and revealed their 82% homology with functional genes involved in human diseases. This makes them a potential in vitro model to study human diseases (Miserocchi et al. 2017). Heilmann et al. (2015) studied the underlying mechanism of metastasis which was thought to be challenging under in vitro environment using cell lines derived from the melanoma tissues of transgenic zebrafish. These principles could be utilized to generate biotherapeutics from fish cell lines. In vitro culture of keratocytes from the explant cultures of scales derived from zebra fish was used to study the cell

migration (Rapanan et al. 2015). This provided a unique model for wound healing mechanism and reepithelialization in cutaneous tissues. Many fish-derived cell lines were used to explore the field of fish endocrinology (Hightower and Renfro 1988). Sertoli cell line established from a cyprinid *Gnathopogon caerulescens* was used to study hormones and endocrine-disrupting chemicals (EDCs) (Higaki et al. 2013). Chen et al. (2010) developed five single-cell clone lines from pituitary glands of adult rainbow trout which are capable of expressing genes specific for pituitary hormones such as gonadotropin 1, 2, and somatolactin.

7.3.5 Drug Screening and Development

Cell cultures are applied in the preformulation, formulation, in vivo efficiency evaluation, and dose determination of therapeutic agents/drugs (Villena 2003; Allen et al. 2005). The first phase of drug administration is done on cell lines to test the efficiency and dosage, then to the animals and finally to the humans. Fish cell cultures can potentially play an important role in research and development of drugs aimed to benefit fish and also to identify therapeutic targets such as receptors.

Cell-based assays have become increasingly important for the pharmaceutical industry, not just for cytotoxicity testing of new drugs but also for high-throughput screening of compounds that may have potential use as drugs. This is also used to find out the effective and safe dosage of new drugs. Cell culture systems could be utilized during drug delivery, vaccine design, drug safety, pharmacology, cellular targeting, intracellular delivery, pharmaceutical analysis and quality assurance, and as drug carrier systems. Cell-based screening plays an important role in the drug discovery process. Automation helps to speed up the selection of lead compounds and profiling against cellular targets.

7.3.6 Cell-Based Production of Biologicals

Cell cultures are widely utilized for the mass production of cell-derived biomolecules. Some of the commercially important genetically engineered products include interferons, blood clotting factors, monoclonal antibodies, interleukins, lymphokines, insulin, growth factors, hormones, viruses for vaccine development, enzymes, and anticancer agents.

The ability to transfect or reprogram cultured cells with new genetic material (DNA and genes) has paved way to produce several commercially important products. So far, human- or rodent-derived cells have been a major vehicle for producing biologics including vaccines and various proteins. The cost of growing mammalian cell cultures is high, so research is underway to produce such complex proteins in other animal cells. Fish cell culture systems can take up this challenge by providing high yields of good quality recombinant proteins. Fish cell cultures can act as miniature factories to express substantial quantities of commercially important proteins after being infected with genetically engineered baculoviruses.

Wang et al. (2018) developed a recombinant baculovirus system for the successful delivery of multiple genes to fish cell lines that pave the way for generating stable transgenic cells for gene manipulation studies. The recombinant baculovirus system contained two promoters, namely, cytomegalovirus (CMV) and white spot syndrome virus (WSSV) immediate-early genes, and followed by a puromycin green/red fluorescent protein that were incorporated into five different fish cell lines fin, kidney, and bladder cell lines from *Mylopharyngodon piceus*, spermatogonial cells of *Oryzias latipes*, and embryonic fibroblast cell line from *Danio rerio*. There were previous reports of transient delivery of DNA into fish cell lines using baculovirus, but none of them were stable (Wagle and Jesuthasan 2003; Yan et al. 2009). The first semi-cloned fish, Holly was developed by transferring haploid ES cells nuclei into mature oocytes (Yi et al. 2009; Hong et al. 2011). Holly was reported to show normal fertility and successful transfer of germline for three subsequent generations.

Although many simpler proteins can be produced using recombinant technology in bacterial cultures, more complex proteins that are glycosylated must be made in animal cells. While cultured cells can be used to produce many important products, three areas are generating the most interest. The first is the large-scale production of viruses for use in vaccine production. The second is the large-scale production of cells that have been genetically engineered to produce proteins that have medicinal or commercial value. The third is the use of cells as replacement tissues and organs. Artificial skin for use in treating burns and ulcers is the first commercially available product.

Also, for the production of mABs (monoclonal antibodies), in vitro methods are preferred due to the ease of culture and less economic consideration compared with the use of animals. In fact, more than 90% of the mABs is produced using in vitro methods. The ability to generate hybridomas has stimulated the use of the in vitro methods for mABs production. The in vitro produced mABs are increasingly utilized in diagnostic tests for the identification of small quantities of specific antigens.

Human cell lines are used to produce numerous FDA-approved therapeutic proteins that are used to control an array of human disorders such as hemophilia A, B, anemia, septicemia, type 2 diabetes, rheumatoid arthritis, multiple sclerosis, and rabies viral infection (Dumont et al. 2016). Similarly, some of the FDA-approved biotherapeutics are produced from non-human engineered cell lines. For example, plant cells are used for the production of the enzyme, *taliglucerase alfa*. Additionally, cervical cancer vaccine from insect cells; monoclonal antibodies, enzymes, cytokines, meningitis vaccines, collagenase, pneumococcal vaccine, and botulinum toxins from bacterial culture; monoclonal antibodies, cytokines, enzymes, hormones, and clotting factors from Chinese hamster ovary cell line are also reported. Most of these are approved in the US and European countries (Dumont et al. 2016). Similar efforts could be ventured using fish cell cultures.

7.3.7 Cancer Research

The basic difference between a normal cell and a cancerous cell can be studied using cell cultures. Normal cells can be transformed into cancer cells using radiation, chemicals, and viruses. Thus, the mechanism and cause of cancer can be studied. Cultured cancer cells can also serve as a test system to determine effective drugs to selectively destroy cancer cells. Studies on cell culture have contributed tremendously to basic research in cancer biology (Mehta et al. 2012). Cell lines are used to study the pathway of malignant progression, induction of cellular apoptosis, DNA methylation, histone modifications, tumor suppressor gene expressions, and functions of various carcinogenic chemicals.

Fish cell lines are used in cancer biology to study the mechanism of activation of procarcinogens, molecular damage, and DNA repair activity (Grist et al. 1986). Fathead minnow cells (FHM), cell lines from goldfish erythrophoromas, and goldfish fibroblast cell line RBCF-1 were used to study the mechanism and activation of procarcinogens and subsequently the damage and repair of genetic materials (Grist et al. 1986; Hightower and Renfro 1988). Primary cell cultures of rainbow trout were used for investigating the carcinogenic effects of aflatoxin B (Bailey et al. 1982).

7.3.8 Parasitology

Cell cultures were also used to study parasites infecting fish. One of the most common ectoparasite of fish, *Ichthyophthirius multifiliis*, was cultured in EPC cell line. The in vitro study supported the attachment and the transformation of various stages involved in the complete life cycle of the parasite (Nielsen and Buchmann 2000). Buchmann et al. (2000) studied the nonspecific response of epithelial cell cultures (EPC) to encapsulate and degrade the fish parasite *Gyrodactylus derjavini* without the involvement of leukocytes. Primary cultures of macrophage cells derived from the kidney tissues of Atlantic salmon and chinook salmon were used to investigate the phagocytic activity toward microsporidian parasite *Loma salmonae* (Shaw et al. 2001).

Macrophage cells derived from the head kidney of ayu were used to study the lectin-mediated phagocytosis of *Glugea plecoglossi* spores (Kim et al. 2000). Kou et al. (1995) developed a permanent cell line EP-1 from the tissues of Japanese eel elver (*Anguilla japonica*) infected with *Pleistophora anguillarum*. EP-1 was found to be persistently infected with *P. anguillarum* and was used to study the microsporidian development within host cell. Wongtavatchai et al. (1994) studied the in vitro multiplication of salmonid microsporidian parasite *Enterocytozoon salmonis* in leukocytes cells from salmonids. Culture media containing human recombinant interleukin-2 (HrIL-2) and polyclonal mitogens supported the in vitro growth of leukocyte cells and proliferation of the parasite (Wongtavatchai et al. 1994). Bedrnik and Vavra (1972) used FHM cell lines to study *Encephalitozoon cuniculi*, a mammalian parasite. Primary cultures of rainbow trout kidney were used

to study comparative development of two different microsporidians infecting AIDS patients and salmonid fish, respectively (Desportes-Livage et al. 1996).

7.3.9 Stem Cell Research

Embryonic stem (ES) cells are undifferentiated cells derived from developing embryos (Till and McCulloch 1961; Evans and Kaufman 1981; Thomson et al. 1998). These pluripotent cells are found useful in biodiversity conservation and marine biotechnology studies (Hong et al. 1996). Fish ES cell lines are used as a vector for efficient transfer of foreign DNA into the germ cells of an organism and therefore have the potential for use in biotechnology. Hong et al. (2004) developed a spermatogonial cell line from testis of adult medaka fish which was able to undergo meiosis and produce viable sperm via spermiogenesis. This finding points out the prospective to reiterate spermatogenesis using in vitro system. Especially, with the hybrid catfish (♀ channel catfish × ♂ blue catfish, *I. furcatus*) production, the blue catfish are sacrificed for sperm collection. Development of a blue catfish spermatogonial cell line could be of potential benefit to the industry. Here, gene targeting and germ cell transplantation are noteworthy, and a lot of progress is happening in these areas (Hong et al. 2011). Embryonic germ cell transplantation was successfully used for surrogate production in salmonids (Yoshizaki et al. 2010). The molecules and exosomes released by stem cell culture are harvested for the purposes of therapeutic development.

The ES cell lines have been established from medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) (Yi et al. 2009; Ciarlo and Zon 2016). Tumors can also serve as sources of ES cell lines. *Epithelioma Papulosum Cyprini* (EPC) and rainbow trout liver (RTH-149) cells are derived from epithelioma and hepatoma, respectively (Fijan et al. 1983; Lee et al. 1993). In vitro culture of cells from the blastula stage of zebra fish embryos was able to express genes introduced through transfection procedures followed in mammalian cell cultures. The observation made in this study supported the potential for alteration of genotype and phenotype of cultured cells (Collodi et al. 1992).

7.3.10 Regenerative Therapy

Cell culture systems can produce functional cells or tissue analogues in large scale that can be used as replacement tissue or organs. Mori et al. (2019) reported the development of limb buds from in vitro culture of murine pluripotent stem cells (PSCs) that is capable to differentiate into forelimb or hindlimb. This finding creates the scope for artificial tissue and organ development from cell cultures. Subsequently, research is going on artificial organ culture such as liver, kidney, and pancreas. Organ culture research is conducted using both embryonic and adult stem cell cultures as these cells can differentiate into many different types of cells and organs. Reconstitution of skin following severe burns is considered the most

successful application of cell-based regenerative therapy. In this regard, fish cell cultures are experimentally utilized for producing artificial skin to treat patients with burns and ulcers.

7.3.11 The 3D Cell Cultures

The 3D cell systems are an advanced approach to cell culture technology that replicate the animal in vivo more closely than traditional 2D culture. Studies report reduction or complete loss of some critical receptors and signaling molecules in 2D cell culture techniques (Hayward et al. 1995; Novaro et al. 2003; Pickl and Ries 2008; Yang et al. 2000). However, in 3D cell culture, cells are permitted to grow or interact with their surroundings in all three dimensions similar to in vivo conditions. The 3D spheroids of rainbow trout (*Oncorhynchus mykiss*) cell lines, RTG-2, and RTS-11 were successfully developed and tested for their efficiency to propagate *Saprolegnia parasitica* spores that resembled in vivo infection (Faber et al. 2021).

For the 3D cell culture technology, semisolid matrices (collagen or matrigel) or synthetic biomaterials (polyethylene glycol gels) are used recreating cell interactions that exist in vivo. The incorporation of extracellular matrix (ECM) components into cultures has strikingly improved the morphology, adhesion, polarity, differentiation, and gene expression in 3D cultures (Kenny et al. 2007; Pampaloni et al. 2007; Yamada and Cukierman 2007; Sung et al. 2013; Xu et al. 2013). It is speculated that these differences are because materials used in 3D cultures provide structural and chemical components that are similar to ECM found in vivo (Wolf et al. 2009).

The 3D cell culture in the form of spheroids allows growth in all three dimensions and is physiologically relevant (Desoize et al. 1998). There is evidence that organization of cells in spheroids, rather than a monolayer, better reflects the in vivo behavior of tumors (Hirschhaeuser et al. 2010; Shield et al. 2009; Weiswald et al. 2015). While there are several techniques to generate spheroids, most commonly cells are placed in a 2D or 3D environment that encourages cell-cell adhesion rather than matrix adhesion (Santini et al. 1999). With growing recognition of the importance of 3D microenvironment, spheroids also can be generated in various biomaterials to achieve spheroid-ECM interactions (Hsiao et al. 2009; Loessner et al. 2010; Ong et al. 2010).

The 3D cell cultures raise the possibilities for the study of complex physiological processes in vitro. These models are biochemically and physiologically similar to in vivo conditions and could provide a more predictive analysis that is critical in drug screening, discovery, and cancer biology (Tsuruga et al. 2008; Ballester et al. 2019). The suitability of the system is evidenced by the fact that primary hepatocytes could be maintained for 3 weeks in a 3D collagen gel matrix, which otherwise would have perished sooner (Schippers et al. 1997).

Recently, the use of primary cells in combination with 3D culture is emerging as the new gold standard to mimic cellular microenvironments more closely in vitro and to achieve improved results. These specialized models can achieve a more biologically relevant approach and provide more reliable data. Hence, there is a

need for the development of fish primary cells in 3D culture technologies, especially within the pharmaceutical drug discovery pipeline, to generate large numbers of relevant models for drug screening. The ability to use more physiologically relevant in vitro models with host-derived primary cells could accelerate the discovery and development of targeted treatments. These systems are key to the advancement of 3D cell culture in drug discovery and biomedical research.

7.3.12 CRISPR/Cas9 Genome Editing

Cell culture is used in knockout studies, where certain genes are inactivated and their effects are traced. Dehler et al. (2016) carried out the first gene editing using CRISPR/cas9 system in fish somatic cell lines. Chinook salmon embryo (CHSE-214) was genetically engineered to create CHSE-EC cell line, which is capable to express geneticin and hygromycin resistance using knockout technology. Ma et al. (2018) used CRISPR/Cas9 gene editing to knock out the DNA sequence of grass carp Junctional Adhesion Molecule-A, gcJAM-A (an immunoglobulin that acts as a reovirus receptor) in grass carp kidney cells (CIK). Successful knockout was evident from suppressed CPEs and reduced multiplication of grass carp reovirus in CIK cell culture following infection with two genotypic varieties of grass carp reovirus (GCRV). Liu et al. (2018) reported successful gene editing using gRNA-Cas9 ribonucleoprotein (RNP) complex in medaka embryonic cell lines. This method was found to have potential for gene function studies in fish cell lines with gene editing efficiency of 62% in diploid cells and nearly 50% in haploid cells as observed in medaka cell lines.

Gratacap et al. (2020) developed protocols for successful CRISPR gene editing in CHSE-214 cell line using lentivirus transduction which could be used to manipulate diseases resistance in salmonid species. Chang et al. (2013) and Hwang et al. (2013) successfully carried out genome editing with RNA-guided Cas9 nuclease in zebrafish embryos.

7.3.13 Cell-Based Fish

Cell culture systems can function as a key source to provide both new products and new ways of producing existing agricultural products like milk, cultured meat, fragrances, and rhino horn from cells and microorganisms. Therefore, it is considered as a way of achieving animal-free culture. Considering the adaptation of fish cell culture to in vitro growth conditions in terms of tolerance to hypoxia, high buffering capacity, and low temperature, an advanced approach toward the sustainability of global fishery resources is the production of cell and tissue culture-based seafood through bioreactor culture (Rubio et al. 2019; Potter et al. 2020; Miller 2020). Benjaminson et al. (2002) used tissue engineering for the in vitro culture of skeletal muscle of goldfish that resembled the fillet from a fibroblast fish cell line to use in space travel.

7.3.14 Others

Cell cultures from fish nervous tissue were used to study the fish neuronal regenerative capacity (Jacobson and Gase 1965). The fibroblast cell line from the fin tissues of the blue-striped grunt was used in radiation biology research, as the cells exhibit photoreactivation (Regan and Cook 1967). Fin cell lines from rainbow trout (RBCF) were used in cellular mechanism-based studies like heat-shock protein synthesis and thermosensitivity (Mitani et al. 1989). Subsequently, much progress in radiation research has been achieved with two fibroblast cell lines from the goldfish caudal fin: CAF-MM1 (Mano et al. 1982) and RBCF-1 (Shima et al. 1980). These cells contained high photo repair activity and little excision repair for UV-induced lesions (Mano et al. 1982; Shima et al. 1981; Shima and Setlow 1985; Mitani et al. 1990). Generally, CAF-MM1 cells were more resistant to ionizing irradiation than mammalian cells (Mitani and Egami 1980, 1982; Mitani 1984, 1986).

Radiosensitivity appeared not to be related to ploidy because primary fin cell cultures of diploid, triploid, and tetraploid goldfish showed similar sensitivity (Mitani 1986). Ryan et al. (2008) tested the adaptive responses of three different fish cell lines CHSE-214 (Chinook salmon), RTG-2 (rainbow trout), and ZEB-2J (zebrafish) to various doses of gamma radiation and found that no adaptive responses were showed by these cell lines. Mutagens can be added to cell cultures for inducing mutations. Useful mutants that are tolerant to pollutants, toxins, salts, etc. can be selected for further research.

Dietary metabolism of polyunsaturated fatty acid was studied using Atlantic salmon cell line (AS) by Ghioni et al. (1999). Likewise, the effect of dietary fatty acids from sunflower, menhaden oils on eicosanoid production, fatty acid composition, and in vitro locomotion of neutrophils were studied using leucocytes derived from head kidney of *Oncorhynchus mykiss*, rainbow trout (Ashton et al. 1994). Morin et al. (2020) used rainbow trout hepatoma cells (RTH-149) as models to reveal the underlying mechanism of amino acid regulated pathways in rainbow trout. In a recent study, Lescat et al. (2020) used fibroblast cell line from medaka fish (*Oryzias latipes*) to demonstrate that chaperone-mediated autophagy (CMA) pathway involving lysosomal proteolysis, which was thought to be present only in mammals and birds, exists in fish. This study was a breakthrough in fish metabolism and showed an insight into the evolutionary relationship of vertebrates including fish, mammals, and birds.

Fish cell cultures are employed in disease diagnosis to standardize and validate diagnostic methods (Villena 2003; Allen et al. 2005). The primary cultures of leukocytes derived from kidney tissues of *Cyprinus carpio* were used to analyze immunomodulating effect of two tetracycline derivative antibiotics such as oxytetracycline and doxycycline in different doses (Grondel et al. 1985).

Cell culture-mediated in vitro assays were used to study the innate and immune response of fish to various pathogens (Villena 2003). RTG-2 cell lines in culture were induced to produce interferon following an infection with infectious pancreatic necrosis virus (IPNV) and purified through ultracentrifugation and chromatographic techniques. The interferon produced from fish cell was able to exhibit antiviral

activity against active infection of infectious hematopoietic necrosis virus or infectious pancreatic necrosis virus (de Sena and Rio 1975). Ganassin and Bols (1998) reported the development of macrophage like cell line from the splenic tissue of rainbow trout, which was able to produce lysozyme like activity to *Micrococcus lysodeikticus* evidenced by the zone of clearance in culture plates.

Cell cultures are also used to simulate in vitro models for the screening of immunostimulants. Similar study was carried out using two different stromal cell lines from kidney and spleen tissues of rainbow trout. Cultured stromal cells expressed immunoregulatory genes following stimulation with immunostimulants such as lipopolysaccharide from *Salmonella typhimurium*, levamisole, and poly (I:C) (Fierro-Castro et al. 2013). Fibroblast cells are less differentiated and therefore can accommodate and express foreign genes more easily than specialized cell lines such as B cells (Bouchard et al. 1989). Vitellogenin synthesis in primary cultures of fish liver cells is used as endpoint for in vitro screening of the (anti) estrogenic activity of chemical substances.

Cell lines are used as models to study the assembly of myofibrils (van der Venand Fürst 1998) and in biopharmaceutical industry as a platform for the generation of stable cell lines for r-protein production (Pham and Durocher 2006). The potential utility of fish cell lines for transgenic and genetic manipulation studies was identified from the fluorescent signals produced when transfected with pEGFP vector DNA (Qin et al. 2006; Ye et al. 2006; Parameswaran et al. 2007; Zhou et al. 2007; Ahmed et al. 2008; Ku et al. 2009).

The ability of RTgill-W1 to withstand hypo- and hyper-osmotic conditions and their optimal growth capacity at room temperature makes these cells an ideal model for in vitro aquatic toxicology as well as model systems to study fish gill function and gill diseases. RTgill-W1 support growth of paramyxoviruses and orthomyxoviruses like salmon anemia virus. RTgill-W1 also supports growth of *Neoparamoeba pemaquidensis*, the causative agent of amoebic gill disease. The cells have been used to understand mechanisms of toxicity, ranking the potencies of toxicants, and evaluating the toxicity of environmental samples. These cells are also valuable for high-throughput toxicogenomic and toxicoproteomic studies which are easier to achieve with cell lines than with whole organisms. RTgill-W1 cell line could become a valuable complement to whole animal studies and in some cases as gill replacements in aquatic toxicology.

Whether continuous fish cell lines have been neoplastically transformed in vitro has not been addressed so far. This research is hampered by the absence of appropriate in vivo tumor assays (Rausch and Simpson 1988). Shima et al. (1980, 1987) injected goldfish RBCF-1 cells into nude mice and X-irradiated goldfish but found no observable tumors. Chen and Kou (1987) demonstrated that nine different fish cell lines were unable to grow in soft agar. FHM cells did not grow in soft agar or form tumors in an immune-suppressed lizard (Rausch and Simpson 1988). In contrast to these results, two cell lines developed from medaka tumors, melanoma and hepatoma, were capable of initiating tumors when injected into the appropriate inbred strain of medaka (Etoh et al. 1987). The EPC cell line from hypertrophic skin lesions of the carp was able to grow in soft agar (Fijan et al. 1983). Few attempts

have been made to directly transform fish cells. Malignant transformation was not brought about by the transfection of RBCF-1 with human c-Ha-ras (Hayasaka et al. 1990).

Fish cell lines should be amenable to the repertoire of techniques for genetically modifying animal cells. Fish cells can be fused with one another and with mammalian cells (Yip and Bols 1982; Bols et al. 1984). Microcells have been prepared from goldfish RBCF-1 and fused with human cells (Shima et al. 1987). DNA has been transfected into fish cell lines by calcium-phosphate precipitation, polybrene procedure, and electroporation (Friedenreich and Schartl 1990; Isa and Shima 1987; Helmrich et al. 1988; Mitani et al. 1990; Zafarullah et al. 1988, 1989). Although mutants have not been isolated, fish cells have been transfected with a dominant selectable gene: xanthine guanine phosphoribosyltransferase (Isa and Shima 1987). A human growth hormone (GH) gene under control of the human metallothionein promoter has been transfected into fish cell lines, where GH mRNA was expressed but not GH (Friedenreich and Schartl 1990).

RBCF-1 has been transfected with xanthine guanine phosphoribosyltransferase (Isa and Shima 1987) and the c-Ha-ras oncogene (Hayasaka et al. 1989). The presence of 06-methylguanine acceptor protein, which is involved in the removal of adducts from DNA, has been demonstrated in RBCF-1 (Grist et al. 1986). Clones of RBCF-1 have been used to study hsp synthesis and thermosensitivity (Mitani et al. 1989). With the parental cell line of CAF-MM1, changes in membrane fluidity have been demonstrated in cells cultured at different temperatures (Tsugawa and Lagerspetz 1990). The mudminnow cell line (ULF-23H) had characteristics suitable for clastogenicity assay (Park et al. 1989).

7.4 Advantages and Disadvantages of Fish Cell Cultures

Fish cell culture systems have the advantages of defined but pliable physiochemical environment, cost-effectiveness, easiness to handle, and infinite source of cells that exhibit a high degree of homogeneity and avoid the ethical constraints of using animals in research.

While the applications and advantages are numerous, there are certain disadvantages as well. Cell lines could mutate during serial culture and are prone to genotypic and phenotypic drift. This is particularly common in frequently used cell lines, especially those that have been deposited in cell banks for many years. Subpopulations may arise and cause phenotypic changes over time by more rapidly growing clones within a population (Bahia et al. 2002; Burdall et al. 2003). For example, bioinformatic analysis of proteomic phenotypes revealed that the Hepa1–6 cell lines were deficient in mitochondria, reflecting rearrangement of metabolic pathways when compared to primary hepatocytes (Pan et al. 2009). This is a cause for serious concern, especially if such cell lines are to be regarded as valid models for evaluating the pathobiology of cancer progression and/or the response to novel drug therapies.

Another concern is misidentification or cell line cross contamination. For example, in the human biomedical field, a large number of cell lines were contaminated with HeLa cells (Nelson-Rees et al. 1981). The increasing misuse of "false" cell lines is a huge concern, with the observation that of 252 new cell lines deposited at the German Cell Line Bank, nearly one-fifth of these (18%) were found to be cross-contaminants (MacLeod et al. 1999; Masters 2002; Hughes et al. 2007; Horbach and Halffman 2017). The channel catfish ovary (CCO) cells previously available from cell repositories (ATCC) has recently been reported as contaminated by brown bullhead (BB) cells (Ford et al. 2021). Therefore, presently there are only limited number of channel catfish (*Ictalurus punctatus*) cell lines, and none from blue (I. furcatus), or hybrid catfish (♀ *I. punctatus* × ♂ *I. furcatus*) available from ATCC.

Several biological pathways cannot be represented by cell lines, which limit the application of cell lines in certain research areas. Primary cells and cell lines could show variability in drug dose, thus data acquired through cell lines has to be adjusted or cannot easily be replicated in an in vivo model. In vitro systems have the inability of binding assays to distinguish agonism and antagonism or dependence of cell proliferative responses on serum factors. An additional problem with many in vitro models is the lack of metabolic competence. The hepatocyte VTG assay can detect estrogenic compounds, but it is not certain whether it avoids false-negative results and whether it can reliably detect weak estrogens.

Primary cultures have the potential to harbor resident pathogens (Wolf 1988). Although fish cell lines can shelter mycoplasma (Schultz et al. 1986), they are usually free of biological contamination. Olsson et al. (1990) directly compared rainbow trout primary hepatocyte cultures and liver cell line (RTH-149) on the regulation of metallothionein (MT) synthesis. Based on the results, primary hepatocyte cultures were judged superior for understanding the regulation of MT synthesis.

7.5 Future Research Potential

Lack of appropriate fish cell cultures hinders the isolation of species- and tissue-specific pathogens. Meanwhile, all the established cell lines are not available to the repositories and fish as a source of cell lines remains unexplored. Evolution of cell line as a standard research agent demands the use of standardized media, reagents, equipment, quality control protocols, proper characterization, and documentation of developed cell lines.

Exploiting the 3D cell culture technology for long term in vitro maintenance of organoids derived from the snake venom gland to obtain venom can be useful to produce antivenom and other therapeutics (Puschhof et al. 2021). Some of the clades of fish such as chimeras, sharks, sting rays, silurid catfish, surgeonfish, stone fish, and rabbitfish are known to produce highly poisonous toxins (Pandey and Upadhyay 2020). Fish toxins are biologically relevant components that are capable to exhibit enzymatic, antimicrobial, cytotoxic, hemolytic, cardiovascular, neuromuscular, antimicrobial, and anticancerous activities (Ortiz et al. 2015). For this reason, fish venoms are found beneficial to use in wide range of pharmacological, therapeutic,

and pesticidal applications (Church and Hodgson 2002; Pandey and Upadhyay 2020). The above method can be applied to produce fish venom for use in biomedical research.

Luciferase-labeled reporter cell lines are used for biological mechanism studies and drug development. These advanced models provide a relatively simple, robust, and highly sensitive means to measure biological processes and to assess drug efficacy in live animal models through bioluminescence imaging. Toxicologists need the high biological relevance of primary cells and the proliferative capacity of cell lines for standard, predictive assays. Two of the major challenges that many scientists experience when developing a cell-based assay include obtaining cells with high biological relevance and then producing or procuring enough cells to run the assay without introducing cell variability. The hTERT-immortalized primary cells are genetically modified to exhibit the growth characteristics of a continuous cell line at the same time maintaining the physiology of a primary cell. These cells solve the problem of limited biological relevancy in cell-based assays. Such cells of fish origin will have huge research potential in durably and correctly recapitulating in vivo physiology.

Using misidentified or cross-contaminated cell lines in experiments can invalidate experimental results; therefore, authenticating cell lines should be part of cell culture workflow. Cross-contamination of cell lines has persisted as a result of mishandling and the lack of attention to best practices in tissue culture. These disadvantages question their use in biomedical research as they are likely to produce unreliable and inconsistent results that are irreproducible or induce additional studies of questionable value. For cell lines to be used as models in a meaningful way, they must be well-characterized before embarking on a plethora of research. Identification and characterization of cell cultures can be conducted by HLA typing, karyotyping, isoenzyme typing and DNA fingerprinting using multi-locus probes, and short tandem repeat (STR) profiling (Masters et al. 2001). Mycoplasma infection can chronically affect the well-being of cells in culture without being detected by visual observation; multiple screening methods can be used to identify contamination. Choosing the correct cell model will add efficiency and productivity to the research. When culturing stem cells or primary cells, certain considerations regarding the choice of media and reagents must be taken. Tools such as proliferation assays and cryo-containers can aid the maintenance of cell health.

7.6 Conclusion

In the last four decades, cell culture systems have transitioned from being merely a research tool into the foundations of several life science fields and its use is continuing to expand rapidly. In vitro models are replacing animals in many tests and assays in addition to avoiding several regulatory concerns. Its enormous potential in the fields of stem cell and targeted therapy has hardly started to be realized. Increased number of cell culture-based products generated is a driver for improved and faster process development technologies. Across the world, scientists are trying

to improve the cell lines for enhanced growth, product synthesis, energy metabolism, glycosylation characteristics, etc. A whole range of genomic and proteomic approaches are used in this regard as well. Undoubtedly, cell cultures are likely to be the key technology for foreseeable future.

There is considerable ethical pressure on scientists to reduce or eliminate the use of animals in research. Fish cell cultures have proven to be a successful biological alternative to the use of animals in research. The number of fish cell lines deposited in the reference laboratories represents only a small fraction of the total established cell lines from all over the world. The increasing importance of fish cell lines suggests that the researchers should be encouraged to deposit more cell lines in repositories, which would be beneficial to the global research community to use cell lines as an alternative for animal research. In addition, the specificity of the pathogens demands the establishment of several fish cell lines from different tissues, organs, and species allowing disease diagnosis and the study of species- and tissue-specific responses.

The current strategies for the development of fish cell lines for the recombinant expression of therapeutic proteins remain empirical to a large extent. Continued improvements in selection procedures are expected for finding highly productive cell lines. Product quality may be achieved through genetic engineering of the host cells or by optimizing culture conditions. Concerns over the use of cell lines have resulted in a growing need for primary cells in a variety of applications from basic research to drug discovery. Often, primary cells are combined with newer technologies such as 3D cell culture given a recent surge within the research community to use better reagents to improve research. Since 3D cell culture systems with primary cells are showing great promise for biomedical research, more research progress in this area will be appreciated.

Misidentified or cross-contaminated cell lines in experiments can invalidate research efforts. Therefore, authenticating cell lines should be part of cell culture. Moreover, cell cultures should be considered as a standard research agent and be given the proper care and quality control measures that include the use of standardized media, enzymes, and other commercially available laboratory reagents. Properly standardized in vitro assays are valuable as high-throughput screening tools that provide relevant data.

While there are some problems associated with fish cell cultures, the benefits and applications outweigh the disadvantages. The end goal of their use is to help in improving aquaculture production by assisting in early disease diagnosis and being a part in developing efficient management strategies against infectious pathogens. Toward this, cell cultures from economically relevant fish species need to be developed to use in fish virus disease diagnosis, development of vaccines, and identification of antiviral agents. Cell cultures developed from several fish tissues can serve as an efficient tool in the diagnosis of uncharacterized viruses leading to the development of efficient pathogen-targeted management strategies. Despite the huge diversity in fish species, the fish cell culture field remains largely unexplored. There is a scarcity of the host-specific cell lines in the aquaculture research field. Although the fish cell culture sector requires a lot of improvements, they are widely

applied recently in many research areas. Future research will depend on improvised 3D cultures which are physiologically relevant and can provide more accurate responses. The guidelines from other animal cell culture research could be adopted in the fish cell culture sector toward achieving these goals.

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Recent Advances in Antimicrobial Peptides to Improve Fish Health

8

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Abstract

Antimicrobial peptides (AMP) belong to the innate arm of immunity and serve as critical defence weapons across the living system. They have a multidimensional role in regulation of immunity which includes their direct antimicrobial response, chemoattraction, immune cell recruitment, stimulation of cytokine secretion and even triggering the adaptive immune responses. AMP-mediated regulation is intricate as these molecules keep a check on proinflammatory cytokines to prevent cytotoxicity in host. These host-derived peptides represent a divergent family in fish and have been tailored to initiate the appropriate response for the pathogen profile encountered by the species. The five classes of fish AMPs—piscidins, β-defensins, hepcidins, cathelicidins and histone-derived peptides—have been investigated for their antimicrobial effects against a wide variety of fish pathogens. The aquaculture industry often resorts to vaccines or antibiotics for evading fish disease; however, the therapeutic dosage of vaccines has the risks of cytotoxicity in host, while the latter promotes the already looming threat of antibiotic resistance. Under these circumstances, the exploitation of the broad-spectrum antimicrobial response of AMPs is not only an effective but also a safe means for combatting pathogenic infections in fish. Interestingly, the fish AMPs have also been found to generate effective immune response against various human pathogens and cancer cells, and this area has been extensively studied

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for its direct application in human health. However, there is a dearth of reports on their field application for amelioration of fish health. This chapter is focussed on fish AMPs, and it highlights their role in nurturing fish health and their potential for development of therapeutics.

Keywords

Antimicrobial peptides · Microbes · Immune response · Fish pathogen

8.1 Historical Overview

It was almost a century ago that Alexander Fleming chanced upon the discovery of the first antimicrobial protein—lysozyme. Amidst the chaos of the First World War, he happened to forget about a petri dish inoculated with his nasopharyngeal mucus from when he was suffering from a cold. A fortnight later, he found this petri dish covered with bacterial colonies in all areas except where his mucus had been inoculated. This led him to deduce the presence of an enzyme in the mucus that inhibited the growth of bacteria. Later, he also detected the presence of lysozyme in hair, nails, saliva and tears. Further, his discovery of penicillin, which also earned him a Nobel Prize, shifted the attention towards antibiotics. The 1980s decade reignited the interest in innate immune mechanisms and thence began the series of discoveries of diverse antimicrobial peptides (AMPs) from the animal kingdom (Tan and Tatsumura 2015). The identification of insect cecropins—P9A and P9B—from the pupa of *Hyalophora cecropia* with broad bactericidal activity debunked the myth that the insect immune system was significantly different from that of vertebrates (Steiner et al. 1981). This was followed by the discovery of α-defensins from human neutrophils and magainins in *Xenopus laevis* (Ganz et al. 1985; Zasloff 1987) that were effective against bacteria, fungi as well as protozoans. The latter also went through a series of clinical trials as an effective treatment for diabetic foot ulcers but were rejected in the phase III clinical trials (Ramirez-Acuña et al. 2019). It was only a decade later that the antimicrobial activity of peptide, pardaxin, was discovered in a fish species (*Pardachirus marmoratus*) (Oren and Shai 1996). Since then, numerous peptides have been identified in the natural systems and even custom-designed for synthesis. The antimicrobial peptide database (APD) was created in 2003 to manage the curation of natural AMPs. The 2015 version of this database contains around 2619 peptides that have been found to be effective against a range of pathogens from bacteria, fungi, viruses to parasites and even against cancer (Wang et al. 2016). Another manually annotated database is DRAMP with a collection of more than 19,000 peptides and includes clinical, patented as well as synthetic AMPs (Kang et al. 2019). In the age of climate change and intensive efforts to meet global food security in lieu of growing population, the aquatic ecosystems have also been marred by high prevalence of infections. The instances of multidrug resistance in cultured fishes (both edible and ornamental) are constantly rising due to the use of antibiotics by fish farmers to combat these episodes of infections, and consequently, their

consumption by humans also poses a concern for public health (Preena et al. 2020). The pleiotropy and specificity of AMPs render them as an effective tool for treatment of these infections. The lurking threat of antimicrobial resistance (AMR) has boosted the investigation of fish AMPs as potential alternatives to antibiotic-based therapy. Advances in machine learning protocols and genetic algorithms have also accelerated the pace of de novo sequence design and optimization of AMPs for enhanced biological activity (Cardoso et al. 2020). In this chapter, we review the fish AMPs and their functional significance for physiology as well as their application in aquaculture.

8.2 What Are Antimicrobial Peptides (AMPs)

AMPs are evolutionarily ancient members of the innate immune system found to be ubiquitously present across the living kingdom from bacteria and plants to animals. They are oligopeptides of less than 100 amino acids bearing a net cationic charge in the range of +2 to +11, attributed to a rich composition of hydrophobic amino acids like lysine and arginine (Bahar and Ren, 2013; Lei et al. 2019). In nature, they are synthesised in two ways—(1) by non-ribosomal mechanism (as seen in bacteria) and (2) by ribosomal mRNA translation (seen in all organisms including bacteria) (Hancock and Chapple 1999). They may be expressed constitutively or induced by a specific external stimulus. AMPs are synthesized as precursor proteins with signal peptide that undergoes hydrolytic cleavage (Hancock and Sahl 2006). Their structural and functional diversity endows upon them a versatile range of functions, primarily broad-spectrum antimicrobial activity. Besides antimicrobial functions, the immunomodulatory capability of AMPs has also led them to be referred as host defence peptides (Hemshekhar et al. 2016).

8.3 AMPs in Fish

As lower vertebrates, the fish have a more robust innate immune repertoire than the adaptive immune components which have become more complex and nuanced in the course of vertebrate evolution. The skin, being the first line of defence, is covered with mucus in fish. This skin mucus is armed with AMPs which provides protection against pathogenic infections. Pardaxin was the first AMP to be isolated from a fish species, and it shared a high degree of structural identity with insect cecropin and melittin (Oren and Shai 1996). Another peptide, misgurin isolated from *Misgurnus anguillicaudatus* was also found to be structurally identical to cecropin and frog dermaseptins and magainins (Park et al. 1997). Multiple fish AMPs have been identified in various species ever since, in fish mucus, saliva, circulatory system and even gut microbiota (Dong et al. 2017; Chaturvedi et al. 2020). The amino acid composition of peptides determines their structure and biological function. The most common fish AMPs are cationic α-helical peptides; however, there are exceptions of non-cationic peptides as well, such as piscidin 2 in *Gadus morhua*, SHβAP in

Katsuwonus pelamis and EC-hepcidin 1/2 in *Epinephelus coioides* (Valero et al. 2020).

8.4 Classification of AMPs

The classification of AMPs is tedious and to an extent, ambiguous, due to the vast diversity of this family. They have been classified based on (1) source, (2) amino acid composition, (3) structure and (4) activity (Table 8.1).

1. *Based on source*: The APD3 database classifies the naturally occurring AMPs based on six life domains that include bacteria, archaea, protists, fungi, plants and animals. Approximately, 75% AMPs in this database are sourced from animals, with amphibians alone accounting for 38% of the repertoire (Wang et al. 2016). Cathelicidins and defensins are the major representatives from the mammalian AMP family, while the amphibian AMPs, though relatively shorter than the mammalian counterparts, include multiple families like magainins, brevinins, dermaseptins, nigrocins, ranatuerins and tigerinins (Varga et al. 2019). There are 117 fish AMPs in ADP3 congregation, representing majorly two families—piscidins and hepcidins (Valero et al. 2020).
   The nomenclature of AMPs in fish lacks clarity mainly due to the independent discovery and naming pattern followed over decades. The naming based on the genus or order of the source organism has not accounted for phylogenetic

Table 8.1 Classification of antimicrobial peptides (AMPs)

S.No.	Basis of classification	Categories
1.	Based on Source (*APD3 classification)*	Bacterial AMPs
		Archaeal AMPs
		Protistan AMPs
		Fungal AMPs
		Plant AMPs
		Animal AMPs
2.	Based on amino acid composition	Proline-rich AMPs
		Glycine-rich AMPs
		Tryptophan and Arginine-rich AMPs
		Histidine-rich AMPs
3.	Based on structure	α AMPs
		β AMPs
		αβ AMPs
		Non-αβ AMPs
4.	Based on activity	Antibacterial AMPs
		Antiviral AMPs
		Antifungal AMPs
		Antiparasitic AMPs

proximity among the possibly similar peptides from two species, thereby also creating a hurdle for distinct classification of the fish AMPs (Muncaster et al. 2018).

2. *Based on amino acid composition*: There are four categories of Amps based on amino acid rich species in the primary sequence—proline-rich, glycine-rich, tryptophan- and arginine-rich and histidine-rich (Huan et al. 2020).
   Proline-rich AMPs are also referred to as PrAMPs and bear short motifs with proline and arginine, though with a low-sequence-level conservation in the family. In spite of being effective against various multidrug resistant strains, they have also been associated with a low cytotoxic profile. The probable cause for this is that its antimicrobial effect is not mediated by destruction of bacteria by membrane damage, rather by engagement with the ribosomal machinery to inhibit protein synthesis. PrAMps like oncocin and Bac7 show an overlap in the binding sites within the ribosomal exit tunnel which helps them to disrupt translation by either blocking the binding of aminoacyl-RNA to peptidyl transferase or by binding to the release factors during the termination phase of translation (Mardirossian et al. 2019). Glycine-rich AMP contains approximately 14−22% glycine residues. The presence of this proteinogenic amino acid also impacts the structure of these peptides, and consequently the mechanism of action, as seen for salmonid cathelicidin-derived peptide (D'Este et al. 2016). Glycine-rich AMPs are common to a wide variety of species, especially insect classes which have coleoptericin in Coleoptera, diptericins in Diptera, hemiptericin in Hemiptera, hymenopterans in Hymenoptera and gloverins in Lepidoptera (Mylonakis et al. 2016). Histidine-rich peptides show potent membrane permeation which enables better antimicrobial activity. Various studies have been carried out on synthetic peptides where replacement with histidine residues has led to improved activity. For example, the replacement of tryptophan or arginine with histidine in the peptide (RRWWRWWRR) was seen to modulate its membrane binding energy and also lower its cytotoxicity (Bacalum et al. 2017). Tryptophan- and arginine-rich AMPs are usually cell-penetrating peptides. The aromatic residue tryptophan and the cationic arginine enhance the efficacy of binding to the anionic part of the bacterial membrane via ion pair-π interactions (Huan et al. 2020). The non-cationic AMPs in fish have a distinct amino acid composition with the presence of aspartic and glutamic acid residues (Lai et al. 2002). Cationic AMPs like piscidins exhibit an enhanced composition of isoleucine, histidine and phenylalanine residues; however, it is the positive charge conferred by arginine and lysine which is responsible for antimicrobial activity (Valero et al. 2020). Song et al. (2012) were able to demonstrate the antimicrobial activity of three non-cationic peptides isolated from pepsin hydrolysate of *Setipinna taty*. Unlike cationic peptides, these AMPs have an amphiphilic structure to mediate membrane interaction with pathogens. In some cases, the peptide interaction with the membrane is facilitated by metal ions via formation of cationic salt bridges (Valero et al. 2013).
3. *Based on structure*: Based on the composition of secondary structure, AMPs have been classified into four types—α (e.g. LL-37, magainin), β (e.g. human

α-defensins), αβ (e.g. human β-defensins, drosomycin) and non-αβ (e.g. indolicidin, drosocin). As the name implies, each category is composed of the designated secondary structural element like α-helix for α-peptides, β-sheets for β-peptides, both for αβ peptides and linear extension/random coil structure for non-αβ peptides. Majority of AMPs belong to the first three categories with ADP containing approximately 398, 98 and 98 entries from α, β and αβ families, while the latter non-αβ category has only 9 entries (Wang et al. 2016). The peptides from α family usually tend to lack structural organization in aqueous medium but attain its amphipathic helical form on coming in contact with biological membranes. The conformation of the peptide is such that the hydrophobic residues are embedded in the tail of the helical core and the charged side is faced towards the surface for lipid interaction on the membrane. The hydrophobicity of the tail and its continuity determine the extent of membrane insertion and thereby the antimicrobial potency of the AMP (Nguyen et al. 2011). On the contrary, β-peptides remain ordered in aqueous medium and do not undergo a major change on membrane contact due to the rigidity imparted by disulphide bonds between the β-sheets (Mahlapuu et al. 2016). The amino acid preference also varies for the two families, with lysines and arginines being more prominent in α- and β-AMPs, respectively. The non-αβ peptides also show a preference for arginine along with histidine, proline and tryptophan, and despite lacking a secondary structure, they fold into amphipathic helices upon membrane interaction, similar to α-peptides (Nguyen et al. 2011). The lysine/arginine ratio is the highest for α-AMPs, followed by αβ, β and non-αβ AMPs. Additionally, cyclic peptides and lasso peptides have also been reported, with more complex topologies in structure (Huan et al. 2020).

Linear α-helical peptides in fish include chemokine-derived peptides, epinecidins, gaduscidins, grammistins, mononecidins, pardaxin and piscidins. Gaduscidins are putative peptides that are closely related to piscidins phylogenetically (Valero et al. 2020). The cysteine residues in the β-sheet AMPs form disulphide bonds resulting in formation of near cyclic conformation which is critical for the peptide's antimicrobial activity (Chaturvedi et al. 2020). Fish cathelicidins, defensins and hepcidins are the most prominent members of this group, with the latter exhibiting the highest number of disulphide bridges.

4. *Based on activity*: Depending on activity, the AMPs have been classified into antibacterial, antifungal, antiviral and antiparasitic peptides. This classification scheme essentially accounts for the target interacting with the AMP. A range of AMPs have been shown to be effective against both gram-negative and gram-positive bacteria including cecropins and defensins. Several reports are available on antibacterial functions of fish AMPs as well, which are mediated by bacterial membrane interaction and degradation. The function of these peptides is known to exhibit species and target-specific variation (Masso-Silva and Diamond 2014). While JF-1 peptide in *Paralichthys olivaceus* does not show any antimicrobial function, JF-2 peptide shows antibacterial response against gram-positive

Lactococcus garvieae and *Staphylococcus aureus* and gram-negative species *Escherichia coli*. Similar studies have been also been carried out in *Oreochromis niloticus*, wherein despite multiple copies of hepcidin being present in the species, only TH2-3 seems to show a strong induction by lipopolysaccharide (LPS) and TH1-5 and TH2-2 do not (Huang et al. 2007). The multiple copies of hepcidin open speculations for the involvement of non-antimicrobial peptides in alternate function. In a recent study conducted on *Dicentrarchus labrax* hepcidin, its two copies *Hamp1* and *Hamp2* were seen to exhibit distinct functions. While *Hamp2* showed a direct participation in antimicrobial activity, *Hamp1* mediated iron homeostasis via ferroportin (Neves et al. 2017).

Viral infections, though ignored initially in the field of aquaculture, have caught eyes due to viruses like nervous necrosis virus (NNV) that have proved to be detrimental to the sustainable culture of economically relevant fish species. Kuan et al. (2012) developed a biosensing mechanism for the screening of the binding affinity of AMPs to NNV to distinguish the peptides showing effective response against the virus. Epinecidin-1 and hepcidin 1−5 from *Epinephelus coioides* have shown potential as in vivo treatment agents against NNV infection. Combinatorial treatment with both AMPs decreased the viral load and enhanced the survival of the NNV-infected fish (Wang et al. 2010). Epinecidin-1 from *E. coioides* also exhibited effective virucidal activity against foot-and-mouth disease virus from Picornaviridae family (Huang et al. 2018). In salmonids, however, the reports on direct antiviral activity of AMPs are scarce. Though recombinant rainbow trout β-defensin was able to reduce the level of viral infection by 80−90%, this was speculated to be mediated by upregulation of type1 IFN response (Brunner et al. 2020).

Broad-spectrum activity of various fish AMPs previously associated with antibacterial responses was unveiled when investigated against fungal infections. Cathelicidin *cath2* from rainbow trout known to be effective against *Aeromonas salmonicida* were also significantly upregulated in response to *Saprolegnia parasitica* (De Bruijn et al. 2012). Similarly, histone-derived H2B peptide isolated from epidermal mucus of *Gadus morhua* was potent against both bacterial (*Aeromonas hydrophila*) and fungal (*Saprolegnia* spp.) species (Bergsson et al. 2005). The antifungal activity of AMPs like piscidin-2 is mediated by disruption of membrane activities, as reported in *Morone saxatilis* (Sung et al. 2008).

Antimicrobial functions of fish AMPs have proven effective against various parasites of fish as well as humans. The best example for this is epinecidin-1 from *E. coioides* which exhibits cytotoxicity towards *Trichomonas vaginalis* and *Candida albicans*, both of which are known infectious agents in fish and humans (Pan et al. 2009). Synthetic piscidin-2 from *M. saxatilis* also exhibited potency against parasites including *Amyloodinium ocellatum*, *Cryptocaryon irritans*, *Ichthyophthirius multifiliis* and *Trichodina* spp. (Colorni et al. 2008). Similarly, piscidin-1 from *Oplegnathus fasciatus* and *G. morhua* demonstrated antiparasitic functions against

Miamiensis avidus and *Tetrahymena pyriformis* (Ruangsri et al. 2013; Umasuthan et al. 2016). The above listed studies only touch the tip of the iceberg in terms of the screening of specificity of AMPs against pathogenic agents. Nevertheless, the above-listed examples aptly convey the multifarious nature of responses of fish AMPs.

8.5 Mechanism of Action of AMPs

The mechanism of action of AMPs has been widely studied to pave the road towards the development of potent therapeutics. As per initial research, AMPs were though to act only via membrane interaction. However, with the elucidation of their role in immune modulation, their mechanism was classified into (1) direct killing and (2) immune modulation, wherein direct killing may occur either by membrane targeting or non-membrane targeting mechanisms (Kumar et al. 2018) (Fig. 8.1).

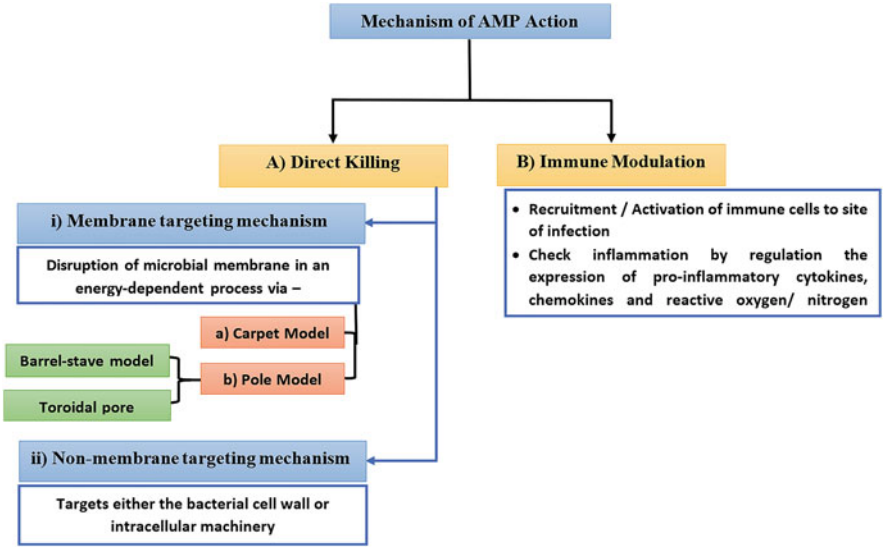

Fig. 8.1 Mechanism of action of antimicrobial peptides

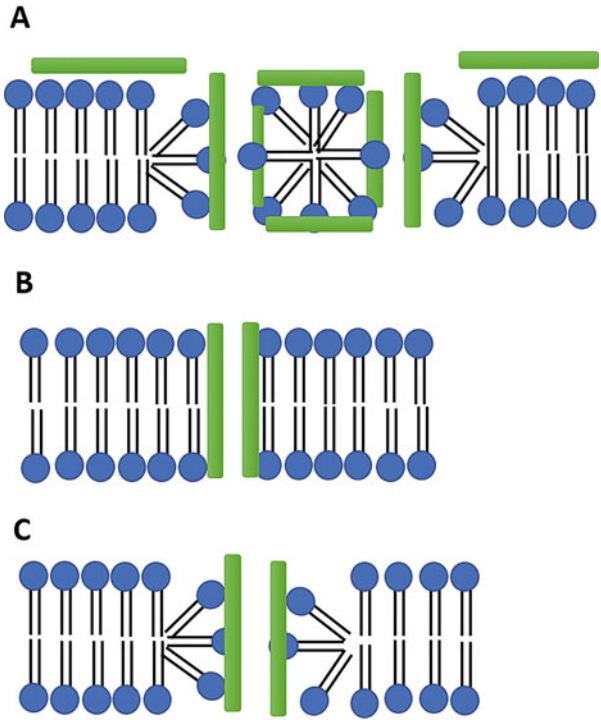

Fig. 8.2 Models hypothesized for membrane targeting by AMPs: (**a**) carpet model, (**b**) barrel-stave model, (**c**) toroidal pore model

8.5.1 Direct Killing

1. *Membrane targeting mechanism*: The membrane targeting mechanism of direct killing involves the permeabilization of the microbial membrane in an energy-dependent process. The description of this mechanism has been conventionally elaborated via two models—(a) carpet model and (b) pole mode (Huan et al. 2020) (Fig. 8.2).
   (a) *Carpet model*: This model was proposed by Pouny et al. (1992) and is also referred to as detergent-like model. The AMPs organize themselves parallel to the lipid bilayer with the hydrophobic end towards it. This accumulation continues up to a threshold. Finally, a detergent-like effect is created resulting in formation of micelles for disintegrating the membrane (Murrell et al. 2018; Huan et al. 2020) . Dermaseptins, cecropin and LL-37 are some peptides that act via this mechanism of bilayer micellization (Lyu et al. 2019).
   (b) *Pole model*: The pole model is further divided into (i) barrel-stave model and (ii) toroidal pore model (Fig. 8.2).
      (i) *Toroidal pore model*: The toroidal pore model also known as wormhole model was given by Hancock and Chapple (1999). In this, AMPs are adsorbed perpendicularly to the cell membrane leading to membrane depolarization (Shabir et al. 2018). This alters the membrane curvature by formation of transmembrane toroidal pores of 1−2 nm diameter,

ultimately causing cell death. The examples from this category include magainin, mellitin, lacticin Q, etc. (Huan et al. 2020).

Nevertheless, in in vivo systems, these mechanisms are not as distinct as in theory. Wimley and Hristova (2011) have also proposed an interfacial model wherein the imperfectly segregated polar and non-polar groups in an amphipathic peptide endow with conformation of an interfacial peptide. The partitioning of this peptide on the lipid bilayer moieties perturbs them, leading to transient pore formation. The fluid domains result by micelle formation, due to which this model is often referred to as an extension of carpet model (Perrin et al. 2014).

(ii) *Barrel-stave model*: Similar to the toroidal pore model, the AMPs here aggregate perpendicular to the lipid bilayer as multimers. This resembles a barrel-stave structure which penetrates into the membrane to form a pore lined with hydrophilic residues along the lumen and hydrophobic ones facing the membrane lipids (Chaturvedi et al. 2020). This results in outflow of the cytoplasm followed by cell death. In order to span the lipid bilayer, the minimal residue length of peptides from this category is approximately 8 and 22 for α-helix and β-sheet AMPs, respectively. The peptides acting via this mechanism are relatively rare e.g. protegrins, alamethicin and pardaxin (Kumar et al. 2018).

The two pore models are distinct at the molecular level of pore formation. The toroidal pore lacks peptide-peptide interactions unlike the barrel-stave pore where these interactions are crucial for multimer formation. The toroidal pore also needs deeper penetration into the lipid membrane to perturb the integrity of the lipid bilayer, therefore promoting membrane hydration. On the contrary, the barrel-stave pore does not alter the net arrangement of the lipid bilayer or its hydration as it spans the membrane without inducing a significant curvature. Further, the mode of interaction of AMPs with the toroidal pores is essentially electrostatic, while both hydrophobic and electrostatic interactions are required for the barrel-stove pore (Li et al. 2017).

2. *Non-membrane targeting mechanism*: AMPs from this category act by targeting either the bacterial cell wall or its intracellular machinery. The former is usually achieved by trapping the key ingredients in the recipe of cell wall synthesis. AMPs may also bind to the precursors of the molecules required for assembly of the cell wall (Kumar et al. 2018). Charge-based interactions with the cell wall components like LPS or peptidoglycan (PG) are effective in mediating the effect of AMPs. Lipid II is a highly conserved molecule that serves as a building block for PG biosynthesis. Cationic AMPs like disulphide-stabilized defensins are known to shut off the supply of lipid II, thereby inhibiting cell wall synthesis (Münch and Sahl 2015). A transcriptomic study conducted on three designer peptides (thrombocidin-19 (TC19), TC84 and bactericidal peptide 2 (BP2)) synthesized based on human bactericidal permeability increasing (BPI) protein demonstrated a cell envelope stress response in *Bacillus subtilis*. A mutant study confirmed that this stress response was attributed to delocalization of essential

proteins of the cell synthesis pathway which does not allow the repair of the cell envelope post bacterial membrane perturbation (Omardien et al. 2018). Non-membrane targeting mechanism is also seen in case of human cathelicidin LL-37 against the fungus, *Candida albicans*. At low concentration, the peptide prevents the adhesion of the *C. albicans* to mice cells by binding to carbohydrate moieties (manna, chitin, glucan) of the fungal cell wall (Tsai et al. 2011).

8.5.2 Immune Modulation

AMPs are also capable of modulating immune functions for promoting host defence. They are one of the foremost molecules that encounter the pathogen during infection. So apart from the direct microbe interaction, they also recruit other immune cells like dendritic cells, leukocytes and mast cells to the site of infection. They activate and promote differentiation of white blood cells and stimulate angiogenesis. Synthetic AMPs are also known to check inflammation by downregulating the proinflammatory cytokine levels and modulating the expression of chemokines and reactive oxygen/nitrogen species (Kumar et al. 2018).

8.6 Parameters for AMP Interaction with Microbes

The mechanism of AMP interaction also varies depending on the biological properties of the bacterial membranes. Both Gram-negative and gram-positive bacteria show distinct differences in the physicochemical properties of their cell envelopes. The gram-positive bacterial membrane consists of an outer cross-linked peptidoglycan layer with a surrounding matrix of teichoic acid. On the other hand, in gram-negative bacteria, the outermost layer is composed of lipopolysaccharide (LPS) followed by a thin peptidoglycan layer. The negatively charged phosphate groups on LPS cater to salt bridge formation with divalent metal ions like Ca^{2+} and Mg^{2+}. The resulting electrostatic network acts a hindrance to the permeation of hydrophobic antibiotics. Therefore, the movement of AMP into gram-positive bacteria is a one-step process involving entry through the nanopores of the peptidoglycan layer. The AMP build-up in the peptidoglycan layer also promotes favourable interaction between negatively charged teichoic acid and the positively charged AMPs (Vollmer et al. 2008). For Gram-negative bacteria, however, the AMP entry requires disruption of two layers. It is initiated by the perturbation of the salt bridges of the LPS layer by hydrogen bond formation between phosphate groups of outer layer and cationic AMPs. The AMPs then advance into the periplasmic space to get absorbed on the inner cytoplasmic membrane of the bacteria. This is also the rate limiting step of this process wherein a threshold concentration of AMPs leads to loss of electrostatic potential and eventual destabilization of the cytoplasmic membrane (Li et al. 2017). Additionally, the physicochemical properties of the AMP-like peptide length, tertiary structure in solution and at the membrane, net ionic charge

and the number of hydrogen bond donors also determine the nature of interaction with the microbe (Li et al. 2017).

It is noteworthy that the microbes also manipulate their properties to escape the AMP defence mechanisms. For example, the bacteria may either express proteins with net cationic charge on the cell surface to repel the positively charged or secrete negatively charged molecules in the environment to trap the cationic AMPs extracellularly. Gram-negative bacteria may resort to teichoic acid modification on its outer layer or L-lysine modification in its inner layer to escape AMP interaction. Similarly, gram-negative bacteria undergo LPS modification either with aminoarabinose or by acylation of its lipid A subunit. Even if the AMP manages to perfuse to the intracellular space, some may be degraded by bacterial proteases. These mechanisms are a layout for host-pathogen coevolution, as proposed by the Red Queen hypothesis (Bahar and Ren, 2013).

8.7 Classes of Fish AMPs

Fish AMPs have been divided into five classes, namely—piscidins, β-defensins, hepcidins, cathelicidins and histone-derived peptides. The following sections discuss each of these AMPs in detail. Their spectrum of their antimicrobial activity is enlisted in Table 8.2.

Table 8.2 Antimicrobial functions of fish AMPs against various pathogens (bacteria, fungus, virus, parasite)

AMPs in fish	Antimicrobial Activity				References
	Ab	Af	Av	Ap	
Class I piscidins (Piscidin1, 2, 3)	✓	✓	✓	✓	Falco et al. (2009), Valero et al. (2013)
Class II piscidins (Piscidin4, 5)	✓	NA	NA	✓	Salger et al. (2016), Zheng et al. (2021)
Class III piscidins (Piscidin6, 7)	✓	NA	NA	✓	Salger et al. (2016), Bae et al. (2018), Raju et al. (2021)
β-defensins	✓	NA	✓	✓	Ruangsri et al. (2013), Shabir et al. (2018), Zhou et al. (2019)
Hepcidins	✓	✓	✓	✓	Wang et al. (2009, 2010), Banerjee et al. (2019)
Cathelicidins	✓	✓	NA	NA	Broekman et al. (2011), De Bruijn et al. (2012)
Histone-derived peptides (H2A)	✓	✓	NA	NA	Fernandes et al. (2002)
Histone-derived peptides (H2B)	✓	✓	NA	NA	Bergsson et al. (2005)

Ab antibacterial, *Af* antifungal, *Av* antiviral, *Ap* antiparasitic, *NA* data not available

8.7.1 Piscidins

Piscidins are amphipathic α-helical peptides with potent antimicrobial effects in fish. They have been structurally and functionally characterized across various fish taxa including *Adrianichthyidae*, *Anoplopomatidae*, *Belonidae*, *Cichlidae*, *Cyprinodontidae*, *Gasterosteidae*, *Fundulidae*, *Moronidae*, *Percichthyidae*, *Sciaenidae*, *Sebastidae*, *Siganidae*, *Sparidae* and *Sygnathidae* (Raju et al. 2021). The first member to be identified from this family was from *hybrid striped bass (Morone chrysops X Morone saxatilis)* (Silphaduang et al. 2006). The piscidin family also includes additional peptides like chrysophsin dicentracin, epinecidin, gaduscidin, moronecidin, myxindin and pleurocidin. Skin, gill and intestine seem to be the predominant expressors of this AMP family; nevertheless, the tissue distribution profile varies species-wide. The expression of this AMP has been traced to embryonic development stages of fish, indicating its role in early defence (Noga et al. 2009).

The evolution of piscidins appears to have taken place under lenient selection constraints and this evidence of positive selection and may be attributed to the need for structural and functional diversification in different fish habitats (freshwater, marine, brackish water). These peptides range between 18 and 46 amino acids in length, and they consist of a N-terminal signal peptide, middle mature piscidin and C-terminal prodomain. They have been known to bear multiple isoforms in the same species. The family is categorized into three classes based on the amino acid length of the AMP (Salger et al. 2016). Class I piscidins include piscidin1, 2 and 3 that are of 22 amino acid residues with high histidine and phenylalanine composition. They are the most effective peptides against gram-positive bacteria among the three classes while also being responsive against viral infections (Falco et al. 2009). In fact, piscidin1 is the most potent antimicrobial across the entire piscidin family and is even responsive against the *Staphylococcus aureus* (resistant strain) (Menousek et al. 2012). However, its application in therapeutics is limited by its high degree of haemolytic activity and cytotoxicity (Lee et al. 2014). Piscidin2 bears broad spectrum activity and is effective at high temperature and salt concentrations, as well (Colorni et al. 2008; Sung et al. 2008). Piscidin3 exhibits weaker antimicrobial response than piscidin1 and 2 but also is the least haemolytic peptide across the piscidin family (Park et al. 2011). Hayden et al. (2015) study has demonstrated complementary mechanisms of action of piscidin 1 and 3, wherein the former acts towards membrane perturbation and the latter is engaged in DNA condensation. It is also the least haemolytic peptide across the piscidin family. Class II piscidins include piscidin4 and 5 with 44−46 amino acids which bear effective potency against gram-negative bacteria and also parasitic infections (Zheng et al. 2021). Their amino acid composition imparts them with β-sheets similar to pattern recognition receptors responsible for binding LPS and carbohydrate moieties on pathogens. Class III piscidins are composed of piscidin6 and 7 with 55 amino acids long sequence. Their higher amino acid residue count imparts them with a coil-β sheet-coil–αhelix structure distinct from the other two classes. Functionally, their potency is low against bacteria but high against protozoans (Bae et al. 2018; Raju et al. 2021).

The piscidins are known to act via toroidal pore formation for disruption of microbial membrane disruption, courtesy of their high amphipathicity and cationic nature (Mehrnejad and Zarei 2010). Their expression may be induced by microbes themselves or microbial components like LPS. Apart from pore formation, they also exhibit immunomodulation by initiating chemotaxis via regulating cytokine and chemokine expression for recruitment of immune cells (Masso-Silva and Diamond 2014). These properties make them highly commendable candidates for usage in commercial therapeutics.

8.7.2 β-Defensins

Fish defensins belong to the β-defensin category and have a β-sheet structure stabilized by disulphide linkages of Cys1-5, Cys2-4 and Cys3-6 patterns (Anooja et al. 2020). While the gene structure of the human β-defensin comprises of two exons and one intron, that of fishes has three exons and two introns. Phylogenetic analysis of human and fish defensins clustered the human hBD-4 with the fish homologues (Cuesta et al. 2011). Fish defensins are composed of 38−45 amino acids, and their net charge may range between +1 and +5. An exception is the *Paralichthys olivaceus* β-defensin which is anionic in nature (Nam et al. 2010). Multiple isoforms of β-defensin have been known to exist in the same species with up to seven genes of the family reported in salmonids. This may be attributed to gene duplication events in the same fish species (Harte et al. 2020).

There is evidence of constitutive expression of fish defensins in early ontogeny stages that are more susceptible to infections. Their expression is reasonably high in organs like head kidney and spleen; however, there is a species-wide variation in their tissue expression profile. Interestingly, significant expression of these AMPs has been seen testis and pituitary of *Epinephelus coioides*, which also broadens the scope of their function to endocrine reproductive regulation (Jin et al. 2010). Fish defensins exhibit moderate antibacterial activity and effective antiviral response against fish-specific viruses. There is still a void with respect to studies on their potency against parasitic and fungal infections (Masso-Silva and Diamond 2014). Similar to piscidins, fish defensins also exhibit immunomodulation like chemoattraction of immune cells. There is evidence of their chemoattractant properties for chemokine-receptor 6 (CCR-6) expressing cells like monocytes, T-cells and dendritic cells (Röhrl et al. 2010). In Atlantic cod, they were also seen to stimulate the phagocytic response of head kidney leucocytes following gram-negative and gram-positive bacterial infection (Ruangsri et al. 2013). IL-22 in salmonids is seen to stimulate the expression of defensins (*defb3 and defb4*). Similar effect is also achieved by B-cell phagocytosis, highlighting that the regulation of β-defensin expression varies depending on the pathogen, cell environment or type (Brunner et al. 2020).

8.7.3 Hepcidin

Fish hepcidin is structurally similar to its human homolog, with hairpin-shaped β-sheets linked by disulphide bridges. However, unlike human hepcidins, fish hepcidins contain four, six or seven cysteines. Fishes also seem to have multiple copies of this peptide unlike mammals which have only a single copy of this gene (Masso-Silva and Diamond 2014).

On the basis of phylogenetic studies, fish hepcidins have been grouped into two clusters of paralogs—HAMP1 and HAMP2. While HAMP1 is orthologous to the mammalian peptide, HAMP2 is limited to acanthopterygians. The origin of HAMP2 is predicted to be post the third round of fish-specific genome duplication. The divergence of HAMP2 in the teleost lineage could be a result of varied fish habitats and low selection pressure. However, fish HAMP1 seems to have evolved under purifying selection (Xu et al. 2012). Although the tissue-based expression profile of AMPs essentially varies across fish species, liver is a prime expressor of hepcidin. The course of their functions may have evolved from defensive AMP in lower vertebrates to an iron regulator in higher vertebrates (Kim et al. 2019). Temporal profiling has traced their expression in fertilized egg as well as early embryos and larvae of *Ictalurus punctatus* (Bao et al. 2005). Fish hepcidins are effective against bacterial, fungal, viral and parasitic infections (Banerjee et al. 2019). In *Oryzias latipes*, hepcidin showed potent response against both gram-negative and gram-positive bacteria (Cai et al. 2012). They have also shown fungicidal activity against *Aspergillus* and *Fusarium* species in the marine fish, *Pseudosciaena crocea* (Wang et al. 2009). The immunomodulatory properties of hepcidin include regulation of expression of immune-related genes in fish including cytokines, lysozymes and candidates of the toll-like receptor pathway. Differential regulation of these immunity genes by different isoforms in the same organism is also seen, as in case of *Oreochromis mossambicus*, where TH1-5 stimulated the genes that were downregulated by TH2-3 (Hsieh et al. 2010).

The role of hepcidin as a key regulator of iron metabolism is well established. The high sequence homology between fish and human hepcidins also translates to their functions. This was demonstrated in *Dicentrarchus labrax* where both HAMP1 and HAMP2 regulate a single ferroportin gene differently (Neves et al. 2017). Iron overload stimulated the expression of HAMP1 and downregulated ferroportin, while a vice-versa response was noted in anaemia. HAMP2 levels, on the other hand, were unperturbed in both cases. Notably, both HAMP1 and HAMP2 were significantly upregulated, and ferroportin level decreased to prevent microbial proliferation. There is evidence for fish hepcidin acting as a sensor for divalent metal ions like Cu^{2+} and Cd^{2+}, as well, which may be an indicator of toxins in the environment (Masso-Silva and Diamond 2014).

8.7.4 Cathelicidins

Cathelicidins are the most divergent among AMPs. They are produced as preproproteins with a N-terminal signal peptide, a conserved cathelin domain and a C-terminal antimicrobial domain. The cathelin domain consists of four cysteine residues forming two disulphide bridges (Maier et al. 2008). Homology-based search using the cathelin domain has led to identification of several homologs of this peptide in various species. The cleavage of the signal peptide releases the AMP which shows high variation in size. Both fish and mammalian cathelicidins share a four-exon gene structure. However, the fish cathelicidin is much linger and divergent than its mammalian counterpart ranging between 47 and 80 amino acids. These C-terminal AMPs have a high positive charge exceeding +10 and are extremely rich in glycine and serine residues (Scocchi et al. 2016). Based on sequence, the salmonid cathelicidins are divided into the CATH-1 and CATH-2. CATH-1 has a single disulphide bond in the mature peptide which is absent in CATH-2. The former also has up to eight copies of six amino acid (RPGGGS) motifs, of which there are only one to two in CATH-2. Scocchi et al. (2009) have also reported another energy-saving form of this AMP, which lacks the cathelin domain. The loss and gain of cathelicidins are evident in the teleost lineage, and the presence of multiple forms of the AMP indicates gene diversification.

Expression studies indicate the presence of cathelicidin transcripts in various tissues like gills, head kidney, spleen, skin and gut; however, the degree of expression varies across species. Similar to other fish AMPs, this peptide is also expressed during the early developmental stages in fish as evidenced by studies in rainbow trout (Broekman et al. 2011). The divergence in sequence is also reflected in the functions of fish cathelicidins evident from the variation in antimicrobial activity profile across fish species. For example, among salmonids, *Oncorhynchus mykiss* cathelicidins show antibacterial response against *Yersinia ruckeri*, but *Salmo salar* cathelicidins do not. Potent antifungal response is also exhibited by cathelicidins in *Gadus morhua* and *Oncorhynchus mykiss* (Broekman et al. 2011; De Bruijn et al. 2012). Their expression is induced by both pathogens and their motifs like LPS or flagellin (Masso-Silva and Diamond 2014). Immune regulation by fish cathelicidins is reported in some salmonid species via modulation of cytokines (IL-1β and IL-8); however, the area requires further investigation (Schmitt et al. 2015; Acosta et al. 2019).

8.7.5 Histone-Derived Peptides

The antimicrobial potential of histone fragments was first established for Buforin I in *Bufo bufo* (Kim et al. 1996). Several histone-derived AMPs have been identified in fish from both N and C termini of histones – H1, H2A, H2B and H6. It is noteworthy that histone H2A derived from *O. mykiss* is the only non-cationic AMP identified in fish. Despite having a neutral charge, it shows an effective response against several bacteria. In contrast, Oncorhyncin II, derived from the same species, has a net neutral

positive charge of +30 and yet shows lower antibacterial potency in comparison to H2A (Brunner et al. 2020). Both the peptides are also known to bear antifungal effects (Fernandes et al. 2002). Immunomodulation by H2A in zebrafish upregulated the expression of antibacterial and MHC-related genes, which is suggestive of mediation of adaptive immune responses by these peptide (Wu et al. 2019).

8.8 Scope of Application of AMPs in Aquaculture

The broad-spectrum antimicrobial activity of AMPs and the lower probability of development of antimicrobial resistance against them escalate their candidacy for therapeutic applications in aquaculture. The results from Pan et al. (2017) study proposed TP3 and TP4 as potential drug candidates for bacterial disease management in hybrid tilapia. Similar results were achieved with the hepcidin, LEAP-2 (liver-expressed antimicrobial peptide) which lowered the ampicillin-induced resistance in *A. hydrophila*, making them more sensitive to the antibiotic (Chen et al. 2020). The supplementation of TP4 expressing yeast cell in the meal of *Lates calcarifer* led to significant enhancement in growth and immunity of the fish (Huang et al. 2020). Another LEAP-2 family AMP identified from *Erythroculter ilishaeformis* has emerged as a promising substitute for antibiotic-based treatment of common aquaculture pathogens, drug-resistant *A. hydrophila* and *Vibrio haemolyticus* (Chen et al. 2020). The peptide was able to clear ampicillin-resistant *A. hydrophila* at five times its minimum inhibitory concentration (MIC) in less than an hour. It also showed synergistic effect for infection clearance with ampicillin, this indicating the scope for combinatorial therapy with AMPs and antibiotics. Epinecidin-1 from *E. coioides* has also emerged as a promising molecule for the treatment of foot and mouth disease virus, a picornavirus known to be a major threat to breeding livestock globally (Huang et al. 2018). Data from this study show the impairment of the infection as well, when treated with AMP at the time of the adsorption of viral particles. Interestingly, supplementation of AMPs from *B. subtilis* in the diet of *E. coioides* was able to improve non-specific immune response and antioxidant status, reiterating the versatility of AMP application (Su et al. 2019). Among the commercial AMPs, moderate levels of dietary cecropin AD successfully promoted intestinal health and disease resistance against *Edwardsiella tarda* without hampering growth or gut microbiota profile (Dai et al. 2020). However, at the highest experimental dosage of 1000 mg/kg, the AMP-supplemented diet inhibited the growth of *Bacteroides* in the intestine and was detrimental to the diversity of the gut flora. In a recent report by Wang et al. (2021), his team was able to create a dietary concoction supplemented with an AMP mix, which not only helped in clearance of *A. hydrophila* infection in *Carassius auratus (var. Pengze)*, but could also enhance growth at low concentrations. One of the most successful vaccines of the aquaculture industry, the Enteric red mouth disease vaccine, was launched in 1976 to combat *Y. ruckeri* infection in salmonids. Recent investigation of its molecular mechanism of action unveiled the active involvement of AMPs (cathelicidin and hepcidin), alongside cytokines and acute phase proteins in

host-mediated response to the vaccine, thus highlighting its significance as a biomarker for aquaculture vaccine development (Wangkahart et al. 2019).

Though these results are encouraging, they need to be followed up with thorough investigation on the impact of AMPs on fish physiology to avoid the risks in field-based commercial application. With the advent of omics-based technologies, it is now becoming easier to span more species for their AMP coverage. High-throughput sequencing of *Betta splendens* transcriptome led to the identification of four AMP families in the species (Amparyup et al. 2020). Similarly, two prosaposin-like peptides were detected via NGS analysis from *Platichthys stellatus* with antimicrobial activity against bacteria, virus and parasite (Choi et al. 2020). The properties of fish AMPs have been amply explored with respect to pathogens relevant to humans; however, their role in aquaculture disease management remains to be intensively explored (Masso-Silva and Diamond 2014).

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Gut Microbiome Research: A New Avenue for Aquaculture Disease Management

9

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Abstract

Aquaculture is considered as one of the fastest growing, efficient and diverse animal protein production sectors globally. With the expansion of aquaculture industry, use of intensive culture methods and introduction of exotic species have greatly increased that have led to the incidence and severity of disease outburst among culturable aquatic species. Considering the role of gut microbiota in digestion, metabolism, health and immunity, modulation of gut microbiota could be a worthwhile control management strategies to counteract reported and emerging pathogens in aquaculture and hence to curb the enormous economic losses. In order to promote sustainable aquaculture and to mitigate the stress mediated through diseases, current knowledge on gut microbiota focusing beneficial and potential pathogens and impacts of gut microbial dysbiosis needs to be extended to generate species-specific gut microbial resources that are linked to modulation of key immune responses and metabolic pathways. Although the databases for environmental DNA (eDNA) and pipelines for the prediction of metagenomic functions of gut microbiota are limited for fishes, however foundational informations are being updated more frequently in recent times to identify core and precise bacteria and evaluating their role in gut health and immune responses. Furthermore, advancement in computer programming and data

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analysis packages have widen the opportunity to identify the link between diseases and particular taxa, indicator or marker bacteria for species specific disease diagnosis and treatments.

Keywords

Aquaculture · Pathogens · Metagenome · Gut microbiota · Immunity

9.1 Introduction

Microbiome is defined as a collection of small microscopic creatures including bacteria, viruses, algae, fungi, archaea and eukaryotes. The microbes are ubiquitous, incredibly diverse and present in almost everywhere in the environment including air, water, soil, fruits and vegetables and in association with other microorganism in animal organs and tissues like the gut, skin, mouth and lungs (Adamovsky et al. 2018). The expansion in microbiome studies for environmental samples has been driven by increased access, reliability and reproducibility of next-generation sequencing (NGS), a culture independent platform, and therefore does not require knowledge of target microbes (Perry et al. 2020).

Anthropogenic and natural incidences result in global changes that pose significant threat to aquatic ecosystems. Extensive aquaculture, climate change, ocean acidification, eutrophication and pollution have impacted microbial communities, host-pathogen interaction and immune defence function of aquatic animal (Sehnal et al. 2021). The changes in environment conditions cause shift in aquatic factors including light, temperature, pH, dissolved oxygen and organic waste and affect the microbial communities in freshwater and oceanic habitats. The natural interaction between microbial communities and aquatic ecosystems is much sensitive wherein disruptions due to symbiotic relationship can bring ecological disturbances (Sehnal et al. 2021). Under the altered environmental conditions, shift in microbial communities can lead to significant impacts on health and immunity of aquatic species. As the aquaculture production and human consumption have increased significantly over the decades in parallel with pollution, the industry must focus on the application of modern technologies to maximise production through sustainable aquaculture.

The gut microbiota plays a crucial role in digestion, in development of immune system and in optimal nutrient absorption of aquatic species. Identifying and monitoring of species-specific gut microbial structure and composition can help in differentiation of beneficial and pathogenic microbes in the gut under altered environmental conditions and diet changes (Xiong et al. 2019). Disruptions, dysbiosis and disturbances of gut microbial compositions lead to the development of various diseases in aquatic animals (Butt and Volkoff 2019). In this review, we aimed to summarise recent studies of aquatic species to identify the role of gut microbiota in diseases and potential disease management strategies to mitigate disease-associated aquaculture losses.

9.2 Gut Microbiota: What Are they?

Traditionally microbes linked to aquaculture productions, aquatic diseases, have been isolated through plate culture. Since a very small percentage of viable microbes in the aquatic environment can be grown in the laboratory (Amann et al. 1995), culture-independent methods have been developed and emerged rapidly to investigate the compositional and functional feature of aquatic microbes. Together with polymerase chain reaction (PCR), quantitative real-time PCR, restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), "omics" technologies including 16S, 18S, internal transcribed spacer (ITS) sequencing, meta-barcoding, metagenomics and meta-transcriptomics are now progressing rapidly providing deep insights into composition and functions of microbiome in the aquatic environment (Ringø et al. 2016). Microbiome is the total collection of microbes, their genome, interactions and functions in a particular environment. Alike other animal, the gut microbiota of aquatic species plays a crucial and indispensable role in physiology, digestion, development, immunity and protection against pathogen. Several studies have been performed recently to identify the gut microbiome of healthy fish and external factors associated to changes in microbial interactions and immunity of fish and crustaceans (De Bruijn et al. 2012). As most of the studies have been focused on diets, rearing conditions and genotype of aquatic species linked to gut microbial changes, not much information is currently available on the interaction and correlation between gut microbiota, immunity and diseases (Nie et al. 2017, Xiong et al. 2019).

9.3 Overview of Gut Microbiota in Aquatic Animal

Overall gut microbiota of aquatic animals is dominated by *Proteobacteria*, *Firmicutes*, *Tenericutes*, *Bacteroidetes* and *Fusobacteria* (Talwar et al. 2018; Wang et al. 2018). Higher abundance of *Tenericutes* differentiates crustacean gut microbiota from fish (Dong et al. 2018; Foysal et al. 2019; Zhang et al. 2020). The gut microbial diversity of aquatic animals is species-specific and depends on various factors including habitat, ecology, physiology, feeding nature and rearing environment (Zhang et al. 2020). The gut microbiota of saltwater aquaculture species are dominated by Gram-negative bacteria *Aeromonas*, *Acinetobacter*, *Bacteroides*, *Pseudomonas*, *Vibrio*, *Flavobacterium* and *Photobacterium*, whereas freshwater species have higher abundance for *Bacillus*, *Pseudomonas*, *Enterobacter*, *Clostridium*, *Flavobacterium*, *Fusobacterium*, *Cetobacterium* and *Lactobacillus* (Talwar et al. 2018). Some of the bacteria are ubiquitous in the gut environment with higher taxonomic values and systematically associated with digestion and metabolism and hence defined as core or resident microbiota of animals (Roeselers et al. 2011).

9.4 Pathogen in the Gut of Aquatic Animals

Monitoring of core and pathogenic bacteria can be used as an indicator to analyse the health status of an aquatic animal. Pathogenic microbes are integral part of the fish gut, and despite their existence, they often do not cause diseases. Infection or progression to diseases results in altered core microbiota, diversified keystone taxa, much complicated bacterial network, enrichment of pathogen and upregulation of gene functions for disease pathways (Foysal et al. 2021; Rajeev et al. 2021), a process called dysbiosis (Moya and Ferrer 2016). Overall, disturbance of *Firmicutes* and *Bacteroidetes* in the fish and *Firmicutes* and *Tenericutes* in the crustacean gut are associated with most of the diseases, predispositions and stresses (Table 9.1). The imbalances in the protective microbial communities in the gut may be arise from diets and changes in the rearing environmental conditions that include temperature, pH, dissolved oxygen, organic waste, seasonal and climate changes, antibiotic use and pollutions. Resident or core microbes in the gut can prevent pathogen by three mechanisms, niche exclusion, competition for essential resources and antibiosis (Banerjee and Ray 2017). In niche exclusion, some probiotic bacteria colonise in the mucosal tissue and occupied the infection site. Iron is essential for growth and microbes obtain iron through iron-chelating compound, siderophores. Resident microbes usually compete with pathogen through higher affinity for iron or by feeding siderophores produced by pathogen (Ahmed and Holmström 2014). Resident microbes, specifically from *Firmicutes*, can also produce certain type of antimicrobial compound to antagonise pathogen, a process referred antibiosis. For instances, lactic acid bacteria can produce cell-free supernatant that can protect fish from *A. salmonicida* infection (Ringø and Holzapfel 2000).

9.5 Model for Colonisation of Gut by Microbes

The process of colonisation of the gut by microflora begins with stochastic assembly followed by a deterministic trend. Stochastic assembly is also referred to as neutral assembly in lieu of the random environmental factors seeding the population during early developmental stages of the host. This population is finally driven in a determined direction during the adult phase by non-neutral parameters like host selective pressure, host-microbe interaction and microbe-microbe interaction (Talwar et al. 2018). The gastrointestinal tract provides a conducive environment for the housing microbes within. However, the screening of microbes fit to retain shelter within the gut is based on various microbial adaptations. The quintessential of these requirements is the ability to adhere to the mucosal epithelium of the wall of the gut. Several bacterial species also possess the capability of biofilm formation, which helps them evade unfavourable conditions within the gastrointestinal tract (Kalia et al. 2017). Further adaptations in the gut microbes include enzyme synthesis to maximise nutrient utilisation and the presence of defensive tools for hostile conditions like bacteriophage strike (Ley et al. 2006). The principle of the survival of the fittest rules here as well, which allows the best adapted microbes to become

Table 9.1 Phyla/genera reported as potential threat to host

Taxa/genus	Species	Disease biomarkers	References
Vibrionales and *Flavobacteriales*	Shrimp (*Litopenaeus vannamei*)	Reduction of *Verrucomicrobiales*, *Alteromonadales*, *Alphaproteobacteria* and *Planctomycetales* by *Gammaproteobacteria*, *Vibrionales*, *Flavobacteriales* and *Mycoplasmatales* in the shrimp gut is the sign of white faces syndrome (WFS) and acute hepatopancreatic necrosis diseases (AHPND)	Chen et al. (2017), Dai et al. (2020), Zhou et al. (2019)
Fusobacteria	Shrimp (*L. vannamei*)	*Proteobacteria* and *Fusobacteria* have higher abundance in healthy shrimp. Decrease in *Firmicutes* and *Bacteroidetes* is linked to white spot virus infection (WSSV) in shrimp	Wang et al. (2019)
Vibrio, *Aeromonas* and *Shewanella*	Crucian carps (*Carassius auratus*)	Bacterial community in "red operculum" infected fish is less diversified and more stable than healthy fish, dominated by *Vibrio*, *Aeromonas* and *Shewanella*. *Cetobacterium* and *Clostridium* are healthy core gut microbiota of crucian carp	Li et al. (2017)
Vibrio spp.,	Marron (*C. cainii*)	Potential pathogen for crayfish. Negatively correlated to immunity by downregulating gene associated with immune response of crayfish. Indicator bacteria of stress for marron aquaculture	Foysal et al. (2020)
Aeromonas	Largemouth bronze gudgeon (*Coreius guichenoti)*	Overwhelming abundance of *Proteobacteria* (86 ± 12%) and *Aeromonas* (81 ± 17%) was detected in fish suffered from furunculosis. Significant drop of *Actinobacteria*, *Firmicutes* and *Tenericutes* was observed in the diseased fish gut. In healthy fish, *Aeromonas* OTUs represented by only one-third of total sequences	Li et al. (2016)
Streptococcus, *Leuconostoc* and *Weissella*	Rainbow trout (Oncorhynchus mykiss)	Overrepresented *Streptococcus*, *Leuconostoc* and *Weissella* in the gut fed plan-based diet can prevent enteric red mouth disease in rainbow trout caused by *Yersinia ruckeri*. Gut microbiota is positively correlated to protective immune response	Ingerslev et al. (2014)

(continued)

Table 9.1 (continued)

Taxa/genus	Species	Disease biomarkers	References
		against *Yersinia ruckeri* infection in rainbow trout	
Vibrio	Seahorses	*Vibrionaceae* including *Vibrio ponticus* and *Vibrio neptunius* are abundant in intestinal-diseased seahorses. *Rhodobacteraceae*, *Alcaligenaceae* and *Serratia* are healthy intestinal flora of seahorses	Li et al. (2015)

permanent inhabitants of the gut and are referred to as autochthonous microbes. The transitory or temporary inhabitants are contributed by surrounding water or from feed variation during host dispersal and are referred to as allochthonous organisms (Rieu et al. 2014). During studies, the two communities of autochthonous and allochthonous microbes are derived separately. While the former is derived from the scraped sample of the mucosal epithelium, the latter is profiled from the faecal samples and offers a higher species richness as compared with the autochthonous counterpart (Gajardo et al. 2016).

9.6 Determinants of Gut Microbiota Profile

The determinants affecting the gut microbiota profile include both abiotic and biotic factors. The impact on the microbial diversity or population consequentially also affects the host's function and metabolism.

9.6.1 Abiotic Factors

9.6.1.1 Environmental Factors

The initial development of the fish embryos occurs within the egg, and it is only after hatching that the larval stages have the novel opportunity of interaction with microbe-populated environment. From here on, the environmental factors like water temperature, salinity and its inherent microbe population play an essential role in contributing to the microbial diversity of the fish gut. There is evidence of variation in the composition of microbial population between freshwater and marine habitats. The studies indicate that the freshwater fish gut is predominantly populated with *Aeromonas* and *Pseudomonas* species, while in the marine species, *Vibrio* species is largely present (Vatsos 2017). The salinity of water is also evidenced to induce significant changes in microbiome composition. The assessment of the impact of 3% and 7% salinity on the *Esox lucius* fry deduced differences in abundance levels of *Proteobacteria* classes in the two groups, with *Alphaproteobacteria* being prominent in the latter group and *Betaproteobacteria*

in the former. They also correlated their results with higher mortality of the fry reared in 7% salinity conditions and suggested 3% salinity levels to be more conducive for fish larval development (Dulski et al. 2020). Schmidt et al. (2015) was also able demonstrate the impact of salinity in significantly altering the gut microbiome of *Poecilia sphenops*. Similar results were also seen in farmed Atlantic salmon, where the farmed fish in freshwater had a varied diversity profile of microbes from those acclimated in seawater (Dehler et al. 2017). However, in both the groups, a core unit of 19 OTUs was maintained, suggesting their involvement in basic fish gut functions. Another study on salmon itself also found a shift in microbiome composition in context of its migration from freshwater to seawater, thereby indicating the microbial profile in freshwater individuals to be immature (Rudi et al. 2018).

Temperature also impacts the microbiota and becomes a significant factor for cold-blooded organisms like fish. Huyben et al.'s (2018) study found an inverse correlation between rearing water temperature and the relative abundance of *Firmicutes* in *Oncorhynchus mykiss*. The relative abundance of *Proteobacteria* is also seen to vary in fish gut microbiota composition in response to temperature (Sepulveda and Moeller 2020). A shift was seen in the relative abundance of *Gammaproteobacteria* within the gut microbial flora of *Salmo salar,* with increase in temperature. The population of *Acinetobacter* plummeted, while that of pathogenic *Vibrio* rose (Neuman et al. 2016). Similar observations have also been made for the abundance of *Gammaproteobacteria* in *Seriola lalandi* (Soriano et al. 2018). Seasonal fluctuations tend to alter the microbial composition of gut in various fish species, in turn, harming the health of the fish due to contraindications like gut dysbiosis (Huyben et al. 2018). Seasonal variations in gut microbiota have been observed in tilapia and its hybrid counterpart. In the latter, the presence of *Flavobacterium*, *Micrococcus* and *Pseudomonas* species is exclusive to the winter season only (Al-Harbi and Uddin 2004).

Another factor under the realm of environmental factors is rearing condition of the fish. Fish may be derived from open-water sources or bred for aquaculture in indoor aquariums or cage facility. The comparative analysis of gut microbial profile of wild versus captive Malaysian Mahseer (*Tor tambroides*) suggested a higher diversity for the former (Tan et al. 2019). In case of wild fish, the properties of the open water source are dynamic as they are constantly influenced by effluents from natural or anthropogenic sources. Therefore, it may be surmised that the constant change in water quality also affects the microbial diversity of the fish habitat. There is evidence of shared OTUs in the microbial assembly of fish gut and surrounding water (Butt and Volkoff 2019). Presence of pollutants and toxins like pesticides, heavy metals and antibiotics has also been seen to cause significant shifts in abundance profiles of OTUs in carps and zebrafish. Acid rain may alter the pH of water, and a simulation of the same was seen to have detrimental impacts on the skin and gut microflora of *Colossoma macropomum* (Sylvain et al. 2016). Interesting, the gut microbiota population was more resilience to the acidic pH than the skin microbes. The contribution of factors like geographical location and ecological weaken in the favour of host selective pressure (Talwar et al. 2018).

9.6.2 Biotic Factors

9.6.2.1 Host-Derived Factors

The host-derived factors impacting gut microbial profile of fish include developmental stage, sex, feeding habits, genetics and species phylogeny. There are numerous reports citing the both inter- and intra-species variations in gut microbiota (Butt and Volkoff 2019). Interspecific variations are even evident in the organisms reared in the same conditions. The intestinal bacterial profile of bighead carp, grass carp, silver carp and blunt snout bream was significantly different, despite being reared in the same conditions (Li et al. 2012). It is noteworthy that the eukaryotic microorganism profile did not show any significant variation in this study. Sex-specific differences in gut microbe composition have also been reported in *Gasterosteus aculeatus* and *Perca fluviatilis* (Bolnick et al. 2014). These differences have been attributed to host-microbe interactions and diet-related variations, but the molecular basis of understanding based on sex steroids or sex-specific genes is still lacking. The age and developmental stage of fish also plays a considerable role in governing the structure of gut microbiota in fish. In Gibel carp, the initial stages of development are governed by environmental filtering and in the later stages; as the diversity of the microflora increases, these factors become less involved (Li et al. 2017b). Bacterial communities in fish have identified at as early as the fertilised egg stage in grass carp (Wang et al. 2018). The progression of bacterial OTUs shifted from *Proteobacteria* in the egg stage to *Bacteroidetes* in the larvae. The autochthonous microbial profile in this case was composed of *Chitinophagaceae*, which persisted from the egg stage to the first ingestion stage of the larva. Additionally, within the individual organism itself, the microflora diversity shows a shift across the various regions of the gut. Despite the maintenance of a core autochthonous community in mid and hind gut, the two regions showed a noticeable variation in beta-diversity of the occupants on gut wall and housed contents (Nielsen et al. 2017).

The genetic backdrop of the host is also crucial for the diversity of the gut microbial profile of the fish. A study conducted on closely related filter feeding carps reared in identical conditions found that the host genetics impacted gut microbiota and metabolite profiles. Though considering that these are herbivorous species, the prominent genera of cellulose-degrading bacteria (*Aeromonas, Bacteroides, Clostridium and Pirellula)* were identified in all the species, which may be accounted for by the factor of phylogenetic proximity (Li et al. 2017a). In another interesting study, Li et al. (2018) bred a hybrid fish lineage from an herbivore (*Megalobrama amblycephala*) and a carnivore (*Culter alburnus*) parent and assessed the microbiota assembly of parents versus hybrid progeny. They referred to the host genetics that led microbiota variation as subgenome domination wherein both the reciprocal hybrids showed higher similarity of gut microbial profile with the herbivore parent. In another report by Bledsoe et al. (2018), two genetically distinct strains of the family *Ictaluridae* showed no significant variation in the gut microbiota assembly or abundance, thus suggesting limited impact of host genetics in regulation of gut microbial structure. Based on these reports, one may say that the

hypothesis on the extent of impact of host genetics on host microbiota is still inconclusive.

9.6.2.2 Feeding Behaviour of Host

The feeding behaviour plays a major in establishment of the microflora structure in the gut of an aquatic species. For example, the microbial diversity shows alterations across herbivorous, omnivorous and carnivorous species. A plant-rich diet requires higher digestive capability, which is fulfilled via enhanced production of exogenous enzymes like cellulase, lipase and protease. In freshwater herbivore, grass carp, bacterial communities majorly included *Actinomyces*, *Citrobacter, Clostridium* and *Leuconostoc* (Wu et al. 2012). *Aeromonas, Bacillus, Citrobacter, Clostridium I*, *Leptotrichia* and *Pseudomonas* are some of the common cellulose digesting groups found in the gastrointestinal tract of herbivore fish (Liu et al. 2016). Marine herbivore *S. fuscescens*, on the other hand, mainly thrives on sea algae, which metabolise differently from other plant diets. A higher representation of short-chain fatty acid producing Phyla *Bacteroidetes, Firmicutes,* and *Proteobacteria* is seen in the hind gut of this species. The enhanced presence of short-chain fatty acids in the hind gut contributes towards fermentation of complex polysaccharides into simpler compounds that can be easily absorbed by the host (Jones et al. 2018). In another marine algalvore species, *A. triostegus,* the most abundant genera in both mid and hid gut were *Epulopiscium* and *Brevinema* (Parata et al. 2020). The study suggests that the former genus is involved in seeding symbiotic bacterial community in the gut and is hosted by surgeonfish via feeding (mainly coprophagy). In contrast to the higher requirement of amylase and cellulase by herbivorous fish, the carnivorous species need higher protease (trypsin) activity, the demand for which is sufficed by *Cetobacterium* and *Halomonas* (Liu et al. 2016). The study found a mix of bacterial genera (*Cetobacterium and Leptotrichia*) from both herbivore and carnivore groups in the gut microflora of omnivore fish species. This is a clear indication of trophic level acting as one of the key determinants of gut microbiota structure.

9.7 Role of Gut Microbiota

9.7.1 Digestive Functions

There are ample studies that directly correlate the gut microbiota with regulation of appetite, digestion and metabolism. However, most of these studies related to the microbiota–gut–brain axis have been restricted to mammals, and very limited reports are available in fish. The phylogenetic diversity of the phylum constituted by fish and the degree of variation with respect to habitat and feeding habits has also widened the scope for nuanced variations in context of regulation of digestive functions (Butt and Volkoff 2019). The gut microbes have been demonstrated to exhibit commensalism with other microbes and the host. They produce an array of digestive enzymes (amylase, cellulase, esterase, protease, phosphatase and lipase) that aid the breakdown of carbohydrates, lipids, protein and cellulose (Perry et al.

2020). They short-chain fatty acids produced by the anaerobic microbes play a vital role in fermentation of algal diets. They have also been associated with the synthesis of amino acids and vitamins in fish as seen in *Oreochromis niloticus*, *Icatlurus punctatus* and *Carassius auratus* where the enrichment of *Bacteroides* and *Clostridium* caused an increase in the level of vitamin B12 (Sugita et al. 1991). Most of the studies conducted in fish include diet variations in fish followed by assessment of the feeding rates and gut microbiome. The varsity of combination of dietary supplements and fish species has led to several studies but inconclusive results due to inconsistent parameters. The supplementation of *Lactobacillus acidophilus* in the diet of *Carassius auratus* led to the downregulation of a ghrelin, a hunger-inducing hormone (Hosseini et al. 2016). Similar pattern of decrease in appetite was also seen in zebrafish fed with probiotic (*Lactobacillus rhamnosus*) as a dietary additive (Falcinelli et al. 2016; Falcinelli et al. 2018). On the other hand, the supplementation of dietary Galactooligosaccharide (GOS) in *C. auratus* caused an increase in ghrelin expression leading to an enhanced appetite in the fish (Miandare et al. 2016). Further, no effect was seen in the feeding rates of *Cyprinus carpio* that fed a diet supplementation with fructooligosaccharide (FOS) (Hoseinifar et al. 2014). Since digestion and metabolism go hand in hand, an alteration of gut microflora also triggers changes in the metabolic pathways of the nutrients. The supplementation of *B. clausii* in the feed of *Paralichthys olivaceus* improved growth performance and feed efficiency of fish, which was attributed to enhanced appetite in the fish (Ye et al. 2011). The regulation of lipid metabolism by the gut microbes is crucial for maintenance of energy homeostasis within the host. Sheng et al. (2018) used germ-free and antibiotic-treated (depleted gut microflora) zebrafish models to demonstrate that loss of gut microbes leads to increase in expression levels of host genes key to lipid catabolism and cholesterol synthesis. On the other hand, the control fish showed an upregulation of genes involved in lipid absorption or biosynthesis. These reports highlight the importance of gut microbiota in maintaining the energy and balance and health of the host.

9.7.2 Immunity and Stress Response

The link between gut microbiota and immunity is even more prominent in fish due to its direct contact with water from where they draw both oxygen and food. A healthy gut microflora is known to protect the fish from the colonisation of pathogenic microbes. This phenomenon is also referred to as "colonization resistance" wherein the commensal residents of the host's gut compete with the pathogens for their niche to prevent their unfavourable proliferation (Butt and Volkoff 2019). The gut microbes also participate in development and maturation of gut-associated lymphoid tissue (GALT). A disruption of gut microbiota often triggers GALT activation and inflammation. Here is still a long to get a wholesome understanding the commensal mechanisms by which they offer colonisation resistance (Lazado and Caipang 2014; Wang et al. 2018). This has paved way for application of probiotics as immunomodulators for controlling infection and mortality of fish in the aquaculture

sector. The most commonly used genera of probiotics in aquaculture are *Bacillus* and *Lactobacillus*. They are reported to enhance the immune indices (expression of inflammatory cytokines, phagocytic activity, immunoglobulin levels and number of goblet cells in mucosal layer) of the fish to provide better resistance against disease (Perry et al. 2020). The regulation occurs at the interface of both immune and adaptive arms of immunity (Lazado and Caipang 2014). The host goes a step ahead to recognise and coat the gut bacteria with secretory immunoglobulins to prevent them from disrupting the gut epithelium, as seen in case of *Gasterosteus aculeatus* (Zhang et al. 2010). The fish are subjected to various stress factors in different surroundings. For example, in the wild, the factors are environmental pollutants or temperature fluctuations, while in a culture set-up, the stress may be confinement, high animal density and malnutrition. The stress response is mediated by the hypothalamic–pituitary–adrenal (HPA) axis, which stimulates the secretion of corticotrophin-releasing hormone (CRH) followed by the secretion of adrenocorticotropic hormone (ACTH) and finally culminates into secretion adrenal glucocorticoids that act to adjust the homeostasis energy for coping mechanism. Stress factors can lead to disintegration of gut lining and change the properties of mucus, which disrupts the microbiota profile, thus making way for opportunistic pathogens (Butt and Volkoff 2019). When Asian sea bass were subjected to starvation, they showed an increase in the abundance of *Bacteroidetes* and diminished *Betaproteobacteria* in their gut (Xia et al. 2014). The functional analysis of the differentially expressed gene in the microbiome also highlighted an upregulation of antibiotics. Additionally, the upregulated expression of immune-related genes in the host corroborates the preparation of the species for a stress response. Exposing Atlantic salmon to mild confinement stress led to an enrichment of *Clostridia* and *Gammaproteobacteria*, while the abundance of *Carnobacterium sp* deteriorated. Interestingly, the authors have attributed this disruption of gut microbiota increased levels of cortisol in faecal sample of the fish (Uren Webster et al. 2020).

9.7.3 Neuroendocrine and Reproductive Functions

The microbiota–gut–brain axis is bidirectional communication system that relays to and fro signals between the gut and central nervous system. It was seen that 4 weeks of administration of *Lactobacillus rhamnosus* IMC in zebrafish diet created differences in shoaling behaviour (Borrelli et al. 2016). At the molecular level, significant variation was noted in the levels of genes involved in serotonin synthesis and signalling, with contrasting expression patterns in gut and brain. The gut microbiota shows an increase in *Firmicutes* and a decline in the *Proteobacteria* population, which is an indicator of improved gut health in zebrafish. The results suggested that the probiotic induced a psychotropic effect via the microbiota–gut–brain axis that boosted the exploratory behaviour and shuttling activity. The same probiotic has also been reported to enhance the reproductive health of zebrafish by accelerating growth and sex differentiation (Carnevali et al. 2012). The treated fish in this experiment exhibited higher gonadosomatic indices (GSI) and upregulated

expression of female reproductive genes luteinizing hormone receptor, the vitellogenin, estrogen receptor α, membrane progesterone receptors α and β, growth differentiation factor9 (gdf9) and bone morphogenetic protein15. The authors implicated the possible involvement of kiss and leptin receptor system in accelerating sexual maturation in treated zebrafish. Though this aspect of correlation of microbiota and neuroendocrine response requires deeper understanding and exploration in fish, the revelation of its underlying mechanisms carries huge potential for aquaculture industry.

9.8 Manipulation of Gut Microbiota and its Application in Aquaculture

The research on gut microbiota of fish has become immensely valuable for the aquaculture community of the growth and immune parameters of the species. The results of these studies have emerged as preventive measures to combat the emergence of infections and diseases in fish. The enhancement of immunity and in turn pathogenic resistance is also environmentally beneficial as it discourages the widespread use of antibiotics that is a major cause of antimicrobial resistance. This has been proven in salmonid species wherein addition of oxytetracycline to the fish feed resulted in increase in abundance of the microbes resistant to the administered drug (Navarrete et al. 2010). The microbiota of the antibiotic-treated group exhibited lower diversity in comparison with the control group, which consisted of the genera *Acinetobacter, Bacillus, Brevundimonas, Flavobacterium, Mycoplana* and *Psychrobacter*. The gut microbe profile of the treated group, on the other hand, was mainly comprised *Aeromonas*, the opportunistic pathogen with high frequency of genes coding for antibiotic resistance. Similar trend was also reported in *O. niloticus,* where prolonged exposure to oxytetracycline disrupted the microbiota assembly and also ameliorated the risk factor for consumer health (Limbu et al. 2018).

The manipulation of gut microbiota is a promising alternative to antibiotics for fish health management, and the application is in the form of prebiotics, probiotics and symbiotics. Probiotics are alive or dead microbes or microbial components that act by varied mechanisms to promote the quality of health of host or its environment. Most commonly used probiotic genera are *Bacillus*, *Carnobacterium, Clostridium, Enterobacter, Lactobacillus, Pseudomonas* and *Saccharomyces*. The probiotic prevents infection by inhibiting the adhesion of pathogen and secretion of unfavourable metabolites (Lazado and Caipang 2014). Their administration has also been associated with improvement in growth, feed efficiency and reproduction. Probiotic consortia have also been quite a success in comparison with usage of a single strain product (Butt and Volkoff 2019). *Bacillus* has also shown potential for bioremediation of aquatic environment by removal of waste from water (Soltani et al. 2019). The list of commercially used probiotics has been listed in Table 9.2. Prebiotics are nondigestible fermentable compounds that promote the functions of probiotics. Some commonly used prebiotics are carbohydrate derivatives like inulin,

Table 9.2 Commercially available probiotics for aquaculture

Commercial name and company	Microbe composition
Aqualact (*Biostadt India)*	*Bacillus spp., Lactobacillus spp.* and *Saccharomyces sp.* (also contains additional enzymes, vitamins, etc.)
Eco-pro water probiotic (*Biostadt India)*	*Rhodopseudomonas palustris* and *Rhodobacter capsulatus*
Environ-AC power (*Biostadt India)*	*Bacillus sp., Nitrosomonas sp., Nitrobactor sp., Nitrosomonas sp.*
Aquastar (*biomin*)	*Bacillus sp.,* Enterococcus *sp., Lactobacillus sp., Pediococcus sp.*
Eco-pro (*PVS laboratories)*	*Bacillus subtilis, B. licheniformis, B. megaterium, Nitrosomonas, Nitrobacter, Trichoderma viride, aspergillus oryzae*
Blue tide Gutpro aqua (*Ceva Polchem*)	Consortium of gram positive, sporulating and non-soprulating lactic acid bacteria
PowerShrimp™ (Qb-labs)	Seven *Bacillus sp.* strains
FeedTreat™ (Keeton industries)	Information *N/A*
PondSafe® (*Novozymes*)	Five *Bacillus Sp.*
Engest *(microtack*)	*Bacillus subtilis, B. megaterium, B. licheniformis*
Prolacto (*drug international*)	*Bifidobacterium bifidum, Lactobacillus acidophilus, L. bulgaricus* and prebiotic (fructooligosaccharides)
Grobact (*tropical biomarine system*)	*Bacillus coagulans, Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, L. rhamnosus, Saccharomyces boulardii, Streptococcus thermophilus*
Prosol (*Prosol chemicals*)	*B. longum, L. acidophilus, L. plantarum, L. rhamnosus, L. salivarius*
Sanolife PRO-F FMC (*INVE aquaculture*)	Information *N/A*
Hydroyeast aquaculture (*Agranco Corp*)	*Bifidobacterium sp., L. acidophilus, Streptococcus faecalis, yeast*

levan, fructooligosaccharides (FOS) and mannanoligosaccharides (MOS) (Yukgehnaish et al. 2020). They have been proven to be beneficial in enhancing growth performance, stress and immune response in several fish species. The combinations of probiotics and prebiotics are referred to as synbiotics and their administration boosts the survival, metabolism and immunity in fish. However, the limitation with their wide-scale application in aquaculture is that a pre-, pro- or synbiotic exhibits broad variation depending on its chemical nature, dosage and the species. Therefore, the results from the diet-supplementation experiments are inconclusive and require intensive laboratory testing before being tested on field.

9.9 Conclusions and Limitations

Dysbiosis of gut microbiota by pathogen has been shown to disrupt bacterial infection in the gut, which leads to induction of ecological niche for invasion by pathogen. Given to the importance of gut bacteria in health and immunity of aquatic animals, introduction and enrichment of beneficial bacteria could be a promising approach in protecting fish from infections. In recent years, lot of studies have been conducted on gut microbiota of aquatic animals resulting in enrichment of sequence databases. Therefore, in future, it is possible to identify the link between diseases and specific species and to optimise the gut microbiota accordingly to avert pathogen (Fig. 9.1). At this moment, the resolution of high throughput sequencing (HTS) is one of the major limitations to identify microbes at lower taxonomic level as it

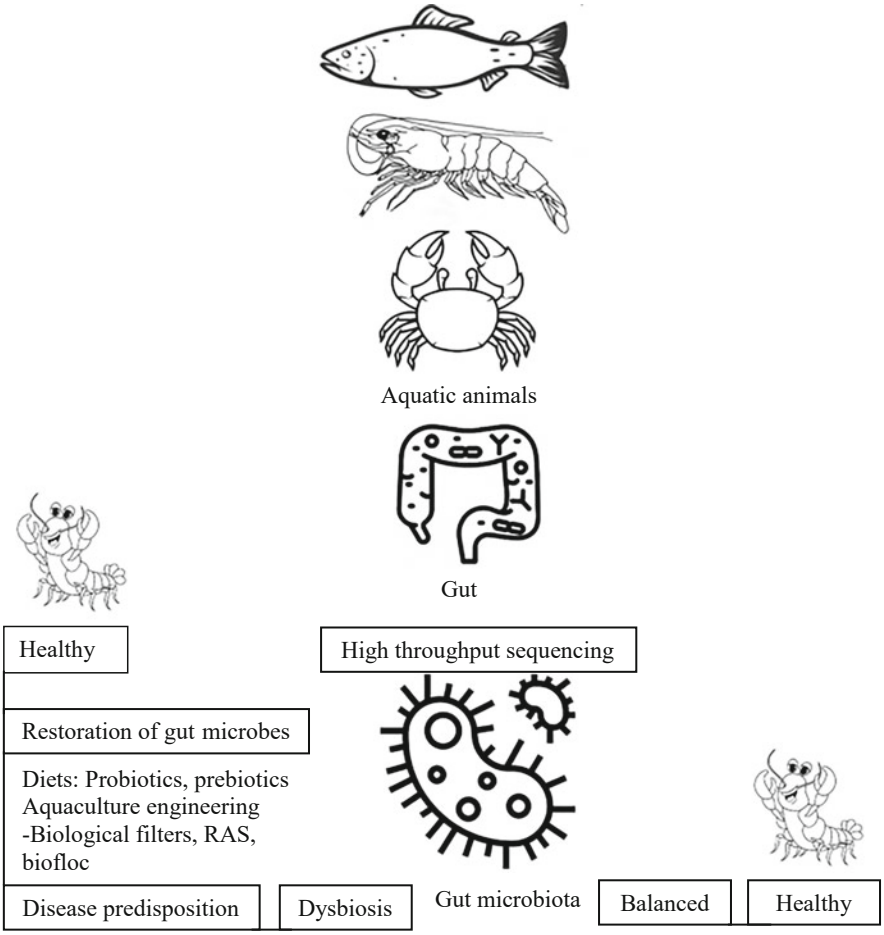

Fig. 9.1 Management and monitoring of gut microbiota to mitigate diseases in aquatic species

entirely depends on information generated from 500 bp of 16S rRNA. Additionally, the databases for environmental DNA (eDNA) are still underdeveloped compared with human DNA (hDNA), and hence the information about the functional compositions and metabolic features of 16S rRNA data is not entirely reliable. However, with the advancement of various HTS platforms, volume of sequence data generated for eDNA and subsequent modification of databases and pipelines, the issues related to reliability, dependency and preciseness could be fixed in upcoming years that would facilitate rapid diagnosis and prediction of the course and prognosis of diseases.

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Recent Understanding of Immunological Defence in Freshwater Pearl Mussel for Better Health Management

10

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Abstract

The production of cultured pearl represents a major industry in many countries including India. The species that belong to the genus *Lamellidens*, *Hyriopsis* and *Cristaria* are some of the important pearl-producing bivalve organisms in cultured condition. Due to the rising demand for cultured pearl, the intensification of culture practices of freshwater mussel is adopted by many aquaculture farms, resulting in disease outbreaks and economic losses. Due to filter-feeding behaviour, bivalves are sensitive to surrounding environments. In recent year, substantial strides have been taken up to explore the deeper understanding of immunological defence mechanisms of freshwater mussel. Many cellular and humoral parameters have been deciphered by various researchers. The updated knowledge of the defence system of mussel is considered as a potential intervention strategy in mussel farming to overcome diseases and make farming more profitable. This chapter focuses on recent information on major diseases and related defence mechanisms of mussels which may be of help for the unceasing expansion of aquaculture enterprises that encompasses cultured pearl production.

Keywords

Lamellidens marginalis · Freshwater mussels · *Hyriopsis* · Disease · Defence mechanism · Immunity

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10.1 Introduction

The freshwater bivalve molluscs that belong to the family Unionidae are highly economically important in the aquaculture industry particularly for freshwater pearl farming. Globally, four species of mussel namely *Hyriopsis cumingii, H. schlegelii, Cristaria plicata* and *Lamellidens marginalis* are commonly regarded as freshwater pearl-producing bivalve molluscs and economically important for countries like China, Japan and India. *L. marginalis* is extensively dispersed in ponds, lakes and rivers of Indian water (Saurabh et al. 2014). In addition, hybrid mussel (*H. cumingii x H. schlegelii*) constitutes an important part of the production of quality pearl (Fiske and Shepherd 2007). In India, more than 52 species of mussels have been documented from diverse freshwater resources. *L. marginalis, L. corrianus* and *Parreysia corrugata* are among the mussels used to make freshwater pearls (Janakiram 2003). The high demand for pearl in the market paves way for intensified culture. However, due to the intensification of culture practices and related climate change, various molluscs diseases are recognised in different countries, causing a decline in pearl production and posing a threat to the mussel culture. Therefore, mechanisms underlying the immune response in freshwater pearl mussel against different pathogenic organisms must be explored for developing useful prophylactic measures in future. It is a well-established fact that immunity is the most important physiological contrivance present in aquatic organisms for protection against a broad range of infection and maintenance of internal homeostasis. Nonspecific natural immunity, which is an innate defence system that renders the host resistant to infection by initiating intracellular molecular signalling cascades, is extremely important for bivalves (Gerdol and Venier 2015). Nonspecific type of immune defence can be found in all living multicellular organisms and are conserved across kingdoms (Danilova 2006). When any pathogen attacks a mussel, it is the nonspecific immune response, which forms the first line of defence and acts immediately to eliminate the invading pathogen or any other stressors. Thus, the ever-increasing interest in bivalve aquaculture by farmers and entrepreneurs endue a major impetus for the study of pearl mussel undelying immune defence mechanism (Fig. 10.1) against invading pathogens to obtain a deeper knowledge of the host-pathogen relationship. The immune defence of the bivalve is mediated by both humoral and cellular components (Song et al. 2010). The present chapter describes empirical disease scenarios in pearl mussels, worldwide and a brief about various components of the cellular and humoral immune system involved in mussels' immune defence.

10.2 Diseases of Freshwater Pearl Mussel

The pearl mussel residing in freshwater habitat is generally considered as a less susceptible species to disease problems as compared to marine bivalves. Nevertheless, few reports are available on the role of different pathogens causing disease outbreaks in freshwater pearl mussel. However, intensification of culture practices and exploitation of mussel for pearl production led to different disease outbreaks in

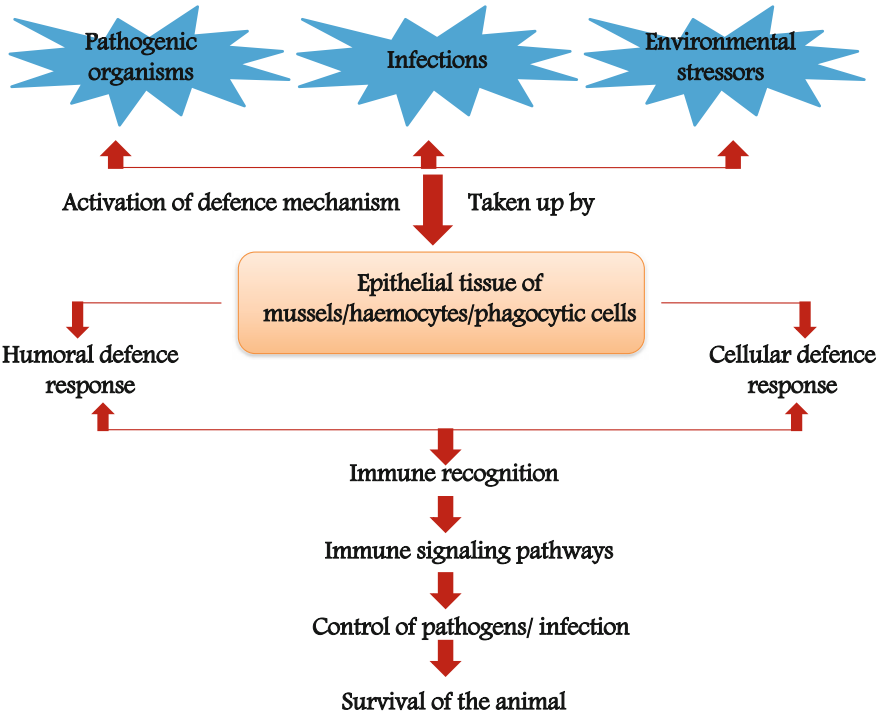

Fig. 10.1 A simple flow diagram showing immunological defence responses in freshwater mussel

cultured conditions. Diseases are found to impact commercial mussel production adversely. Grizzle and Brunner (2009) described many infectious ailments of freshwater mussels and other freshwater bivalve molluscs. Bacteria are generally considered as opportunistic pathogens and seem to take the upper hand when the immune status of the organism becomes compromised. *Aeromonas* is generally regarded as one of the destructive pathogens known, causing mortality to freshwater fish (Sahoo et al. 2008), freshwater prawn (Sung et al. 2000; Chand and Sahoo 2006), crayfish (Jiravanichpaisal et al. 2009; Yang et al. 2018), soft-shelled turtle (Chen et al. 2013), scallop (De Silva et al. 2019), clam (Dahanayake et al. 2019) and marine bivalves (Olafsen et al. 1993). *Aeromonas* virulence factors include biologically active compounds, adhesives metabolites, extracellular enzymes and toxins that cause mass mortality in aquatic species (Cahill 1990; Hossain and Heo 2021). Zhong et al. (2016) reported that *A. veronii* SJ-2 was associated with large-scale mortality of pearl mussel *H. cumingii*. The pathogenic strain of these bacteria caused lesions on various organs in the mussel, resulting in a gradual deterioration of normal physiological metabolism, which ultimately leads to mortality. Recently, Yang et al. (2020) isolated *Stenotrophomonas maltophilia* and *A. veronii* from the haemolymph and tissues of pearl mussel, *H. cumingii*, causing massive mortalities of cultured pearl mussel. In the freshwater mussel *H. cumingii*, Wang et al. (2013a) found a link

between SNPs in the interferon regulatory factor 2 (IRF-2) genes and resistance to *A. hydrophila*. In another study, Wang et al. (2013b) established a link between SNPs in the superoxide dismutase 3, extracellular (SOD3) gene and *A. hydrophila* resistance in the pearl mussel, *H. cumingii*. Wu et al. (2017a) reported toxic cyanobacteria, *Microcystis aeruginosa* and hypoxia-induced histopathological changes in the gills, digestive glands and stomach of the pearl mussel, *H. cumingii*, resulting in necrosis and mortality. When pearl mussel was challenged with three pathogens (*A. hydrophila*, *Streptococcus agalactiae* and *Pseudomonas fluorescens*), overt clinical symptoms such as tissue oedema, columnar cell disarrangement and epidermal detachment were identified, and the infection also adversely affected host immunity (Yang et al. 2021). The information related to viral infections in freshwater pearl mussel is very diminutive. Only one viral disease was reported in pearl mussel, *H. cumingii*. Light and transmission electron microscope (TEM) analysis of tissues from diseased bivalve mussels identified the etiological agent as bisegmented ambisense RNA arenavirus under the family Arenaviridae, which is subsequently termed as *H. cumingii* plague virus (HcPV) (Zhang 1986, 1987). Lei et al. (2011) examined the histopathological changes including lesions in different organs, cell vacuolisation accompanied with some empty areas and necrosis of some cells in tissues of mussel artificially infected by the plague virus. Stomach, digestive gland and intestine are identified as primary target organs of the plague virus.

Parasites and other infectious agents generally cause lesions that include inflammation and regressive phenomenon. Recently Paul et al. (2018) recorded *Glossiphonia complanata* infection in freshwater pearl mussel, *L. marginalis* that caused heavy mortality of cultured stock of mussel. Further, Pradhan et al. (2019) reported malachite green as the most effective means to control leeches with a better survival rate of mussel (Figs. 10.2, 10.3 and 10.4).

10.3 Epithelial Barriers as First Line of Immune Defence

The first line of defence includes structures, which form stable physical and biological barriers against pathogenic and environmental stressors. The outer shell of the pearl mussel is made up of hard substances that covers the internal soft tissue and protect the organism from various stressors or injury. Further, the skin and mucosal layer attached to the inner shell of the mussel constitute the second most important immune defence barrier. The most important function of host mucus is to prevent the attachment of bacteria, fungi, or parasites to the epithelial surfaces (Sahoo 2006). Generally, all molluscan epithelia produce mucus, which plays a crucial role in lubrication and feeding and acts as a shield against environmental stress and physical-biological barriers to infections (Allam and Raftos 2015). The rate of mucus secretion may increase after infection or physicochemical insults. In mucus, the presence of a large array of antimicrobial factors viz., agglutinins, lysozymes and protease have been reported (McDade and Tripp 1967; Brun et al. 2000; Xing et al. 2011). In addition, the space between shell and mantle tissue is

Fig. 10.2 Healthy mussel, *L. marginalis*

filled with extrapallial fluid, which harbours abundant haemocytes that not only provide protection against injury but also help in biomineralisation and shell deposition (Wilbur 1964; Allam and Paillard 1998; Fisher 2004,). Hydrolytic enzymes such as lysozyme and peptidases have also been reported in the extrapallial fluid that help to neutralise several pathogens (Allam and Raftos 2015).

10.4 Nonspecific Humoral Defence Molecules

The humoral defence factors in bivalve freshwater pearl mussel include the following:

10.4.1 Antioxidant Enzymes

In bivalves, a network of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) have been identified, and their response to pathogens and stress has been studied in freshwater pearl mussel *L. marginalis* (Chakraborty et al.

Fig. 10.3 Leech infection in freshwater mussel

Fig. 10.4 Leech collected from mussel

2013) and *H. cumingii* (Xia et al. 2019; Yang et al. 2021). Different stressors in the aquatic environment might increase the intracellular generation of reactive oxygen species (ROS) capable of causing damage; yet, these networks of enzyme defence systems may be able to detoxify their damaging effects on organisms (Livingstone

et al. 1990; Kumar et al. 2014). Superoxide dismutase (SOD) is a constitutive protein that catalyses the conversion of the superoxide (o_2^-) radical into oxygen and hydrogen peroxide. The SOD activity was modulated in pearl mussel, *H. cumingii*, following exposure of an organism to ammonia-N (Xia et al. 2019). Yang et al. (2021) found that SOD activity was decreased in the haemolymph of *H. cumingii* after bacterial challenges. In the pearl mussel *H. cumingii*, Hu et al. (2015) found a positive link between toxic algae, *Microcystis aeruginosa* and SOD activities, as well as a negative association between hypoxia and SOD activities. Interestingly, Li et al. (2010) delineated enhanced SOD activity in serum of *H. cumingii*, while decreased activity has been observed in haemocytes after the introduction of the pearl nucleus in the body cavity of the mussel. Ray et al. (2020) found that copper oxide nanoparticle exposure reduced SOD activity in the haemocytes of the Indian freshwater pearl mussel *L. marginalis*.

Catalase (CAT), an antioxidant enzyme, is utilised to demonstrate the biological influence on the redox status of bivalve organisms when they are exposed to environmental stress, making it an important biomarker to measure the immune response (Song et al. 2010). This enzyme aids in the enzymatic breakdown of hydrogen peroxide into water and molecular oxygen, which protect cells against hydrogen peroxide toxicity (Duan et al. 2015). One molecule of catalase has also been reported to convert 40 million molecules of H_2O_2 to water and oxygen every second (Ho et al. 2004). Modulation of catalase activity was noticed in freshwater pearl bivalves *L. jenkinsianus obesa* and *Parreysia corrugate* exposed to lead (Brahma and Gupta 2020). Ray et al. (2020) reported inhibition in the activity of catalase in the haemocytes of Indian freshwater pearl mussel, *L. marginalis*, exposed to copper oxide nanoparticle. It has been speculated that an increase in generation in peroxidant molecules in response to the toxin and a decrease in the activities of the antioxidant enzyme might be one of the signs of a shift in oxidative status and related stress in *L. marginalis*. The exposure of inorganic arsenite caused a decline in catalase activity in the haemocytes and digestive tissues of *L. marginalis* (Chakraborty et al. 2013). Variation in the activity of catalase was observed in *H. cumingii* exposed to ammonia-N (Xia et al. 2019). Inhibition of the activity of catalase in *H. cumingii* was noticed when exposed to harmful algae *M. aeruginosa*, hypoxia and thermal stress (Hu et al. 2015; Liu et al. 2020).

The antioxidant enzyme glutathione peroxidase (GPX) catalyses the conversion of hydrogen peroxide and organic hydroperoxides to corresponding alcohols and water (Kutlu and Susuz 2004). In general, two isoforms of GPX have been identified: selenium-dependent GPX, which catalyses the reduction of both organic and inorganic peroxides like hydrogen peroxides and selenium-independent GPX, which exclusively catalyses the reduction of organic peroxide (Song et al. 2010). In bivalves, GPX is activated by bacterial infection, hydrogen peroxide and heavy metal exposure and to have a role in oxidative damage prevention and immunological defence (Soldatov et al. 2007; Qu et al. 2019). The activity of GPX in *H. cumingii* has been detected when exposed to harmful algae *M. aeruginosa*, hypoxia, high pH and thermal stress (Hu et al. 2015; Liu et al. 2020). Catalase, superoxide dismutase

and peroxidases have a central role in enzymatic detoxification (Muradian et al. 2002).

Glutathione S-transferase (GST) is a phase II biotransformation enzyme that catalyses the conjugation of glutathione to a wide range of hydrophobic substances by forming a thioether bond with their electrophilic centre. It is found primarily in the cytosol (Song et al. 2010; Liu et al. 2017). The modulation of GST gene expression under oxidative stress is representing an adaptive response, so its expression is considered an important biomarker of organisms exposed to oxidative stress (Park et al. 2009). To date, the activity of GST has been detected in pearl mussel, *L. marginalis* (Chakraborty et al. 2013) and *H. cumingii* (Xia et al. 2019). Furthermore, transcriptome data from another freshwater pearl mussel, *C. plicata*, reveals the existence of oxidative stress enzymes such as glutathione peroxidase and glutathione-S-transferase (Patnaik et al. 2016).

Overall, antioxidant enzyme activities in mussels are commonly utilised as biomarkers of oxidative stress and damage to mediate the animals' well-being and health. Furthermore, a multi-biomarker approach that includes biological impacts, anti-oxidative enzyme activity and easily measured behavioural aspects could be useful diagnostics in the assessment of stress-related disorders in freshwater bivalves (Brahma and Gupta 2020).

10.4.2 Lysozyme

Lysozyme (muramidase, EC 3.2.1.17) is a critical defence molecule in organisms' innate immune systems, providing primary defences against pathogens and other stressors (Saurabh and Sahoo 2008a, b). Lysozyme breaks the β (1 → 4) linkages between N-acetylmuramic acid and N-acetylglucosamine in Gram-positive bacteria's cell walls (peptidoglycan layers), thus inhibiting intrusion of the host epithelial layer. Bacteriophages, bacteria, plants, invertebrates and vertebrates, as well as animal secretions such as mucus and saliva, have all been reported to contain lysozyme (Jollès and Jollès 1984). Chicken-type lysozyme (c-type) includes stomach lysozyme and calcium-binding lysozyme, goose-type lysozyme (g-type), plant-type lysozyme, bacterial lysozyme, T4 phage lysozyme (phage-type) and invertebrate type (i-type) lysozyme, which are the six forms of lysozyme (Beintema and Terwisscha van Scheltinga 1996; Fastrez 1996; Prager and Jolles 1996; Saurabh and Sahoo 2008a, b; Ren et al. 2012; Bassim et al. 2015). Invertebrate type (i-type) lysozymes, which differ from insect c-type lysozymes but are comparable to vertebrate c-type lysozymes, are found in the bivalve (Nilsen and Myrnes 2001). The mollusc's digestion and innate immunity are aided by i-type lysozymes. In the freshwater pearl mussel, *H. cumingii*, four different forms of lysozyme genes belonging to the i-type have been uncovered (Ren et al. 2012). In another study, Wu et al. (2013a) revealed an i-type lysozyme gene in *C. plicata*, another significant freshwater pearl mussel. *C. plicata's* i-type lysozyme cDNA is 763 bp long, encoding a 160-amino-acid protein with a predicted molecular mass of 17.8 kDa and an isoelectric point of 6.07. The optimum pH and temperature for *C. plicata*

lysozyme were 5.5 and 50 °C, respectively. It is expressed in haemocytes, hepatopancreas and gill of mussel. Further, the expression level of lysozyme was found to be modulated when artificially challenged with *A. hydrophila* (Wu et al. 2013a). In pearl mussel, *H. cumingii*, four separate lysozyme genes (HcLyso1 to HcLyso4) were identified, each encoding a protein with 144, 144, 161 and 228 amino acids (Ren et al. 2012). Glu and Asp catalytic residues are needed for efficient enzyme activity, according to multiple alignments of all four genes.

Further, downregulation in the expression level of lysozyme was found against *Vibrio* or *Staphylococcus* bacterium challenge (Ren et al. 2012). Hong et al. (2006) reported an increased expression of lysozyme gene in basophilic cells of digestive gland tubules of *H. cumingii* after injection with bacterial DNA. Table 10.1 shows the tissue distribution and characteristics of freshwater pearl mussel lysozyme.

10.4.3 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are tiny, gene-encoded cationic peptides that serve as an essential innate immune effector molecule in organisms across the evolutionary spectrum and offer protection against wide varieties of the pathogen (Novoa and Figueras 2012). According to their amino acid sequences, secondary structures and functional similarities, AMPs are divided into four groups: (1) peptides lacking cysteine residues and forming amphipathic α-helices, (2) peptides containing cysteine residues with one to six intra-molecular disulphide bonds, (3) peptides with a variable structure rich in normal amino acids like proline and (4) peptides formed by hydrolysis of large inactive or low-activity proteins (Song et al. 2010). Approximately 20 AMPs have been identified from bivalves, viz., defensins, mytilins, myticins, mytimacins and big defensins that belong to cysteine-rich subgroups, whereas myticalins belong to a linear/α-helical subgroup (Bouallegui 2019). Defensins are effector AMPs identified in marine bivalves' innate immune systems (Díaz 2010) and freshwater pearl mussels including *L. marginalis* (Estari et al. 2011), *H. schlegelii* (Peng et al. 2012), *H. cumingii* (Ren et al. 2011) and *C. plicata* (Patnaik et al. 2016). Peng et al. (2012) described the molecular characterisation and immunological activity of defensin in the freshwater pearl mussel *H. schlegelii.* The Hs-defn of *H. schlegelii* is 410 bp long, including a 51 bp 5'UTR, a 161 bp 3'UTR with an mRNA instability motif (AATAAA) and a 198 bp open reading frame (ORF) encoding a 65-amino-acid polypeptide (Peng et al. 2012). It is expressed in haemocytes, gill, hepatopancreas, mantle and intestine and is found to be stimulated against *A. hydrophila* challenge. Another antimicrobial peptide, theromacin, was reported from the pearl mussel, *H. cumingii* (Xu et al. 2010). Theromacin cDNA from mussel was found to be 547 bp long, with a 294-bp open reading frame encoding a 97-amino-acid peptide and a 61-amino-acid putative mature peptide in the deduced peptide sequence. It is found in the liver, foot, gills, adductor muscle, heart, mantle, intestine, and haemocytes, and its titre increases after gram-positive and gram-negative bacteria are challenged.

Table 10.1 The distribution of lysozyme and its characteristics in freshwater pearl mussel

Scientific name	Common name	Lysozyme type	Lysozyme gene	Distribution	Accession Number (s)	Number of amino acids	References
Hyriopsis cumingii	Triangle sail mussel/ triangle shell mussel	i-type	HcLyso1	Hepatopancreas, haemocytes		144	Ren et al. (2012)
			HcLyso2	Hepatopancreas, haemocytes		144	
			HcLyso3	Haemocytes, hepatopancreas, gills, mantle		161	
			HcLyso4	Haemocytes, hepatopancreas		228	
Cristaria plicata	Cockscomb pearl mussel	i-type	CpLYZ1	Pallium, gill, haemocytes	AFN66527	160	Wu et al. (2013a)
			CpLYZ2	Pallium, gill, haemocytes	AFN66526	161	Dai et al. (2015)

10.4.4 Heat Shock Proteins (HSPs)

HSPs are a group of ubiquitous proteins that help organisms to cope with stress and protect them from cellular damage caused by the environment (Wang et al. 2013c). The molecular mass of principal HSPs range from 15 to 110 kD and members of HSP22, HSP60, HSP70 and HSP90 have been reported in bivalves (Song et al. 2010). Heat shock protein 90 (HSP90) is well characterised in pearl mussel *H. cumingii* (Wang et al. 2017). The mussel HSP90 cDNA is 2659 bp long, with 3′ and 5′ untranslated portions and a 2187 bp open reading frame that encodes a 728 amino acid protein. The expression of HSP90 in *H. cumingii* has been shown to increase in response to temperature fluctuations, cadmium exposure and bacterial infection (Wang et al. 2017). In the pearl mussel, *C. plicata*, Patnaik et al. (2016) found HSP60, HSP70 and HSP90, as well as minor HSPs (HSP10, HSP20 and HSP40).

10.4.5 Complement System

Complement is an innate immunity humoral component involved in immune surveillance and pathogen elimination. The complement system is constituted by more than 40 plasma proteins that act as enzymes or binding proteins (Sarma and Ward 2011). The classical complement pathway (CCP), alternative complement pathway (ACP) and lectin pathway, which are triggered by the interaction of mannose-binding lectin (MBL) with mannose-rich polysaccharides, are the three unique complement pathways. Complement component 3 (C3) is the key mediator among all complement proteins, keeping the complement system alert, activating all known complement activation pathways, fuelling complement response amplification and exerting direct opsonic and cystolic effects on microbial pathogens and synchronising downstream innate and adaptive immune effector molecules to achieve effective pathogen defence in animals (Dunkelberger and Song 2010; Ricklin et al. 2016). C3 gene has been well characterised in pearl-producing mussel, *H. cumingii* (Wang et al. 2019) and plays an important role in alloimmune responses and intricate complement activation in mollusc during tissue allograft.

10.4.6 Cytokines

Bivalves' defence mechanisms are regulated by a cytokine-triggered network similar to that found in vertebrates (Philipp et al. 2012; Bassim et al. 2015). Immunity, inflammation and haematopoiesis are all controlled and facilitated by cytokines, which are small proteins that trigger cell surface receptor complexes (Wu et al. 2013b). Interleukin (IL), interferons (IFN), tumour necrosis factor (TNF) and chemokines are examples of these proteins (Adzigbli et al. 2020). Tumour necrosis factor α (TNF α) is a type of cytokine produced by the bivalve's haemocytes in response to infections (Li et al. 2012). In *H. cumingii* tumour necrosis factor

receptor-associated factor 6 (TRAF6) was reported to be induced after *A. hydrophila* and Lipopolysaccharides stimulation in the gills and haemocytes. The full open reading frame of TRAF6 from *H. cumingii* has a 1965-bp region and encodes a 654-amino-acid predicted protein (Huang et al. 2018). Interferons (IFNs) are cytokines that function during virus infection, suppress the cell cycle, and regulate the immune system (Tailor et al. 2006). Interferon regulatory factor 2 (IRF-2) is a multi-functional transcription factor found in the pearl mussel *H. cumingii*, belonging to the IRF family (Wang et al. 2013a, b), is linked to *A. hydrophila* resistance, and could be important in the genetic improvement of pathogen resistance in freshwater mussels. The mussel IRF-2 cDNA sequence was 2688 bp long and encoded a 329 amino acid protein. Interleukin (IL)-1, also known as cytolytic T-lymphocyte-associated antigen 8, is a critical inducer of pro-inflammatory cytokine release, and granulopoiesis plays an important role in the transplantation of invertebrate tissue. The induction of IL-17 in the pearl mussel, *H. cumingii*, was time-dependent, representing the several stages of alloimmune events during pearl sac development in the mussel (Zhang et al. 2016). The mussel IL-17 cDNA sequence has a 567-bp open reading frame that encodes a 188-amino-acid-residue putative protein. Other mediators found in bivalves include transforming growth factor-beta, allograft inflammatory factor-1 (AIF-1), and macrophage migration inhibitory factor (MIF) (Lelong et al. 2007; Zhang et al. 2013; Rosani et al. 2019).

10.4.7 Lectins

Invertebrates' lectins play an important role in their hosts' immune defence in a variety of ways. The principal component of invertebrate immunity is the ability to differentiate between self and non-self-particles. In the biological membrane of the host, lectins are present in soluble form or coupled with specific carbohydrate recognition domains (CRDs) and play a key role in self/non-self-recognition and complement-mediated opsonisation, agglutination and death of invading microorganisms (Sharon and Lis 2004; Song et al. 2010).

10.4.8 Proteinase Inhibitor

Proteomics data of shell matrix revealed the existence of proteinase inhibitor in different mollusc species (Gerdol and Venier 2015). Furthermore, the mussel *Mytilus edulis* and oyster *Pinctada maxima* have many possible peptide fragments having a Kunitz-like type II domain in their mantle transcriptomes (Jackson et al. 2010; Freer et al. 2014). Zhang et al. (2014) revealed a Kazal-type serine proteinase inhibitor in the pearl oyster *P. fucata*. Recently, Jin et al. (2019) reported the presence of a Kunitz proteinase inhibitor from *H. cumingii* that participated in the antibacterial process during the development of a pearl sac and caused calcium carbonate overgrowth. Further, it was also observed that the Kunitz proteinase inhibitor was required for the creation of the nacreous layer.

10.4.9 Cellular Defence Mechanism

Bivalves possess an 'open' circulatory system in which the haemolymph bathes all of the organs before returning to the heart via sinuses and respiratory systems (gills) (Song et al. 2010). Bivalve circulating haemocytes play a critical role in the phagocytosis of microorganisms and the creation and exocytosis of an array of bioactive chemicals. The haemocytes not only execute inflammatory-type reactions such as stimulation of oxidative metabolites and synthesis of microbicidal protein but are also involved in wound healing, transportation, digestion and excretion of important nutrients (Cheng 1996; Saurabh and Sahoo 2008a, b; Wang et al. 2012; Le Pabic et al. 2014; Li et al. 2018). A summary of different types of haemocytes and their key features in freshwater pearl mussel is presented in Table 10.2.

Phagocytosis is a typical cellular defence reaction that is widely acknowledged as a critical and crucial mechanism for removing germs and foreign particles from the body. Phagocytosis includes an attachment to the foreign body followed by ingestion and its destruction (Sindermann 1971; Söderhäll and Cerenius 1992). It might happen as a result of receptor-mediated endocytosis or nonspecific hydrophobic interactions between the cell membrane and the target particles (Yoshino 1986). During phagocytosis, a variety of reactive oxygen intermediates (ROIs) are formed. When pathogens invade an organism, they activate the NADPH-oxidase enzyme, which reduces oxygen molecules and generates a plethora of ROIs, including superoxide anion (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1o_2$) (Holmblad and Söderhäll 1999). Pathogens are particularly hazardous to these substances. This phenomenon, known as respiratory burst activity, plays a vital role in the microbicidal activity and pathogen clearance from the body (Song and Hsieh 1994). Many bivalves, notably the pearl mussel *L. marginalis* (Anisimova 2013; Ray et al. 2020) and *H. cumingii* (Hong et al. 2006; Liu et al. 2020), have been found to produce superoxide anion in their haemocytes.

The prophenoloxidase (proPO) system is a dominant component of the bivalve defence system influencing the behaviour of the cell, stimulating the secretion of important biomolecules and neutralising pathogenic agents. The phenoloxidase (PO) is the major enzyme produced during the activation of the proPO system. This enzyme is critical for the melanisation process that occurs during infection, nodule and capsule development around invading microorganism (Johansson and Soderhall 1989). This enzyme contains copper pigment, which catalyses the oxidation of phenolic compounds such as L-3, 4-dihydroxyphenylalanine (L-DOPA) to quinones, which are then polymerised non-enzymatically to form the dark pigment melanin (Nappi and Vass 1993). Melanin biosynthesis is controlled and mediated by a family of enzymes known as phenoloxidases, which includes metalloproteins like tyrosinases, catecholoxidases and laccases (Walker and Ferrar 1998). It was also reported that the laccase enzyme has a multidimensional role in bivalves including immune defence, antioxidant activity and detoxification. Further, it has been assumed that laccase might be used as ecological potential biomarker against environmental stress (Luna-Acosta et al. 2017). The decrease in PO activities and the occurrence of diseases in bivalve invertebrates were shown to have a strong

Table 10.2 Summary of different types of haemocytes in freshwater pearl mussels

Species	Haemocyte types	Features	Reference
Cristaria plicata	Large granulocytes	• Nucleus/cytoplasm (N/C) ratio (0.35)	Xie et al. (2011)
		• Size (13.18 μm)	
		• Shapes are circular, ovoid or elongate serpentine	
	Small granulocytes	• Nucleus/cytoplasm (N/C) ratio (0.39)	
		• Size (9.11 μm)	
		• Cytoplasmic granules 0.1-0.3 μm	
	Hyalinocytes	• Nucleus/cytoplasm (N/C) ratio (0.47)	
		• Size (7.56 μm)	
		• cytoplasm with a few electron-dense granules	
	Lymphoid haemocytes	• Nucleus/cytoplasm (N/C) ratio (0.80)	
		• Size (4.68 μm)	
		• Large nuclei with much of the heterochromatin in the central position	
Lamellidens marginalis	Blast like cell	• Nucleus/cytoplasm (N/C) ratio (0.79)	Chakraborty et al. (2013)
		• Size (9.05 μm)	
		• Cells have a scarce filopodial extension	
	Agranulocyte	• Nucleus/cytoplasm (N/C) ratio (0.41)	
		• Size (12.06 μm)	
		• Ovoid to spherical	
	Hyalinocyte	• Nucleus/cytoplasm (N/C) ratio (0.38)	
		• Size (8.57 μm)	
		• Ovoid in shape; hyaline cytoplasm with few granules	
	Granulocyte	• Nucleus/cytoplasm (N/C) ratio (0.54)	
		• Size (10.1 μm)	
		• Spherical or oval having granular cytoplasm	
	Asterocyte	• Nucleus/cytoplasm (N/C) ratio (0.27)	
		• Size (10.53 μm)	
		• Variable morphology and forms projecting filopodia	
Hyriopsis cumingii	Granulocytes	• Nucleus/cytoplasm (N/C) ratio (0.14)	Yang et al. (2021)
		• Size (10.86 μm)	
		• Granular cytoplasm	
	Hyalinocyte	• Nucleus/cytoplasm (N/C) ratio (0.19)	
		• Size (9.77 μm)	
		• Cells spherical with no granules	
	Spindle cell	• Nucleus/cytoplasm (N/C) ratio (0.14)	
		• Size (15.05 μm)	
		• Shape Fusiform	

(continued)

Table 10.2 (continued)

Species	Haemocyte types	Features	Reference
	Thrombocyte	• Nucleus/cytoplasm (N/C) ratio (0.25)	
		• Size (11.64 μm)	
		• Having slim pseudopodium	
	Lymphocyte	• Nucleus/cytoplasm (N/C) ratio (0.36)	
		• Size (8.32 μm)	
		• Spherical and small in size	

correlation, which occasionally resulted in host mortalities (Luna-Acosta et al. 2017). Chakraborty et al. (2013) reported decreased PO activity in the haemocytes and digestive tissue of *L. marginalis* under prolonged exposure to arsenic. Further, it was also reported that phenoloxidase of haemocytes declined significantly upon exposure to different doses of copper oxide nanoparticle in *L. marginalis* (Ray et al. 2020). Hong et al. (2006) reported prompt response of proPO in *H. cumingii* post-challenge with bacterial genomic DNA suggesting PO-system as a sensitive and efficient component of innate immunity against bacterial infection in molluscs.

10.5 Immune Recognition

The ability to discriminate between self and non-self-particles is a crucial component of invertebrate immunity. Immune recognition is one of the most crucial phases in the immune response, and it plays a critical role in the immune system's ability to discriminate non-self-molecules from self-molecules (Song et al. 2010; Yang et al. 2019**).** When specialised soluble or cell-bound pattern recognition receptors (PRRs) are recognised and bound to pathogen-associated molecular patterns (PAMPs), immune responses are triggered (Medzhitov and Janeway 2002). Many PRRs have been detected in bivalves, including peptidoglycan recognition proteins (PGRPs), C1q domain-containing (C1qDC) proteins, gram-negative binding proteins (GNBPs), C-type lectins, galectins, thioester-containing proteins (TEPs), scavenger receptors (SRs), lipopolysaccharide and β-1,3-glucan-binding proteins (LGBP), fibrinogen-related proteins (FREPs), complement homologues and Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Song et al. 2010; Zhang et al. 2012; Allam and Raftos 2015; Bouallegui 2019; Yang et al. 2019).

Both invertebrates and vertebrates have peptidoglycan recognition proteins (PGRPs), which are crucial pattern recognition receptors (Yang et al. 2017, 2019). It was first identified from the haemolymph and cuticle of the silkworm, *Bombyx mori* as a 19 kDa protein. It inherits affinity for peptidoglycan (PGN) and is able to trigger the phenoloxidase cascade (Yoshida et al. 1996). In the recent past, several PGRP genes have been identified in molluscs (Zhang et al. 2007; Itoh and Takahashi 2008). The short type PGRP is found in a variety of bivalve species, including pearl mussels, and the deduced amino acid sequence contains 235 residues at the

C-terminus (Yang et al. 2017). It is expressed in the hepatopancreas, gonad, nephridium, gill and foot and has antibacterial activity against Gram-negative and Gram-positive bacteria (Yang et al. 2013).

In the freshwater pearl mussel, *H. cumingii*, complement C1r/C1s, Uegf and Bmp1 (CUB) domains are only seen in extracellular and plasma membrane-related proteins. A CUB domain-containing protein that contains 2280 bp complete cDNA was mainly expressed in hepatopancreas followed by gills and upregulated by bacteria, virus and viral analogues, suggesting its biological role in the host defence system of molluscs (Huang et al. 2021). In pearl mussel *H. cumingii*, four C1q domain-containing (C1qDC) proteins have been reported, which are widely distributed in haemocytes, hepatopancreas, gill, mantle and foot (Zhao et al. 2016a) and are regulated by bacteria, suggesting their role in anti-bacterial immune defence. In a subsequent study, Huang et al. (2016) revealed a novel C1qDC that could bind to Gram-negative and gram-positive bacteria as well as various PAMPs such as LPS, which is a significant constituent of Gram-negative bacteria's outer membrane, and PGN, which forms the outer wall of Gram-positive bacteria (Takeuchi et al. 1999; Girardin et al. 2003).

C-type lectins (CTLs) are involved in the recognition of self and non-self-molecules, microbial agglutination, phagocytosis induction, pathogen encapsulation and elimination in a variety of mollusc species, including the freshwater pearl mussel, *H. cumingii* (Kong et al. 2011). The mussel CTL cDNA sequence was 1558 bp long, with a 1281-bp open reading frame that encodes a 426-amino-acid-residue putative protein (Huang and Ren 2019). Zhao et al. (2016b) described novel C-type lectins with four CRDs from *H. cumingii* that are involved in antimicrobial peptide control. Study shows that CTLs can attach to bacteria, lipopolysaccharide (LPS) and peptidoglycan (PGN). Galectins are members of the lectin superfamily, and they have one or more carbohydrate recognition domains on their surface that produce several sugar-binding sites. The involvement of galectin from the pearl mussel *H. cumingii* in the innate immune response against pathogenic bacteria was disclosed by Bai et al. (2016). The galectins were also expressed during pearl sac formation and play a role in the healing of the wound caused during implantation. Recent work by Zhao et al. (2018) showed three galectin homologues from pearl mussel, *H. cumingii*. They're mostly found in the mussel's hepatopancreas and gills, and they're thought to be pattern-recognition receptors. Putative lectin sequences such as tandem-repeat galectin, C-type lectin, sialic-acid binding lectin, fucolectin and immulectin-3 were uncovered in the transcriptome of the freshwater pearl bivalve *C. plicata* (Patnaik et al. 2016). Tandem-repeat galectins are an acute-phase protein implicated in bivalve mollusc immune defence against the bacteria *Vibrio* sp. (Zhang et al. 2011).

The Gram-negative binding protein (GNBP) family includes members that bind Gram-negative bacteria, lipopolysaccharides (LPS) and β-1, 3-glucan (Christophides et al. 2002). By increasing cellular biosynthesis and the production of proinflammatory cytokines and other bioactive metabolites or by fast turning on extracellular complement, coagulation and fibrinolytic pathways, LPS can activate a wide range of immunological molecules in the host (Munford 2005). In pearl mussel

H. cumingii, two isoforms of lipopolysaccharide-binding protein and bactericidal permeability-increasing protein (LBI/BPI) (designated as HcLBP/BPI1 and HcLBP/BPI2) were cloned and described. HcLBP/BPI1 and HcLBP/BPI2 had full-length cDNA sequences of 1887 and 2227 bp, respectively, and encoded two secreted proteins with 501 and 518 amino acid residues (Hu et al. 2017). The upregulation of LBI/BPI in bivalves after exposure to *A. hydrophila* and LPS suggested that the gene may have different functions in bacterial mediating immunological responses.

Membrane proteins that belong to the toll-like receptor family have an extracellular domain made up of leucine-rich repeats (LRR) and a conserved cytoplasmic domain (Magor and Magor 2001). Toll-like receptors (TLR) network systems stimulate the generation of cytokines, cell differentiation, reactive nitrogen and oxidative radicals (Aoki and Hirono 2006). After detecting PAMPs (pathogen-associated molecular patterns), TLRs initiate intracellular signal transduction, which results in the expression of genes involved in inflammation, antiviral responses and dendritic cell maturation (Rebl et al. 2010; Saurabh et al. 2011; Sahoo 2020). The toll gene was first recognised in fruit flies as a gene required for embryonic dorsal-ventral development, but later it was eventually discovered that it has a function in antifungal and antibacterial defence of *Drosophila* (Nüsslein-Volhard and Wieschaus 1980; Leulier and Lemaitre 2008). Nevertheless, characterisation of TLRs has been done in many aquatic invertebrates including mussel and oyster (Wu et al. 2017b; Xu et al. 2019). TLR diversity in invertebrates has been uncovered using genomic data, which could be attributed to a variety of evolutionary mechanisms such as retrotranscription, gene duplication, rapid gene expansion, and alternative transcript splicing (Gomez-Chiarri et al. 2015; Hu et al. 2019). Three unique TLR genes (HcToll1, HcToll2, and HcToll3) have been identified in the freshwater pearl mussel, *H. cumingii*, and their expression has been demonstrated to be upregulated upon bacterial or viral assault (Ren et al. 2013, 2014; Zhang et al. 2017). Zhang et al. (2017) reported invasion of Gram-negative bacteria being recognised by HcToll3 in mussel and subsequently regulated different downstream AMP gene expression.

10.6 Immune Signalling Pathways

TLR signalling pathway, NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling system, mitogen-activated protein kinase (MAPK) signalling cascade and complement pathway are among the signalling systems for efficient immune response documented in bivalve (Song et al. 2010; Adzigbli et al. 2020). When bivalves are subjected to high infections, PRRs activate a variety of signalling pathways that trigger the systemic immune response and the production of responsive effectors. Some of the major pathways known to be induced during pathogen invasion in bivalves and related with antiviral and antibacterial immune responses include Janus kinase/signal transducer and activation of transcription (JAK/STAT) (Bassim et al. 2015). For most invertebrate species, only one STAT was reported (Huang et al. 2015), whereas Dai et al. (2017) reported three STAT

pathways from pearl mussel, *H. cumingii*, which regulate the expression of antimicrobial peptides. They reported that the expression of all three STATs induced against *S. aureus* or *A. hydrophila* in the haemocytes, hepatopancreas, gill, mantle and foot of the mussel.

Myeloid differentiation primary response protein 88 (MyD88), tumour necrosis factor receptor-associated factors (TRAFs), IL-1 receptor-associated kinase (IRAK) and adapter-like protein (MAL, also known as TIRAP) are the most well-known components activated in the TLR pathway of bivalves (Bassim et al. 2015). In the Toll/TLR pathway of innate immunity, MyD88 is recognised as a 'central linker' in the activation of downstream signals (Wen et al. 2013) and is ubiquitous and conserved in the animal kingdom. It plays a role of an adaptor protein that links TLR and IL-1 receptor-mediated signal transduction (Jiao et al. 2020). Ren et al. (2014) reported two MyD88 genes in the pearl mussel, *H. cumingii*, that play a critical role in antibacterial innate immunity. Among the TLRs signalling pathway components, tumour necrosis factor receptor-associated factor 6 (TRAF6) is a multifunctional protein that is conserved from *Drosophila* to human (Walsh et al. 2015; Mao et al. 2017). TRAF6 acts as a central intracellular signal adapter molecule that mediates the tumour necrosis factor receptor superfamily and the interleukin-1 receptor/Toll-like receptor family in vertebrate and invertebrates (Qiu et al. 2009; Kongchum et al. 2011). In pearl mussel, *H. cumingii*, the transcriptome of TRAF6 was reported to be upregulated post *A. hydrophila* infection and lipopolysaccharide stimulation in gill and haemocytes of the animal. Furthermore, after mantle implantation, its expression was found in the pearl sac, gills and haemocytes, implying a function in innate immune responses against microbes and the alloimmune system in the pearl mussel (Huang et al. 2018).

Exploring the role of other important signalling pathways in freshwater pearl mussel may be an interesting research area that could be useful to our comprehensive understanding of bivalve immunity particularly freshwater pearl mussel immunity.

10.7 Strategies for Sustainable Development of Freshwater Pearl Mussel Farming

For sustainable development of freshwater pearl farming, the following possible strategies are to be followed:

- Creation of state of art research institute dedicated to research related to freshwater pearl culture, breeding, water quality, nutritional requirements and health management.
- Emphasis should be given to the application of robotics, electronics, statistical, computer technology and artificial intelligence for the sustainable development of the pearl farming industry.
- Live aquatic animal movement should be supervised following Asian Regional Technical Guidelines on Fish and Shellfish Health Management (FAO 2000).
- The national strategy on listing infectious diseases of fish and shellfish including molluscan species and their causative agent, developing diagnostics, health

certification, quarantine and institutional-governmental-farmer cooperation may be strengthened.

- Level I diagnostic facilities at the field level (gross examinations), level II diagnostics at state levels (use of parasitological, bacteriological, mycological and histopathological tools) and level III (diagnostics at research institute/university/central level, i.e. laboratory observations using immunology, molecular biology and electron microscopy) should be strengthened.
- Research into the development of swift and sensitive diagnostic devices and methodologies, as well as disease modelling and forecasting, should be bolstered.
- Establishment of proteomics and genomics laboratory for the exploration of newer molecules to strengthen the immunity of freshwater pearl mussel.
- Biosecurity, disease surveillance, reporting and disease zoning may be planned.
- Initiation of a collaborative research program with the international organisation working on bivalve aquaculture.
- Nevertheless, emphasis should be given to the development of skilled manpower to cater to the need of the pearl mussel farming communities of India.

10.8 Conclusion

The intensive culture practices of pearl mussel raised in freshwater environment occasionally trigger the development of diseases outbreaks, which led to the loss of cultured stocks. Hence, knowledge of major diseases and the underlying immunological defence of freshwater pearl mussels are crucial for the sustainable development of the cultured pearl industry. In case, where outbreaks of infection are periodic and can be foreseen, then immunostimulants, probiotics and herbal therapy may be applied in prolepsis of events to elevate defence mechanisms and thus prevent further losses from infection. Furthermore, having a quarantine facility on the farm site helps to prevent disease pathogens from being transferred with live aquatic animal movements. A quarantine programme, which serves as the first line of defence against potential negative consequences due to entry or transfer of exotic fish and shellfish, including molluscs, should be prioritised. In addition, genomics, proteomics, transcriptomics and immunological tools can be utilised in freshwater pearl mussel research to further characterise the new immune molecules, signalling pathways and their interaction, responsible for modulation of mussel immunity to produce healthy mussels with quality pearl production.

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Prebiotic–Synbiotic Nexus: Critical Dietary Role in Aquaculture

11

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Abstract

Aquaculture as a major food-basket industry has been emphasized to proclaim tremendous growth over the decades. However, continuous supply of aqua-food needs subsequent intensification program that have challenged the success of the industry with emerging diseases. For many decades, probiotic application is considered as safe and environmentally sound practice for disease control. The success of prebiotic (nondigestible carbohydrates) use and its potential to act in synergism with probiotic in animal and human nutrition has paved its way for aquaculture applications. The concept of synbiotic is relatively new, and a plethora of work is reported in both fish and shellfishes of commercial importance. The linked immune enhancement and the mechanism of action need to be understood for furtherance of the research arena in this promising area of health enhancement. Looking into this, the chapter provides an insight of the prebiotic and synbiotic use in aquaculture with due synthesis of the reported works across the globe referring the action and mechanisms of these biotic components.

Keywords

Prebitoic · Synbiotic · Immunnity · *A. hydrophila* · Growth · Stress · Fish

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11.1 Introduction

In modern aquaculture practice, nutritional biotechnology offers a promising solution to the present crisis of feed- and disease-related problems. Nutrient optimization for better feed efficiency and utilization is one means for reducing the feed inputs through better nutrients absorption. In addition, it helps in a minimal release of nutrient waste into the culture system, thus avoiding problems of water quality deterioration. In this regard, studies regarding functional substances promoting better health and integrity of intestinal lining of fish are of utmost need. Moreover, study of the intestinal micro-flora of fish, which benefits the host in many ways, viz., nutrient digestion through production of secondary metabolites, exclusion of pathogenic flora through competition, is also important. Furthermore, the stress received in midst of intensive farming program is also exercising a great reduction in growth and welfare of the cultured species. In this context, better immune status of the cultured fishes in order to counteract the opportunistic micro-organisms in the culture environment is the most essential aspect of health management program. The use of both plant- and animal-based immune stimulating agents/substances in aquaculture practices is a decade old practice. Research findings related to the beneficial use of micro-organisms isolated both from the host system and surrounding environment have led to the modern concept of "probiotics." Probiotics are originally defined as the organisms and substances that contribute to the intestinal microbial balances (Parker 1974). Probiotics of the genus *Bacillus* and *Lactobacillus* are the most important bacteria in fish nutrition and health management. On the other hand, success stories in both human and animal nutrition have led the fishery researchers to explore the potential of "prebiotics" in growth and health promotion in fishes. Based on definition of Gibson and Roberfroid (1995), prebiotics are a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health. Among all the prebiotics evaluated for human and animal nutrition, only few had been selected for research in fishes. Fructooligosacchrides (FOS) along with mannan oligosaccharides (MOS) are the most intensely studied oligosaccharides in fishes. The individual role of both probiotic and prebiotic is studied and verified in fish models across the globe. The simultaneous use and the synergism through association of these biotic components proved beneficial and have wide application in both human and animal nutrition, which paved way for its application in fishes. Synbiotics is a combination of probiotic and prebiotic that has been introduced since 2005 for enhancing the immune responses of fish (Panigrahi et al. 2005). Over the past 15 years, numerous studies on uses of synbiotics to improve aquatic animals' health are reported. The overview of the different prebiotic and its combination with potent probiotics to understand the synbiotic nexus is highlighted in this chapter.

11.2 Synbiotic: Existing Concept and Scope

As per the most accepted definition given by Gibson and Roberfroid (1995), synbiotic represents "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare." Conversely, Kolida and Gibson (2011) supplemented the existing definition stating that both synergistic and complementary approaches should be taken into consideration for synbiotic application in animal feed. The general nexus between any two components used in biological science benefit the host through three possible means: *additive*, *synergism*, or *potentiation*. Additive effect occurs when the effect of two ingredients used together approximates to the sum of the individual ingredient effects. In case of synergism, it is said to occur when the combined effect of the two products is significantly greater than the sum of the effects of each agent administered alone. The term potentiation is used differently, some pharmacologists use potentiation interchangeably with synergism to describe a greater than additive effect, and others use it to describe the effect that is only present when two compounds are concurrently (Table 11.1).

In a latest debate comprising panel of experts held during International Scientific Association for Probiotics and Prebiotics (ISAPP) (May 2019), the definition of synbiotics has been reviewed further (Swanson et al. 2020). After a sequence of discussion and consensus, the panel updated the existing definition of a synbiotic to a newer version. The updated definition defines synbiotic as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host." The panel thus concluded that defining synbiotics as simply as a mixture of probiotics and prebiotics could holdback the innovation pointing the synbiotics that are designed to function cooperatively. In other words, a functional synbiotic should be designed at doses below those at which the probiotic or prebiotic could independently exercise health benefits in a host. It also may happen that a specific microbe might not possess probiotic functions even at high dosages owing to competition or other ecological effects but, in the presence of a suitable substrate, could exert health benefits. Likewise, a novel substrate, in spite of high doses might not by itself provide benefits, but when combined with a selected live microorganism(s), its potency is well enhanced. Such formulations comprise a live microorganism and a substrate that depend on the presence of one another and function in concert.

Considering this, the overall synergistic effect of the used probiotic is primarily based on its visual effect on the host immunity, while the prebiotic is based on the potential for stimulating growth and activity of the probiotic. On the other hand, the relationship is merely considered a complementary effect when the former is chosen based on specific effects on the host, while the latter is independently chosen to selectively improve host microbiota. For example, prebiotics like β-glucans in parts of synbiotics directly improve immune functionality, regardless of the possible effect on host gut microbiota, while other studies reported that β-glucans help as substrates

Table 11.1 Dietary administration of synbiotics in aquatic animals

Fish species (g)	Duration of the study	Probiotic component and dose	Prebiotic component and dose	Outcomes	References
Finfishes					
Rainbow trout, *Oncorhynchus mykiss*:13.2 ± 0.25 g	12 weeks	*Enterococcus faecalis* @ 1%	MOS @ 0.4%	↑ Immune responses and disease resistance	Rodriguez-Estrada et al. (2009)
Yellow croaker, *Larimichthys crocea*:7.82 ± 0.68 g	10 weeks	*B. subtilis* 0,0.42 × 10^7 and 1.35 × 10^7 CFU g^{-1}	FOS 0.2 and 0.4 g kg^{-1} diet	→ Interaction ↑ SGR and FER at each FOS level and disease resistant against *vibrio harveyi* with 1.35 × 10^7 CFU g^{-1} *B. subtilis.*	Ai et al. (2011)
Cobia, *Rachycentron canadum*: 10.1 ± 0.5 g	8 weeks	*B. subtilis* 0,1 × 10^{10} and 2 × 10^{10} CFU g^{-1}	Chitosan 3 and 6.0 g kg^{-1} diet	1.0 g kg^{-1} *B. subtilis* + 6.0 g kg^{-1} chitosan is adequate for growth, innate immune response and protection against *v. harveyi.*	Geng et al. (2011)
Japanese flounder *Paralichthys olivaceus*: 21 g	8 weeks 56 days	*B. clausii* 10^7 cells g^{-1}	FOS + MOS 2.5 g + 2.5 g kg^{-1} diet	Highest WG ↑ Intestinal protease and amylase activity ↑ Growth performance and health benefits	Ye et al. (2011)
			MOS 5 g kg^{-1} diet	↑ Intestinal protease activity ↑ Growth performance and health benefits.	
Rainbow trout fingerlings (4.59 ± 0.2 g)	60 days	Synbiotics biomin IMBO, (*Entercoccus faecium*, 5 × 10^{11} CFU/kg) + FOS)		↑ Growth performance, SR and FE ↑ Total serum protein	Mehrabi et al. (2012)

		60 days @ 0, 0.5, 1 and 1.5 g kg^{-1} diet.			
Hybrid Surubim, *Pseudoplatystoma* sp.: Avg. weight 76.3 g	15 days	*Weissella cibaria* (CPQBA 001-10 DRM 02) (7.87 ± 0.2 log CFU g-^{1})	Inulin @0.5%	Manipulation of intestinal microbiota; improved hemato-immunological parameters	Mouriño et al. (2012)
Labeo fimbriatus fingerlings: 2.50 ± 0.16 g	60 days	*B. subtilis* 10^4 CFU g^{-1} diet	MOS 5 g kg^{-1} diet	↑ Growth performance, SR and FE	Pawar et al. (2013)
Gilthead Sea bream, *Sparus aurata L.*: 50 g	4 weeks	B. subtilis @10^7 cfu g^{-1}	Inulin @ 10 g kg^{-1}	No effect on the intestinal absorptive area. ↓ Bacterial diversity	Cerezuela et al. (2013)
Trachinotus ovatus: 10.32 ± 0.46 g	8 weeks	*Bacillus Subtilis* @ *5.62 × 10^7 CFU* g^{-1} diet	*Fructooligosaccharide (FOS) 0.2%* kg^{-1} diet	↑ SGR ↑ Immune responses and disease resistance	Zhang et al. (2014a)
Megalobrama terminalis: 30.5 ± 0.5 g	8 weeks	*Bacillus licheniformis* @10^7 CFU/g^{-1} diet	*Fructooligosaccharide* (FOS) 3 g kg^{-1} diet	↑ Growth performance, SR, intestinal ↑ Enzymes activities as well as microvilli length	Zhang et al. (2014b)
Huso huso juvenile: 26.45 ± 0.19 g	8 weeks	Biomin IMBO (biomin, Herzogenburg, Austria) (*Entercoccus faecium* 5 × 10^{11} CFU kg^{-1} + Fructooligosaccharide)		↑ Innate immunity	Akrami et al. (2015)
Salmo trutta caspius (~ 9 g)-	7 weeks	BetaPlus®@ 1 g kg^{-1} diet (BioChem Co., Karlsruhe, Germany), contained a 1:1 ratio of spores of *B. subtilis* (DSM 5750), and *B. licheniformis* (DSM 5749), at concentration: 5.12 × 10^{12} (CFU kg^{-1} per strain)	Isomaltooligosaccharides (IMOS) @ 2 g kg^{-1} diet	↑ Growth, SR, intestinal microbiota, and some hemato-immunological parameters	Aftabgard et al. (2017)

(continued)

Table 11.1 (continued)

Fish species (g)	Duration of the study	Probiotic component and dose	Prebiotic component and dose	Outcomes	References
Cirrhinus mrigala juvenile: 2.87 ± 0.01 g to 3.26 ± 0.05 g	60 days	*Bacillus subtilis* (15% x10^7 CFU ml^{-1})	Mannan oligosaccharide (MOS) 0.6% kg^{-1} diet	↑ Innate immunity, antioxidant activity and disease resistance	Kumar et al. (2018)
Anguilla japonica: 9.00 ± 0.11 g	8 weeks	*Bacillus subtilis WB60 0.5 × 10^7 CFU g^{-1} diet*	Mannan oligosaccharide (MOS) 5 g kg^{-1} diet	↑ Growth performance, non-specific immune responses, intestinal morphology and disease resistance	Lee et al. (2018)
Catla catla: 5.05 ± 0.45 g	60 days	*Bacillus subtilis ATCC 6633 @10^6 CFU g^{-1} diet*	Mannan oligosaccharides (MOS)@ 0.4% MOS (4 g kg^{-1})	↑ Growth performance	Gupta et al. (2019)
Oreochromis niloticus:30.7 ± 0.31 g	45 days	Synbiotic @ 0.7 or 1.5 g/kg^{-1} diet (Lacto forte: Beta glucan 30 g, MOS 30 g *Enterococcus faecium* 6 × 10^{12} CFU, *Bacillus subtilis* 6 × 10^{12} CFU, and dextrose to 1 kg.)		Short term feeding @ 0.7 g/kg: No influence on growth performance and immune response while long term feeding with a high dose @ 1.5 g/kg has a negative impact on fish health status resulted in marked intestinal degeneration at the top surfaces of villi, underlying muscular degeneration, blood congestion, and massive leucocyte infiltration.	Ismail et al. (2019)
Anguilla japonica: 12.8 ± 0.47 g	12 weeks	*Bacillus subtilis* KCTC 2217 *or Bacillus licheniformis* KCCM 11775 @ 10^8 CFU/g diet	*Mannan oligosaccharide* (MOS*) or Fructooligosaccharide* (FOS) @ 5 g kg^{-1} diet	↑ WG and SGR ↑ Disease resistance against *Aeromonas hydrophila*	Park et al. (2019)

				↑ Intestinal morphology, and immune related gene expression	
Labeo rohita fingerlings (2.50 ± 0.16 g)-60 days		*Bacillus circulans PB7 (BCPB7)* 10^6 CFU g^{-1} diet	FOS 10 g kg^{-1} diet	↑ Stress tolerance against low pH	Singh et al. (2019)
Shell fishes					
Western king prawns, *Penaeus latisulcatus* 4.63 ± 0.39 g	84 days	*Pseudomonas synxantha* and *P. aeruginosa*	Bio-Mos® and β-1,3-D-glucan	Improvement in the growth, SR and immune response	Hai and Fotedar (2009)
Shrimp, *Litopenaeus vannamei*: 1.75 g	28 days	*Bacillus* OJ (0, 10^8 and10^{10})	IMO 2 g kg^{-1} diet	(10^8 + 0.2) best combination Higher immune response and disease resistance against white spot syndrome virus	Li et al. (2009)
European lobster, *Homarus gammarus*: Zoeal I	30 days	*Bacillus* spp. Artemia enriched with probiotics (100 mg l^{-1} *bacillus* spp.)	MOS Artemia enriched With 2 mg l^{-1}	After 18 dph ↑ Growth performance. Cost effective in terms of survival and added benefits of improved growth compared to their individuals supplementation	Daniels et al. (2013)
Shrimp, *Penaeus japonicas* 5.20 ± 0.15 g.	–	*B. licheniformis* 10^8 CFU g^{-1} diet/ *B. subtilis* 10^8 CFU g^{-1} diet/ *B. licheniformis* + *B. Subtilis* @ 10^8 CFU g^{-1} diet each	IMO 2 g kg^{-1} diet	↑ Intestinal microflora, ↑ Immunological parameters ↑ Resistance against *Vibrio alginolyticus*	Zhang et al. (2010)
Echinoderms					
Sea cucumber, *Apostichopus japonicus*:5.06 ± 0.10 g	8 weeks	*Bacillus subtilis* 56 days (0, 1.82 × 10^7 or 4.95 × 10^7 CFU g^{-1} diet)	FOS 0, 2.5 or 5 g kg^{-1} diet	↑ Total coelomocytes counts, intestinal microbiota composition ↑ Immune response and disease resistance *Vibrio splendidus*	Zhang et al. (2010)

(continued)

Table 11.1 (continued)

Fish species (g)	Duration of the study	Probiotic component and dose	Prebiotic component and dose	Outcomes	References
Sea cucumber, *Apostichopus japonicus*:3.72 ± 0.16 g-	50 days	*Microencapsulated B. subtilis* (0.2, 0.4 and 0.8%), 0.2% *B. subtilis* + 0.4% or 0.8% FOS	FOS (0.4, 0.8 and 1.6%)	→ Interaction → SGR and disease resistance against *Vibrio splendidus.*	Sun et al. (2011)
Sea cucumber, *Apostichopus japonicus*: 4.92 ± 0.02 g	8 weeks	*Bacillus* TC22 0, $10^7$and 10^9 CFU g^{-1} diet	FOS 0 and 0.5 g kg^{-1} diet	↑ Immune response and disease resistance against *Vibrio splendidus.*	Yancui et al. (2011)

MOS mannan oligosaccharide, *FOS* fructooligosaccharide, *IMO* isomaltooligosaccharide, *SGR* specific growth rate, *WG* weight gain, *FE* feed efficiency, *SR* survival rate, *FER* feed efficiency ratio
Symbols indicates an increase (↑), decrease (↓), or no effect (→) on the specified parameters

for complementary growth and multiplication of the probiotic bacteria (Guzman-Villanueva et al. 2014). Thus, the common definition given by Gibson and Roberfroid in general deviate the actual of prebiotic–synbiotic nexus, which vary with the type of prebiotic.

11.3 Prebiotics Use in Aquaculture

Prebiotics are nondigestible constituents that are metabolized by specific health-promoting bacteria like *Lactobacillus* and *Bifidobacterium*. These bacteria are thought to be beneficial to the host's health and growth by reducing the presence of intestinal pathogens and/or altering the production of health-related bacterial metabolites (Roberfroid 1993; Gibson and Roberfroid 1995). For example, short-chain fatty acids (SCFA) are among the health-associated bacterial metabolites, which are generally thought to be beneficial to colonic health. Prebiotics are nondigestible carbohydrates classified as monosaccharides, polysaccharides, or oligosaccharides based on their molecular size or degree of polymerization (number of monosaccharide units). Despite the fact that the first study on prebiotics in aquaculture was published in 1995 (Hanley et al. 1995), researchers have gained a better understanding of the importance of commensal intestinal microbiota in fish intestines over the last decade. Since then several research have been performed, and a summary of the outcomes is given in Tables 11.2, 11.3, 11.4 and 11.5. To date, the most common prebiotics validated in fish include inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), MOS, GOS, xylooligosaccharides (XOS), arabinoxylo-oligosaccharides (AXOS), isomaltooligosaccharides (IMO), GroBiotic-A, and trans-galactooligosaccharides (TOS). These additives are mostly extracted from plants, and such dietary fibers are rarely found in fish diets, particularly for carnivorous fish. Therefore, the prebiotic ability of oligosaccharides and other dietary fiber may be useful in aquaculture to promote gut health and the presence of beneficial gut bacteria while suppressing potentially harmful bacteria. Microbial levan, a type of fructan, composed of repeating five-member fructofuranosyl rings connected by β-(2→6) link is considered as a suitable prebiotic for aquaculture. Dietary administration of microbial levan as feed supplement has demonstrated to augment growth performance, hemato-immunological and physio-biochemical response, and thermal tolerance and to maintain liver homeostasis under fipronil toxicity in fishes (Gupta et al. 2008, 2010, 2011, 2014, 2013, 2015). Molecular mechanism of disease protection has revealed remarkable increase of pro-inflammatory cytokine genes (IL-1β, TNF-α, IL-12p40, TLR22, β-2 M and IFN-γ); however, decline of regulatory gene (IL-10 and TGF-β) was found in pathogen provoked rohu (Gupta et al. 2018, 2020a, b). Recently, Gupta (2021) demonstrated improvement in glycogen level, serum cholesterol, triglyceride, LPO, HSP-70, myeloperoxidase content, and total immunoglobulin (Ig) in *C. carpio* due to dietary supplementation of microbial levan.

Table 11.2 Dietary administration of inulin in aquatic organisms

Fish species (g)	Duration of the study	Inulin dose	Outcomes	References
Finfishes				
Grass carp, *Ctenopharyngodon idellus:* 24.6 ± 3.5 g	2 weeks	i. p (10 mg kg $^{-1}$ body weight	→ Susceptibility against *Aeromonas Hydrophila* and *Edwardsiella tarda*	Wang and Wang (1997)
Tilapia, *Tilapia aureus*: 21.8 ± 3.3 g	2 weeks	i. p (10 mg kg $^{-1}$ body Weight)	→ Susceptibility against *A. hydrophila and E. tarda*	Wang and Wang (1997)
Arctic charr, *Salvelinus alpinus* L.: 218 g	4 weeks	150 g kg $^{-1}$	Intestinal cell damage	Olsen et al. (2001)
Siberian sturgeon, *Acipenser baerii*: 213 ± 0.7 g	1 month	20 g kg $^{-1}$	→ Total SCFA and lactate ↓ Butyrate ↑ Gas production	Mahious et al. (2006a)
Turbot (*Psetta maxima*) larvae	1 month	20 g kg $^{-1}$	↑ GR Effects on gut microbiota (*bacillus* and *vibrio*)	Mahious et al. (2006b)
Atlantic salmon, *Salmo salar* L.:172 g	3 weeks	75 g kg $^{-1}$	→ Intestinal cell damage ↑ Intestinal growth and relative mass of the gastrointestinal (GI) tract → Hydrolytic and absorptive capacity	Refstie et al. (2006)
Arctic charr: 218 g	4 weeks	150 g kg $^{-1}$	↓ TVC Microbiota – Control: *Pseudomonas, Psychrobacter glacincola, Carnobacterium divergens, micrococcus, Staphylococcus, streptococcus* Microbiota – Inulin: *Bacillus, Carnobacterium maltaromaticum, staphylococcus, streptococcus* Different colonization pattern on enterocytes surface	Ringø et al. (2006)
Atlantic salmon: 172 g	3 weeks	75 g kg $^{-1}$	→ TVC control ↓ *Marinilactibacillus psychrotolerans, C. maltaromaticum,*	Bakke-McKellep et al. (2007)

(continued)

Table 11.2 (continued)

Fish species (g)	Duration of the study	Inulin dose	Outcomes	References
			Enterococcus faecalis Inulin ↓ *Pseudoalteromonas, micrococcus* → Intestinal cell damage	
Gilthead seabream, *Sparus aurata* L.: 175 g	1 week	5 and 10 g kg^{-1}	Significant inhibition in phagocytosis and respiratory burst in leucocytes	Cerezuela et al. (2008)
Juvenile beluga, *Huso huso*:16.14 ± 0.38 g	56 days	1 to 3 g kg^{-1}	↓ WG, SGR, PER, energy retention, FE, PE	Reza et al. (2009)
Nile tilapia (*Oreochromis nilotics*) (11 ± 0.2 g)	2 months	5 g kg^{-1}	↑ Growth and survival ↑ Hematocrit, nitroblue tetrazolium, and lysozyme	Ibrahem et al. (2010)
Red drum (*Sciaenops ocellatus*) 7 g	56 days	10 g kg^{-1}	↑ Growth performance and highest lysozyme activity ↑ Microvilli heights in pyloric caeca, proximal and mid-intestine	Zhou et al. (2010)
Rainbow trout, *Oncorhynchus mykiss*:~150 g	49 days	5 and 10 g kg^{-1}	↑ WG, body energy and calcium content ↓ Intestinal population of *vibrio spp.*	Ortiz et al. (2012)
Common carp, *Cyprinus carpio* fry: 0.55 ± 0.02 g	7 weeks	5 and 10 g kg^{-1}	→ On growth performance and diet utilization ↑ SR and lipid content of carcass	Eshaghzadeh et al. (2014)
Nile tilapia, *Oreochromis niloticus*: 0.55 ± 0.03 g	8 weeks	0, 0.2%, 0.4%, and 0.8%	Inulin in the diet @ 0.4% at 16 psu salinity improved growth and alleviated hypersaline induced oxidative stress	Zhou et al. (2020)
Nile tilapia, *Oreochromis niloticus*: 1·19 ± 0·01 g	10 weeks	5 g kg^{-1}	Alleviates adverse metabolic syndrome and regulates intestinal microbiota composition in case of fish fed with high carbohydrate diet	Wang et al. (2020)
Shell fishes				
Indian white shrimp, *Fenneropenaeus indicus* Larvae (Mysis-I to	–	Artemia echrich (0-14 dph) 60 mgL^{-1}	→ Growth performance and survival	Hoseinifar et al. (2010)

(continued)

Table 11.2 (continued)

Fish species (g)	Duration of the study	Inulin dose	Outcomes	References
III): 4.43 ± 0.30 mm				
Indian white shrimp Postlarvae (PL1 to 8) 5.42 ± 0.82 mm	–	Artemia echrich (14-22 dph) 50 mgL^{-1}	→ Growth performance 50 mgL^{-1} Selco +50 mgL^{-1} inulin ↑ survival	

i.p intraperitoneal injection, *TVC* total viable counts, *SCFA* short-chain fatty acids, *WG* weight gain, *SGR* specific growth rate, *PER* protein efficiency ratio, *PE* protein efficiency, *FE* feed efficiency
Symbols indicates an increase (↑), decrease (↓), or no effect (→) on the specified parameter

11.4 Host Immunity and Role of Prebiotic–Synbiotic

The defense system of an aquatic vertebrate including fish referred to as immune system acts immediately upon invasion by foreign agents, mostly the harmful microorganisms, viz., viruses, bacteria, fungi, and protozoa, apart from different stressors. The response of the host is activated and controlled in a way to minimize the pathological consequences. When exposed to an antigen, the innate immune response of an animal gets triggered, which includes humoral components (complement system, lysozyme, acute phase proteins, antimicrobial peptides, interferon, lectins, proteases, protease inhibitors, or eicosanoids) and cellular elements (monocyte-macrophages, granulocytes, natural killer, and nonspecific cytotoxic cells). Despite the inherent immune build in the host, the role of dietary immune-stimulating factors is quite important due to the severe level of stress and pathogen load in midst of aquaculture intensification program coupled with poor management. Over a long period, immune stimulants like probiotics are known to provide effective protection in fishes. The concept and application of dietary prebiotic in human and animal nutrition has further paved way for its parallel application in fishes. The role and mechanism of action of the feed prebiotic and synbiotic in fishes have been not widely analyzed and need research.

11.5 Prebiotic as Immune Modulator

The use of immunostimulants in fishes could enhance nonspecific defense and elevate the resisting power to several infectious disease through enhancing the innate humoral and cellular defense mechanisms. Prebiotics are basically the nondigestible food ingredients that will selectively and beneficially influence the host organism by stimulating the growth and/or activity of selected/limited number of bacterial population in the gut. Overall, the proliferation of beneficial bacteria in host gut provides protection against invading pathogens through competitive exclusion for adhesion

Table 11.3 Dietary administration of fructooligosaccharides (FOS) and short-chain fructooligosaccharides (scFOS) in aquatic animals

Fish species (g)	Duration of the study	FOS and scFOS dose	Outcomes	References
Finfishes: FOS				
Hybrid tilapia (*Oreochromis niloticus* ♀· *Oreochromis aureus* ♂): 57 g	58 days	0, 2 and 6 g kg^{-1}	→ GR ↑ Survival ↑ Nonspecific immunity	He et al. (2003)
Turbot larvae	1 month	20 g kg^{-1}	↑ Growth rate effects on gut microbiota (*bacillus* and *vibrio*)	Mahious et al. (2006b)
Atlantic salmon: 200 ± 0.6 g	4 months	10 g kg^{-1}	→ Feed intake, growth, or digestibility	Grisdale-Helland et al. (2008)
Red drum (*Sciaenops ocellatus*) (10.9 ± 0.2)	4 weeks	10 g kg^{-1}	↑ Feed efficiency, serum lysozyme, and IC-SOD → Survival against *Amyloodinium ocellatum*	Buentello et al. (2010)
Turtles: FOS				
Soft-shell turtle *Trionyx sinensis*	100 days	0,1.5 and 2.5 g kg^{-1}	↑ GR at 0.25% inclusion ↑ SOD activity at 0.25% inclusion ↓ Lysozyme activity	Ji et al. (2004)
Finfishes: scFOS				
Hybrid tilapia: (5.6 ± 0.02 g)	8 weeks	0.8 or 1.2 g kg^{-1}	↑ GR, feed intake, FCR → Survival and condition factor ↑ *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *lactobacillus* spp., *Streptococcus faecalis*	Hui-yuan et al. (2007)
Hybrid tilapia: 1.24 ± 0.01 g	8 weeks	1 g kg^{-1}	↑ Uncultured bacterium clones and *Thiothrix eikelboomii*	Zhou et al. (2009)
Common carp: 3·23 ± 0·14 g	7 weeks	2-3 g kg^{-1}	WBC, RBA, THC, LAB, SUR, SR (↑) FNW, WG, SGR, CF, FCR, LYM, NEU, MON, LEU, HCT, Hb (↔)	Hoseinifar et al. (2014b)
Common carp: 20 ± 2 g	12 weeks	2.5 -5 g kg^{-1}	↑ WBC, GRA, LYM, MON, PLA	Abdulrahman and Ahmed (2015)
Common carp: 550 ± 20 mg	7 weeks	5 g kg^{-1}	LI, AMY, SUR, LAB (↑) FNW, WG, SGR, FCR, CF (↔)	Hoseinifar et al. (2015a)
Shell fishes: scFOS				

(continued)

Table 11.3 (continued)

Fish species (g)	Duration of the study	FOS and scFOS dose	Outcomes	References
White shrimp (*Litopenaeus vannamei*) (75.4 ± 0.8 g)	6 weeks	0.25, 0.5, 0.75, 1, 2, 4 and 8 g kg^{-1} -	→WG, FCR and survival scFOS affected gut microbiota	Li et al. (2007)
White shrimp (0.17 g)	8 weeks	0, 0.4, 0.8, 1.2 And 1.6 g kg^{-1}	↑ GR, feed intake, feed conversion scFOS affected gut microbiota	Zhou et al. (2007)

SOD superoxide dismutase, *IC-SOAP* intracellular superoxide anion production, *WG* weight gain, *GR* growth rate, *SGR* specific growth rate, *PER* protein efficiency ratio, *PE* protein efficiency, *FCR* feed conversion ratio, *FE* feed efficiency, *AMY* amylase, *SUR* Survival, *LAB* lactic acid bacteria, *FNW* final weight, *LI* lipase, CF condition factor, *NEU* Neutrophils, *PLA* platelets, *GRA* granulocytes, *THC* total heterotrophic bacteria, *RBA* respiratory burst activity
Symbols indicates an increase (↑), decrease (↓), or no effect (→) on the specified parameter

sites; production of organic acids like formic acid, acetic acid, lactic acid, hydrogen peroxide etc.; and other anti-microbial factors such as antibiotics, bacteriocins, siderophores, and lysozyme, which effectively modulate the physio-immunological responses of the fish (Nayak 2010). As far as the innate immune response of a fish is concerned, neutrophils are important components that are produced in the head kidney of fishes. They exert their antimicrobial activities through various mechanisms. Either they manifest intracellular uptake and killing of the pathogens by phagocytosis process or effectively degranulate the antimicrobial substances or release trapping substances known as neutrophil extracellular traps (NETs). NETs are primarily composed of nuclear DNA fibers connected to antimicrobial peptides and stabilize proteins such as histones and are released in response to pro-inflammatory signals or the pathogens stimulations (Remijsen et al. 2011). In addition to stabilizing NET formation against pathogenic degradation, prebiotic such as β-glucan has been shown to elicit NET formation in kidney cells of carps (Brogden et al. 2014). Pathogens like *A. hydrophila* have capacity to degrade NETs using nuclease activity. Apart from this, the pattern recognition receptors (PRRs), namely, beta (b) glucan receptors and dentin-1 receptors, which are expressed in macrophages, have direct interactions, which mediate the immune-modulation of supplemented prebiotics (Yadav and Schorey 2006). These receptor interactions finally activate the signal transduction molecules like NF-KB, which stimulate the immune cells. Additionally, the available saccharides can also interact with the PRR format of microbe-associated molecular patterns (MAMPs) as teichoic acid, peptidoglycan, glycosylated protein, or the capsular polysaccharide. The mechanism of action of prebiotic on fish is represented in Fig. 11.1.

Prebiotic mostly work on the host through manipulation of the gut micro-biota, which are the key players in modulating the immune function of fish. Moreover, the

Table 11.4 Dietary administration of mannan oligosaccharides (MOS) in aquatic animals

Fish species (g)	Duration of the study	Dose	Outcomes	References
Finfishes				
Hybrid tilapia: 8.1 g	58 days	0, 2, and 6 g kg^{-1}	→ GR ↑ Survival and ↑ Nonspecific immunity	He et al. (2003)
Gulf sturgeon, *A. oxyrinchus desotoi*:130 g	5 weeks	0 and 3 g kg^{-1}	→ Growth performance, FCR, and Gross gastrointestinal morphology	Pryor et al. (2003)
Hybrid tilapia: 9.8 g	80 days	0, 1.5, 3 and 4.5 g kg^{-1}	→ Growth parameters and body indices Dry matter and protein contents of fillets increased with increasing rates of MOS	Genc et al. (2007a)
Rainbow trout:30 g	90 days	2 g kg^{-1}	↑Growth and survival ↑ Antibody titer and lysozyme activity in one trial →Bactericidal activity	Staykov et al. (2007)
European sea bass: 33.7 ± 7.7 g	67 days	20 and 40 g kg^{-1}	↑ Growth →FCR ↑ Lipid vacuolization ↓ Presence of *Vibrio alginolyticus* on head kidney	Torrecillas et al. (2007)
Channel catfish, *Ictalurus punctatus* (~16 g)	4 weeks	2 g kg^{-1}	→Growth performance, hematology Or immune function → Survival against *Edwardsiella ictaluri*	Welker et al. (2007)
Rainbow trout: 37.5 ± 1 g	90 days	0, 1.5, 3 or 4.5 g kg^{-1}	1.5 g kg^{-1} ↑GR 1.5 g and 3 g kg^{-1} ↑ Intestinal villi →FCR, hepatosomatic index, intestinal morphology	Yilmaz et al. (2007)
Red drum, *Sciaenops ocellatus* L.:500 g	3 weeks	10 g kg^{-1}	↑ ADC values of protein and organic ↓ ADC value of lipid	Burr et al. (2008)
Atlantic salmon: 200 ± 0.6 g	4 months	10 g kg^{-1}	↓Oxygen consumption ↓ Protein and › energy concentration in the whole body	Grisdale-Helland et al. (2008)

(continued)

Table 11.4 (continued)

Fish species (g)	Duration of the study	Dose	Outcomes	References
Rainbow trout:13.2 g	12 weeks	0 and 4 g kg^{-1}	↑Growth ↑Hemolytic and phagocytic activity ↑ Mucus weight ↑Survival against *Vibrio anguillarum*	Rodriguez-Estrada et al. (2009)
Nile tilapia:13.6 ± 0.7 g	45 days	0, 2, 4, 6, 8 and 10 g kg^{-1}	→Hematological parameters ↓ Daily feed consumption with increasing level	Sado et al. (2008)
Nile tilapia: 0.82 g	3 weeks	0, 2, 4, and 6 g kg^{-1}	↑Weight, length and average daily growth of fish fed 4 and 6 g ↑Survival against *Streptococcus agalactiae*	Samrongpan et al. (2008)
Cobia (*Rachycentron canadum*) larva	13 dph	0.2%	↑ Larval survival ↑Microvilli alignment ↓ Supranuclear vacuoles	Salze et al. (2008)
Rohu, *Labeo rohita*: 4.5 ± 0.07	2 months	0,10, 20, 40 g kg^{-1}	10 g kg^{-1} ↑ growth and survival against *Aeromonas hydrophila* infection	Andrews et al. (2009).
Common carp (1.11 ± 0.05 g)	90 days	5 g kg-^{1}	WG, CF, PER (↑)	Atar and Ates (2009)
Marron (*Cherax tenuimanus*) 10.44 ± 0.20 g 4.44 ± 0.20 g and 94 ± 2.17 g	30 days (infection) 112 days (air) and 42 days (NH3)	0, 0.2 and 0.4%	↑ Survival, health status and immunity under the bacterial infection and stress conditions caused by air and NH3 exposures.	Sang et al. (2009)
Red drum (*Sciaenops ocellatus*) (10.9 ± 0.2 g)	4 weeks	10 g kg^{-1}	10 g kg^{-1} diet is adequate ↑ the feed efficiency and disease resistant against *Amyloodinium ocellatum* infection	Buentello et al. (2010)
Rainbow trout	8 weeks	2 g kg^{-1}	↑ Absorptive surface in the posterior gut region ↑Microvilli density and microvilli length	Revived by Ringø et al. 2010
Platy (*Xiphophorus maculatus*) 0.60-0.61 g	26 weeks	Immunogen® (B-glucan and MOS) 0,5, 10. 15 g kg^{-1}	15 g kg^{-1} ↑ reproductive performance ↑ Survival of fry	Abasali and Mohamad (2011)

(continued)

Table 11.4 (continued)

Fish species (g)	Duration of the study	Dose	Outcomes	References
Sharpsnout seabream, (*Diplodus puntazzo*) 100 g	114 days	8 g kg^{-1}	→ Growth performance →Apparent digestibility	Piccolo et al. (2011)
Common carp (*Cyprinus carpio*) (11.12 ± 0.55 g)	8 weeks	Immunogen® (B-glucan and MOS) 0, 0.5,1,1.5 and 2.5 g kg^{-1}	1 to 1.5 g kg^{-1} ↑ the FE, GR and bacterial resistance	Ebrahimi et al. (2012)
Gibel carp (6.87 ± 0.21 g)	8 weeks	4.5 g kg^{-1}	IgM, LZY, ACP (↑) FNW, WG, SGR, FCR, CF (↔)	Akrami et al. (2015)
European sea bass, *Dicentrarchus labrax* (L.):45.95 ± 0.60 g	8 weeks	4 g kg^{-1}	↓ Cumulative mortality, against gut inoculated V. anguillarum ↑Innate immune response	Torrecillas et al. (2012)
Nile tilapia, *Oreochromis niloticus (L).* 6.46 ± 0.02	12 weeks	0.05, 0.1, and 0.2% bio-Mos®.	(↑) WG, SGR, FCR, PER, EU (↑) Body protein content and hematological responses Antagonistic activity pathogenic *Aeromonas hydrophila* infection	Ahmad et al. (2013)
Shellfishes				
European lobster, *Homarus gammarus*	Stage IV	*Enrichment of artemia nauplii with* bio-MOS	↑Larval survival ↓ Early survival and morphological development of early juvenile stages	Daniels et al. (2006)
European lobster, *Homarus gammarus*	40 days	*Enrichment of artemia nauplii with* fluorescently abled bio-MOS	Potential breakdown of MOS by artemia → Survival and growth	Daniels et al. (2007)
Tiger shrimp, *Penaeus semisulcatus*: 0.34 g	48 days	0, 1.5, 3 and 4.5 g kg^{-1}	3 g kg^{-1}↑ growth, feed conversion and survival No detrimental effect was noted on hepatopancreas tissue	Genc et al. (2007b)

dph days posthatching, *ADC* apparent digestibility coefficient, *WG* weight gain, *GR* growth rate, *SGR* specific growth rate, *FCR* feed conversion ratio, *FE* feed efficiency, *FNW* final weight, CF condition factor, *LZY* lysozyme
Symbols indicates an increase (↑), decrease (↓) or no effect (→) on the specified response

Table 11.5 Dietary administration of β-glucan in aquatic animals

Fish species (g)	Duration of the study	Dose of β-glucan	Outcomes	References
Finfishes				
Common carp: 185.5 ± 35.1 g	50 days	5 g kg^{-1}	Hb, WBC, LYM, MON, GLU, ALB, CHO, TSP, LAC, IMG (↑)	Dobšíková et al. (2013)
Common carp: 93.2 ± 24.9 g	14 days	10 g kg^{-1}	Diversity of the intestinal microbiota (↑)	Jung-Schroers et al. (2015)
Mirror carp: 7 g	4 weeks	10 g kg^{-1}	Total heterotrophic bacteria (↑)	Kühlwein et al. (2013)
Mirror carp: 11.1 g	8 weeks	10–20 g kg^{-1}	WG, SGR, FCR, HCT, MON (↑) CCCP, CCL, RBC, WBC, GLU, TSP, ALB, LYM, GLU (↔)	Kühlwein et al. (2014)

WG weight gain, *SGR* specific growth rate, *FCR* feed conversion ratio, *Hb* hemoglobin, *HCT* hematocrit, *RBC* red blood cells, *WBC* white blood cells, *GLU* glucose, *LYM* lysozyme, *MON* monocytes, *CCL* carcass composition lipid, *CCCP* carcass composition crude protein, *TSP* total serum protein, *ALB* albumin, *IMG* immunoglobulin production

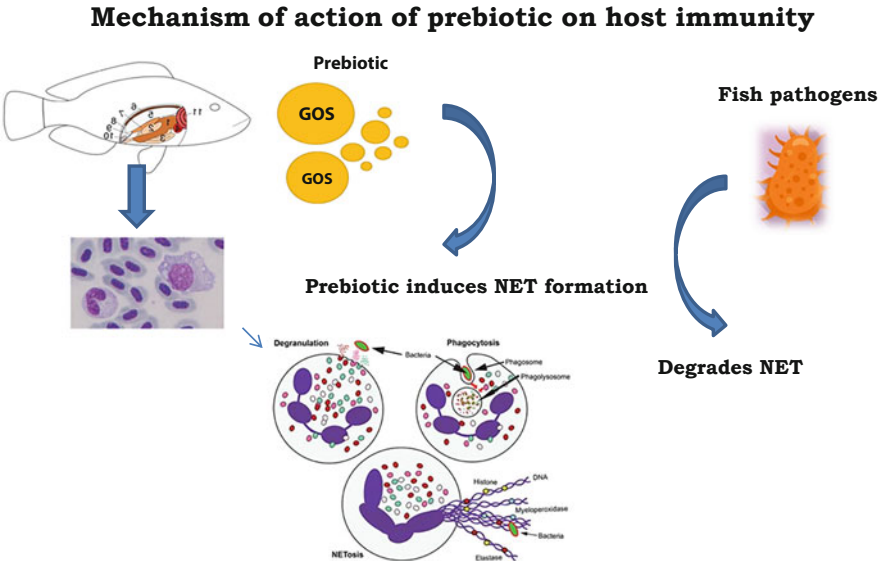

Fig. 11.1 Mechanism of action of prebiotic on fish

effects of prebiotics on fish mucosal immunity is also considered and researched upon. Thus, the action of dietary prebiotic follows two major routes. Firstly, it directly stimulates the innate immune system, and secondly, it also enhances commensal microbiota development (Kühlwein et al. 2013). For instance, the studied time-dependent effect of β-(1, 3) (1, 6)-D-glucan on both allochthonous and autochthonous microbial communities in carp gut by Kühlwein et al. (2013) revealed the influence of the prebiotic on composition of the carp intestinal microbiota. Nevertheless, the resulting effect seems to vary in several studies with both positive and negative outcome. This could be possible due to several related factors apart from the combination (probiotic + prebiotic) used in a particular species like the amount of the prebiotic in the diet, its solubility, type of fish species, the water temperature, and duration of the study. For example, dietary MOS supplementation in common carp fingerlings at 1–1.5 g kg^{-1} could improve the growth, feed efficiency, and resistance to *A. hydrophila* infection (Ebrahimi et al. 2012), whereas the improvement was nonsignificant in case of other species like channel catfish (*Ictalurus punctatus*) (Welker et al. 2007), Nile tilapia (*Oreochromis niloticus*) (Hisano et al. 2007), and grass carp (*Ctenopharyngodon idella*) (Shaker 2011).

11.6 Synbiotic Application in Fishes

Rahimnejad et al. (2018) studied the effects of dietary supplementation of GOS prebiotic and *Pediococcus acidilactici* MA18/5 M probiotic in combination as synbiotic in rockfish (*Sebastes schlegelii*). The authors reported a comprehensive and coherent proof of health benefits on circulating innate immunity, skin mucus, and serum bacteriostatic activity with a higher survival level performed through an in vivo bacterial challenge. The observed combined effect substantiate the statement by Tallarida (2011) who stated that "when the combined effect (of two or more drugs) is greater than that predicted by their individual potencies, the combination is said to be synergistic."

11.7 Synbiotic and Skin Mucosal Immunity

The antimicrobial action of the skin mucus is a functional property of great significance, which is a result of a complex array of immune molecules and mechanisms acting in concert (Esteban 2012). The skin mucus operates the innate immune function in terms of immunological indices like complements, lysozyme, Igs, protease, and lectins and thus plays a pivotal part to be considered as the primary defense conferring protection to the fish against pathogens. The physiological and nutritional status is known to affect the bactericidal activity of skin mucus (Subramanian et al. 2007). Rahimnejad et al. (2018) reported that skin mucus had an overall stronger bacteriostatic activity compared with serum against the marine pathogenic bacteria (marine pathogenic bacteria: *Vibrio harveyi*, *V. anguillarum*, and *Photobacterium damselae* subsp. *piscicida*) tested, thereby confirming the biochemical barrier

function of skin mucus against invading pathogens. Further, they observed that the bacteriostatic activity of skin mucus was marginally modulated and significantly reduced against *V. harveyi* in the synbiotic diet and speculate that it may have steamed from the high baseline antimicrobial activity of skin mucus, which warrants further studies. Their study concluded that the tested synbiotic formula could modulate skin mucosal antimicrobial functions in a pathogen-specific manner indicating varying degree of practical benefits according to the local pathogen population. The study by Azimirad et al. (2016) found that administering of FOS and *P. acidilactici* to angelfish, *Pterophyllum scalare* alone or in combination modulated skin mucus immune responses. Also, dietary supplementation of *Saccharomyces cerevisiae* (Sheikhzadeh et al. 2012) and XOS (Hoseinifar et al. 2014a, 2014b) increased antibacterial properties of the rainbow trout, *Oncorhynchus mykiss* and the Caspian kutum, *Rutilus frisii kutum*, respectively. Parallel to this, earlier research confirmed an elevated antibacterial activity following administration of heat-killed *L. plantarum* and β-glucan in red sea bream (*Pagrus major*) (Dawood et al. 2018), *L. acidophilus* in black swordtail (*Xiphophorus helleri*) (Hoseinifar et al. 2015a). Similarly, improved resistance to in vivo bacterial challenge was reported in synbiotic administration containing *B. subtilis* + chitosan in cobia, *Rachycentron canadum* (Geng et al. 2011), and *B. subtilis* + FOS in ovate pompano, *Trachinotus ovatus* (Zhang et al. 2014a, b).

11.8 Role in Stress Amelioration

Aquatic animals, like any other animals, are constantly and regularly exposed to several external and internal environmental quality alterations with subsequent compensatory and adaptive responses to encounter the external stimuli. The strength and duration of the stress perceived often threatens the health, well-being, and survival of the fishes. The disturbances caused by the so-called stressors, which can be physical, chemical, biological, and/or social, will lead to overall imbalance of the homeostasis of the internal physiological function and external environment. The fish immune system is one critical physiological setup that has the capacity to protect the overall integrity and homeostasis of the organisms by monitoring the alterations in cells or tissues caused by the induced stressors. Nutritional approach for stress amelioration is quite promising in the sense that the active components can be directly incorporated through the diet of fish. The case of synbiotic feeding and its effective capacity in encountering water-borne contaminants has been studied narrowly. However, there are few reports that demonstrate the potential of dietary synbiotic formulations to successfully reduce environmentally significant pH and nitrite levels. In a report by Singh et al. (2019), the synbiotic combination of *Bacillus circulans* PB7 and FOS could mitigate low pH (5.5) in *Labeo rohita* juveniles. The authors correlate the enhanced growth and health of fishes fed synbiotic under low pH stress to the ability in maintaining an endogenous microbiota, which further enhances the immune function. Further, it is also possible that supplemented synbiotic helps in faster proliferation of the probiotic through the substrate provided

by FOS. The measure of the antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) also revealed nonsignificant changes under the applied stress, indicating ameliorating capacity of the synbiotic. To connect the positive result, many of the probiotic strains possess antioxidant properties, which are linked to heteropolysaccharide fractions derived from the probiotics and increased mRNA levels (Gao et al. 2013). In another study, Singh et al. (2013) reported the capacity of synbiotic against nitrite induced challenges in the same fish.

11.9 Regulation of Key Immune Genes Post Synbiotic Feeding

A precise understanding of the mediated immune response through any dietary immunosupplement like synbiotic feeding can be measured by the expression level of key immune genes, which are expressed fondly against stress or external pathogenic invasion/injury. Among several genes, interleukin 1β (*IL-1β*) is one key mediator in response to pathogen invasion or tissue injury. It protects the host organism from infection by regulating the development of proinflammatory cytokines such as IFN- and IL-8 by monocytes/macrophages. It can stimulate immune responses through activation of lymphocytes or by inducing the release of other cytokines that are capable of activating macrophages, natural killer (NK) cells, and lymphocytes. The chemokine interleukin 8 (*IL-8*) represents a small secreted cytokine involved in control of the trafficking of immune cells. Other cytokine like tumor necrosis factor α (TNFα) plays key role in host immune responses and inflammatory processes (Alejo and Tafalla 2011).

The single use of prebiotic in fishes with subsequent immune-related gene expression changes in response to dietary feeding is summarized in Table 11.4. Increased gene expression of lysozyme and TNFα in the head kidney of rainbow trout, *O. mykiss* is reported upon feeding commercial prebiotic Immunogen® (containing β-glucan and MOS) (Ahmadi et al. 2014) and Vitacel® (fermentable fiber) (Yarahmadi et al. 2016). In other cases, β-glucan slightly upregulated IL8 in the head kidney of Caspian trout and in the livers of Nile tilapia (Jami et al. 2019; Salah et al. 2017), whereas MOS 0.16% downregulated TGFβ and upregulated Ig, MHC-II, TCRβ, and caspase-3 in juvenile in European sea bass, *Dicentrarchus labrax* (Torrecillas et al. 2015).

Many study demonstrated effectiveness of synbiotic against lone use of prebiotic. The related finding on regulation of key immune genes through synbiotic feeding is depicted in Table 11.1. In a study by Mohammadian et al. (2019), administrated dietary β- glucan, MOS, and *Lactobacillus casei* showed varied expression level of the above genes in *C. carpio* head kidneys, which contradicts against earlier reports possibly due to an appropriate indigenous bacterial colonization (microflora alteration) in the intestine (Panigrahi et al. 2005). The authors reported that the applied probiotic, *L. casei*, could not colonize as strong as autochthonous bacteria resulting in low expression levels, whereas, in a recent study, Dawood et al. (2020) observed an enhancement in the production of cytokines (IFN-γ and IL-8) in Nile Tilapia fed

MOS, establishing the importance of using MOS to improve the ability of fish to counteract with inflammation, similar to those reported in *C. carpio* (Mohammadian et al. 2019) and *Seriola dumerili* (Fernández-Montero et al. 2019).

11.10 Protection against Pathogenic Challenge

The paradigm shift in farming accompanied by intensification of stocking density led to frequent outbreak of several bacterial, viral, and fungal diseases, which is quite alarming. Among several pathogens of concern, *A. hydrophila*, which is a gram-negative, motile, rod-shaped pathogenic bacterium, has caused severe havoc in the aqua industry affecting several aquatic finfishes (Harikrishnan and Balasundaram 2005). *A. hydrophila* outbreaks lead to high mortality rates in cultured fishes, either by induction of internal hemorrhages and general bacteremia (acute form), and skin ulcers and underlying necrosis of the musculature (chronic form) (Derome et al. 2016). As a protective approach, antibiotics are used in commercial farming; however, the incidence of antimicrobial-resistant *Aeromonas* strains, suppression of host's immune function, environmental contamination, and food safety concerning human nutrition are major challenges (Zhang et al. 2014a, b; Banerjee and Ray 2017). There are several works reported on the application of synbiotics for protection against *A. hydrophila* (Table 11.1). The individual role of probiotics and prebiotics in aquatic animals has received enough interest, especially for fortification against major infectious diseases through potentiating the host nonspecific immunity against potential pathogens of interest (Merrifield et al. 2010). For example, feeding a single dose of probiotic *Lactobacillus acidophilus* (1 g kg^{-1} feed containing 10^{10} CFU), prebiotics, viz., yeast (1%), β-glucan (0.1%), GOS (1%), and MOS (0.2%), could stimulate immunity and provide protection of *Channa striata* fingerlings to *A. hydrophila* challenge. The protective feature of some prebiotic like yeast and β-glucan is mostly linked to their glucan contents, which are reported to increase disease resistance by stimulating nonspecific immune system (Burgents et al. 2004). Similarly, Geng et al. (2011) demonstrated synergistic of dietary chitosan and *B. subtilis* in cobia in resistance against *Vibrio harveyi* during a 56 days trial. Conversely, no synergistic effect was observed between *B. subtilis* and FOS with regard to the resistance of yellow croaker against *V. harveyi* challenge (Ai et al. 2011). Synbiotic formulations are able to provide effective protection against challenges from pathogenic bacteria. In one of the studies by Ye et al. (2011), the immune response of Japanese flounder, *Paralichthys olivaceus*, was found significantly higher in those fed synbiotic combinations of FOS and MOS with *Bacillus clausii*, compared with singular effect. Even though the actual mode of action of dietary synbiotics on fish immune response remain unrevealed, recent findings points to the possible production of SCFA by probiotic through the microbial fermentation when supplied in combination. Further, higher in vitro production of butyric acid as in case of synbiotic partnership between PA and GOS against combination of the former with prebiotic like FOS and XOS suggest a specific association between pre- and probiotic in its determination of the overall effect

(Hoseinifar et al. 2015b). It is also established that significant level of butyrate secretion acts as an important energy source for epithelial cells and has beneficial effect on intestinal health and resistance against pathogens (Maslowski and Mackay 2011). However, the effect shown by synbiotic formula may vary depending upon the dose of pro−/prebiotics, the host fish, and duration of administration. To support this, several studies reported no remarkable effects on immune parameters. For example, dietary administration of symbiotic *Bacillus subtilis* + chitosan in cobia (*Rachycentron canadum*) (Geng et al. 2011), *Weissella ci- baria* + inulin in hybrid surubim (*Pseudoplatystoma* sp.) (Mouriño et al. 2012), *B. subtilis* + FOS in juvenile large yellow croaker (*Larimichthys crocea*) (Ai et al. 2011), and *B. subtilis* and FOS also showed no such beneficial role. The non-functionality and low efficacy of synbiotic as exampled above may arise possibly due to the inappropriate prebiotic administration as the required substrate in a synbiotic formula or very low dose level for fermentation by selected prebiotic to produce the SCFA (Hoseinifar et al. 2015b). In this midst, delineation of the exact mechanisms of action of dietary synbiotic on both systemic and mucosal immune response is an obvious future thrust area.

11.11 Conclusion

In the present scenario of aquafeeds, application of prebiotics and synbiotic additives is a novel concept to improve well-being of cultured animals. In the context of feed additives, the current review emphasizes the dietary role of prebiotic–synbiotic nexus in the promotion of growth, feed efficiency, gene regulation, stress amelioration, immune-modulation, and disease resistance of cultivated aquatic animals. The indicative references cited in this chapter clearly illustrate the critical contribution of these biotics to the general health and well-being of cultured organism. Although nearly a dozen of prebiotic substrates from a variety of sources and chemical structures have been evaluated for aquatic animals worldwide, across studies, commonly used prebiotic substrates include mannan oligosaccharide (MOS), fructooligosaccharide (FOS), and inulin, while species from the genera *Bacillus* and *Enterococcus* are the most commonly used live microorganisms within the formulations tested. In addition, dietary administration of a commercial synbiotic using Biomin IMBO, Lacto Forte, and Beta Plus® is reported to consist of monospecies or multispecies biotics. It is documented that dietary prebiotic applications differ greatly (0.8 g to 150 g/Kg^{-1} diet) among aquatic animals, with the optimal prebiotic requirement for desired outcomes noted to be in the range of 1.5 g to 10 g Kg^{-1} diet for the majority of the species studied (finfishes, shellfishes, and echinoderms). However, probiotic inclusion levels vary from 10^4 to 10^{12} CFU g^{-1} diet, with optimal requirement rages between 10^4 and 10^8 CFU g^{-1} diet for most of the species studied. Regardless of the duration of the administrations of the biotics, few studies have identified no effects or negative effects of high inclusion levels in the diet of aquatic animal (Refstie et al. 2006; Mahious et al. 2006a; Ringø et al. 2006; Ismail et al. 2019); it is also evident that different prebiotics modulate growth, immune response, and other physiological parameters

in different manner. Thus, a thorough investigation is needed to fully understand their impact, appropriate dose requirement, duration of the administration, composition and structure of biotics, etc.

Progress of previous studies over two decades has shown that biotic applications are useful tools for improving the health status and production of fish; moreover, consensus statement on the definition and scope of synbiotics (Swanson et al. 2020) has given further insight to synbiotic research, particularly complementary synbiotic vs synergistic synbiotic. Several studies using combined biotics (synbiotic) have shown comparatively higher health outcomes than individual applications. Therefore, the selection of biotic additives with a precise dose is not only crucial for the design of synbiotics but also animal and consumers safety.

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Biofloc Technology: An Eco-Based Emerging Avenue in Aquaculture Health Management

12

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Abstract

Intensified aquaculture is becoming a major stake in the global demand for animal protein. The enhancement in the aquaculture practices and management of resources can reduce the environmental impact on the aquatic system. This helps in the transition of traditional culture practice to the intensified sustainable culture system. On this note, biofloc technology is considered as one of the sustainable intensification practices to culture aquatic animals for increasing production and immunity with minimal water usage. This system is growing faster and getting attention currently due to its advantages and augments large-scale production. The microbial community improves various aspects in biofloc system like recycling of the nitrogen metabolites through in situ bioremediation, improving water quality, producing microbial protein, and inducing the immune system of the cultured animals. Biofloc has a high nutrition profile, which is an excellent alternative as a feed ingredient and reduces the feed conversion ratio as it provides natural feed in the system. The diversified microbial community in biofloc plays a key role in inducing disease resistance against the pathogenic bacteria. The constant exposure to natural probiotic microflora in the environment induces the immunity of the animal by competitive exclusion and quorum sensing of beneficial microbiota. Therefore, it increases overall performances and productivity when compared with the conventional system. This current review provides an insight into the biofloc technology, microflora assemblages, and their function/role in gut microbiome maintenance, improving immunity, disease resistance, and overall productivity in nursery and grow out aquaculture systems.

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Keywords

Aquaculture · Sustainable · Intensification · Biofloc · Heterotrophic · Probiotic

12.1 Introduction

The human population's continuous growth demands alternate protein sources, and there is lot of expectation from sustainable intensification in aquaculture. The limited supply of fish food from native fishing generated interest in alternate food production sources, leading to the sustainable aquafarming industry's growth. Aquafarming guarantees the fulfillment of the world's food security, and presently, aquaculture is a predominant food produced next to agriculture, supplying sufficient protein demands (around 20%) to the world food market. The Aquafarming industry uses a variety of aquatic organisms, including fishes, mollusks, and crustaceans. According to FAO (2018), aquaculture represents around 47% of the global fish production of 171 million tonnes, inclusive of fish (>54 million tonnes), mollusks (>17 million tonnes), crustaceans, and other products (eight million tonnes). Also, at the estimated value of 362 billion dollars of the total first-sale value of fisheries and aquaculture production, aquaculture represents 232 billion dollars. Aquaculture is the fastest-growing food sector with annual growth rate of 5.8% from 2001 to 2016 (Tičina et al. 2020). Even though the aquaculture industry guaranteed food and job, it faces many threats mainly because of disease and environmental pollution. Feed costs and access to specific pathogen-free broodstock are some of the other important issues faced by the industry.

Intensive aquaculture often uses an enormous amount of chemicals in the form of water treatment compounds, pesticides, antibiotics, disinfectants, fertilizers (urea and triple superphosphate), and hormones. So the expansion of intensive coastal shrimp farming uses overstocking, and subsequent overfeeding leads to more effluent discharge enriched with ammonium, nitrate, phosphates, and other micronutrients to the environment. Coastal aquaculture synonym with shrimp aquaculture is a multibillion-dollar industry with production touching 7 lakh metric tons with export earnings of 45,000 crores from India recently (2018–2019). However, disease prevalence like running mortality syndrome (RMS), early mortality syndrome (EMS), and *Enterocytozoon hepatopenaei* (EHP), which has reached an epidemic proportion in Asian shrimp aquaculture, is a greatest challenge. *The risk of pathogenicity and environmental impact can be avoided in many ways such as through better management practices, control of effluent discharge, wastewater treatment, less application, or complete avoidance of chemical additives. This could be possible by following the novel technique like biofloc technology (BFT). This is an eco-based approach that promises a healthy culturing environment for preventing diseases.*

12.2 The Concept and Definition

Biofloc is a consortium of particulate matter formed predominantly by a biota of aerobic and heterotrophic bacteria, protozoa, microalgae, metazoan, exoskeletons, feces, and remains of dead organisms. The diverse microbial community present in the biofloc system acts like natural probiotics and stimulates nonspecific immune activity. It is a proficient alternative culture method based on the growth of beneficial microbes in the culture environment. The unconsumed feed materials and other organic nutrients are continuously recycled by microbes and enable host animal consumption. This system improves water quality, avoids the accumulation of toxic metabolites such as ammonia, and requires zero or limited water exchange, which reduces the water consumption, competitive exclusion that evades the growth of pathogenic organisms, reduced effluent discharge, and eco-friendly (Panigrahi et al. 2018, 2020a; Emerenciano et al. 2013a, b, Fig. 12.1).

The balanced carbon and nitrogen sources stimulate microorganisms' growth, including bacteria and planktons, which will use the ammonium and other nitrogenous and phosphorus matters for their development. This will lead to microbial proteins and microbial biomass (Avnimelech 1999; Schneider et al. 2005; Panigrahi et al. 2019b).

The stimulated bacterial growth utilizes the ammonium more quickly than the normal nitrification process. It was estimated that the heterotrophic bacterial growth and biomass yield through ammonia immobilization were ten factors higher than those observed in normal nitrifying bacteria (Hargreaves 2006). The heterotrophic biomass yield was also calculated as the 0.5 g biomass C/g substrate C used (Eding et al. 2006).

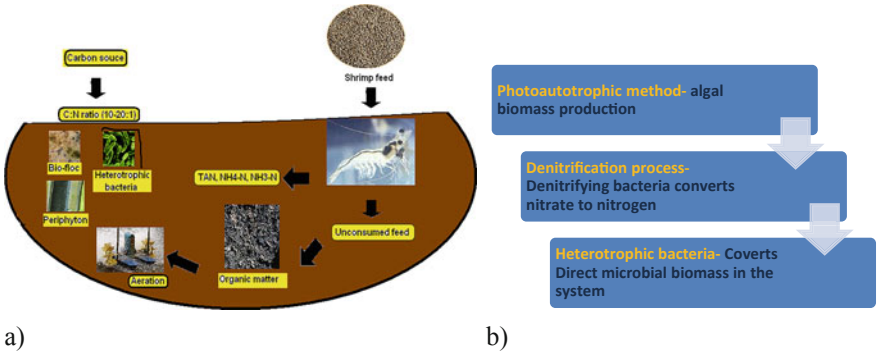

Fig. 12.1 (**a**) Schematic diagram showing the concept of biofloc in a grow out system and (**b**) nitrogen conversion pathway in biofloc system

12.3 Nitrogen Recycling, Water Quality, and a Stress-Free Environment

As the biofloc technology utilizes zero or low water exchange, the nitrogenous output especially ammonia raised from the feed and other inputs needs to be recycled continuously. Due to the protein metabolism, the level of ammonia is increased, and even at low concentration, it is toxic to the animal (0.025–1.0 mg/ml (Li et al. 2007; Chen et al. 2006; Emerenciano et al. 2017; Jiménez-Ojeda et al. 2018); subsequently, this causes stress, affects the reproductive performance and appetite, favors the growth of pathogenic bacteria, impairs immune response, and increases mortality rate (Liu and Chen 2004; Zeitoun et al. 2016; Wicks et al. 2002; Zhang et al. 2018). They exist in either ionized (NH_4^+) or unionized ammonia (NH_3) form and physical parameters such as salinity, pH, and temperature, which determine the equilibrium status of both forms (Lin and Chen 2001; Luo et al. 2020). Nitrogen recycling in a typical aquaculture environment is often reported as a slow process due to many factors, including heterotrophic microorganisms (Sigee 2005; Stein and Klotz 2016; Jiménez-Ojeda et al. 2018).

In general, nitrogen recycling is taking place in three different ways: photoautotrophic algal assimilation, chemoautotrophic bacterial nitrification, and heterotrophic bacterial assimilation (Luo et al. 2020). Algal assimilation is practiced in ex situ, and it is light-limited (Aquilino et al. 2020); moreover, excess algal bloom causes toxicity to animals. The other method is microbial-based chemoautotrophic bacterial nitrification. This works in fixed-cell bioreactor attached to recirculating aquaculture systems. There are two groups of bacterial consortia, namely ammonia oxidants such as *Nitrosomonas* sp., which utilize unionized ammonia converting it into nitrite, and nitrate oxidants such as *Nitrospira* sp., which convert the nitrite to nitrate. This is possible in stagnant stat condition, requiring a bioreactor equipped with a biofilter (Ruiz et al. 2020; Luo et al. 2020). The next method is ammonia assimilation by heterotrophic bacteria, which rapidly converts ammonia into bacterial protein and other metabolites without involving the conversion of nitrite and nitrate (Huang 2019; Panigrahi et al. 2019b, 2020a, b). When compared with the first two methods, heterotrophic bacterial-based biofloc technology now widely emerged all around the world for the culture of fish and shrimp. In intensive aquaculture systems, the production cost is the primary concern, and also feed inputs and water management affect the stocking density of the fish and shrimp. A heterotrophic microbial-based culture system does not require additional facilities like a recirculation system and considerably reduces water consumption.

12.4 C/N Ratio Manipulation and Carbon Sources Requirement

The optimization of the C/N ratio is key for the biofloc system. As the fish/shrimp ponds are rich in the microbial community, the inorganic nitrogen added through the feed can be assimilated by the microorganism and converted into microbial protein through an adjustment of the C/N ratio (Avnimelech 1999; Xu et al. 2013; Panigrahi

et al. 2018; Chakrapani et al. 2020; Elayaraja et al. 2020). The optimum C/N ratio in an aquaculture system can be maintained (C/N ratio 12–15:1 is optimal for biofloc production) by adding different locally available cheap carbon sources and/or reducing protein percentage in the feed. Under optimum C/N ratio, inorganic nitrogen is immobilized into the bacterial cell, while organic substrates are metabolized. In a typical brackish water pond, only 20–25% of fed protein is utilized by the fish/shrimp, the rest of which goes as waste in the form of ammonia and other metabolites. Maintaining the appropriate C/N ratio is required for optimum heterotrophic population, usually, the higher C/N ratio stimulates the system to shift from autotrophic to the heterotrophic bacterial community (Avnimelech 1999). The conversion not only reduces toxic metabolites and maintains water but also improves the feed conversion ratio, immunity, and productivity of cultured species (Long et al. 2015). It is not only required for generating the bloom but also colonizes the bacteria subsequently. Thus, there is a higher demand for suitable carbon sources; it should be cost-effective, economically viable, available all through the year, and as well competent for the biofloc system. So far, molasses is the most researched and used carbon source in biofloc system for fish/shrimp culture, as it was reported for improving water quality and enhancing immunity, survival, and productivity on *Farfantepenaeus brasiliensis* (de Souza et al. 2014), and better immune response was reported when challenged against APHND causing *V. parahaemolyticus* on *Fenneropenaeus indicus* (Megahed et al. 2018). Similarly improved African catfish' reproductive performance (*Clarias gariepinus*) with comparable fecundity rate and eggs quality besides growth performance and water quality (Ekasari et al. 2016) also highly recommended carbon source for the low salinity culture of Nile tilapia fingerlings (de Lima et al. 2019). Though molasses has many pluses, the usage is not legal in many countries. Owing to finding an alternative for molasses was mandatory. Many researchers tried and tested the potentially equal carbon source from agricultural by-products such as rice bran, wheat bran, and soybean meal, which are available universally (Ekasari et al. 2014; Serra et al. 2015; Xu et al. 2016; Panigrahi et al. 2019c). It was reported that a 22% higher yield and 15% lower feed conversion rate resulted in rice bran compared to molasses used in the biofloc-based culture of *Litopenaeus vannamei* (Vilani et al. 2016). And plant-based carbon sources such as rice flour, wheat bran, cellulose (El-Husseiny et al. 2018), wheat flour (Rajkumar et al. 2016), millets and multigrain (Panigrahi et al. 2019c), and distillery spent wash a by-product of sugar (Yuvarajan 2020) are reported as a carbon source for the biofloc-based fish and shrimp culture. Besides simple carbon sources such as poly (β-hydroxybutyrate-β-hydroxyvalerate) (Liu et al. 2019), wheat milling by-product (Mansour & Esteban 2017), dextrose (de Lorenzo et al. 2016a, b), sucrose (Zhang et al. 2017), glucose (Liu et al. 2018a, b), sodium acetate, (Luo et al. 2014), and glycerol (Dauda et al. 2018), spoilage date extract is proposed as an alternate carbon source to molasses results in better growth performance and augments immunity in biofloc-based *Litopenaeus vannamei* culture (Abbaszadeh et al. 2019). Tapioca flour was used as a carbon source in *Labeo rohita*, and it enhances growth and survival. Biofloc generation with tapioca flour has the advantages of improving humoral immunity and increases the relative percentage

survival of *L. rohita* when challenged with *A. hydrophila* (Verma et al. 2016) and in the culture of *Pelteobagrus vachelli* (Deng et al. 2018). The main downside of the agriculture by-product usage in the biofloc system is dispersal and efficiency. Simple carbon sources like glucose and glycerol can diffuse easily, and it will be available rapidly for the growth of heterotrophic bacteria and subsequent floc development, but it needs excess carbon source supplementation to the culture tank (Dauda et al. 2017; Chakrapani et al. 2020). The complex carbon derived from agricultural by-products such as rice bran or wheat bran is having a complex form due to its nutritional content and the dispersion of this carbon source unhurried, but they promise to give a stable response to the culture medium (Khanjani et al. 2017).

The optimum C/N ratio will be varied according to the cultural conditions. Most of the experimental results indicated that of C/N ratio of 10:1 to 15:1 was found optimum for different fishes and shrimp (Panigrahi et al. 2018; Liu et al. 2018a, b; Chakrapani et al. 2020), and a C/N ratio above this level either unaffected or reduce the growth performance of the cultured species (Xu et al. 2016; Panigrahi et al. 2018).

12.5 Advantages of Adopting BFT in the Shrimp Culture System

Health Advantages

- **Zero or minimal water exchange**—BFT encourages better biosecurity and pathogen exclusion.
- **Stress-free environment—**Continuous aeration in the system mixes water, thus avoiding stratifications and stable water.
- **The diverse beneficial bacteria—**Bacterial community in the biofloc can stimulate nonspecific immunity and limit the establishment of pathogenic strains.
- **Probiotic action**—Bioflocs can act as a natural probiotic, which could act internally and/or externally against, *Vibrio* sp. and ectoparasites, diverse aerobic gut flora reducing pathogenic bacteria (*Vibrios*).
- **Removal of settled and suspended solid waste—**These wastes are removed from the biofloc system, thus preventing any risk of disease from the sludge.
- **Priming of the immune system** of the host helps in immunomodulation and disease resistance in the animals reared in this system.

Nutritional Advantage

- **Natural productivity—**Augmentation of natural food and improvement of FCR.
- **Protein is utilized twice—**Lower protein creates a better heterotrophic environment.
- **Nutrient-rich**—The high protein-lipid rich nutrients in biofloc, including fatty acids protects against oxidation, vitamins, phospholipids, and highly diverse "native protein," could be utilized continuously.
- **Broodstock gonad formation**—Help in building reserve energy and superior reproductive performance.
- **Reduced costs** (15–20% lower cost of production) including 30–50% cost savings in feed.

Environmental Advantages

- Zero/minimal water exchange system.
- **Bioremediation**—Heterotrophic bacteria can reduce toxic metabolites (NH_3 and NO_2).
- **Diurnal stress**—Diurnal changes (pH, O_2, and CO_2) in water, and turn the stress is reduced.
- **Environmental friendly approach** reduced protein requirement, fish meal usage, and water/nutrient discharge.

12.6 Microbial Assemblage and Function

Heterotrophic microbial assemblage and function is the central dogma of the biofloc mechanism. The nutritive source of the environment (outside or inside the host) is the main factor deciding the microbial composition. Studying the structure and function of biofloc and gut microbes related to the source of carbon and C/N ratio will give the perfect mechanism of symbiosis because they often proved to participate not only in digestion but also in preventing the pathogens (Llewellyn et al. 2014). The carbon source is the most influential factor for microbial colonization. For example, the total heterotrophic count, *Bacillus,* and fungal count were varied between molasses and spoilage date extract that are used as a carbon source for the cultivation of *Litopenaeus vannamei*. Spoilage date extract results comparatively high count of above said microbial population (Abbaszadeh et al. 2019).

Compared with the shrimp intestine, biofloc resulted in more diversity of heterotrophic bacteria (Cardona et al. 2016). Usually, the intestine is a rich nutrient source that attracts the bacterial population (Sullam et al. 2012). But in the case of the biofloc system, nutritional content will be rich surrounding water so the species richness is automatically high in biofloc water (Cardona et al. 2016). Heterotrophic microbial assembly competitively inhibits the growth of pathogenic microorganisms in the biofloc system. Soybean molasses in the mixture decreased the total *Vibrio* count compared to clearwater for the rearing of *L. vannamei* (do Espírito Santo et al. 2017). Likewise, metagenomics of the intestine of *Litopenaeus stylirostris* revealed that the abundance of *Gammaproteobacteria Vibrionales* is relatively low in biofloc reared shrimp than clear water reared shrimp (Cardona et al. 2016). This may be attributed to the antibacterial effect of the heterotrophic bacterial population. Most of the intestinal heterotrophic bacteria of *L. vannamei* reared in different biofloc system varying with carbon sources resulted in reducing biofilm of *Vibrio* and confirming the competitive growth against pathogens (Panigrahi et al. 2019d). Carbon source not only influences the heterotrophic bacterial count but also have an impact on the microalgae. Cassava starch influenced more percentage of Chlorophyceae than sugar and molasses. Simultaneously, sugar and molasses allow more Cyanophyceae growth than Cassava starch used for the biofloc-based culture of *Oreochromis niloticus* (Silva et al. 2018). Recently, light is also an influential factor with carbon sources; molasses with light sources increased the richness of beneficial flora such as

Paracoccus. But in the absence of the light, genus resulted in the growth of harmful *Leucothrix* (Jiang et al. 2020).

The heterotrophic bacteria in the BFT proved to improve the water quality by recycling ammonia. They use toxic ammonia for their cellular activities and may produce protein bioactive compounds such as poly-beta-hydroxybutyrate (PHB), carotenoids, polysaccharides, etc. (Zhao et al. 2016; Panigrahi et al. 2019b). Production of bioactive compounds varied with the type of carbon sources, and these compounds positively correlated with the immune and antioxidant status of the animal (Zhao et al. 2016). Shrimp's gut is exposed to natural microflora, which provides nutritional and immunological benefits, especially on preventing the infection from the pathogen by competitive exclusion, neutralization of toxins, and bactericidal activity. This also results in improving the animal's overall health enhancement, animal's physical condition, appearance, average size, weight of the animal, and restoration of reduced appetite and feed consumption.

The biofloc system was also found to improve the digestive enzyme activity as it guaranteed continuously available food. Activities of digestive enzymes such as protease, lipase, amylase, and cellulase were found to increase in different animals reared in biofloc system (Xu et al. 2013; Long et al. 2015; Yu et al. 2020a). This may be attributed to the enzyme-producing ability of heterotrophic bacterial assemblage in the intestine.

The gut microbiome also plays role in aquatic animal health; in general, the host animal gut is colonized with beneficial bacteria from the environment. The beneficial bacteria colonization resists the growth of pathogenic microorganisms (Lawley and Walker 2013; Panigrahi et al. 2019d). Moreover, colonization resistance is supported by the microbiota derived through the bioactive compound from the biofloc. Changes in the gut microbiome can be an indicator of disease, and the presence of biofloc could alter the microbiome of the cultured species that could even resist the pathogenic bacteria and virus (Holt et al. 2020; Huang et al. 2020).

Carbon sources positively influenced the colonization of enzyme-producing bacterial flora in the intestine. The majority of intestinal heterotrophic bacteria of *L. vannamei* reared in different biofloc systems found to produce protease, amylase, lipase, xylanase, and cellulase than the microflora of control group animals. However, the percentage differed with the type of carbon sources used (Panigrahi et al. 2019c).

12.7 Biofloc Technology and Immunity Enhancement

In aquaculture, animal health is an important criterion as it can affect the growth and cause mortality. Prevention of disease is a better strategy than treating it. Presently, there is a ban and increased regulation over the use of antibiotics in aquaculture considering the safety (Ajadi et al. 2016). Since the ban of antibiotics in aquaculture, the need for alternatives is required to use probiotics, prebiotics, synbiotics, and microbial-based system (Ventola 2015; Ajadi et al. 2016; Buruiana et al. 2017; Chauhan and Singh 2019; Dauda 2020). To avoid antibiotics, bacteria are

recognized as a useful tool to fight such diseases, minimizing the rampant use of these drugs. The biofloc system acts as a natural source of probiotic bacteria (Ferreira et al. 2015; Panigrahi et al. 2020b), which acts as an immunostimulant source (Xu and Pan 2014, Anand et al. 2014, Kumar et al. 2018,). This improves the animal's immunity and reduces the prevalence of the disease. The complex microbial interaction in the biofloc enhances the immune response of the cultured animals (Kim et al. 2014; Panigrahi et al. 2018). Biofloc technology can be seen more as a mechanism, which provides shrimp/fish a chance to keep the immune system active at all times, as they get exposed to various microbes.

In complex communities, these microbes facilitate competitive strategies, like competitive exclusion, or harm the other microbes either by fighting or producing toxins (Hibbing et al. 2010). Further, they can inhibit the proliferation of pathogens (Fig. 12.2).

The associated-microbes are involved in assimilating and converting the nutrients as consumable microbial protein (Porchas-Cornejo et al. 2011a, b). In addition to this, the microorganisms and their cell wall components or metabolites in biofloc have been involved as immunostimulants and improves the nonspecific immunity and antioxidation status of fish and crustacean (Anand et al. 2014; Balzaretti et al. 2017; Dennis-Wall et al. 2017; Chen et al. 2018). The dietary supplements of biofloc also improve the shrimp immunity and stimulate the innate immune response in shrimp (Anand et al. 2014; Promthale et al. 2019). It is also reported that the microbes in the system uphold the immune activity and the digestive and metabolic activity in shrimp (Browdy et al. 2001; Samocha et al. 2007; Suita et al. 2015; Correia et al. 2014).

Shrimp's immune system is considerably different from other vertebrates. Shrimps lack adaptive immunity, and they entirely rely on innate immunity for their defense against the pathogen (Vazquez et al. 2009). This has a direct impact on shrimp farming. Consequently, new disease outbreaks are being expected in shrimp farming especially with white spot syndrome virus, early mortality syndrome, and yellow head virus. The first line of defense in shrimp starts when the pathogenic bacterial cell wall components of microorganisms such as bacteria and fungi consist of lipopolysaccharides (LPS), peptidoglycan (PG), and b − 1, 3-glucans (BG) are coming in contact with the shrimp immune system. These are the main triggering agents for activating the shrimp's immune system (Tepaamorndech et al. 2020; Qiao et al. 2020). Biofloc is an intricate system with an abundance of microbes. This can act as an immune stimulator (Fig. 12.3) in the cultured species (Ju et al. 2008).

Whereas in fish, the immune system holds on between the innate and adaptive immune response (Sajali et al. 2019). Mainly the immune response starts when an injury or a pathogenic invasion (Magnadóttir 2006; Kim et al. 2014; Ekasari et al. 2014; Liu et al. 2019), and they rely on a nonspecific immune activity like macrophages, (Yin et al. 2018) neutrophils, and nonspecific cytotoxic cells. Nonspecific immune response assisted widely circulated in mucus, serum, and ova of the fish, the produced glycoprotein in fish mucus when the bacterial load is increased in the surrounding environment (Van der Marel et al. 2010; Yu et al. 2020b). A variety of humoral factors like cytokines (Li et al. 2019), anti-proteases, peroxidases, and lysozymes are also delivered when they encounter a pathogen.

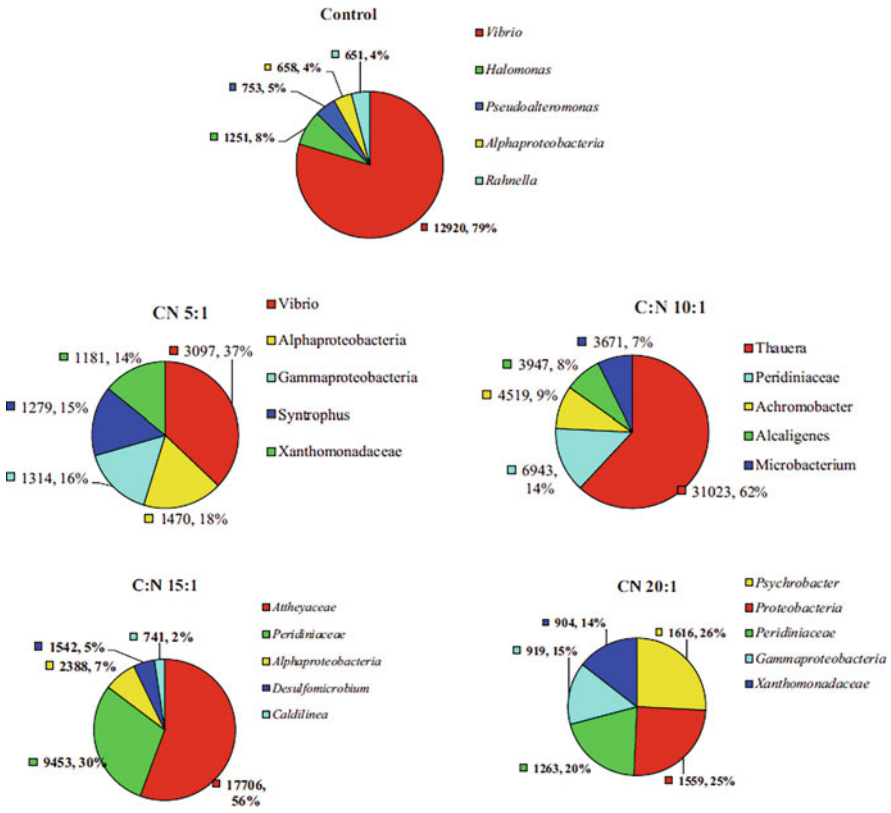

Fig. 12.2 The increase in the C/N ratio decreases the percentage of *Vibrio* and dominance of beneficial microbes' phyla was observed in *P. vannamei* culture (Source: Panigrahi et al. 2018)

Lysozyme acts as a vital fish immune marker due to its anti-inflammatory and antiviral property and possesses intense bacteriolytic activity against gram-positive and gram-negative bacteria (Saurabh and Sahoo 2008; Elayaraja et al. 2020).

12.8 Immune Gene Expression

The immunological potential level of biofloc reared animals was reported that the microbial cell components and their metabolites can act as immunostimulant that enhances the shrimp innate immune system and provide improved protection against pathogens. It has been already established that bacteria and some microalgae produce exopolysaccharides (EPS) such as uronic or pyruvic acid under a stressed condition, and these polymers are responsible for the adhesion and aggregation of dispersed cells of bacteria and microalgae called biofloc.

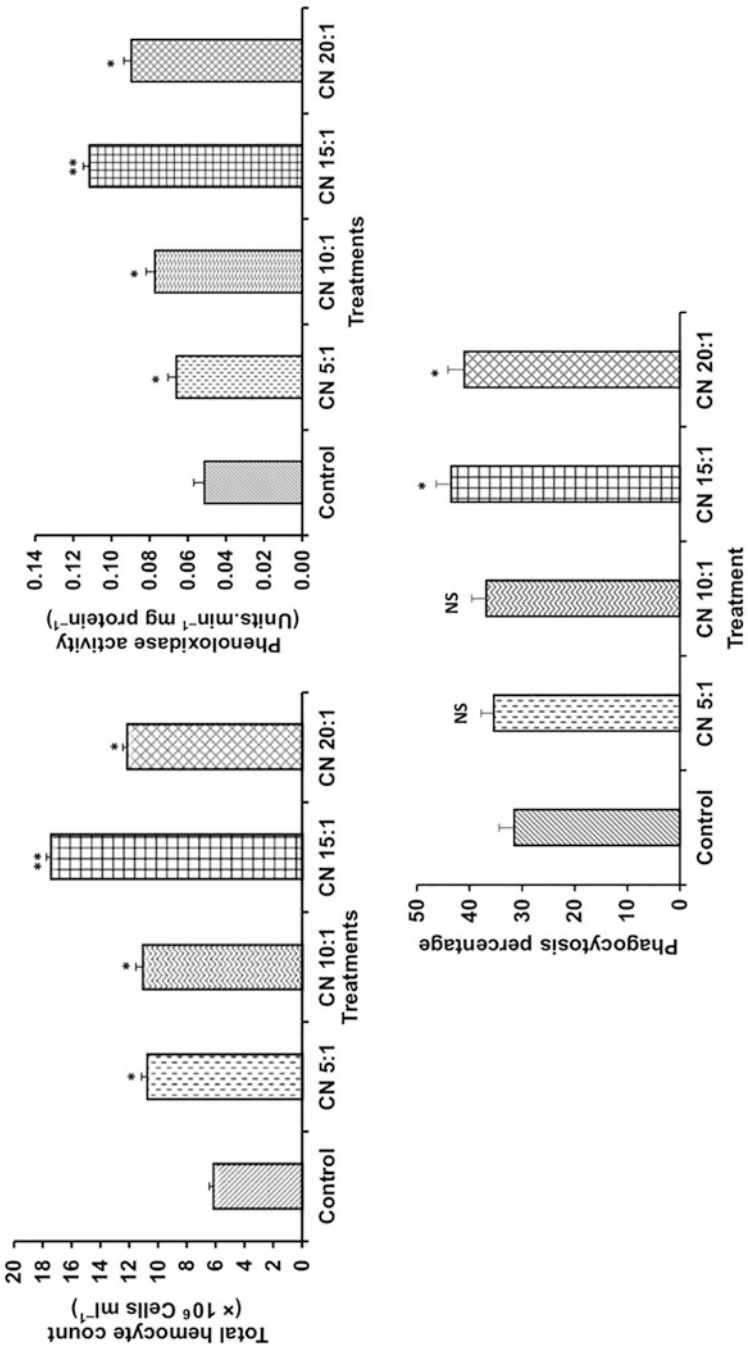

Fig. 12.3 Total hemocyte count, phenoloxidase activity, and phagocytosis activity increased with the increased C/N ratio in shrimp *P. vannamei* culture (Source: Panigrahi et al. 2019a)

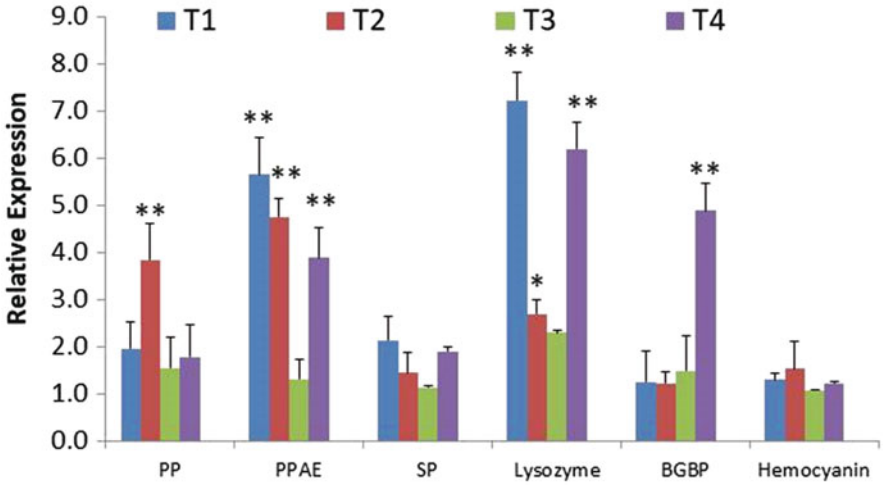

Fig. 12.4 Relative expression of immune related gene in shrimpreared in biofloc with different carbon sources and error bar showing standard deviation of three replicates

Transcripts of target immune genes can be measured by qPCR. Q-PCR results for an experiment on biofloc shrimp production with different carbon sources revealed appreciably enhanced mRNA expression of certain genes. Prophenoloxidase, phenoloxidase activating enzyme, serine proteinase, lysozyme β-glucan binding protein, and hemocyanin also shown to play a crucial role in the enhancement of immunity. Propheoloxidase activation is a major part of the immune system in shrimp. The upregulation of this gene indicates that the bacteria-associated biofloc play a major role in enhanced immune activity in the shrimps (Panigrahi et al. 2018) (Fig. 12.4).

A similar study revealed six genes that are involved in the innate immune response of shrimp proPO1 (prophenoloxidase 1), proPO2 (prophenoloxidase 2), PPAE (prophenoloxidase activating enzyme), SP1 (serine protease), mas (masquerade-like serine proteinase), and ran (ras-related nuclear) through mRNA expression (Kim et al. 2014). Gene expression measured in mysis, postlarvae, and adult *P. vannamei* was found to be enhanced in the presence of biofloc. Our studies suggest that microbes associated with bioflocs may enhance the expression of certain immune-related genes and metabolic pathways (Panigrahi et al. 2018, 2019b, c,).

12.8.1 Disease Resistance

There is a diversity of microbes present in the system, which is both beneficial and pathogenic to the cultured species and quorum sensing of the microbes that significantly elevates the number in the system. This antagonism activity between the pathogen and other heterotrophic bacteria limits the pathogen to multiply. *Vibrio* is

an opportunistic bacterium; they may grow at a higher rate during the initial culture period, but as the biofloc water matures, the system builds a strong diverse environment against the pathogenic bacteria.

In one of our study, a trial was conducted to check the immunity of biofloc reared shrimps by challenging them with the known pathogen *V. parahemolyticus.* The bacterial suspension for the challenge has been prepared as per the protocol and when ABW of 15–18 g biofloc reared shrimps were injected with pathogens and evaluated against normal saline injected shrimps. The cumulative mortality was found to be significantly lower in the BFT animals (Panigrahi et al. 2019a).

12.8.2 Bioflocs as Biocontrol Measure

The "natural probiotic" effect of biofloc could act internally and/or externally against, *Vibrio* sp. and ectoparasites. The regular addition of carbon in the water is known to select for polyhydroxyalkanoates (PHA) accumulating bacteria, which produces biodegradable polymer storage products, like poly-ß-hydroxybutyrate (PHB), having antibacterial or biocontrol properties that provide immunity to the host. Though the biofloc concept is yet to be practiced by many in India, probiotics is an established business at present in Indian aquaculture (Fig. 12.5).

Probiotic interventions of *L. rhamnosus* and *B. subtilis* were found to be advantageous in terms of better growth and survival rate, and the expression of certain immune-related genes in response to microbial interventions was significantly upregulated explaining the possible immunomodulation and in turn better protection in fish (Panigrahi and Azad 2007). Beneficial communities in the biofloc system control the pathogenic *Vibrio* population (Crab et al. 2012).

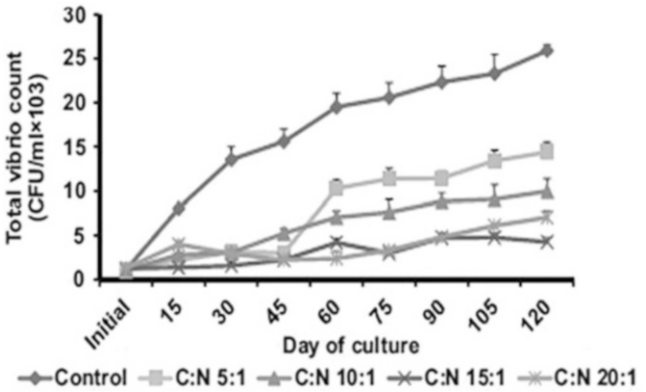

Fig. 12.5 Mean values of total presumptive vibrio count in water samples of *L. vannamei* culture in fifteen days interval of four CN ratios groups and control groups. Values are mean (±*SD*) of three replicant tanks per sampling time in each group. (Panigrahi et al. 2019a; Aquaculture research. 50(1): 29-41)

Many researchers tested the immunity of biofloc grew finfishes and shellfishes against the pathogenic strains. The reports includes artemia that was challenged against V*ibrio harveyi* (Crab et al. 2010), infectious myonecrosis virus (IMNV), and WSSV (Ekasari et al. 2014); *Labeo rohita* against *A. hydrophila* (Verma et al. 2016); tilapia fish the virulent strain of *A. hydrophila* (Haridas et al. 2017; Elayaraja et al. 2020), *Vibrio harveyi* on *P. vannamei* (Liu et al. 2018a, b; Panigrahi et al. 2018), and African catfish (*Clarias gariepinus*) was challenged against *A. hydrophila* (Dauda et al. 2018); APHND causing *Vibrio parahemolytics* on *P. vannamei* was challenged in biofloc condition (Hostins et al. 2019); and *P. vannamei* and Nile tilapia (*Oreochromis niloticus)* are challenged against APHND causing *Vibrio parahemolyticus.* (Sajali et al. 2019). *Oreochromis niloticus* is challenged against *Streptococcus agalactiae.* (Ekasari et al. 2015; Van Doan et al. 2020), etc. The animals cultured in the biofloc system have the potential of fighting against the pathogenic microbes from a variety of bacteria to virus. However, mortality was observed during the challenging trail; the rate of survival in all above-mentioned findings was significantly higher compared to the control system. Thus, it proved that the immune system in the animals grown in biofloc was robust, and growth was augmented.

Biofloc system can be maintained for the nursery and grow out system (Serra et al. 2015). In shrimp culture, the nursery is an intermediate phase between early post-larval and juvenile stages of shrimps, and here, small rearing system with high stocking density can be maintained for a short period of 20–28 days. This system can have more control over it than directly stocking the post-larvae in the grow out ponds. The main advantage of having a nursery can get consistent growth, better feed utilization, less water usage, and good survival (Fóes et al. 2011; Wasielesky Jr et al. 2013; Panigrahi et al. 2020a). Many farmers are interested in having a biofloc nursery because of the biosecurity and disease-free condition (Panigrahi et al. 2020a). This technology has been tried and tested on various aquatic species to fine-tune to apply for better aquaculture production (Khanjani and Sharifinia 2020).

12.9 Conclusion

In conclusion, biofloc technology is an intensive and sustainable system, which upholds many advantages. As the system follows zero water exchange, it can maintain biosecurity and minimal usage of water. The percentage of feed can be lower (Panigrahi et al. 2019a) as biofloc serves as supplementary feed for the cultured species; eventually, it becomes cost-effective for the farmers (Panigrahi et al. 2019a). The natural probiotic effect in biofloc provides antigens to trigger the immune response in the gut. Many reports suggest that a wide range of beneficial microbes or their cell wall components and metabolites is improving innate immunity and can be employed in the health management of fish and shellfishes. Biofloc is reported to be rich in natural sources for bioactive compounds and microbes; thus, it is proved to be an efficient immune inducer in the shrimp/fish. The disease outbreaks in the aquaculture sector can be avoided if proper biosecurity measures and methods

are employed for culturing the aquatic species. Biofloc system adding several benefits could to be used successfully by many farmers, and still many kinds of research are testing the system for controlling the excessive floc and microbial load and sludge management for refinement in the system.

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Metabolomic Advances in Fish Nutritional Research and Health Management

13

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Abstract

Aquaculture production has become one of the fastest-growing quality animal protein-producing enterprises, contributing significantly to satisfying increased demand for animal protein by providing barely half of all fish and shellfish consumed directly by humans. As consequences of the intensification of aquaculture for meeting the demand, high feed input, reckless use of antibiotics and drugs/chemicals, water quality deterioration, climate change, poor growth, and disease outbreak could be a major threat in fish culture. The majority of farmed fish is lost each year, resulting in significant economic losses owing to disease outbreaks in diverse culture systems, making farming unprofitable and unsustainable in the long run. Metabolomics is a technique for assessing metabolites in a living system holistically and systematically, and it employs a system biology approach to evaluate the biochemical processes of complex organisms in terms of nutrition and health conditions. Metabolomics strives to find biomarkers emblematic of physiological reactions of live samples such as whole organisms, tissues, and cells to ambient or culture conditions by using metabolite profiles as fingerprints. We have tried to highlight some of the most current uses of metabolomic developments in fish nutrition research and health management to

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solve challenges across the entire production cycle of an organism, including post-harvest quality control.

Keywords

Metabolomics · Fish nutrition · Health management · Aquaculture

13.1 Introduction

Aquaculture production has become one of the fastest-growing animal food-producing sectors, contributing significantly to fulfilling the growing need for animal protein by supplying nearly half of all fish and shellfish consumed directly by people. Fish and fishery products provide an average of 35 calories per capita per day in terms of high-quality nutritional sources and readily digested animal proteins, which explains the high consumption (FAO 2020). As a result of its expanding relevance, the aquaculture industry has faced numerous obstacles in producing safe and high-quality fish on a long-term basis. Intensification of aquaculture for meeting the demand, high feed input, water quality deterioration, climate change, poor growth, and disease outbreak could be a major threat in fish culture. The majority of farmed fish is lost each year, resulting in significant economic losses owing to disease outbreaks in diverse culture systems, making farming unprofitable and unsustainable in the long run. Antibiotics and drugs/chemicals used indiscriminately in the culture system frequently cause buildup in the aquatic environment, harm to other creatures, toxicity to the host animal, growth reduction in fish, disruption of the natural reproductive cycle, and financial loss. Residues buildup in fish tissues, posing a health risk to humans who eat the fish. Diverse omics technologies, like genomics, transcriptomics, and proteomics, have been employed to explore the interactional response between different disease-causing agents and fish hosts in recent years. Metabolomics, a new and emerging omics technology, has lately been used to study fish metabolic responses to heavy oil, anoxia, hypoxia, microbial illnesses, pesticides, zero fish meal, and fish oil-based diets. Greater growth rates of farmed species, the higher nutritional content of aquafeeds, improved stock health, and reduced environmental impacts have all been made possible by innovative technology, many of which have been taken from other disciplines. Metabolomics has the potential to be a useful method for identifying and characterizing the metabolomes of any fish or food product. Multiple features of fish can be investigated and biomarkers for their welfare recognized using a metabolomic method, assuring sustainable fish growth and thus the quality and safety of aqua food. Recent metabolomic applications in aquaculture have demonstrated enormous potential for tackling problems across the entire production line, from hatchery production to post-harvest quality control. During the last decade, metabolomics has been implemented in aquaculture with a spectrum of uses in diets and nutrition (Grandiosa et al. 2018, 2020; Huynh et al. 2018), immunology and disease impacts (Nguyen et al. 2019, 2020a, b; Nguyen and Alfaro 2020), environmental stress (Huo et al. 2019; Li et al., 2019; Nguyen and Alfaro 2020), ecotoxicology (Li et al. 2017;

Nguyen et al. 2018a), and post-harvest handling (Alfaro et al. 2019; Nguyen et al. 2020a, b).

13.2 Metabolomics

Systems biology is a multidisciplinary method of studying biological processes at the cellular, tissue, and organism levels. The whole genome, transcriptome, proteome, and metabolome are all studied using "omics" technology. Metabolomics is a form of omics that focuses on characterizing, identifying, and quantifying small molecule (<1500 Da) metabolites in the metabolome at high throughput (German et al. 2005). As a result, metabolomics is frequently employed as a sophisticated analytical method to get a deeper understanding of the molecular mechanisms underpinning aquatic creatures' responses to nutrition, external stresses, infections, and developmental processes. The metabolome is the collection of all tiny molecules, metabolites, or chemicals present in a cell, organ, or organism, according to a formal definition. Tiny molecules include peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids, minerals, and just about every other chemical that a cell or organism can use, ingest, or make. Metabolomics identifies biomarkers/chemical signatures indicative of physiological responses of living samples such as whole organisms, tissues, and cells to ambient or culture conditions using unique metabolite profiles. Metabolites provide real-time information on what is going on at the metabolic and physiological levels since they are the most sensitive to environmental changes (Patti et al. 2012). Unexpected issue or risk areas can be recognized using biomarkers, and corrective action can be taken for future management. Though metabolomics in aquaculture is still in its infancy, it has already found widespread application in a variety of fields and applications, including mammalian toxicology, plant chemistry, human nutrition, environmental sciences, food quality, clinical disease diagnostics, and microbial metabolomics, as well as drug discovery. In recent years, metabolomics in aquaculture has become a burgeoning topic, assisting aquaculture in achieving its major goal of increasing production scale while maintaining a high-quality, long-term product. Fish metabolomic study could aid in the investigation of metabolome changes caused by disease, crowding, hypoxia, malnutrition, or other environmental conditions such as pollution, poisons, and temperature fluctuations that might disrupt normal metabolism in the body (Fig. 13.1).

13.2.1 Advantages of Metabolomics over Other Omics Technology

Metabolomics has the following advantages over other omics technologies:

- Metabolomics is the study of metabolites, which are the end products of biological regulating systems that are extremely vulnerable to outside stimuli. These profiles can be thought of as biological systems' final response to genetic or environmental change (Fiehn 2002).

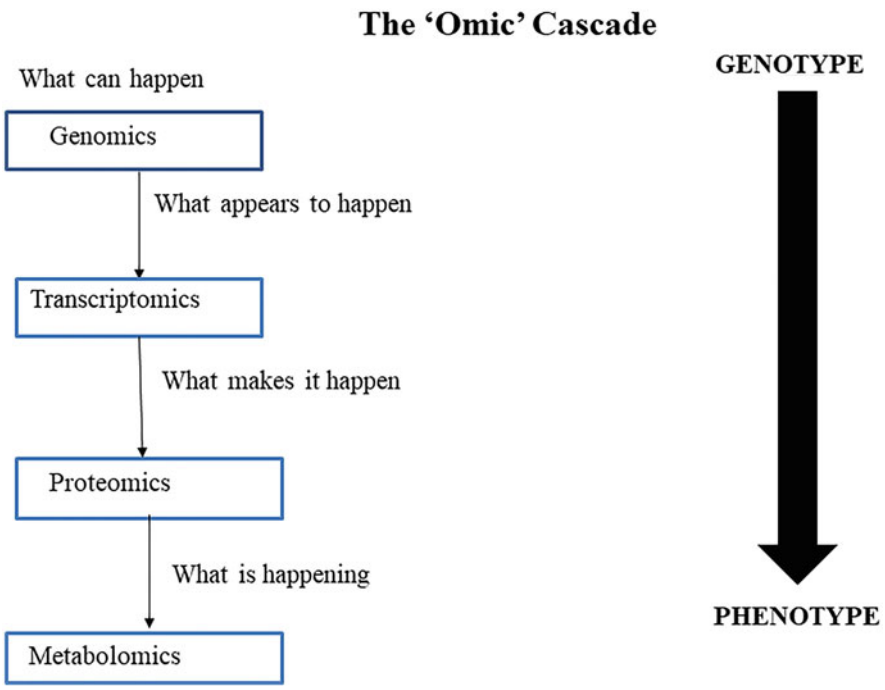

Fig. 13.1 'Omics' cascade depicts the genotype to phenotype continuum and defines genomics, transcriptomics, proteomics, and metabolomics

- In comparison with proteome and transcriptome investigations, metabolomics often requires less sample preparation and shorter turnaround times from sample collection to data interpretation, lowering costs.
- Because metabolites have significantly fewer types/classes than genes or proteins in many species, metabolite data processing is often simpler (Wang et al. 2006; Lu and King 2009).
- Non-invasive bodily fluids/solids, like plasma and faeces, can be used in metabolomics research, which may be very useful in fish investigations. Furthermore, without destroying a sample, a variety of analytical procedures can be applied (Alfaro and Young 2018). When biological material is restricted and/or several studies are to be performed on a single sample with the goal of data integration, this is particularly valuable.
- When compared with other "-omic" techniques, metabolomics has several advantages, the most important of which is its biological proximity to the system's phenotype, allowing for quick detection of system perturbations in the metabolome.

13.3 Basics of Metabolomic Techniques Used in Aquaculture

Metabolomics is a promising method for biomarker discovery since it involves both focused and non-targeted analysis of endogenous and exogenous small-molecule metabolites (<1500 Da). Metabolomics is a global metabolic profiling framework that combines high-resolution analytics (typically NMR and MS) with chemometric statistical tools like principal component analysis (PCA) and partial least squares (PLS) to produce a comprehensive picture of both endogenous and xenobiotic metabolism. Small-molecule biomarkers such as peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids, and inorganic substances represent the functional phenotype of a cell, tissue, or organism. The physical and chemical properties of the molecules listed above are extremely diverse, and they exist in a wide concentration range. Technological breakthroughs in metabolomics have enabled the separation and identification of these tiny molecules. These cutting-edge technologies, which include accurate high-resolution MS, NMR, CE, HPLC, and UPLC technology, can detect metabolites in a matter of minutes. A number of analytical systems, including NMR, Fourier transform infrared spectroscopy (FTIR), and MS coupled to separation techniques, such as NMR, GC-MS, LC-MS, FT-MS, and UPLC-MS, have been used for metabolomic applications.

13.4 Sample Collection and Preparation for Metabolomic Study

Because the metabolome can vary extremely quickly in response to slight changes in the environment, extreme caution should be exercised when collecting the sample by limiting biological, technological, and experimental variability. Collected samples must be representative of the biology under study and appropriate for the study's specific research goals. It's also crucial to choose the right sample material. Different tissues (e.g., muscle, gills, liver, and pancreas) go through different metabolic processes depending on their role. Even after the metabolome has been taken from the body, it remains in a highly dynamic state in tissues and biological fluids. The ability to accurately measure the metabolome requires the rapid termination of enzyme activity. As a result, metabolic processes within samples must be stopped, or quenched, as quickly as feasible during collection in practically all metabolomic studies. To avoid enzymatic activity recovery, the conventional strategy for quenching metabolism in animal tissues is to freeze samples in liquid nitrogen and store them at or below −80 °C or lyophilize them. The most important aspect of any metabolomic investigation is sample preparation, and sample preparation techniques differ depending on the type of biological material obtained and the analytical platform to be used. Regardless of the method, the metabolite extraction process should be quick and reliable, with as little sample degradation and metabolite alteration as possible (Allwood 2013). For efficient sample extraction, while maintaining the chemical properties of the sample, tissues and cells must be broken down either by grinding in a liquid N2-cooled mortar and pestle (Rosenblum et al.

2005; Viant et al. 2005) or by an electric tissue homogenizer directly in the extraction solvent (Warne et al. 2001; Pears et al. 2005). Methods for metabolite extraction range from simple one-step solvent extraction to more complex approaches requiring multiple phases and/or chemical synthesis steps. Sample preparation and introduction methods for biological samples encompass direct injection, liquid–liquid extraction (LLE), solid-phase extraction (SPE), supercritical fluid extraction, accelerated solvent extraction, microwave-assisted extraction, protein precipitation, and membrane methods such as dialysis or ultracentrifugation. The different types of solvent extraction method include the following:

1. Using a mixture of methanol, water, and chloroform to extract polar and/or nonpolar metabolites.
2. Polar metabolite extraction using methanol alone or in combination with water.
3. Perchloric acid is used to retrieve polar metabolites.

There is no single perfect approach to extract all classes of metabolites with high efficiency due to the enormous range of metabolites found inside tissues, many with widely varying physical and chemical properties. Perchloric acid is commonly used to precipitate proteins and extract hydrophilic metabolites for metabolic fingerprinting research. To extract hydrophilic metabolites, polar organic solvents such as methanol, ethanol, acetonitrile, and acetone are generally combined with water (Coen et al. 2003; Kim et al. 2004; Stentiford et al. 2005a). Hydrophobic metabolites can be extracted using chloroform (Choi et al. 2004; Stentiford et al. 2005b).

13.5 Analytical Tools for Measuring Metabolomes

There is currently no one adaptable platform that can analyze all metabolites inside a sample due to the complexity of metabolites and the high number of metabolites present. Depending on the aims and scope of the investigation, the type of sample material collected, the available sample mass, the accessibility of analytical platforms, and the cost involved, multiple techniques may need to be selected and used to partially overcome the shortcomings of single-analysis techniques. Nuclear magnetic resonance (NMR), mass spectrometry (MS), Fourier transform-infrared spectroscopy (FTIR), and MS coupled to separation techniques, such as NMR, GC-MS, LC-MS, FT-MS, and UPLC-MS, are the most often used high-throughput and high-resolution systems for metabolomics studies. While NMR spectroscopy is best for analyzing bulk metabolites and GC-MS is best for analyzing volatile organic compounds and derivatized primary metabolites, LC-MS can be used to analyze a wide range of semipolar molecules, including many secondary metabolites of interest. LC-MS is a popular instrument because it avoids chemical derivatization. For the identification and quantification of metabolites, MS-based metabolomics offers great selectivity and sensitivity, and when combined with improved and high-throughput separation techniques, the complexity of metabolite separation can be reduced. MS-based approaches, on the other hand, necessitate a sample preparation

phase that can result in metabolite loss. To examine the global metabolome, it is ideal to use various techniques at the same time, such as GC-MS, LC-MS, or NMR.

13.6 Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) is a spectroscopic analytical technique that can uniquely identify and quantify a wide range of organic substances in the micromolar range. It identifies atomic nuclei's distinctive spin characteristics. When nuclei with specific magnetic properties are submerged in a magnetic field, they align with (low energy state) or against (high energy state) the field. The application of extremely particular radio frequency pulses to the nuclei causes a "spin flip," which is a change in the energy state (Savorani et al. 2013). Nuclear shielding is a tiny change in the intensity of the applied magnetic field caused by the existence of other nuclei and chemical bonds surrounding a nucleus. A chemical shift occurs when nuclei within a metabolite absorb radiation at slightly different frequencies as a result of this shielding. The sample's distinct spectrum or "fingerprint" is created by combining all of these various frequencies. Furthermore, more sophisticated spin interactions under varied pulse settings can reveal a wealth of information about a molecule's chemical bonding and composition. NMR's main benefit is that it is largely automated and nondestructive, allowing samples to be used for further research while also providing extremely reliable and repeatable readings. Separation of metabolites before detection is not required, and just a minimal amount of sample preparation is required, saving both money and time. Metabolite fingerprinting, profiling, and metabolic flux analysis have all been done with it. The limited sensitivity of NMR makes it unsuitable for the investigation of large numbers of low-abundance metabolites, which is a fundamental restriction for comprehensive metabolite profiling. NMR can be particularly valuable in drug discovery and development since it offers extensive information about a compound's structural alteration as a result of metabolism.

13.7 Mass Spectrometry

Mass spectrometry (MS) is a technique for determining the molecular weights of compounds. Molecules in a test sample are transformed into gaseous ions, which are then separated and identified in a mass spectrometer based on their mass-to-charge (m/z) ratio. The mass spectrum is a graph showing the ions' (relative) abundances at different m/z ratios. The ion source, mass analyzer, and detector are the three parts of a mass spectrometer (Glish and Vachet 2003). Different steps involved in all mass spectrometers include:

1. Production of ions in the gas phase.
2. Acceleration of the ions to a specific velocity in an electric field.

3. Separation of the ions in a mass analyzer.
4. Detection of each species of a particular m/z ratio.

Electron ionization and electrospray ionization are the most often utilized ionization procedures in metabolomics research (Lei et al. 2011). MS can be used to analyze biological materials either directly without prior metabolite separation or after chromatographic separation. Direct MS techniques are quick; however, they have low ionization efficiency and ion suppression. MS-based metabolomic techniques often require the separation of metabolites by chromatography or electrophoresis before MS detection to reduce the complexity of the sample matrix and improve the sensitivity and selectivity of the analysis. The most often used procedures for this purpose are gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE). These instruments are referred to as hyphenated platforms when they are used together (GC-MS, LC-MS, and CE-MS). MS approaches can have exceptionally high sensitivity or at least detection limits.

13.8 Fourier Transform Infrared (FTIR)

The vibrational fingerprints of wide metabolite functional groups can be measured using Fourier transform-infrared (FTIR) spectroscopy, a type of vibrational spectroscopy that uses lower resolution devices (Moore et al. 2014). In metabolic fingerprinting and metabolomics research, FTIR is a typical analytical tool. Because distinct absorption bands may be ascribed to individual molecular bonds, FTIR spectra can be used as a fingerprint to offer extensive information on the chemical structure and composition of substances. Infrared radiation is transmitted through a sample in IR spectroscopy. The sample absorbs some of the IR radiation, and some of it passes through (transmitted). The resulting spectrum depicts the sample's molecule absorption and transmission, resulting in a molecular fingerprint. The FTIR technique is faster than other procedures, requires a small sample size with minimal or no preparation, does not require the use of solvents, and is more cost-effective.

13.9 Applications of Metabolomics in Nutritional Management

Aquaculture confronts a daunting task in improving feed appropriateness and supporting global fish production growth. Aquaculture, as a burgeoning animal protein-producing business, must evolve dramatically to improve its reliability to meet world demand for fish, while catch fisheries production has nearly stagnated in recent decades (FAO 2020). Because of its well-balanced nutrients and high digestible proteins, high-quality fish meal (FM) is used as a primary nutritional ingredient in the majority of cultured fish. Overreliance on fishmeal (FM) in aquafeed formulations, on the other hand, is seen as one of the primary impediments to the

aquaculture sector's long-term viability, due to supply shortages and price disparities (Van Vo et al. 2015). As a result, aquaculture nutritionists around the world are working hard to identify nutritionally adequate and sustainable alternatives to fishmeal (FM) for fish feed formulation. As a result, feed components derived from terrestrial crops have been thoroughly investigated as FM alternatives (Hardy 2010). As a result, aquaculture must compete for terrestrial feedstuff with cattle, the fuel industry, and direct human consumption, raising concerns about aqua farming's impact on world food security (Troell et al. 2014). Furthermore, greater levels of plant protein sources in the diet resulted in growth retardation, lowered immunity, altered intestinal architecture, and oxidative stress (Ng et al. 2019; Xu et al. 2016). Some supplements/functional additives are used in the feed mix to address this issue. By interfering with digestion and intestinal function, added nutrients should not harm fish growth and physiology (Krogdahl et al. 2015). As a result, precise characterization of alternative feed ingredients/supplements is required to fully comprehend their impact on fish metabolism and suitability for optimal growth and immunity. The traditional method of evaluating new feed formulations is first determining the analytical composition and digestibility of the feed, followed by examining its impact on fish growth, feed consumption, and other zootechnical characteristics. However, while these traditional approaches are useful for demonstrating the major impact of feedstuffs and feed on fish growth, they may be insufficient for understanding the influence of feeds on fish metabolism and the mechanisms that underpin it. At the level of genes, transcripts, proteins, and metabolites, omics technologies allow a novel holistic view of a biological system. Nutrigenomic techniques, which study the relationship between nutrients and specific gene expression, have grown in importance in recent years, leading to novel discoveries such as the regulation of genes involved in protein, lipid, and carbohydrate metabolism in fish that have given plant-based diets (Panserat et al. 2009a, b; Geay et al. 2011). Nutrigenomics, on the other hand, has the same limitations as transcriptome methods. What happens is partly unknown because post-transcriptional changes and protein functions are not explored. Proteomics has been utilized to better understand the molecular pathways that fish use to respond to external stimuli, such as nutritional supplements, and these discoveries can be utilized to improve feed formulation and optimization. Metabolomics, on the other hand, focuses on a global set of metabolites within the biological system and provides data on metabolic activities. By combining a feeding trial with metabolomic investigations of tissues and biofluids, new insights into feed and nutrient effects could be gained. Metabolomics was utilized as a system biology approach to investigate the effects of dietary nutrients on fish growth by comparing the metabolite profiles of various tissues from different dietary regimens (Schock et al. 2012a, b; Abro et al. 2014a, b; Wagner et al. 2014a, b). Metabolomics can be used to figure out how a particular diet affects fish physiology. It aids in the selection of the appropriate feeds for optimal growth, based on their compatibility with fish metabolism, to maintain a positive link between product quality and feed conversion efficiency. Metabolomics is intended specifically to analyze metabolic reactions to nutritional deficits or excesses, and it may provide in-depth mechanistic insights to help build optimal feeding regimens (Table 13.1).

Table 13.1 Applications of metabolomics in different aspect of fish nutrition

Sl. No	Technology applied	Objectives of the study	Species [Tissue]	Remarks	References
Assessment of fish freshness and quality					
1.	1H NMR	Assessment of freshness	Gilthead Sea bream (*Sparus aurata*)	Differential metabolites identified as potential biomarkers of freshness and spoilage	Melis et al. (2014)
2.	(HR-MAS) NMR	Assessment of fish rreshness and quality	Sea bream, sea bass, trout, and red mullet	Fish freshness and quality markers such as K value and trimethylamine nitrogen (TMA-N) concentration can be determined quickly.	Heude et al. (2015)
3.	^{1}H-NMR	Quality assessment of the fish reared in the different culture system	*Sparus aurata,* flesh	Glycogen (a stress indicator), histidine, alanine, and glycine were all measured and showed considerable variations depending on the aquaculture system and storage times.	Picone et al. (2011)
4.	^{1}H-NMR	Before and after simulated gastrointestinal digestion, nutrient discrepancies between two types of freshwater fish	Crucian carp and snakehead fish, soup	Different health functions, such as taurine for enhancing immunity and alanine for increasing bodily energy levels, may be aided by metabolic alterations in digested fish soups	Cao et al. (2020)
Comparison between wild and farmed fish					
5.	NMR	Discrimination of wild and cultured fish	Sea bass, skin, and muscle	When compared with wild fish, there is a significant decrease in EFA and an increase in mono- and di-unsaturated fatty acids (MUFA and DUFA)	Mannina et al. (2008)
6.	^{1}H NMR	Differentiate wild and farmed fish and classification of origin	*Sparus aurata*, muscle	Lipid spectra	Rezzi et al. (2007)
7.	NMR	Classification of fish based on muscle fiber	Atlantic salmon, muscle	Polar and nonpolar extract	Gribbestad et al. (2005)

8.	GC-MS	Differentiation between different culture system	Mandarin fish, serum	33 metabolites were significantly different between RAS and pond groups and can be used as a biomarker	Xiao et al. (2020a, b)
Identification of a nutritional biomarker					
9.	UHPLC-HRMS	Identification of malnutrition biomarkers	Gilthead seabream (*Sparus aurata*), serum of fasted fish		Gil-Solsona et al. (2017)
10.	MS	Investigate metabolism pattern of starved fish	Rainbow trout, muscle, liver, and serum	Polar and nonpolar	Baumgarner and Cooper (2012)
11.	MS	Metabolic profiling of fish fed low protein diet fraction	Grass carp, liver, plasma	Polar compound profiling	Jin et al. (2015)
12.	NMR	Effects of food deprivation in juvenile rainbow trout	Rainbow trout, plasma, liver and muscle	The most apparent reactions were altered plasma lipoprotein levels and tissue-specific patterns of fatty acid mobilization, indicating the importance of lipids as the principal energy source during fasting	Kullgren (2010)
13.	NMR	Characterization of fish nutritional biorhythms	Leopard coral grouper, muscle	Branched-chain amino acids were involved in energy production in the muscular tissues of fasting fish. Furthermore, diurnal rhythms were seen in glycolysis, TCA cycles, and purine metabolic components	Mekuchi et al. (2017)
Effect of functional feed additive					
14.	1^{H}NMR	Explore the effect of dietary sesamin	Atlantic salmon, liver, muscle	The liver and white muscle metabolism in fish are affected by high levels of sesamin, which elevates metabolites mostly linked with energy metabolism	Wagner et al. (2014a, b)

(continued)

Table 13.1 (continued)

Sl. No	Technology applied	Objectives of the study	Species [Tissue]	Remarks	References
15.	HPLC–MS	Effect of partially protected butyrate supplementation on growth and intestinal metabolism	*Sparus aurata*, intestine	The availability of various critical amino acids and nucleotide derivatives was increased when butyrate was supplemented	Robles et al. (2013)
16.	1H-NMR	Metabolic effect of dietary taurine supplementation	*Epinephelus coioides*, intestine	Taurine supplementation enhances energy utilization and amino acid uptake, as well as protein, lipid, and purine synthesis and fish development	Shen et al. (2019)
	Effect of dietary manipulation				
17.	^{1} H NMR	Evaluation of feather meal as an alternative protein source in aquafeed	*Oncorhynchus mykiss*	Metabolic changes are caused by a higher amount of FTH inclusion	Jasour et al. (2017)
18.	^{1}H NMR	Study the effect of gelatinized starch and raw starch	*Dicentrachus labrax*, muscle	Increased glycine and phenylalanine	Jarak et al. (2018)
19.	NMR	Evaluate the efficacy of reduced fishmeal diets for growth	Cobia, serum	ANFs may have disrupted the metabolism of a plant-based component	Schock et al. (2012a, b)
20.	^{1}H NMR	Effect of fishmea-based diet, diets containing size-fractionated fish protein hydrolysate and plant protein-based diet	Turbot, liver, muscle	Changes in the metabolic profile of the liver and muscle in response to various treatments	Wei et al. (2017)
21.	^{1}H NMR	Evaluation of decontaminated fishmeal and fish oil from the Baltic Sea as promising feed sources	Arctic char, muscle, liver	When compared with treated Arctic char, those fed decontaminated fishmeal and fish oil had changes in their metabolic profile and gene expression related to energy metabolism and hepatic toxicity	Cheng et al. (2016)
22.	^{1}H NMR	Effect of dietary SBM substitution on growth performance, serum biochemistry and metabolism	Hybrid sturgeon, liver, serum	The altered phenylalanine, tyrosine, and tryptophan pathways suggested that SBM diets caused substantial liver damage	Yue et al. (2019)

23.	^{1}H and ^{2}H NMR	Effects of a high starch content diet on hepatic glycogen synthesis as well as the muscle and liver metabolome	Asian seabass, muscle and liver	In starch-fed fish, the relative content of muscle alanine (ala), a crucial intermediary in glycolysis, gluconeogenesis, and the Krebs cycle, increased significantly	Palma et al. (2020)
24.	DART–TOFMS	Effect of supplemental feeding with cereals (triticale) on the composition of muscle metabolites	Common carp, muscle	In comparison with fish given only natural food, supplemental feeding resulted in higher levels of pyroglutamic acid, glutamine, and proline in the supplemental feeding group (plankton and benthos)	Cajka et al. (2013)
25.	^{1}H-NMR	Effect of insect (*Hermetia illucens*) protein extract on metabolism	Rainbow trout, liver, muscle, gut mass, blood	Through the simultaneous provision of balanced free amino acids and energy substrates in muscle, efficient metabolic utilization of dietary free amino acids toward protein synthesis is achieved	Roques et al. (2020)
26.	NMR	Fish meal replacement with fungal material zygomycete	Arctic char, liver	When fish were fed diets containing the majority of the protein from fish meal or zygomycete biomass, a metabolic fingerprint was comparable	Abro et al. (2014a, b)
27.	UPLC-QTOF-MS	Effect of dietary oxidized fish oil on lipid metabolism and plasma metabolomics	Largemouth bass, plasma	Phospholipid and sterol metabolism changes, as well as a decrease in the unsaturated degree of membrane phospholipids and fatty acids, an increase in the levels of oxidized cholesterol and phospholipid in plasma, and repression of bile acid synthesis	Xie et al. (2020)
Effect of environmental perturbations					
28.	FTIR	Interactions between dietary factors and seasonal temperature variations	*Sparus aurata*, liver	Dietary changes can help to reduce seasonal temperature differences	Silva et al. (2014)

(continued)

Table 13.1 (continued)

Sl. No	Technology applied	Objectives of the study	Species [Tissue]	Remarks	References
29.	NMR, ICP-MS	Effect of environmental variation on diversity in aquatic ecosystems	Yellowfin goby and juvenile Japanese seabass, muscle, and fin	The mineral makeup of body muscle and fin tissues differs between species	Yoshida et al. (2014)
29.	NMR	Effect of elevated temperature on growth performance, growth- and appetite-regulating hormones and metabolism	Atlantic salmon (Salmo salar), plasma, liver, mesenteric fat	Substantial metabolic changes at a suboptimal temperature concomitant with impaired food intake and growth was observed in	Kullgren (2013)
30.	LC-MS	Physiological responses to cold and starvation stresses	Yellow drum, liver	Cold and/or hunger stress elicited different physiological responses. Glutamate and GSSG were the most prevalent metabolites produced as a result of various stressors.	Jiao et al. (2020)
31.	LC-MS	Metabolic response to long term salinity exposure	GIFT tilapia, gill	Under salinity stress, 12-hydroxyeicosatetraenoic acid and choline are greatly reduced, while adenine, Lys-pro, and inosine are greatly increased	Qin et al. (2021)
32.	LC-MS	Metabolomic responses to toxic ammonia and thermal stress	*Litopenaeus vannamei*, Hemolymph	A change in hemolymph amino acid and arachidonic acid metabolism was observed, and other stress-related metabolite indicators	Duan et al. (2021)
33.	^{1}H NMR	Metabolomics response to inking stress	*Sepia pharaonic,* liver, gill, and muscle	Ink stress causes amino acids, organic osmolytes, nucleotides, energy storage molecules, and apparent tissue-specific metabolites	Jiang et al. (2021)
34.	UHPLC-MS	To better understand the regulatory mechanisms underpinning melatonin's stimulatory influence on astaxanthin and lipid coproduction during inductive stress	*Haematococcus pluvialis,* algae	Identification of novel biomarkers that aid in the buildup of astaxanthin and lipids in algae, such as intermediates in glycolysis, the TCA cycle, and γ-aminobutyric acid (GABA)	Zhao et al. (2021)

13.10 Metabolomics in the Management of Fish Health

Fish health is an important part of aquaculture welfare that is influenced by any negative changes in the environment, such as stress and sickness caused by pathogen infection (Segner et al. 2012; FAO 2016). Disease management is also a significant concern for long-term aquaculture operations. Metabolomics has shown great promise in better understanding disease susceptibility and host-pathogen interactions (Solanky et al. 2005; Guo et al. 2014; Ma et al. 2015; Peng et al. 2015), disease characterization (Stentiford et al. 2005a, b; Southam et al. 2008), and treatment efficacy determination (Cheng et al. 2016; Su et al. 2014). The host's energy metabolism, osmotic control, oxidative stress, cell signalling pathways, and respiratory processes are all affected by pathogen exposure. A changed metabolic profile can be utilized to determine an organism's health condition and can aid in understanding pathogenesis and immune response. Metabolomics has been applied comprehensively in several aspects of health management, including the metabolic response of shrimp to pathogen invasion (Wu et al. 2017a, b; Ning et al. 2019), toxicity and environmental stress (Li et al. 2017; Chen et al. 2019; Xiao et al. 2019), and super-intensive grow-out conditions (Schock et al. 2013). The hepatopancreas of white leg shrimp *L. vannamei* infected with the microsporidian *Enterocytozoon hepatopenaei* (EHP) revealed downregulation of that energy metabolism pathway, according to a study (Ning et al. 2019). In the EHP-infected groups, 49 unique metabolites were discovered, which could be employed as a biomarker to distinguish between EHP-challenged and healthy groups. Nguyen et al. (2021) looked at the metabolic responses of penaeid shrimp to *Vibrio parahaemolyticus* caused acute hepatopancreatic necrosis disease (AHPND). GC-MS was used to produce the hemolymph metabolome of *Penaeus vannamei* challenged with *V. parahaemolyticus* and control shrimp (not exposed to the pathogens). The examination of the pathways revealed Infection with *V. parahaemolyticus* produces major changes in amino acid metabolism, the TCA cycle, and gluconeogenesis pathways, as well as their intermediates. TCA cycle intermediates such as cis-aconitic acid, citric acid, fumaric acid, isocitric acid, and succinic acid were found to be upregulated, which is generally associated with a high metabolic rate, higher energy demand, and an immunological response (Nguyen et al. 2018b, c, 2018b, c**;** Song et al. 2019). Increased glucose, which may be used as an energy source to maintain immunological response, was seen in the hepatopancreas of *Litopenaeus vannamei* infected with WSSV and aberrant amino acid and fatty acid metabolism (Wu et al. 2017a, b). Solanky et al. (2005) compared the metabolite profiles of plasma collected from Atlantic salmon challenged with virulent *A. salmonicida* to saline-injected and unfed control groups using NMR-based metabolomics. Different NMR spectra (metabolite profiles) were detected for each of these groups, and distinct metabolites were found. For the identification of infected and noninfected persons, a metabolomic-based technique can be developed. In a minimal-exchange, superintensive, and biofloc system, Schock et al. (2013) used NMR-based metabolomic approaches to evaluate the condition of shrimp health throughout the whole production cycle, from the nursery phase through

harvest. Tissue-specific metabolic alterations were discovered, primarily in the areas of energy metabolism and nitrogen detoxification. Guo et al. (2014) employed a GC/MS-based metabolomic technique to find biomarkers that differentiated life from death in crucian carps infected with *Edwardsiella tarda*. The most important metabolites distinguishing survival from death in these *E. tarda* infected fish were increased unsaturated fatty acid production, particularly palmitic acid, and decreased fructose and mannose metabolism, particularly D-mannose. The metabolic pathways linked to antibiotic resistance have been widely studied using metabolomics (Jiang et al. 2019; Liu et al. 2019; Zhang et al. 2019; Li et al. 2020).

13.11 Conclusion

Metabolomics is a powerful, new science with a lot of potential in aquaculture because it provides a global view of metabolism by identifying many metabolites involved in biological responses of organisms exposed to various circumstances like nutrition, environment, and disease. An improved understanding of metabolic pathway variation aids in the identification of biomarkers and the development of effective nutritional and health management methods that support optimum growth and long-term aquaculture output.

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Vaccines to Prevent Diseases in Aquaculture

14

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Abstract

Preventive measures are very important and have become a part of sustainable and healthy aquaculture. Aquaculture involves the culture of huge number of animals, and therapy is not an option, and hence disease prevention by vaccination is an important strategy. Vaccination helps in control and spread of diseases, thereby reducing the application of antibiotics. Vaccination increases the resistance to diseases and provides protection to unvaccinated fish through herd immunity. Vaccination is generally a safe and economically acceptable preventive measure, and hence, it has become a common practice in modern aquaculture. Vaccines are of several types and administered in different ways; each have their pros and cons. Several adjuvants are also used in vaccines to enhance the efficacy of the existing formulations. Vaccines thus help to strengthen the quote "prevention is better than cure."

Keywords

Vaccine · Adjuvants · Immune cells · Diseases · Fish

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14.1 Introduction

Tropical countries in Asia contribute the major portion of global aquaculture production, and India stands second in the world. Aquaculture has the potential to meet the everincreasing demand for quality protein food through its highly nutritious species of aquatic animals. India has the immense potential for both horizontal and vertical expansion to increase the production capacities of different fish species. Diseases are considered major limiting factors to achieve the targeted growth in the sector. Hence, it is essential to develop sustainable and cost-effective health management system in Indian aquaculture. Disease prophylaxis through vaccination is the proven approach extensively practiced both in medical and livestock sectors globally. Present increase in life expectancy in humans and leading livestock production is attributed to disease prevention measures through vaccination programs. Vaccines are developed and are available against several aquatic pathogens, which have helped to prevent several aquaculture diseases (Brudeseth et al. 2013; Carmen and Forlenza 2016). As of today, there are 28 licensed fish vaccines available against various fish pathogens (22 bacterial diseases and 6 viral diseases) (Brudeseth et al. 2013). Recent developments in the area of nanoencapsulated adjuvants for immersion and oral vaccines are expected to help in the development of vaccine formulations for effective immunization of fishes. Additionally, disease prevention using vaccination will help in reducing the use of antibiotics and other chemicals, which are of public health concerns due to possible residues and development of antibiotic resistance. Further, the loss due to disease incidence will reduce and add to the assured production.

Prophylactic approach in aquaculture will reduce dependence on antimicrobials, disinfectants, and anti-parasitic drugs, which are known to be harmful to the host and environment in a long run. Vaccination program could achieve the reduction of annual antibiotic usage from 50 metric tons to 746.5 kg over a period of 10 years in Norwegian Atlantic salmon industry, while the production increased by sixfold in the same period. Though globally, vaccination is practiced extensively in aquaculture for control and prevention of diseases; no commercial vaccine technology is available in India.

Indian aquaculture is mainly dominated by carp culture, and disease problem due to *A. hydrophila* is predominant. Despite the severe economic loss limited reports are available on development of *A. hydrophila* vaccine in Indian major carps. Vibriosis is one of the most prevalent fish diseases caused by bacteria of the genus *Vibrio*, affecting several marine and freshwater fishes; however, information on anti-vibrio vaccines is scanty. Similarly, *Streptococcus iniae* and *F. columnare* vaccine in Tilapia need to be developed. *E. tarda* infection in cultured pangasius is prevalent, and vaccines have to be developed evaluated for use in Indian aquaculture.

Among viral diseases, viral nervous necrosis leads to considerable economic losses in brackishwater/marine aquaculture. Vertical transmission of VNN from broodfish to young larvae could be controlled by maternal antibody transfer. Prevention and control of VNN can be achieved through immunizing broodstock by injection, larvae by immersion, and fingerlings by oral administration. Outbreaks of

tilapia lake virus (TiLV) have been reported in Ecuador, Israel, Colombia, Egypt, Thailand, China, and Malaysia and recently in India. Control of TiLV in intensively cultured tilapia in tanks, cages, and RAS could be achieved through injectable vaccines. Emerging transboundary viral diseases due to RSIV and ISKNV, which are reported recently in India (Girisha et al. 2019, 2020), need to be prevented by developing vaccines. To achieve the sustainable aquaculture growth in the country, it is essential to provide the emphasis on development of safe and cost-effective prophylactic methods like vaccination against economically important diseases. Several experimental vaccines need to be developed for industrial applications.

The first fish vaccine was reported in 1938 in polish language (Snieszko et al. 1938), against *A. punctata*, providing protection to carps followed by a report by Duff, who reported protection against *A. salmonicida* in trout (Duff 1939, 1942). However due to the advent of antibiotics and chemicals, the disease control was largely done by chemotherapy and application of antibiotics, and the first commercial licensed fish vaccine was applied in 1976 in the United States against yersiniosis for salmonid fishes. The realization of ill effects of antibiotics and the fact that "prevention is better than cure" has boosted the concept of vaccine development and application. Traditional vaccines are prepared basically by isolation of a pathogen, inactivate, and inject as explained by Louis Pasteur (Zhao et al. 2014). However, the modern-day scientific developments have made it possible to identify specific protein and peptide antigens, which can induce specific immunity without having any side effects, but with low immunogenicity (Smith et al. 2015). Immunogenic adjuvants are often essential to enhance the immunogenicity of these specific antigens (Petrovsky and Aguliar 2004; Corradin and Giudice 2005; Evensen et al. 2005; Evensen 2009). Vaccines in aquaculture have proved as a safe and efficient method of disease prevention.

14.2 Fish Immune System

Fish immune system consists of innate and adaptive components. Vaccination is practiced to induce protective immunity against specific pathogens in the immune system of an organism (Pulendran and Ahmed 2011; Brudeseth et al. 2013; Sahdev et al. 2014). Upon infection, innate defense mechanisms like surface barriers, growth inhibitors, enzyme inhibitors, lysins, precipitins and agglutinins, nonspecific cellular factors like phagocytes, phagocyte activating molecules, natural cytotoxic cells, eosinophils, basophils, mast cells and inflammation are activated immediately to fight against the pathogen in a nonspecific manner (Magnadottir 2006; Secombes and Ellis 2012). However, adaptive immunity needs time to develop memory cells and provide specific immune response to fight off the target pathogen (Tort et al. 2003; Secombes and Ellis 2012). Adaptive immune system consists of three main aspects, mediated by lymphocytes: humoral immunity, cell-mediated immunity, and immune memory. In humoral response, B-cells produce immunoglobulins (Ig). Till date, three types of Igs (IgM, IgD, and IgT) are characterized and reported from fishes (Hansen et al. 2005; Hu et al. 2010; Ballesteros et al. 2013). T-cells play a

major role in cell-mediated immunity in combating a pathogenic infection, and these are required to provide protection against several aquatic pathogens. Specific immune response mainly relies on immunological memory, which helps to mount a robust immune response during pathogen encounter, which forms the basis of a successful vaccine strategy (Secombes and Ellis 2012). Development of vaccines includes the incorporation of components, which can trigger both innate and adaptive immune systems, which is essential for a successful vaccine development.

14.3 Vaccine Development

Several steps are involved in a vaccine development to get a final product. Vaccines should be safe for the organism and also to the end user through the food chain, provide protection against several strains in several species, provide 100% protection, provide protection for entire culture period, easy application, easily available, and should be economical.

Identification of the causative organism and the detailed understanding of the epidemiology of disease is very important in vaccine development. Identification of the pathogenic organism with its detailed characterization will help in vaccine development, and the understanding of its epidemiology will help to determine when to vaccinate. The detailed understanding of the pathogen will help to develop methods and schedule for vaccination. Further, the information on the economic significance of disease will help to design the vaccination programs. The next step and the most important step are to develop the challenge model to reproduce the disease and its signs to evaluate the developed vaccine.

In principle, the vaccine efficacy is evaluated by comparing the survival of vaccinated fish with that of nonvaccinated group against the pathogen challenge.

The survival due to vaccination is expressed as relative percentage survival (RPS). The formula to calculate RPS is given below:

$$\text{RPS} = \{1 - (\%\text{mortality in vaccinated fish}/\%\text{mortality in control fish})\} \times 100.$$

A mortality level of at least 60% is expected in nonvaccinated control groups to get a reliable result. RPS values above 60% are considered good for a vaccine. To establish RPS, it is always recommended to have a good pathogen challenge model, which can induce mortality in target species. If a challenge model is established, prototype vaccines can be developed and tested. Vaccine development has advanced from being a conventional science of using whole pathogen to using only the required protein and peptide antigens to induce specific immunity against the target antigen. Vaccinology is an interdisciplinary science combining microbiology, immunology, and molecular biology. Vaccines can be formulated as inactivated vaccines either adjuvanted or not, live attenuated vaccines, subunit vaccines, recombinant vaccines, synthetic vaccines (peptide vaccines), or DNA vaccines.

14.4 Vaccine Adjuvants

> "Any substance that acts to accelerate, prolong, or enhance antigen-specific immune responses when used in combination with specific vaccine antigens can be termed as adjuvants."

Adjuvants are mainly defined by their chemistry and mode of action, and they are a major factor in determining the efficacy of a vaccine (Evensen et al. 2005; Schijns and Tangeras 2005).

Adjuvants can enhance the efficacy of a vaccine by:

- Prolonging the duration of immune responses.
- Reducing the amount of antigen required.
- Modulating the specificity of antibody responses.
- Stimulating the cellular immune responses.
- Increasing the immunogenicity of a weak antigen.
- Promoting the induction of mucosal immunity.
- Activating the immune mechanism of an immuno-compromised.

Oil adjuvants are the most common adjuvants used by aquaculture industry. The mode of their action is to act as a reservoir to hold antigens in oil globules at the site of injection and release in a controlled manner over a period of time (Anderson 1997). This helps in inducing innate immune responses like inflammatory response, leading to the release of cytokines, which ultimately stimulate the production of antigen-specific antibodies (Evensen et al. 2005). However, these oil adjuvants in vaccines though very successful, can lead to severe adverse side effects like, inflammation at the site of injection, intra-abdominal adhesions, pigmentation, and granulomas, affecting the overall health of the animal and reducing the carcass quality (Melingen and Wergeland 2002; Midtlyng 1997; Midtlyng and Lillehaug 1998; Sorum 2004). The formation of adhesions and granulomas at or around the site of injection can damage internal organs, thus reducing feed intake and potentially decreasing overall growth (Midtlyng et al. 1996a, b; Poppe and Breck 1997). Hence, it is very important to select an adjuvant with minimum toxicity and side effects for the organism to be administered, and the toxicity and acceptability of these compounds in humans has to be considered as the vaccinated fish is ultimately used for human consumption. Aluminium hydroxide is licensed for human use by FDA, and in 1997, the MF59™ adjuvant is licensed for human use after the introduction of aluminium salts 70 years earlier (Schultze et al. 2008). An ideal adjuvant should be nontoxic and induce immune response with lower doses. To vaccinate a healthy individual or a population, priority should be given to safety aspects (Evensen et al. 2005; Kensil et al. 1991), unless the benefit of vaccine is significantly more than toxicity (Committee for Medicinal Products for Human Use [CPMP] 2004). However, most of the potential adjuvants are reported to be toxic, and toxicity varies among the species (Batista et al. 2011). Hence, it is very important to evaluate the toxicity in the species to be administered. Several,

advanced adjuvants are been designed, and the development of potent, safe adjuvants for aquatic organisms will help to develop efficient vaccines against major fish pathogens.

14.5 Strategies for Vaccination

Vaccination strategies consists of vaccine development, mode of vaccination, and decision on which pathogen to vaccinate against (Lillehaug 1997). Parameters like timing of vaccination, temperature, and size of fish are closely associated and necessary to induce proper immune response.

An optimal vaccination strategy aims to protect the fish against specific pathogenic infections causing aquatic animal diseases. Vaccines should protect the animals to overcome the infection, and the immune response should last for an entire culture period. Ultimately, the vaccine should aim at achieving a favorable solution, economical for the aquaculture industry.

The environment, including water temperature, influences the function of the immune system and the immune response of fish (Bowden 2008). The studies suggest that the immune response of an organism will be active at its ambient temperature. Since, the temperature requirement of different species is so diverse. The vaccination should be done at the ambient temperature of that particular species. The ontogeny of immune system is known for only few fishes, and it is different for each fish, and hence the size to be vaccinated of a particular fish has to be determined after conducting the ontogeny of immune system for that particular fish species.

14.6 Methods of Vaccination

Vaccine may be administered to fish by three basically different routes: orally through feed, by injection, or by immersion.

14.6.1 Oral Vaccination

Oral vaccines are most preferred in aquaculture due to the easy and mass application, without causing any stress to fish. The vaccine is either mixed with the feed, coated on the feed, or encapsulated (Quentel and Vigneulle 1997). When antigens are to be incorporated in feed, heat sensitivity of the antigen should be known. For those antigens to be coated on feed, a coating agent is required to avoid leaching or degradation. For several antigens, various micro- and nanoencapsulation methods are being evaluated. Oral vaccination has the advantage in that it is easy to administer and causes no stress to the fish. However, in most cases, only limited protection can be obtained, and the duration of protection is rather short. Thus, although oral vaccination is the preferred method, there are only few examples of effective oral vaccines.

14.6.2 Injection Vaccination

Injection vaccines can be administered by intramuscular or intraperitoneal injection. Given the possibility of inflammatory reactions at the injection site, most available injection vaccines are developed for intraperitoneal injection. Vaccination by injection is the delivery method generally resulting in best protection. Disadvantages of the method are: it is labor intensive and skilled labor, and specialized equipment are required and difficult to inject smaller fish (Evensen 2009). However, injection vaccines have a number of advantages that make them the preferred method. Injection vaccination provides long duration of protection, i.e., for over a year, and it allows for multiple antigens to be combined in a single vaccine and therefore in a single administration (Evelyn 2002).

14.6.3 Immersion/Dip Vaccination

For immersion vaccination, two application methods exist: (a) dip vaccination and (b) bath vaccination. The antigen uptake is considered to take place via the gills, the skin, and the lateral line, and possibly also via the intestine (Nakanishi and Ototake 1997). The fish may be dipped for a short period (30 s to 1 min) in a relatively concentrated vaccine solution or may swim for a prolonged time period in an extensively diluted mixture. The vaccine solution may also be sprayed onto the fish. Immersion vaccination is particularly convenient for small fish, fry and fingerlings, which are impractical to handle for injection. The limitations of immersion vaccination are that the duration of immunity is not very long and booster vaccination is required when the disease prevails over longer periods.

14.6.4 Future

Aquaculture production is the fastest growing food production sector globally and has expanded from being negligible to fully comparable with capture productions. The global aquaculture production is reported to be 73.8 million tons in 2014 and is increasing steadily (FAO 2016). In order to meet the increasing human population and to meet the demand for fish as a nutrition source, the aquaculture systems are getting super intensified. These intensified systems are leading to the emergence and reemergence of aquatic pathogens causing devastating losses. Development of suitable prophylactic measures to mitigate these outbreaks is an important area of research in aquatic animal health management and development and application of vaccines play a major role (Brudeseth et al. 2013).

The development and manufacture of vaccines for aquatic species is a complex process. Some of the important elements to be considered when developing vaccines and vaccination strategies include:

1. Fish farming technology (intensive production of a particular species with good management).
2. Etiology and epidemiology of the diseases (identification and characterization of the disease-causing agent).
3. The ontogeny of the immune system (identification of the earliest time to vaccinate and available windows for vaccination).
4. Efficacy and safety of the product, preferably applicable to multiple fish species.
5. A good return on investment for the fish farmer. Until now, the first three elements have not yet been established for most of the Asian species.

The future of vaccine development focuses on new technologies, entirely different of inactivated whole cell vaccines adjuvanted with oils. Improved formulations containing new adjuvants with recombinant proteins or peptide antigens, nanovaccines, and plant-based vaccines will be developed. DNA vaccines will also likely be part of the future vaccine portfolio. Immunization through the mucosal surfaces will be refined and improved in coming years, probably with a focus on improved local responses that can evoke an immune protection at the site of entry of the pathogen or the primary replication site.

Nanoparticles (NPs) are used as adjuvants and delivery systems in many experimental vaccines developed for aquatic organisms. Nanoparticles exhibit very interesting properties, different from their original materials in having increased relative surface area and quantum size effects, having greater applications in biomedicine field (Yildirimer et al. 2011). Nanoparticles are taken up by cellular endocytosis mechanism (Zaman et al. 2013; Zhao et al. 2014) and increase the ability of antigen presentation (Oyewumi et al. 2010; Kim et al. 2014; Shaalan et al. 2016). The solubility, stability, targeting, biocompatibility, and permeability of vaccines increases with the application of nanotechnology in vaccine development (Frohlich 2012; You et al. 2012; Doll et al. 2013; Lai et al. 2013). The convergence of nano- and biotechnology has made a significant progress in modern day biomedical science (Pankhurst et al. 2003; Tissot et al. 2008; Zhao et al. 2014). Application of nanotechnology in the field of vaccinology has given rise to a new field of science called "Nanovaccinology" (Mamo and Poland 2012; Zhao et al. 2014). Nanovaccines are formulated with antigens either encapsulated within or adsorbed on to the surface of nanoparticle against which an immune response is desired (Gregory et al. 2013; Zaman et al. 2013). Nanovaccines are advantageous in protecting antigens by encapsulation from degradation, and aid in site-specific delivery of antigens, enhance bioavailability, and reduced side effects (Zolnik et al. 2010; Gregory et al. 2013; Zaman et al. 2013). Oral nanovaccines are very important for mass vaccination.

Further, the concept of plant-based vaccine production using plant genetic engineering technique is a novel vaccine development system that can aid to develop large scale, inexpensive vaccines. Several prototype vaccines are available against various antigens, engineered using plant and plant products. Presently, a plant-based vaccine against new castle disease has been approved by US Department of Agriculture (USDA) for poultry. The prospects of developing plant-based vaccines for

fish are high due to the less stringent regulatory issues for animals, and also enough proof of concepts is available in animal vaccine studies if not exactly in fish. Plant-based vaccine research is lagging behind in fish vaccinology.

14.7 Conclusion

Vaccines play a major role in sustainable and healthy aquaculture by preventing diseases. With the advancement in science, new forms of efficient vaccines and vaccine adjuvants will also emerge. Given the nature of aquaculture, the method of vaccination has to be simplified and more focus on mass vaccination with minimum or without stress has to be given more importance.

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A Progress on Biotechnological Advances in Immunostimulants and Gene Interaction in Fishes

15

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Abstract

Evaluation of supplemented immunostimulating diets in fishes has frequently taken into account several common innate immune parameters such as respiratory burst activity, lysozyme, RBC and WBC count, myeloperoxidase activity, and alternative complement system etc. Efforts have also been simultaneously made in deducing the molecular mechanism of the immune response based on the interaction of marker genes mainly TLRs, inflammatory cytokine genes such as interleukins, interferons, and tumor necrosis and growth factors, genes encoding tight junction and complement proteins, etc. However, these regular parameters provide very limited scope in addressing the fundamental modulating mechanism with the direct understanding of the stimulated immunological functions and underlying pathways. Therefore, the possible elucidation of the effect of immunostimulating diets on overall immune health of fishes require advances in the biotechnological techniques applied, in the form of modern omics techniques using high-throughput screening tools. This would ignite the expansion of current research on the immune perspective of immune diets, to transcriptome, proteome, and metagenome response analysis in fishes. This

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chapter hence deals with the progress made with the evaluation of immunostimulants, pertaining to induced gene interaction in fishes. The focus here sticks to the elucidation of genes encoding effector molecules chiefly involved in the activation of pathways to coordinate important response mechanisms in different model fish species studied so far.

Keywords

Immunostimulants · Innate immunity · PAMPs · TLRs · Cytokines · Omics techniques

15.1 Introduction

Over the past few decades, the culture system of fisheries has succumbed to pathogen outbreaks due to compromised immunity—a consequence of intensive farming approaches for higher yield and profit margins. The production of fish meal for inclusion in commercial aqua-diets has also led to the overexploitation of wild fisheries. The massive use of antibiotics has gradually led to enhancement in antimicrobial resistance and climate change, and fluctuations in weather patterns. The aquaculture research community is thus exploring various means of confronting these issues, one of the primary ones being dietary manipulation.

Immunostimulants have now become a mainstream application for improving the weight of fish, feed efficiency ratios and disease resistance. They may be chemically synthesized or naturally sourced (plant or animal-based) and may target specific immune pathways or even general immune mechanisms (Vallejos-Vidal et al. 2016). Further, apart from its efficacy, a good immunostimulant must also have low toxicity (i.e., it should not trigger an immune overdrive in host) and bioaccumulation to negate any anti-environmental impact. It should be easily available and cost-effective in the region, and therefore its inclusion in the diet of the same species may also vary based on locally available resources. The timeline of its application in a particular species is also a critical factor as its administration is preventive and not curative; therefore, its inclusion in diets is usually initiated at early stages of fish development for provision of sufficient dosage and time of exposure to generate an effective response against pathogenic infections (Farooqi et al. 2018).

One of the major advantages of preference for immunostimulants over conventional vaccines in aquaculture is their broad-spectrum activity in the host. Most studies focus on investigating the impact of immunostimulants on physicochemical and immune parameters; however, the understanding of the molecular mechanism of their impact on immunity needs more attention. It is only in the recent decade that the researchers have started emphasizing on the molecular pathways of immune systems while testing the effects of immunostimulants on fish. The nonspecific arm of immunity has been a major focus area in this regard, and both its cellular and humoral components seem to be active mediators of the effect of immunostimulants.

Immunostimulants have been widely successful as a sustainable measure of fish health management. Several studies have found their mediation of immune activity via regulation of cytokine levels. The stimulation of the pro-inflammatory cytokines like IL-1, IL-6, and IFN-γ leads to the activation of various immune molecules including acute phase response proteins, pattern recognition receptors, and antimicrobial peptides. They also act as cross-linkers between innate and adaptive arm of immunity by initiating B- and T-lymphocyte recruitment and enhancing markers for antigen processing and presentation (Mehana et al. 2015; Sakai et al. 2021). In the age of omics, it has been more convenient than ever to gauge the impact of a treatment or administration in an organism on physiological pathways. A recent omics-based study by Xue et al. (2019) also implicated the role of dietary additive CpG in the modulation of immune-relevant microRNA levels in Atlantic salmon, thus unveiling a new avenue of molecular level of interaction by this immunostimulant. Further, gut metagenome and its microbial profile have also become critical factors in delineating the immune potential of fish, especially in case of orally administered immunostimulants (Hoseinifar et al. 2019). These advances in technology are also drawing the attention of researchers to the aspect of eco-immunological balance within the host, whereby a higher energy demand in one physiological system is usually countered by a functional compromise in another system like growth, reproduction, or metabolism (Lieke et al. 2019). Therefore, an omics-based approach allows for the testing of a potential immunostimulant not only for its immune-relevant functions but also for its impact on other pathway networks. Caution should also be exercised while transferring laboratory trials to field applications to negate the impact of previously unaccounted parameters in experimental conditions. Though there is a plethora of reports on the potential of a wide range of additives in terms of their immunostimulant activity, the range of their pathogen resistance experiments is mostly restricted to bacterial infections and leaves a void for scenarios under viral, fungal, or parasitic infections (Leike et al. 2020). Additional studies on these aspects would help in deeper understanding of the molecular regulation of immunity by immunostimulants and also provide the aquaculture industry with more alternatives to switch to sustainable means of disease management.

15.2 Benefits of Using Immunostimulant in Aquaculture

The rationale behind the widespread application of immunostimulants in aquaculture lies in its sustainability in maintaining preventive health contrary to the classical therapeutants such as antibiotics, vaccines, and other chemotherapeutants that tend to raise concerns of residual non-metabolism and bioaccumulation within the environment (Harikrishnan et al. 2011). In addition, the immunostimulants are cost-effective and generally obtained from cheap natural sources and hence could be extracted in bulk (Citarasu 2010).

Immunostimulants act as important therapeutic candidates within expanding aquaculture by mediating the stimulation of inherent immunity, most importantly the innate one, in the host fish. The heightened immune activity thus gained is ensured as a result of instigating defense pathways that encourage the phagocytic and bacterial killing mechanisms of macrophages, complement pathways, lymphocytes, and innate cytotoxic cells (Hoseinifar et al. 2020). In this way, the use of immunostimulants enables both the healthy and the immunecompromised, stressed, or diseased fishes to fight against opportunistic pathogens, thereby reducing mortality. This is mediated by the enhancement of the efficacy of antimicrobial substances prior to an anticipated disease outbreak or during early stages of disease development. In addition, these potent supplements also render increased immune stimulation in fry or larval stages. Besides stressors and pathogens, resistance to parasites is also ensured by means of broad-spectrum activities of the immunostimulants in a non-dependent way of the causative agent.

Thus, after looking at the tremendous advantages of the application of immunostimulants, focus now turns toward their effective mode of administration. There are different modes of administration suggested for employing these immunostimulants as clinical agents such as intraperitoneal injection, oral, bathing or immersion, and dietary administration. Most often, these are administered either alone or combination with vaccines to assure improved response with boosted antibody titer and without any adverse side effects (Wang et al. 2017).

15.3 Various Immunostimulants Used in Aquaculture

A wide spectrum of immunostimulants has been examined in aquaculture for their health-promoting role. Their diverse range can be broadly classified into two main categories, namely, immuno-nutrients and immuno-additives, depending on their characteristics (Kiron 2012). These categories broadly refer to the attributing molecule responsible for the immunomodulating effect of the supplement, as demarcated by its specific mechanism of action. They can be further subdivided into categories as depicted below:

1. Immune-nutrients:
   (a) Functional amino acids.
   (b) Dietary nucleotides.
   (c) Fatty acids.
   (d) Antioxidant micronutrients.
      - Vitamin C.
      - Vitamin B6 (Pyridoxine).
      - Minerals.
2. Immune-additives.
   (a) Probiotics.
      - Monospecies probiotics.
      - Probiotic consortia.

(b) Microbial flocculants.
(c) Prebiotics.
  - β-Glucan,
  - Immunomodulatory polysaccharides.
  - Seaweed polysaccharides.
(d) Synbiotics.
(e) Phytobiotics.
  - Herbal plants.
  - Algae.

Further, based on their origin, chemical characteristics, and mode of action, the immunostimulants can be classified as natural or synthetic (Bricknell and Dalmo 2005; Petrunov et al. 2007). Natural immunostimulants can be further bacterial, algae-derived, and animal-derived microbial products, while synthetic immunostimulants could preferably be either nutritional factors such as vitamin C and E; hormones like lactoferrin, interferon, growth hormone, and prolactin; or recombinant cytokines, depending on their source (Shahbazi and Bolhassani 2016). Synthetic immunostimulants, on the other hand, include Levamisole, FK-565, and MDP (muramyl dipeptide).

Natural immunostimulants:

- Bacterial derivatives—β-glucan, peptidoglycan, LPS (lipopolysaccharides), and probiotics.
- Immunomodulatory polysaccharides—chitin, chitosan, levan, lentinan, and schizophyllan.
- Animal derived—Ete (tunicate), firefly squid, Quillaja saponin (scaptree), glycyrrhizin (licorice), and Hde (abalone).

15.4 Action Mechanism of Immunostimulants

Immunostimulants, as stated earlier, principally perform their function by enhancing the immune response mechanisms. The mechanisms of action are highly specific and vary depending on the class of immunostimulants, dosage, method of administration, and duration of exposure. Among the various response mechanisms of the immunostimulants, chiefly involving the change in number or function of corresponding immune cells, the most proven effect is the stimulation of phagocytic cells to enhance their bacterial killing and fungicidal activities. This enhancement in phagocytosis is brought about by either increase in antibody, superoxide, complement, or mitogenic production (Siwicki et al. 1996; Engstad et al. 1992; Thompson et al. 1993; Watanuki et al. 2006). Besides, phagocytosis as the foremost and basic microbe killing mechanism, follows activation of diverse immune cells via cellular communication and production of free radicals and cytokines triggering the inflammatory response. All these processes are complexly interconnected in a way that is

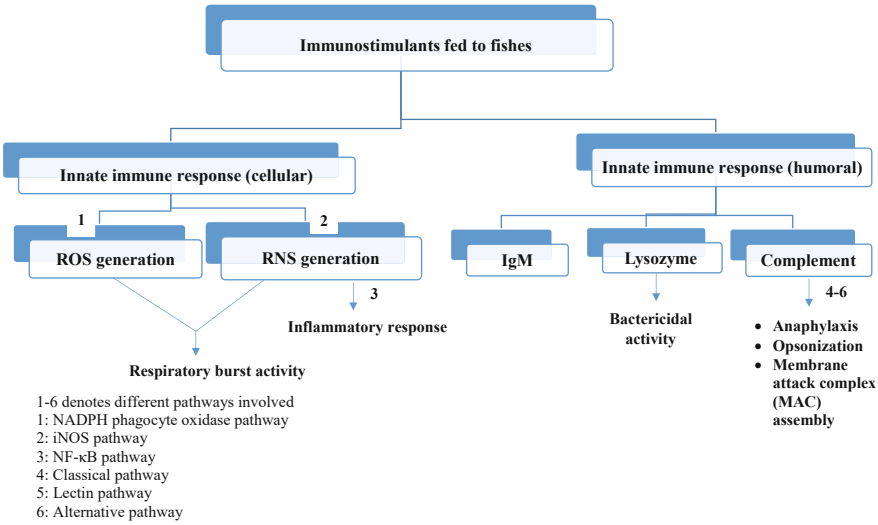

Fig. 15.1 Mechanisms of action of innate immune responses induced by immunostimulants. 1–6 denotes different pathways involved. (1) NADPH phagocyte oxidase pathway, (2) iNOS pathway, (3) NF-κB pathway, (4) classical pathway, (5) lectin pathway, (6) alternative pathway

responsible for the generation of reactive oxygen and nitrogen species mainly through NADPH phagocyte oxidase and inducible nitric oxide synthase (iNOS) pathways (Fig. 15.1).

Different classes of immunostimulants have been known to impact the innate immune responses in fishes at different levels. For example, the foremost barrier cells such as macrophages are activated by probiotics; prebiotics including glucans, chitin, and chitosan as well as linolenic acids; and bacterial components such as lipopolysaccharides (LPS), on pathogenic encounter. Similarly, monocytes have been reported to get effected by substances like plant extracts of Echinacea and vitamins such as vitamin A; neutrophils by β-glucan, zymosan and vitamin A; and mast cells by probiotics. Next, inflammatory agents such as chemokines are mostly stimulated by cell membrane modifiers like detergents including sodium dodecyl sulfate, quaternary ammonium compounds (QAC), and saponins; nutritional factors such as vitamins (C and E) and short chain fatty acids (SCFA); and heavy metals like cadmium and mitogens. Even the arms of adaptive immunity such as B-cells and T-lymphocytes are activated by bacterial components like endotoxins and lipopolysaccharides (LPS) and synthetic immunostimulants like levamisole, Freund's Complete Adjuvant (FCA), Glucans, muramyl dipeptide, and FK-565, respectively. Table 15.1 depicts the targeted genes on incorporation of immunostimulant diets in various model fish species.

Table 15.1 Depicts the targeted genes on incorporation of immunostimulant diets in various model fish species

Effector molecule	Fish species	Pathway involved	Immunostimulating supplement	References
Interferons and signaling factor IFN-γ2	Grass carp (*Ctenopharyngodon idella*)	NF-κB signaling pathway, TOR pathway	Met	Pan et al. (2016)
IFN-γ ↑	Nile tilapia (*Oreochromis niloticus*)	–	Probiotics	Xia et al. (2018)
Type 1 interferons and interferon regulatory factors (IRF)	Rainbow trout	–	AMPs (human alpha defensin1)	Falco et al. (2008)
IFN-γ ↑	Atlantic cod (*Gadus morhua* L.)	–	Mannan oligosaccharide or β-glucan	Lokesh et al. (2012)
IFN-γ2 ↑	Grass carp (*Ctenopharyngodon idella*)	NF-κB	SCFAs	Tian et al. (2017)
IFN-a, IFN-c ↑	Atlantic salmon (*Salmo salar*)	NF-κB	Soya bean meal (SBM)	Marjara et al. (2012)
Interleukin-1 family members and receptors	Jian carp (*Cyprinus carpio var. Jian*)	TOR pathway	IAA	Zhao et al. (2013, 2014), Chen et al. (2015b)
IL-1β ↑	Rainbow trout	–	AMPs (human alpha defensin 1)	Falco et al. (2008)
IL-1β ↑	Hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂)	–	Probiotics	He et al. (2013), Liu et al. (2013)
IL-1β ↑	Common carp (*Cyprinus carpio*)		SCFAs	Liu et al. (2014)
IL-1β ↓, IL-10 ↑	Grass carp (*Ctenopharyngodon idella*)	NF-κB signaling pathway, TOR pathway	Phospholipids	Chen et al. (2015c)
IL-1β, IL-10 ↑	Nile tilapia (*Oreochromis niloticus*)	–	Probiotics	Standen et al. (2013, 2016), Xia et al. (2018)
IL-1β, IL-8, IL-10 (↑)	Atlantic cod (*Gadus morhua* L.)		Mannanoligosaccharide or β-glucan	Lokesh et al. (2012)

(continued)

Table 15.1 (continued)

Effector molecule	Fish species	Pathway involved	Immunostimulating supplement	References
IL-1β, IL-8 ↑	Atlantic salmon (*Salmo salar*)	–	Synbiotics	Abid et al. (2013)
IL-4/13A, IL-4/13B, IL-6, IL-10, IL-11 (↑) IL-1β, IL-8, IL-12p35, IL-12 p40, IL-15 (not IL-17D) (↓)	Grass carp (*Ctenopharyngodon idella*)	NF-κB signaling pathway, TOR pathway	IAA	Su et al. (2018)
IL-10, IL-11, IL-4 ↑ IL-1b, IL-6, IL-8, IL-15, IL-17D, IL-12 ↓	Grass carp (*Ctenopharyngodon idella*)	NF-κB	SCFAs	Tian et al. (2017)
IL-17A, IL-1b,	Atlantic salmon (*Salmo salar*)	NF-κB	Soya bean meal (SBM)	Marjara et al. (2012)
IL-1β, IL-6, IL-8, IL-12p35, IL-15, IL-10, IL-11, IL-4/13A and IL-17D	Grass carp (*Ctenopharyngodon idella*)	NF-κB signaling pathway, TOR pathway	Met	Pan et al. (2016)
IL-8↓, IL-10 ↑	Grass carp (*Ctenopharyngodon idella*)	TOR and NF-κB signaling molecules	IAA (Leu, His, Phe, Trp)	Luo et al. (2014), Wen et al. (2014), Feng et al. (2015), Jiang et al. (2015a, b, 2016)
IL-1b, IL-2, IL-8, IL-4	Japanese sea bass (*Lateolabrax japonicus*)	–	SBM	Zhang et al. (2018)
Tumor necrosis factors (TNFα ↓ and TGFβ ↑)	Jian carp (*Cyprinus carpio* var. Jian)	TOR pathway	IAA	Zhao et al. (2013, 2014), Chen et al. (2015b)
Tumor necrosis factors (TNFα ↓ and TGFβ ↑)	Grass carp (*Ctenopharyngodon idella*)	NF-κB signaling pathway, TOR pathway	IAA	Su et al. (2018)

(continued)

Table 15.1 (continued)

Effector molecule	Fish species	Pathway involved	Immunostimulating supplement	References
TNFα ↓ TGFβ1, TGFβ2↑	Grass carp (*Ctenopharyngodon idella*)	NF-κB	SCFAs	Tian et al. (2017)
TNFα, TGFβ↑	Nile tilapia (*Oreochromis niloticus*)	–	Probiotics (Bacillus spp., Enterococcus spp. and Lactobacillus spp.)	Standen et al. (2016)
TNFα, TGFβ↑	Hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂)	–	Probiotics	He et al. (2013), Liu et al. (2013)
TNFα, TGFβ↑	Common carp (*Cyprinus carpio*)	–	SCFAs	Liu et al. (2014)
Tumor necrosis factors (TNFα and TGFβ)	Grass carp (*Ctenopharyngodon idella*)	NF-κB signaling pathway, TOR pathway	Phospholipids	Chen et al. (2015c)
TNF-α	Juvenile blunt snout bream	TOR pathway	IAA (Leu; Thr)	Habte-Tsion et al. (2015, 2016), Ren et al. (2015)
TNF-α	Rainbow trout	–	AMPs (human alpha defensin1)	Falco et al. (2008)
TNF-α	Turbot (*Scophthalmus maximus* L.)	–	SCFAs	Liu et al. (2019)
TNF-α	Japanese sea bass (*Lateolabrax japonicus*)	–	SBM	Zhang et al. (2018)
TNF-α, TGF-β1 ↑	Grass carp (*Ctenopharyngodon idella*)	TOR and NF-κB signaling molecules	IAA (Leu, his, Phe, Trp)	Luo et al. (2014), Wen et al. (2014), Feng et al. (2015), Jiang et al. (2015a, c, 2016, Pan et al. (2016)
Intestinal TJ proteins (ERK1/2, p38MAPK)	Jian carp (*Cyprinus carpio* var. Jian)	NF-κB signaling pathway	IAA (Ile)	Zhao et al., (2014)
Claudin b, claudin 3, occludin and ZO-1 ↑	Grass carp (*Ctenopharyngodon idella*)	NF-κB and IκB signaling pathway	IAA (Leu, His)	Jiang et al. (2015a, 2016)

(continued)

Table 15.1 (continued)

Effector molecule	Fish species	Pathway involved	Immunostimulating supplement	References
Occludin, zonula occludens 1 (ZO-1), claudin-3, claudin-b and claudin-c↑ claudin-12↓	Grass carp (*Ctenopharyngodon idella*)	NF-κB signaling pathway, TOR pathway	Phospholipids	Luo et al. (2014), Chen et al. (2015b)
Myogenic regulatory factors	Blunt snout bream	GH-IGF-I axis, TOR signaling pathway	Lys	(Cai et al. (2018)
Antioxidant enzymes (SOD, CAT and GPx) ↑	Grass carp (*Ctenopharyngodon idella*)	Nrf2 signaling molecule (Nrf2-Keap1 pathway)	Arg, Met, Trp	Wen et al. (2014), Feng et al. (2015), Jiang et al. (2015c), Wang et al. (2015), Pan et al. (2016), Su et al. (2018)
SOD, CAT, GPx, GR, and glutathione *S*-transferase (GST) activities and GSH	Jian carp	Nrf2 signaling molecule (Nrf2-Keap1 pathway)	Ile	Zhao et al. (2013, 2014)
SOD, CAT, GPx, GR, and glutathione *S*-transferase (GST) activities and GSH	Juvenile blunt snout bream	Nrf2 signaling molecule (Nrf2-Keap1 pathway)	Ile, Thr	Habte-Tsion et al. (2016), Ren et al. (2017)
GST, GPx and CAT	European eel (*Anguilla anguilla*)	Nrf	His	Giuliani and Regoli (2014)
Antioxidant enzymes activities	Jian carp	Nrf	Met	Kuang et al. (2012)

(continued)

Table 15.1 (continued)

Effector molecule	Fish species	Pathway involved	Immunostimulating supplement	References
TCRc, CD4a, CD8b, TGF-b, trypsin, PAR2 and MyD88 genes	Atlantic salmon (*Salmo salar*)	–	Soya bean meal (SBM)	Marjara et al. (2012)
Intestinal transporter genes PepT1, LAT1 and SLC1A5	Japanese sea bass (*Lateolabrax japonicus*)	–	SBM	Zhang et al. (2018)
Complement C3	Grass carp (*Ctenopharyngodon idella*)	–	IAA	Luo et al. (2014), Chen et al. (2015a), Feng et al. (2015), Jiang et al. (2015a), Su et al. (2018)
C3	Orange-spotted grouper *Epinephelus coioides*	–	Probiotics, prebiotics (mushroom beta-glucan mixture (MBG) or sodium alginate)	Sun et al. (2010), Chang et al. (2013), Lee et al. (2017)
TLR2 ↑	Nile tilapia (*Oreochromis niloticus*)	–	Probiotics (Bacillus spp., Enterococcus spp. and Lactobacillus spp.)	Standen et al. (2016)
TLR3	Atlantic salmon (*Salmo salar*)	–	Synbiotics	Abid et al. (2013)

15.5 Signaling Immune Relevant Genes and Their Role in Innate Immunity

1. Pattern recognition receptors
   (a) Toll like receptors (TLRs)
   (b) RIG Like receptors (RLRs)
   (c) Nod like receptors (NLRs)
2. Antimicrobial peptides
3. Complement molecules
4. Lectin family members
5. Cytokines

(a) Interferons and signalling factors
(b) Interleukin-1 family members and receptors
(c) Tumor necrosis factors
(d) Chemokines

15.5.1 Pattern Recognition Receptors

The pattern recognition receptors are the primary surface receptors that perform cell recognition by identifying specific conserved molecular patterns of the invading microorganisms. This triggers multiple signaling pathways that induce host immunity toward eradication of the pathogens.

15.5.1.1 Toll-Like Receptors (TLRs)

The TLRs were the first pattern recognition receptors (PRRs) to be characterized. These are diversified transmembrane protein receptors that mediate the recognition of molecular patterns on invading pathogens called PAMPs and their genetic material. The activation of TLRs initiate a cascade of intracellular signaling events that in turn activate the immune cells leading to production of effector molecules such as cytokines, chemokines, and antimicrobial products responsible for the innate immune response. The TLRs have been widely studied in fishes for elucidating the mechanism of action of immunostimulants toward pathogenic infection.

15.5.1.2 RIG-Like Receptors (RLR)

These are another class of PRRs comprising of three components such as the retinoic acid-inducible gene I (RIG-1), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). These receptors are involved in the recognition of viruses.

15.5.1.3 Nod-Like Receptors (NLRs)

The NLRs are a class of newly identified self-regulated cytoplasmic PRRs that take part in pattern recognition through oligomerization of domains, namely, N-terminal effector binding domain, central nucleotide-binding domain, and C-terminal leucine-rich repeat domain (Chen et al. 2009). This interaction of proteins thus stimulates downstream signal generation by the activation of NF-kB, MAPK (mitogen-activated protein kinase) signaling pathway or caspase-1. The NLRs also ensure immune protection during the embryonic stage.

15.5.2 Antimicrobial Peptide (AMP)

The antimicrobial peptides are the effector molecules that are mainly responsible for specific memory response against a wide array of microbial pathogens (Ravichandran et al. 2010). Major classes of AMPs studied in various fish species include defensins, natural resistance-associated macrophage protein (Nramp),

NK-lysin, hepcidins, piscidins, and cathelicidins. Fish AMPs have been shown to be associated with a wide range of functions including antibacterial, antiviral, antifungal, antiparasitic, and immunomodulatory (Rajanbabu and Chen 2011a, b). These molecules generally play an important role in innate immunity as microbicidal agents by carrying out membrane disruptive and nonmembrane disruptive mechanisms. Further, immunomodulation by AMPs include phenomenon such as chemotaxis, recognition of PAMPs/MAMPs, neutralization of bacterial endotoxins and virulence factors as well as opsonization.

15.5.2.1 NK-Lysin Gene

The NK-lysin gene imparts broad spectrum resistance to pathogens by its lytic ability. The transcripts of the gene have been mainly cloned in channel catfish.

15.5.2.2 Hepcidin

Hepcidins are the most diverse and widely studied class of AMPs in teleosts. They are multifunctional, cationic, cysteine-rich amphipathic peptides. Fish hepcidins have been first reported from hybrid striped bass (Xu et al. 2008). These genes are generally induced on exposure to pathogens and take part in iron regulation via ferroportin-mediated endocytosis and proteolysis.

15.5.2.3 Piscidins

Piscidins are basically helical, amphipathic AMPs expressed by mast cells and phagocytic and eosinophilic granulocytes. The genes encoding them usually get expressed as varied isoforms each with its specific expression pattern within and between species. Piscidins represent a class of potent antimicrobial, antiviral, antifungal, and antiparasitic molecules in fishes.

15.5.2.4 Cathelicidins

These are short peptides possessing a cathelin domain, which selectively target a wide range of pathogens. Cathelicidins were first discovered in Atlantic hagfish, *Myxine glutinosa* (Uzzell et al. 2003). These are usually expressed early in the life cycle of fishes. The genes encoding these peptides are highly variable and are specific to the tissue producing them. In addition, they generally get expressed either ubiquitously or post challenge by pathogens.

15.5.2.5 β-Defensins

β-Defensins are cysteine-rich cationic peptides conferring resistance to specific viruses and bacteria in fishes. They are often characterized by a β-sheet structure stabilized by disulfide linkages. These are known to be the earliest evolved AMPs in organisms. These are constitutively expressed in the skin since early developmental stages, from where they get distributed in vital immune organs like head kidney, gill, and spleen (Zhao et al. 2009; Wang et al. 2012). Immunostimulants, namely, β-glucan, LPS, and peptidoglycans significantly stimulate the expression of β-defensins genes. Besides their role as typical AMPs, these also are home to a wide array of other important properties like immune responsiveness, cell signaling,

immunomodulation, and recruitment of dendritic cells at the infection site (Chaturvedi et al. 2020).

15.5.3 Complement Molecules

Complement molecules are vital immune components representing crucial effector innate immune responses in fishes. They trigger the secretion of pro-inflammatory mediators called anaphylatoxins on activation, leading to opsonization followed by lysis of the ingested pathogen. The complement system represents fully developed aspect of fish's innate immune response and is mediated by classical, lectin, or alternative pathways.

15.5.4 Lectins

Lectins belong to the group of glycoproteins acting as pivotal immune components that induce important immune mechanisms such as phagocytosis, platelet activation, initiation of complement system, and enhancement of lysis by natural killer cells (Osorio and Reis 2011). These molecules mainly carry out agglutination and pathogen inhibiting activities.

15.5.5 Cytokines

Cytokines are low-molecular-weight soluble proteins secreted by activated immune cells such as macrophages, granulocytes, lymphocytes, dendritic cells, mast cells, and epithelial cells in response to pathogen encounter. They modulate and regulate key immune functions via interaction with corresponding receptors on the host cell in an autocrine or paracrine manner. These are further classified as interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors (CSFs), and chemokines.

15.5.5.1 Interferons and Signaling Factors

Interferons are a group of cytokines with varied structures and functions, interacting with cell-surface receptors to mount an immune response. These are mainly endowed with antiviral activity, macrophage activation, and T- or B-cell proliferation. There are different types of interferons, namely, type I, type II, and type III IFNs. Specifically, the interferons are known to increase the phagocytic abilities and stimulate the production of monocytes via interaction with IFN-γ receptors.

15.5.5.2 Interleukins

Interleukins refer to the class of cytokines that regulate the activation and functioning of lymphocytes and phagocytic cells. These play important role in inflammation by acting as pro-inflammatory and anti-inflammatory cytokines.

15.5.5.3 Tumor Necrosis Factor (TNF)

TNFs are an important component of innate immune system in fishes. They play role in effecting the inflammatory response, in turn enhancing host defense against infections. Among different TNFs identified in animals so far, only TNF-α has been reported in fish species. TNF-α is chiefly an important macrophage activating factor (MAF) produced by leukocytes. It also carries out chemotaxis and induces the expression of interleukins and other genes such as cyclooxygenase-2 (COX-2). Besides, it also mediates apoptosis, migration of neutrophils, and respiratory burst activity taking place in the macrophages.

15.5.6 Chemokines

Chemokines belong to the class of small, chemotactic cytokines with chemoattractant properties for recruitment, activation, migration, and adhesion of macrophages and phagocytic cells to the infection sites. In this way, they act as second-order cytokines in effectively translating the innate immune response into the adaptive one. Chemokines have been well characterized in fish species such as rainbow trout, catfishes, flounders, carps, and Atlantic halibut. Further, these are known to chiefly affect production, development and functioning of monocytes via chemotaxin 2 (LECT2) and PaCXCL8l receptors.

15.6 Conclusion

The relevance of immune related genes toward the elucidation of immunological mechanisms in fishes drives the focus of research in recent times, toward molecular identification and functional explorations of key immune marker genes. This presents innate and adaptive immune aspects as perspectives that could be effectively studied with the use of fishes as reference models. Moreover, this could further lead to the exploration of evolutionary relationships from fish to mammals by understanding the interrelationships between signaling genes and supplementing diets that could act as immune enhancers by affecting the expression of these genes. The knowledge about this shall turn out to be more comprehensive with the use of modern OMICS techniques that could help in revealing the cross-talks between major factors affecting immune health and its components.

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Probiotic Supplements in Aquaculture: Latest Developments and Future Trends

16

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Abstract

Aquaculture is one of the fastest growing fish farming venture in the world. With the intensification of culture practices, different methods and technologies have developed simultaneously. Usage of different chemical additives, antibiotics, prophylactics, medicines, etc. has become more widespread for reducing disease risk and to increase production for commercial benefit. Some of this methods, technologies, and substances may bring commercial benefits for producers; but, over the year, adverse health effect of these substances for consumption has become a great concern. The probiotic application instead of other chemotherapeutic drug is more safely, eco-friendly because it is nonantibiotic and alternative source of antibiotic. It fights against different infectious disease by increasing the population of beneficial bacteria. It accelerates the growth, increases immune response, improves the digestibility, and also improves the water quality. The probiotic helps fish to fights against different types of pathogens and improves the anti-bacterial, anti-viral, and anti-fungal properties. To identify probiotic supplements use in aquaculture, their current condition and future perspective different journal and scientific paper are reviewed. The probiotics use in aquaculture is recent trend. But it has not been studied extensively in the field of aquatic environment. This review study provides recent knowledge of the use of probiotic supplements in aquaculture with the latest developments and future prospects.

Keywords

Probiotic · Supplements · Aquaculture · Antibiotic · Disease outbreak

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16.1 Introduction

In recent year, aquaculture has developed worldwide rapidly for meeting the increasing demand of fish as a food. The vastly increasing population is the cause of overfishing from wild stock. It increases the pressure in wild water resources. To mitigate this problem, aquaculture plays a significant role. Aquaculture increases not only the fisheries production but also the economic condition of the country. Nowadays, disease outbreak in aquaculture is causing great loss to the farmers. Different chemical additives and medicines have been used indiscriminately worldwide to reduce risk of disease outbreak as a preventive and curative method, and Bangladesh has been no exemption. The widespread use of antibiotics and different prophylactics has created a great concern due to antibiotic resistance of some bacterial species. Use of different chemical substances may also have a harmful effect on the consumer. Aquaculture provides 56.72% of total fish production in Bangladesh (DoF 2019), which is more than half and its' contribution in total fish production can't be neglected. So the use of such harmful substances is needed to be controlled and reduced concerning consumer health. Different probiotic substances can be used as a replacement as these substances doesn't have any adverse health effect and increase fish growth and improves immune response against any pathogenic substance. Different probiotic substances are already in use in aquaculture and their effect on fish health and against any virulent pathogen needed to be tested in order to avoid any adverse effect on fish. Probiotics are screened by fish gut assessment by testing the effect of bacteria and other substances *in vivo* and *in vitro* simultaneously.

The probiotics are live microorganisms, which are competent to adapt, colonize, and produce within the gut of the host and develop a constructive stability of microorganisms to advance animals' health (Cruz et al. 2012). The numerous benefits of probiotics for growth, defense, and intestinal health of the host were revealed, and broader use of probiotics in aquaculture could prevent diseases, promote growth, and reduce the extensive use of antibiotics (Austin and Austin 2016). Probiotics retard or completely inhibit the development of pathogenic bacteria following a competitive elimination, also boost up the resistance and secretion of mucosal enzymes to stimulate host growth, and they do not cause secondary pollution difficulties (Xia et al. 2020).

To control and compete with pathogenic bacteria and to promote the growth of the cultured organisms, probiotics can be introduced as "bio-friendly agents" into the cultural environment (Farzanfar 2006). Some contemporary studies have clearly validated the beneficial effects of probiotics on immune system modulation, stress tolerance and growth rate of cultivated fishes, African catfish (Al-Dohail et al. 2009), Nile tilapia (Lara-Flores and Olvera-Novoa 2013), Japanese flounder (Taoka et al. 2006), and also increasing interest in south-east Asian aquaculture (El-Haroun et al. 2006).

16.2 Probiotics, Types, Quality, and Function

The word "probiotic" is a modified word of probiotika (Lilly and Stillwell 1965). Probiotics is a term originates from Greek word "Pro" and "Bios" (Schrezenmeir and de Vrese 2001). According to Parker (1974), "Organisms and substances that exert beneficial effects on the host by balancing its intestinal microbes." Fuller (1989) defined probiotic as "live microbial food supplement that benefits the host (human or animal) by improving the microbial balance of the body" and in extreme range of temperatures and salinity variations of probiotics would be performed effectively. It is also found that probiotics are live microorganisms, which, if consumed in acceptable amounts, confer health benefits to the host (Guarner and Schaafsma 1998). It is also defined as "microbial cells administered in a certain way, which reaches the gastrointestinal tract and remain alive with the aim of improving health" (Gatesoupe 1999). Different types of microorganisms are comprised in probiotics. Those are unicellular algae, beneficial bacteria, fungi, yeast, and bacteriophages. The probiotics would be defined for aquaculture as "a probiotic organism can be regarded as a live, dead or component of a microbial cell, which is administered via the feed or to the rearing water, benefiting the host by improving disease resistance, health status, growth performance, feed utilization, stress response, which is achieved at least in part via improving the hosts microbial balance or the microbial balance of the ambient environment" (Merrifield et al. 2010).

On the basis of the mode of application, probiotics are classified as feed and pond probiotics. The feed probiotic is used through the feed supplements. By this method, the probiotic directly finds their way to gut or gastointestine and helps in beneficial microbiota growth to fight against the pathogen. It can be mixed with the feed supplements in two ways: (1) preparing the artificial feed by using probiotics such as pellets, granules, crumbles, flakes, and microencapsulated diets and (2) the natural live organisms, which reared in probiotics used as feed. Live organisms reared in probiotics enrich media as a result it encapsulated by probiotics. This procedure is called bioencapsulation (Nayak 2010b). The pond probiotic is used in water to improve the water environment for unusual stress condition of fish and other aquatic biota. The deteriorate condition is created by low dissolved oxygen, accumulation of dissolved ammonia, nitrite, and also the hydrogen sulfide in the pond sediments. In this case, probiotics create the antagonistic properties and eliminate the pathogenic organisms from waterbody by bio-control process. The probiotics also increase the beneficial bacteria into the waterbody, which are responsible for the breakdown of complex organic matter into simpler form. It helps in bioremediation by controlling or reducing the biochemical or chemical oxygen demands. The oxidizing capacity reduces the toxic elements like ammonia and nitrite and make them harmless (Nayak 2010b). Probiotics work in different ways in aquaculture systems as presented in the Fig. 16.1.

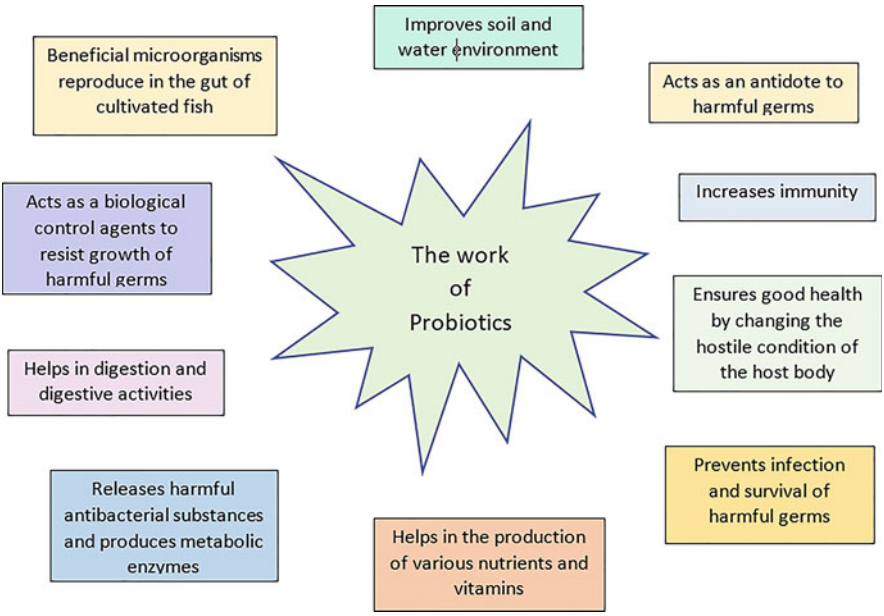

Fig. 16.1 Function of probiotics in different ways in aquaculture systems

16.2.1 Significance of Probiotics

Probiotic amplify the growth of desirable benignant microbiota in the intestinal tract of fishes. The digestible compounds are breakdown by different process during food consumption. It produces vitamins and detoxification of the diet, which helps animate the dearth and improving nutrient, and all those are cause by the help of probiotics (Irianto and Austin 2002). It makes the favorable condition by increasing the production and immune response in fishes, thereby reducing risk of disease (Fig. 16.1). It also helps in maintaining water quality by reducing organic pollutants. According to Mamun et al. (2018), probiotics helps the host by:

- Increasing length of the villus
- Natural killer cells
- Antibodies
- Protease enzyme
- Antioxidant enzyme
- Cytokines
- Complements

The *Bacillus* species reduces metabolic waste in water. Among them, some helps to control the bacterial pathogen, some improves growth, some provides nutrients, some bacteria shows antiviral activity, and some helps to improve fecundity. The

combination of different bacteria together can be more beneficial than single species. It was found that reduction in the outbreak of white spot syndrome virus (WSSV) can possibly by combination of *Pediococcus, Staphylococcus*, and *Haemolyticus pentosaceus* (Leyva-Madrigal et al. 2011). *Bacillus subtilis* and *Lactobacillus acidophilus* combination could increase the hematocrit values and also serum bactericidal activity in *Oreochromis niloticus* (Aly et al. 2008). The live probiotics provide more advantage than inactivated ones.

16.2.2 Selection Criteria and Selection Process for Probiotics

The probiotic selection is a fecund issue. It must identify by maintaining certain qualities (Merrifield et al. 2010; Pandya 2016), which are as follows:

1. Probiotics must help the fish to fight against different pathogenic bacteria. It also should have the fruitful effect on growth, developmental ability, and protectoral criteria.
2. The selection criteria of the probiotic are less harmful for host organisms.
3. It should not show the resistance power and maintain the hereditary traits.
4. It should be efficient for feed, exhibits acid bile tolerance and resistance to gastric juice, and also have the adherence ability to the digestive tract.
5. Probiotic should show the decent sensorial things, have fermented accomplishment, have tolerance to freeze drying, and have great viability during storage and packaging period.

Selection of microorganisms for probiotics is very important and useful because it is important to identify the efficient probiotic organism by isolating the organism, characterization, testing, and lastly certification of the organisms for its probiotic efficiency (Fig. 16.2).

16.2.3 Application of Probiotics

The probiotics can be used in three ways, but mixing with feed additives has been the most common method used in aquaculture (Hai et al. 2009). In aquaculture system, there are different ways by which probiotic application is conducted. It can be applied via dietary supplements or direct application to the water as a form of live feed supplements such as *Artemia*, *Rotifer*, pellet feed, etc. (Fig. 16.3):

1. Can be directly used as feed additives
2. Can be administered through oral
3. By mixing with water

Considering on broad aspect, probiotics are distributed into two categories: (a) gut probiotics, which managed by oral to the fish along with food to increase

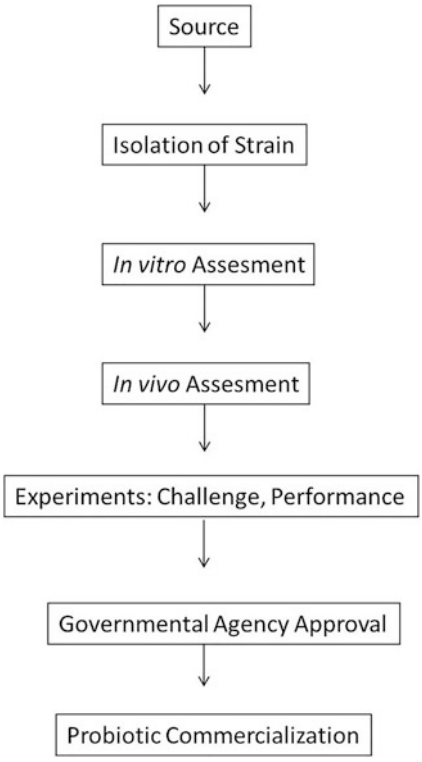

Fig. 16.2 Flowchart of probiotic selection (de Azevedo and Brag 2012)

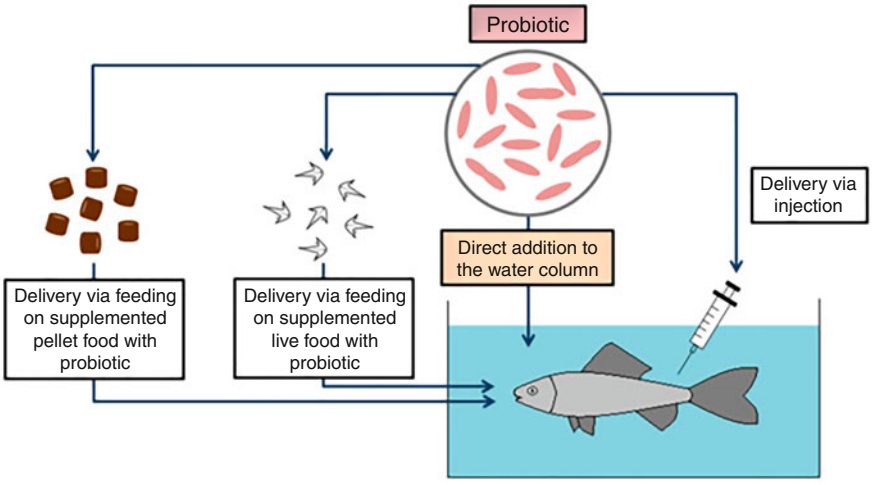

Fig. 16.3 Probiotic application methods in aquaculture (Moriarty 1998; Skjermo and Vadstein 1999)

Table 16.1 Gut probiotics and their beneficiary effects on aquatic organisms (Hasan and Banerjee 2020)

Probiotics name	Beneficial effects	Reference(s)
Lactobacillus rhamnosus	Enhance immunity and reduce disease susceptibility	Nikoskelainen et al. (2003)
Lactobacillus plantarum	Enhance stress tolerance	Taoka et al. (2008)
Lactobacillus rhamnosus	Improve blood quality	Panigrahi et al. (2010)
Streptococcus sp.	Improve feeding efficiency and growth rate	Lara-Flores and Olvera-Novoa (2013)
Bacillus subtilis	Enhance cellular immunity	Sanchez Ortiz et al. (2015)
Bacillus subtilis + *Lactococcus lactis* + *Saccharomyces cerevisiae*	Enhance survival rate, foster metabolism, enhance weight	Abareethan and Amsath (2015)
Bacillus amyloliquefaciens	Enhance antibody concentration, reduce stress	Nandi et al. (2018)
Bacillus subtilis + *Lactobacillus rhamnosus*	Enhance the food digestibility	Munirasu et al. (2017)
Lactobacillus sp.	Reduce pathogen load, provide protection against *Aeromonas hydrophila*	He et al. (2017)
Bacillus cereus	Protect from *Aeromonas hydrophila* infection	Dey et al. (2018)
Bacillus, Arthrobacter, Paracoccus, Acidovorax, etc.	Reduce pathogen load and provide nutrients	Nandi et al. (2018)
Alcaligenes sp.	Enhance volatile short chain fatty acids	Asaduzzaman et al. (2018)

the gut associated beneficial microbial flora (Table 16.1), and (b) water probiotics, the probiotics provided into the water, which helps to excluding of the harmful pathogenic bacteria from the waterbody by using essential nutrients and make the pathogenic bacteria to die in starving condition (Table 16.2).

16.2.4 Probiotic Use as Supplements in Aquaculture

In aquaculture sector, the probiotics are currently most usable agents to increase the growth of the fish with less negative impact (Nicolas et al. 2007; Wang et al. 2008). In fish farming, the probiotics are used to emphasize on fish species, fish size, and condition of the feed adaptation. Nowadays, the commonest probiotics are the yeast, *Saccharomyces cerevisiae*, *Enterococcus* sp., *Lactobacillus* sp., and *Bacillus* sp.; all lactic acid bacteria are used in aquaculture industry (Rahiman et al. 2010). The modification of the gut microflora and replacing the destructive microorganisms from the gut by the use of sufficient quantities of beneficial microbes in feed of the host might fulfill the result. Intestinal balance is enlightening in the animal during

Table 16.2 Water probiotics and their role in maintaining water quality (Hasan and Banerjee 2020)

Probiotics name	Beneficial effects	Reference (s)
Bacillus sp.	Reduces the load of ammonia and nitrite	Porubcan (1991)
Enterococcus faecium ZJ4	Improves water quality and enhances immunity	Wang and Wang (2008)
Lactobacillus acidophilus	Improves water quality	Al-Dohail et al. (2009)
Bacillus NL110, Vibrio NE1	Reduces ammonia and nitrite concentration	Rahiman et al. (2010)
Nitrosomonas sp., *Nitrobacter* sp.	Reduces the concentration of ammonia, phosphates and nitrite in culture pond	Padmavathi et al. (2012)
Rhodopseudomonas palustris, Lactobacillus plantarum, Lactobacillus casei, Saccharomyces cerevisiae	Reduces nitrate load, maintains water pH, and enhances dissolve oxygen concentration	Melgar Valdes et al. (2013)
Paenibacillus polymyxa	Enhances immunity and reduces pathogenic stress	Giri et al. (2013)
Lactobacillus rhamnosus	Reduces pathogen load in culture tank	Talpur et al. (2013)
Pseudomonas sp.	Enhances transcription rate of anti-microbial peptide	Ruangsri et al. (2014)
Bacillus sp.	Promotes the growth of beneficial algae and reduces the growth of harmful algae	Lukwambe et al. (2015)
Nitrosomonas sp., *Nitrobacter* sp.	Reduces pathogen load in culture pond and increases dissolved oxygen content	Sunitha and Krishna (2016)

bacterial colonization in gut, and furthermore extraordinary bacterial strains by utilizing live microbial feed added substance, emphatically affecting the creature, which help battle against the dangerous microorganisms and affecting the organic entities' exhibition (Martínez Cruz et al. 2012). Understanding the development, the increase of the respectable probiotic microbial strains multiplies the stomach-related compounds such as activities of lipases, proteases, and amylases in the gut (Boonthai et al. 2011; Roberfroid 2007). However, the actions of probiotics are as following aspects:

1. Probiotic produce different types of antibacterial compounds; those are bacteriocin, antibiotics, lysozymes, siderophores, proteases, organic acids, and also hydrogen peroxide, which cause sudden shock to pathogenic bacteria (Fuller 1989).

2. Probiotic shows the competitively excluding characters. It competes with the pathogenic bacteria by introducing inhibitory compound and also compete for the space, oxygen, and for nutrients (Fuller 1989).
3. The probiotics make the colony into the fish gut and those colonization followed to the gut wall of fish, which highly show the preventive and inhibitory characters for the pathogenic bacteria to adhere to the gastrointestinal tract.
4. The probiotics produce the potential nutrients, which increase the nutrient in the culture animals.
5. Probiotics compete for the oxygen so that they reduce the availability of the oxygen to the pathogenic bacteria.
6. The probiotics animated the humoral or cellular immune response (Fuller 1989).
7. Probiotics help in increasing or decreasing of the relevant enzyme that's why the microbial metabolism is altered (Fuller 1989).
8. It boosts the lactose utilization, which helps in cancer inhibition. The lactic acid forming bacteria helps to control serum cholesterol.
9. Probiotics detoxify the metabolites, which is produced by the pathogenic bacteria in the intestine.

16.3 Latest Development of Probiotics in Aquaculture

Advancement of probiotics for business use in aquaculture is a multidisciplinary interaction requiring both observational and key examination, full-scale preliminary, and a monetary appraisal of its employments. Probiotics application in aquaculture is of incredible advantage to the host fish, fish farmer, or fish consumer severally. Significantly, probiotics settle the microbial populace of the fish's GI plot through the end of pathogenic microorganisms and expanded edibility and bioavailability of supplements needed for ideal development and great well-being. Farmers should be urged to include probiotics for feed to appreciate the relating benefits it presents. There are several benefits of probiotic in aquaculture, which are described below:

16.3.1 Probiotics as Potential Candidates

Application of probiotics in aquaculture sector is becoming popular for their better and nonpathogenic performance. There are different types of bacteria selected for probiotics, but among them the lactic acid forming bacteria (LAB), Bifidobacterium and streptococcus, are mostly popular (Giri et al. 2013). At present, there are different types of bacteria such as *Aeromonas media*, *Bacillus subtilis*, *Lactobacillus helveticus*, *Enterococcus faecium*, and *Carnobacterium inhibens* are vastly used as probiotics. Those bacteria are meaningfully effective against pathogenic bacteria. On the other hand, it is found that there are some gram-negative facultative symbiotic anaerobic bacteria that also play a significant role, such as *Vibrio*, *Pseudomonas*, *Plesiomonas*, and *Aeromonas*. Those bacteria are found in the gastrointestinal tract (GIT) of fish and shellfish. Apart from these discussed laboratory-based probiotics,

various experimentally approved commercial probiotics are also available in the market, which is also effective in aquaculture (Verschuere et al. 2000) (Table 16.3).

16.3.2 Probiotics for Sustainable Aquaculture

Maintenance of the sustainable aquaculture is very important, but the disease outbreak increases the risk of this sector, which makes the burning concern develop research to mitigate this problem. People use antibiotics, but this creates more problems in this sector. The application of probiotics is safer than antibiotics. According to FAO recommendation, probiotic application is beneficial. It helps to improvement in the aquatic environment and reduces the mortality (Subasinghe 2005). It also increases the resistant against pathogenic bacteria (Irianto and Austin 2002). The favorable effect of the probiotics depends on the application time (Verschuere et al. 2000).

16.3.3 Maintenance of Water Quality

Probiotics have significant capabilities to convert the organic nutrient in the field of the aquaculture, which helps to improve the water environment for fish culture (Wang et al. 2007; Wang and Wang 2008). The nitrogenous compound such as ammonium and ammonia (NH_3) are toxic and main concern for fish culture. Paradigm of this concern the cat fish rearing into the pond (Sahu et al. 2008). The maintenance of water environment probiotics is used in the recent period of time. It is use to mitigate hazardous condition of the water environment and balancing the water quality ($NH_3/NO_2/NO_3$). But the candidates for probiotics is limited (Wang et al. 2007) (Fig. 16.4).

Different photosynthetic bacteria such as *Bacillus*, nitrifiers, and denitrifiers are combined together because of their strong tendency of combination. There are different species of fish culture in diverse condition treated with the probiotics, which sometimes labeled as multifunctional activities (Wang and Wang 2008). Probiotics play a significant role to transforming the organic CO_2, which helps in the maintaining higher production reducing the load of organic carbon and increase the better health of the fish (Fig. 16.4).

16.3.4 Enhancement of Growth and Survival

To improve the growth of different cultivated fish species in the aquaculture sector, probiotics play a great role. For example, *Puntius gonionotus* showed the significant weight gain when *Enterococcus faecalis* causes supplemented with feed at the amount of 10^7 and 10^9 cfu per gram (Allameh et al. 2016). Probiotics combined and colonized into the gastrointestinal gut wall of the fish for long-time application duration. It colonizes because of higher multiplication capacities into the gut wall.

Table 16.3 Commercial probiotics for aquaculture available in the market (Hasan and Banerjee 2020; Rahman et al. 2017)

Product name	Company name	Composition
Prosol	Prosol Chemicals	*Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus plantarum*
Progut	Lincoln Pharmaceuticals	*Yeast cell wall, Mannoproteins, Betaglucans, nucleotides, and peptides*
Lact-Act	Geomarine Biotechnologies	*Lactobacillus sporogenes*
Engest	Microtack	*Bacillus subtilis, Bacillus megaterium, Bacillus licheniformis*
Grobact	Tropical Biomarine Systems	*Lactobacillus rhamnosus, Lactobacillus acidophilus, Saccharomyces boulardii, Bacillus coagulans, Streptococcus thermophilus, Bifidobacterium longum, Bifidobacterium bifidum*
Prolacto	Drug International	*Lactobacillus acidophilus, Bifidobacterium bifidum, Lactobacillus bulgaricus, and fructooligosaccharides*
Probio Diet	Prowin Bio-Tech	*Saccharomyces* sp., *Lactobacillus* sp. *and Bacillus* sp.
Hydroyeast Aquaculture	Agranco Corp	*Streptococcus faecium, Lactobacillus acidophilus, Yeast, Bifidobacterium* sp.
Biotix Plus	Matrix Biosciences	*Lactobacillus* sp.
Aqua Star	Biomin	*Pediococcus* sp., *Lactobacillus* sp., *Enterococcus* sp., *Bacillus* sp.
Natu Rose	Artemia International	*Haematococcus pluvialis*
Enterotrophotic	National Centre for Aquatic Animal Health, India	*Bacillus cereus, Arthrobacter nicotianae*
Pond Plus	Novozymes	*Different kind of heterotrophic bacteria*
Eco-Pro	Biostadt India Limited	*Rhodopseudomonas palustris*
Eco marine	Organic Pharmaceuticals Ltd.	*Bacillus subtilis*, *B. pumilus, B. amyloliquefaciens, B. megaterium*
Profs	Eon Pharmaceuticals Ltd.	*Bacillus* sp. and *Pediococcus* sp.
Aqua gold	Organic Pharmaceuticals Ltd.	*Rhodopseudomonas* sp.
Aqua photo	ACI Animal Health	*Bacillus subtilis* and *Rhodopseudomonas*
pH fixer	CP Aquaculture	*Bacillus* sp.
Super Biotic	CP Aquaculture	*Bacillus* sp.
Super PS	CP Aquaculture	*Rhodobacter* sp., *Rhodococcus* sp.
Procon-PS	Rals Agro Ltd.	*Bacillus* sp. *Rhodococcus*, and *Rhodobacter*
Pond care	SK + F Bangladesh Ltd.	*S. faecalis* and other bacteria

(continued)

Table 16.3 (continued)

Product name	Company name	Composition
AQUA LIFE-S	NAPHAVET Co. Ltd	*Bacillus subtilis, B. licheniformis, B. mesentericus, Lactobacillus acidophilus, Nitrobacter* sp., *Nitrosomonas* sp., *Saccharomyces cerevisiae, etc.*
Everfresh Pro	Blueweight, India	• *Bacillus subtilis, Bacillus licheniformis* • *Bacillus megaterium, Bacillus pumilus* • *Enzymes protease, Amylase, Cellulase* • *Xylanase,* etc.

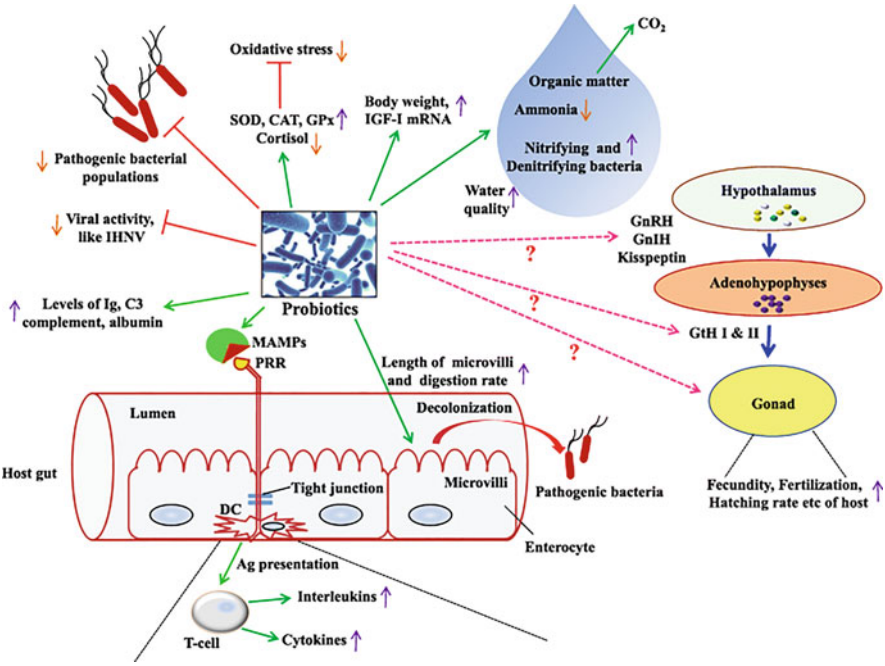

Fig. 16.4 Probiotic performance information in host body (Adopted from Hasan and Banerjee 2020)

Continuous application of probiotics into the aquaculture sector enhances the immunological factors, which helps in reduction of pathogen load into the fish gut mucus layer by increasing the microbial load into the gut (Banerjee and Ray 2017). Probiotics enhance the nutrient of the host body (Hamdan et al. 2016). Probiotics increase the crude lipid and protein and also increase the body weight of Nile tilapia (*Oreochromis niloticus*) treated with *Lactobacillus* sp. into the supplemented feed (Hamdan et al. 2016). Alongside the probiotic-treated feed supplement, different components act a critical part in development improvement of fish, for example, water quality hydrobionts species, protein level, and furthermore the hereditary opposition (Tan et al. 2016).

The species such as *Xiphophorus helleri, Xiphophorus maculates*, and *Poecilia reticulata* showed the increased growth and survival rate when it treated with *Bacillus subtilis* and *Streptomyces* sp. with the feed supplement. The growth performance and the hematological parameters showed the best result of the aquarium reared *O. niloticus* treated with higher amount of probiotics 0.2% dietary supplements into the basal feed of this fish (Hasan et al. 2021). A previous study on the *O. niloticus* showed the highest weight gain at 0.2% probiotic supplemented feed, which was differed from the control group (Chowdhury et al. 2020). The 0.2% inclusion of probiotics dietary supplement increases the growth and increases the production rate and survival rate of *Pangasianodon hypophthalmus* in floodplain cage culture (Chowdhury and Roy 2020).

16.3.5 Upliftment of Nutrient Utilization

The probiotic microorganism influences the gastrointestinal tract of the aquatic animal, which helps in the processing of dietary supplements and produces the energy. The most common probiotics used for gastrointestinal influence are lactic acid forming bacteria (Ringø et al. 2018). The nutrient digestibility increases because of higher amount of digestive enzyme (protease, amylase, cellulose, phytase, etc.). Those enzymes are produced by the influence of probiotics, which alter the gut associated microorganism community of the host (Banerjee et al. 2017; Ghosh et al. 2017). The probiotics *Lactobacillus brevis* and the *Bacillus subtilis* produce digestive enzyme phytase. Some microorganism of the probiotics contributes to produce the fatty acids, minerals, vitamins, and essential amino acids (Nayak 2010a; Newaj-Fyzul et al. 2014).

16.3.6 Role of Probiotics on Bacteriostatic Effects

The bacterial population of probiotics secretes different substances, which have the bactericidal or bacteriostatic impact on both the gram-positive and gram-negative microorganisms. The probiotics produce inhibitory substances, for example, proteinceous substance (lysozyme and various sorts of proteases), and compound substances, for example, (hydrogen peroxide) and iron chelating siderophores (Giri et al. 2013). The LAB-based compound bacteriocins alter between populace relationship impacting by the competition for energy and substance (Kesarcodi-Watson et al. 2008; Ringø et al. 2018).

16.3.7 Prolongation in the Immune System

The probiotics help the aquatic animal by stimulating the immune system. It protects the animal by reducing the disease and pathogen entrance (Dawood and Koshio 2016; Hai 2015). Probiotics increase the immune response, which makes the species

disease resistance and also reduces the malfunction of the carp species (Wu et al. 2015). The probiotic supplement feed containing 10 cfu/g diet and continued for 2 weeks increases the immune impact by combining the microbial related molecular pattern to the pathogen arrangement recognition receptors to immunogenic cells and trigger intracellular action against viral and inflammatory pathogens (Balcázar et al. 2006) (Fig. 16.3). Probiotics also boost up the secretion of the mucosal enzymes and the immune response, which helps in the host growth and prevention from the secondary pollution problems (Xia et al. 2020).

16.3.8 Influence of Probiotics in a Viral Pathogen

The probiotics life forms like *Pseudomonas* sp. and *Vibrios* sp. showed the critical impact on the irresistible hematopoietic putrefaction infection (Sahu et al. 2008). The lymphocytes disease virus also assistant by using probiotics like sporolact (*Lactobacillus* sp.) with the feed supplement of the Paralichthys olivaceus (Harikrishnan et al. 2010).

16.3.9 Probiotics Effects on Reproduction

Probiotics perform a significant character in the field of disease resistant, which is well documented, but the role of probiotic into the reproduction is not well established (Fig. 16.3). There are few research studies on this purpose to demonstrate the role of probiotics in the reproduction of the aquatic animal (Abasali and Mohammad 2011; Ghosh et al. 2008). They used different strains of *Lactobacillus acidophilus*, *B. subtilis*, and *Lactobacillus casei* to demonstrate the probiotics performance on the reproduction. The probiotics play a significant role in the reproduction of the aquatic animal. It influences the reproduction by fertilization, fecundity, gonadosomatic index, and production of the spawn in the female (Abasali and Mohammad 2011). The present studies documented that the probiotics help to increase the daily egg ovulation number compared to control. It increases the hatching rate and faster the embryonic development of the zebrafish (Gioacchini et al. 2013).

16.3.10 Additional Activities of Probiotics

Presently, it is tracked down that the probiotics assist with lessening the pressure chemical focus like cortisol and furthermore actuate the counter oxidative proteins (superoxide dismutase, catalase, and glutathione peroxidase) articulation, which assists with expanding the pressure resilience of the host (Zolotukhin et al. 2018), which are likewise fundamental for the better multiplication execution (Hasan and Banerjee 2020; Hasan et al. 2014) (Fig. 16.4).

16.3.11 Relation Between Probiotics and Food in Aquaculture

The aquaculture supplemented feed is balanced by the probiotics. This is the common practice in the commercial aquaculture. The feed provides the farmers and the consumers to improve the growth performance, production rate, flesh quality, fish immune response, and protein quantity, carcass quality, intestinal health, and also reduce the malformation of the fish (Hai 2015). But large numbers of farmers belong to low income; they are not able to provide this commercial feed. So they face in great loss. They rely on the natural feed so that the growth performance, production rate, and flesh quality is reduced and increased the mortality rate. There are many research proved that the aquaculture sector can increase the profit by using probiotic supplemented diet in the early stage of the fish. It protects the larvae from disease. But probiotic application in this early stage is difficult. There are many researchers that are found to work on this field (Table 16.4).

16.4 Future Perspectives

Nowadays, the probiotic application is becoming popular in the field of aquaculture. In the aquaculture, the probiotics are used to confer different advantages. The application of probiotics is conducted for increasing the growth, stimulates the immune system for better performance, and increases the feed efficiency and also

Table 16.4 Interaction between probiotics and different types of food in fish farming

Fish species larvae	Probiotic feed	Beneficiary effects	References
Scophthalmus maximus	Lactic acid bacteria enriched *Brachionus plicatilis*	Resistant against wide range of *Vibrio* sp.	Gatesoupe (1997)
Sparus aurata	*Lactobacillus fructivorans* and *Lactobacillus plantarum* enriched dry feed or live feed (*Brachionus plicatilis* and *Artemia salina*)	Enhanced colonization on the gut epithelial surface and significantly reduced the mortality rate during larval rearing and fry culture	Carnevali et al. (2004)
Gadus morhua	Life feed enriched probiotic bacteria *Phaeobacter gallaeciensis*	Reduced the pathogenic load during larvae culture	D'Alvise et al. (2012)
Seriola lalandi	Live feed (*B. rotundiformis* and *B. plicatilis*) and *Artemia* sp.) enriched with *Pseudoalteromonas* sp.	Enhanced survival rate of the larvae	Sayes et al. (2018)
Scophthalmus maximus	*Bacillus amyloliquefaciens* enriched *Brachionus plicatilis* and *Artemia sinica*	It improves the microbial community in live feed and ultimately confers the beneficial effects to larvae	Jiang et al. (2018)
Centropomus undecimalis	*Bacillus licheniformis* and *Bacillus amyloliquefaciens* enriched feed	Improved water quality, fish health and rearing tank environment	Tarnecki et al. (2019)

the water quality improvement. There is necessary for the farther studies to understand the proper application and mechanisms of the probiotics in the aquaculture sector. It is important to understand the suitable stage of the probiotic application in early or adult stage by further study. The study of environmental condition and amount of probiotic application in the feed supplement is also very important. The larval stage is more exposed to the environment. So it is important to identify the effect of probiotics on that condition, which amount makes it more appropriate because they grow in different microbial flora in the intestine of the larvae.

Reducing the production cost of probiotics is vital. So that it will be affordable for both poor and middle scale farmer. This is the main concern for future technological developments of probiotics in Bangladesh. Bangladesh is lacking in assessment of screening potential probiotics from gut of different fish species that can help in developing probiotic technologies for certain commercial fish species. Development in this site can be one of the main focuses for future studies of probiotics in Bangladesh. Negative effect of different probiotic substances used in aquaculture in Bangladesh needed to be identified. This is important to identify the solution for making the probiotic viable and stable in to the new food environment that's why it is important to have future studies on new technologies and innovations (Mattila-Sandholm et al. 2002).

The study of the recent development of probiotic extraction technology, formulation, and encapsulation is also very important. By this study, we can identify the better extraction method and identify the biological carrier and barrier, material, and ingredients for making better performance of probiotics. Recent research must be carried out to identify the ingredients by treating there tradition, physical, and enzymatic way to overcome the challenges in probiotics preparation and increasing the potentiality of the probiotic-treated feed supplements. It is important to identify the specific technology for identification of the food ingredients, which is appropriately incorporating with the probiotics.

The probiotic application into the water body must be investigated specifically. It is important to identify the relationship among the quality and quantity of the probiotics used for controlling the complex compound ammonia and nitrogen from the environment of the water body (Skjermo et al. 2015). The yeast plays a significant role as probiotic, but there is lack of information regarding their use in finfish aquaculture. The probiotics play a significant role in the biofloc technology, and it showed the great result in the field of shrimp culture (Hostins et al. 2017; Widanarni et al. 2010). There are different biotechnological tools used for the determination of the immune response of the fish. But there is limited study on the technological tools used for the probiotics impact on the immune system fish (Gupta et al. 2016; Murray et al. 2010; Reyes-López et al. 2015). It is necessary to focus on the study of the probiotic administration through water and obtain the result of the efficiency of probiotics.

The probiotics used in the field of aquaculture mostly collected from the gastrointestinal tract, and after that, it applied to the host body. In the present time, the probiotics are commercially produced by different commercial company for better production of the aquaculture sector. To making the appropriate and beneficial

probiotics for the field of the aquaculture, it is very important to identify the appropriate bacterial strains. Otherwise, the probiotic strains may have mutation problem or they are expensive. Sometimes act as pathogen towards the host and creates stress. There are various studies of the probiotic effectiveness that was studied by different researchers. The result showed efficiency of probiotics in the fields of the aquaculture. More study is needed to specify the effectiveness in aquaculture field. It is very much important to study the quality control of the probiotics, applications, validations, and evaluation methods. It will help to increase the better performance, quality, and functional properties of the probiotics.

16.5 Limitations in the Use of Probiotics

- The treated host or animal may not found the proper dosages of the probiotics due to leaching. So it is important to maintain the proper required dosages in the supplemented feed.
- The probiotic strains, which contain different types of bacteria, may not able to survive in the supplemented feed, because there is extreme temperature and pressure during the preparation of the feed in the extruder.
- Sometimes, there are high organic loads found in the sediments that's why the probiotic loss its efficiency in this condition.
- The exact quantity of the dosage must be calculated according to the water sediment status and for each condition (Nayak 2010b).

16.6 Conclusions

Aquaculture has undergone rapid advancement in the last few years. The main reasons for the increased interest and development of fish farming are due to the recent advances of fish culture techniques. Recently, the use of probiotics for promoting fish well-being, survival, and growth performance increased feed efficacy and enhanced immunity, and disease prevention has gained considerable attention for environment friendly aquaculture (Munir et al. 2016). It is also considered valid option to the prophylactic use of chemicals in aquaculture practices (Merrifield et al. 2010).

The use of different chemicals and antibiotics for controlling disease and increasing production has always been a matter of concern for their residual effect, drug resistance, and immune suppressants and for the adverse effect of residues in the environment. Accumulation of antibiotics and chemical residues in soil and waterbodies is degrading the environment and causing risk for wild populations. Use of a more environment friendly method can avoid these problems and benefit both the consumer and producers. Probiotics are always considered as a natural supplement of fish food that reducing production cost, while avoiding any adverse effect and ensuring consumer health. The European Union has controlled the utilization of anti-infection agents in organic entities for human utilization. As of

now, customers request normal items, which staying liberated from anti-microbial and counterfeit added substances. Accordingly, the utilization of probiotics is a practical option for the hindrance of microorganisms and infectious prevention just as development speed increase in in aquaculture species. The present study will be the educational arrangement of the attractive attributes of probiotics, their method of activity, and valuable impacts on fishes, which can help to culture this fish species more commercially to focus on benefit of aquaculture production and farmers livelihood.

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Heat Shock Proteins (Chaperones) and Role in Aquatic Animal Disease Management

17

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Abstract

Heat shock proteins (HSPs), also known as stress proteins and exogenous chaperone proteins, are a group of highly conserved proteins with different molecular weights, including sHsps, Hsp70, Hsp90, and Hsp60. HSPs are synthesized under normal physiological conditions and in response to stress. As molecular chaperones, HSPs are involved in the folding and assembly of protein polypeptide chains, renaturation of damaged proteins, immune recognition, cell apoptosis, and other physiological activities. In aquatic animals, including fish, crustaceans, and echinoderms, the functions of a variety of Hsp proteins have been reported. In addition to enhancing the anti-stress ability of aquatic animals, Hsps can also play a broader role in various aspects of the epidemic system function, cell apoptosis, and inflammatory process. Exogenous HSPs can also be used as vaccines to increase resistance to pathogens by stimulating the humoral and cellular aspects of the innate immunity of the host. HSPs can also be used as vaccine adjuvant, playing an important role in the development of effective vaccines against aquatic diseases. Therefore, HSPs can be used as an important target site for aquatic animals. By regulating the expression and function of HSPs, it can not only enhance the anti-stress ability of aquatic animals but also play an important role in disease prevention and control.

Keywords

HSPs · Fish · Disease prevention · Innate immunity · Environmental stress

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17.1 Introduction

The heat shock proteins (HSPs) were first discovered by Ritossa in the animal experiment on the larvae of *Drosophila melanogaster* in 1962 (Ritossa 1962). On this basis, further researchers found that the high temperatures can inhibit the synthesis of most proteins, but it starts a new set of protein synthesis at the same time, and this process means the heat shock response (HSR) (Lindquist and Craig 1988). HRS is an adaptive response for the external environment stress, which is ubiquitous in the biological world, and the proteins produced in the HSR are called the heat shock proteins (Sorger 1991). However, because of the continuous accumulation and breakthroughs of the research achievements, the HSPs have not only contained the unilateral biological significance of high-temperature heat shock but also have a great significance for the animals to adjust the environment by producing corresponding stresses to deal with the other adverse factors like hypoxia, heavy metal pollution, reactive oxygen species, salt stress, and pathogen exposure (Beckmann et al. 1990). Therefore, HSPs are a class of evolutionarily highly conserved protein superfamily, which can maintain intracellular homeostasis and protect cell functions when the body received chronic or acute stress. They are widely distributing in the body of eukaryotes and prokaryotes (Sharp et al. 1999). Acting as a molecular chaperone, the HSPs participate in the folding and assembly of protein polypeptide chains, damaged protein renaturation, immune recognition, cell apoptosis, and other physical activities. And they may protect the body by repairing the damaged proteins. The HSPs have a very low expression in normal physiological conditions. However, the HSP synthetic has a sharp increase in cells when it has ultraviolet irradiation, heat shock, heavy metal and other environmental stresses, pathological conditions (virus, bacteria, parasitic infection, fever, inflammation, etc.), and physiological conditions (growth factor, cell differentiation, etc.) (Iwama et al. 1998; Roberts et al. 2010).

The cultivated fish and shellfish are still the important food, nutrition, income, and livelihood source for the billions of people in the world. Though the intensification of aquaculture leads to the significant enhancement of productivity, it also results in the prevalence of disease, including bacteria, fungus, virus, and parasitic pathogen. The outbreak of disease is becoming a significant constraint for the development of this industry in the whole world. Therefore, we should control the outbreak of disease to hold the sustainability of the industry and satisfy the growing demand for animal proteins for the ever-increasing population. At present, it has limited success to prevent and treat aquatic disease in common ways (like antibiotics). Because the bacteria have increased drug resistance and persistence in the environment, they have been questioned for their reuse. Though vaccination has effectively controlled many diseases that impact aquaculture, it must be combined with good farm management, nutrition, and other methods of disease control. Up to now, all kinds of vaccines are developing and coming into the market. However, they can't be used as the universal measures of disease control in aquaculture. We can prevent diseases in all kinds of ways. It's an indispensable tool to control aquaculture diseases, like combining with proper health management, vaccination,

immunostimulation, and other prophylactic treatments (Defoirdt et al. 2004). Therefore, we need to exploit new ways and skills to improve the health of aquaculture species without harming the environment. On the other hand, aquatic animals are the extracorporeal thermal vertebrates living in the aquatic environment with high-temperature conductivity. Water temperature changes, pathogen stimulation, pollution in water, and other stresses may all make a certain difference to the expression of HSPs for aquatic animals. Fish are a convenient model for studying HSR effects in intact organisms whether on a short-time or long-time scale (Kelly et al. 2000). We can research the expression and control of HSPs at all stages of the fish life cycle. Accordingly, this article summarizes the functions and roles of HSPs in aquatic livestock and emphasizes introducing its potentially important role in preventing and controlling aquatic animal immune diseases.

17.2 Classification and Characteristic of HSPs

The HSPs are highly conserved in evolution and highly homologous in the same family. That means they have great significance to other living things' vital movements. According to the degree of homologous and molecular weight from small to large, the HSP family can be divided into five classes: sHSPs, HSP60, HSP70, HSP90, and HSP110 (Lindquist and Craig 1988; Schlesinger 1986). They mainly distribute in the cytoplasm and partial organelles, like mitochondria, chloroplast, and endoplasmic reticulum. Also, a small amount of them is presented in the nucleus. HSPs are molecular chaperone, which is conducive to the folded and oligomerization of nascent peptide, protecting proteins from irreversible denaturation and refolding or degrading damaged proteins, and transfer the proteins to the membrane-bound cell compartment, which can help fight disease. For controlling the diseases in the aquaculture period, we mainly focused on the HSP70 in the HSP family. However, the sHSPs, HSP90, HSP60, and HSP40 seem to improve the pathogen infection. The HSP family, like HSP90 and HSP70, are very important to the folding and assembling of other proteins (Gething and Sambrook 1992). Meanwhile, HSP and other molecular chaperones are also participated in the regulation of the dynamic distribution between folding, translocation, and gathering and have broader roles in immune, apoptotic, and inflammatory processes (Pockley 2003). The sHSPs provide an oligomerization platform for the ATP independent binding of disordered proteins and prevent irreversible degeneration when the proteins are under stress. When the cells are exposing to the chemical or biological toxin, the intracellular or cell surface protein denaturation takes place because of the polar bond weakening and the hydrophobic groups exposing whatever the iatrogenic or microbial active results. These lead to the mistaken folding and aggregating of proteins (Wedler 1987). However, the major function of these molecular chaperone families is combining and folding new proteins through the allosteric rearrangement driven by ATP. Though every molecular chaperone has different structures and mechanisms of action, the HSPs can play a role synergistically by forming the intracellular network combining with chaperonin, co-chaperone protein, and auxilin.

The sHSPs mainly exist in the plants. According to its location in the cells, it can be divided into five categories: cytoplasmic type I and II, which are in the cytoplasm, and the other three categories are apart in the mitochondria, chloroplast, and endoplasmic reticulum. It can't be detected under the optimum growth temperature, but the synthesis of sHSP volume increased rapidly after induced by the heat shock. Normally, the sHSP monomer is constituted by a conservative α-crystal protein domain (the flanks are the amino-terminal sequence and the carboxy-terminal extension) and assembled into the oligomer (Hilario et al. 2011; McHaourab et al. 2009; Sun and MacRae 2005). When the environment pressure is transmitted, the proteins released by the sHSPs may be refolding spontaneously or refolding with the help of ATP-dependent HSP like HSP70 (Ehrnsperger et al. 1997; Lee and Vierling 2000). The sHSPs mainly protect the proteins free from irreversible deformability when exposing to stress conditions.

HSP60 (chaperonin, TRiC, and CCT), the most structurally complex HSP, is composed of two rings positioned back to back, each constructed with eight to nine different but related ATP-hydrolyzing monomers composed of three domains. The HSP60 associates with many different substrates through hydrophobicity, polar, and charged amino acid. The substrates are always combined with the HSP60 in late folding and released when the ATP hydrolysis and ADP + Pi dissociation. The proper folding of cytoskeletal protein and tubulin relies on the HSP60. The HSP60 can make the denatured protein refolding, which is during the period of infection. Also, it may interact with the peptide and protein that are related to the invertebrates' immune response.

HSP70 (molecular weight about 72–80 kDa) is the most widely studied HSP. Its functions are similar to other HSPs. Not only it can play an important role in the transmembrane transport, transposition, assembly, and breakdown of protein complexes and so on as the molecular chaperone (Mao et al. 2003; Rüdiger et al. 1997), but also the induced HSP70 may act as the cytokines to participate in the congenital immunity and acquired immune response in the body (Multhoff 2002). The HSP70s family protein is structurally conservative, and its construction can be divided into the following parts: a highly conservative N-terminal adenosine triphosphatase (ATPase) functional domain (ATP-binding domain), which let the HSP70 has a high affinity to the ATP; a 15 kDa conservative polypeptide-binding domain; a less conservative 10 kDa variable region functional domain approaching the C-terminal; and a C-terminal most positioning motif. There is also a study called the C-terminal of the HSP70s about 30 kDa as substrate-binding domain (SBD). Under normal physiological conditions, the heat shock factor (HSF) combined with the HSP70 protein to form a complex in the body's cytoplasm, and both of them don't participate in the physiological responses. However, the HSF and HSP70 all released from the HSF-HSP70 complex when the body under external stress. And then, the HSP70 is combined with the denatured protein to help restore spatial conformation. At the same time, the HSF is phosphorylated by the protein kinases (like serine-threonine kinase) and forming a trimer that goes into the nucleus. Then, it is combined with the thermal response element sequence of the promoter of the HSP70 gene and has further phosphorylation by protein kinases to start the

expression of the HSP70 gene, which may synthesize more HSP70 to take part in the protein repairing and other functions. The HSP70 has a negative feedback regulation to itself, which means when the HSP70 proteins accumulate to a degree, it may combine with the HSF again and lead to the separation of HSF and HSE, which will inhibit the transcription of HSP70. The synthesis of HSP70 is controlled by environmental, physiology, and pathology reasons. The researches have shown that many kinds of cells, including neutrophile granulocyte, monocytes or macrophages, B cells, epithelial cells, and so on, all can secretion HSP70 proteins. The extracellular HSP70 takes part in the innate and acquired immune response and has a certain protective effect on the cells. When the mammal cells meet bacteria or other immunological stress, they have a high expression of the HSP70 and influence the expression of cytokines in the transcriptional and protein secretion level. The HSP-antigen peptide complex secreted by tumor cells can activate the immunoreaction mediated by CD8+T. Also, the HSP70 can restrain inflammatory responses leading by bacterial epitope. HSP70 can significantly improve the immunocompetence of regulatory T Cells (Treg) and then enhanced the inhibition of the Treg's inflammatory effect and promote the increased secretion of anti-inflammatory factor IL-10 and transforming growth factor-β (TGF-β), accordingly, and reduce the expression of inflammatory factor. Therefore, increasing the expression of HSP70 has the function of treatment and remission to many kinds of immunological diseases.

The HSP90 is the highly conservative cytoplasmic protein in the course of biological evolution and has various biological functions. Under the normal physiological conditions or stress inductions in cells, HSP90 is an ATP-dependent dimer, which is produced in large quantities. It composed with the monomer that has three structural domains. In eucaryons, HSP90 mainly distributes in the cytoplasm, nucleoplasm, endoplasmic reticulum, mitochondria, chloroplast, etc. The homologs of HSP90 are the tumor necrosis factor receptor-associated protein 1 (TRAP1). The HSP90 always exist in the cytoplasm as the form of α-α and β-β homodimer, but it may become the oligomer at a higher temperature (Nemoto et al. 2001). Forming the oligomers can effectively preventing the aggregation with the "client protein" on which they act on. Hypoxic stimulation and transforming growth factor α (TGFα) can all cause the nontraditional secretion of HSP90α. This secretion way makes HSP90α become a potential wound healing agent. There are nearly 100 kinds of proteins are associated with HSP90 in eukaryocyte. Most of these client proteins were related to the functions of cell differentiation, growth, signal transduction, etc. At present, the known client proteins of HSP90 are transmembrane tyrosine kinases, a metastable signaling protein, a mature signaling protein, a chimeric signaling protein, steroid hormone receptor, cell cycle regulator, and so on. HSP90 not only participates in the transportation and updating of proteins but also plays an important role in immune regulation and antigen presentation. Through the production and assembling of the 26S determinant, HSP90 can promote the antigen processing of major histocompatibility complex class I (MHC I) (Yamano et al. 2008). By deepening researches at the molecular level of HSP90, we transform the genes of HSP90. Controlling its expression, secretion, assembling, and other processes can

control the proliferation, differentiation, survival, and apoptosis process, which provide new approaches for immunological therapy and gene therapy of diseases. Researchers also focus on developing HSP90 inhibitors. Via finding the inhibitor with the characteristic of efficient, specific, and slight side effect for the preventing and treatment of tumor and related diseases, it can provide a fully theoretical foundation for annotating life activity phenomenon, mechanism of disease, and so on.

17.3 Regulation of HSPs in Aquatic Animals

17.3.1 Environmental Stress on HSP Expression

Expression of heat shock protein is under the influence of a variety of biological and nonbiological factors; this section will focus on a number of factors expression of heat shock protein. Typically, under conditions of temperature change, in particular heat stress stimuli, can significantly induce a plurality of types of HSPs (Horowitz et al. 1997; Logan and Somero 2011; Purohit et al. 2014). In recent years, many studies have pointed out that changes in salinity in water bodies can also induce the production of Hsp; related content has been researched and reported on species such as *Epinephelus coioides*, *Ruditapes philippinarum, Huso dauricus, Pinctada martensii*, *Pinctada radiata*, etc. (Li et al. 2016; Nie et al. 2017; Peng et al. 2016; Yokoyama et al. 2006). Osmotic pressure changes related to salinity change stress can also induce changes in the expression of *Oncorhynchus tshawytscha* and *Salmo salar* related heat shock proteins (Allen 1993; Palmisano et al. 2000). Exposure to environmental pollutants like heavy metals or environmental toxins is also a factor in aquatic animals inducing heat shock protein expression. Studies have pointed out that increased levels of various heat shock proteins have been measured in fish tissues exposed to environmental pollutants (including pesticides, heavy metals, organics, etc.) (Joseph and Raj 2011; Lee et al. 2006; Rhee et al. 2009). In fish, HSPs are considered as a potential biomarker of pesticide toxicity (Joseph and Raj 2011). In a variety of HSPs, the Hsp70 is most concerned. However, there are other studies that discussed current knowledge on expression of HSPs and concluded that HSP was unreliable as biomarker due to synergistic effects of toxicants and other environmental factors (Kaviraj et al. 2014). Hormones affect the physiological system extensively. Studies have pointed out that hormones may regulate the level of heat shock protein in fish (LeBlanc et al. 2012). Increasing cortisol levels can inhibit the expression of Hsp30 in salmonids gills, Hsp70 in liver and gills of rainbow trout, and Hsp70 in gills of tilapia (Ackerman et al. 2000; Basu et al. 2001; Faught et al. 2017). Exogenous administration of growth hormone at 10 and 100 ng/mL caused an increase in the expression of Hsp70 mRNA in the blood of silver sea bream (Deane and Woo 2005). Studies have also pointed out that the exogenous addition of hexarelin and sulpiride significantly inhibited the expression of HSPs (Deane et al. 2000). Therefore, various non-biological factors, including temperature, salinity, environmental pollutants, and hormones, can significantly

affect the expression level of HSPs, but whether HSPs can be used as a reliable biomarker of environmental pollutants needs further discussion.

17.3.2 Pathogenic Microbial Infection Stimulates the Expression of HSPs in Aquatic Animals

Biological factors affecting the expression of HSP mainly refer to pathogenic microorganisms. Pathogenic microorganisms can have harmful effects on the health of aquatic animal populations in the natural environment. Strongly virulent pathogens may damage intracellular components by releasing cell-lysing substances, thereby changing cell homeostasis and inducing heat shock proteins. Inflammatory pathology caused by pathogen exposure changes physiological processes at the cellular level, such as ion regulation and acid-base balance. Host immune cells (phagocytes and granulocytes) release extracellular substances, such as reactive oxygen species, cationic peptides, lysozyme, and cytokines, which are known inducers of various HSPs and expression of HSP protein.

At present, there have been a large number of research reports on the expression of HSPs in aquatic animals induced by bacterial pathogen infection. Salmon kidney bacteria (pathogens of bacterial kidney disease) infection in *salmon* will enhance expression in liver and kidney of HSP 70 (Forsyth et al. 1997). After *Vibrio anguilla* acutely infected with *rainbow trout* and *sea bream*, the expression of Hsp70 in the liver reaches its peak in a short time (Ackerman and Iwama 2001). *Vibrio alginolyticus* is the main pathogen of *portunus* emulsification disease; it can significantly induce the expression of Hsp70 protein within 3, 12, and 24 h after infection (Cui et al. 2010). *Vibrio harveyi* can induce the upregulation of Hsp90 expression in *Penaeus monodon* (Somboonwiwat et al. 2010). *Penaeus vannamei* after infection with *Staphylococcus aureus* and *Vibrio alginolyticus* can induce the upregulation of HSP-related protein expression in gills and hemolymph (Yan et al. 2014). Another study pointed out that the infection of *Vibrio alginolyticus* can cause changes in the expression of Hsp60 and Hsp70 in *Penaeus vannamei* (Zhou et al. 2010). In the bivalve *Venerupis philippinarum,* the infection of *Vibrio anguillarum* can instantly promote the synthesis of sHsps in blood cells (VpsHSP-1 and VpsHSP-2), which is to protect the proteins that deform when pathogens are exposed (Li et al. 2010). In the case of *Mya arenaria* infected with *Vibrio splendidus*, the expression of Hsp90 in its blood cells was significantly increased after 1 h, but the expression level decreased after 2 h and 3 h (Araya et al. 2010). Hsp22 is a member of the sHsp family. When *Chlamys farreri* responds to *V. anguillarum* infection, CfHsp22 in blood cells is significantly upregulated at the transcription level, suggesting that CfHsp22 play a critical role in response to the bacterial challenge in hemocytes of scallop *C. farreri* (Zhang et al. 2010). A constitutive form of Hsp71 was found in clams, and the expression in hepatopancreas and gills increased by two times under the infection of *Vibrio parahaemolyticus* (Song et al. 2006). Most studies have shown that the expression of HSPs will be upregulated under the infection of aquatic

pathogenic bacteria, suggesting that HSP protein is closely related to the immune defense function of aquatic animals.

In addition to bacterial pathogens, viral infections of aquatic animals also regulate the expression of related HSP proteins. After infection of white-spot syndrome virus (WSSV) in *Penaeus chinensis* and *Scylla serrata*, the transcription level of Hsp70 in the hepatopancreas increased significantly, but the expression of Hsp90 in the hemolymph of *Scylla serrata* was inhibited (Liu et al. 2011; Wang et al. 2006). The expression of Hsp90 will also increase when *Noda virus* infects *Epinephelus obliquus* (Chen et al. 2010). Scophthalmus maximus rhabdovirus (SMRV) upregulates the expression of three Hsp40 genes in *Paralichthys olivaceus* embryo cell lines, significantly stimulates PoHsp40A4, and attenuates the production of PoHsp40B6 and PoHsp40B11 (Dong et al. 2006). When infectious hematopoietic necrosis virus (IHNV) infects *Salmon* embryonic cell line CHSE-214, the high expression of Hsp90 implies the relationship between HSPs and fish diseases (Lee et al. 1996). The expression of Hsp70 in gill was significantly increased when infected with infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Penaeus vannamei*, implicating the HSP-70 as a differential modulator of viral coinfection in shrimp (Vieira-Girão et al. 2012). The induced expression of HSPs of aquatic animals by this pathogenic microorganism is more of a defensive response of the host itself against pathogenic invasion, which also indicates that HSPs have certain correlation with various immune and disease-resistant functions of aquatic animals.

17.4 The Relationship Between HSPs and Immunity of Aquatic Animals

Initially, the response of HSP to cellular stressors was considered to be a short-term functional response with a range of basic housekeeping and cell protection functions (Pockley 2003). However, it has been increasingly recognized that HSPs play important role in the regulation of immune response in vertebrates and invertebrates (DeNagel and Pierce 1993; Pockley 2003; Robert 2003). In particular, in T-cell-mediated immune response, HSPs can act as an intercellular signaling molecule, inducing many cell types to change their activities and producing inflammatory products, such as cytokines and adhesin. Endogenous HSPs in cells are a potentially dangerous signal (Moseley 1998), because they are upregulated under a variety of stress conditions and are released by stress, infection, necrosis, or tumor cells, but not from apoptotic cells. The released HSPs can deliver mature signal peptides to antigen-presenting cells through receptor-mediated interactions. In the mammalian immune response, a particular HSP is a potent inducer of innate and specific immunity. They activate dendritic cells and natural killer T cells, increase antigen presentation to effector cells, and enhance the response of t cells and body fluids to their specific antigens (Segal et al. 2006). Similarly, extracellular endogenous HSP has been shown to trigger translocation of the nuclear factor NF-κB to the nuclei of macrophages and dendritic cells (Valentinis et al. 2008).

In aquatic animals, the effect of extracellular endogenous HSP has not been fully studied; however, since HSPs are one of the oldest and highly conservative protein, it is speculated that endogenous heat shock proteins will also serve as a danger signal to activate innate and/or adaptive immune responses in animals to protect them from (pathogenic) stress. Some studies have shown that HSPs have an effect on the immunity of aquatic animals, that is, it enhances the synthesis of Hsp70. It was found in invertebrate *Artemia* that individuals whose expression of HSP70 was induced by heat and cold stress, after being infected with *Vibrio campbelii*, had a significantly higher survival rate after infection than the control group (Sung et al. 2007; Sung et al. 2009). The survival rate of Artemia embryos after Hsp70 knockdown by RNAi was significantly lower than the control after *Vibrio campbelii* infections (Iryani et al. 2017; Iryani et al. 2020). Enhancing the expression of Asian Green Mussel *Perna viridis* Hsp70 can effectively prevent the infection of *V. alginolyticus* (Aleng et al. 2015). In *Penaeus monodon*, increased expression of Hsp70 by short-term heat stress was consistent with increased resistance to gill-associated viruses (de la Vega et al. 2006).

In addition to the role of host HSPs in mediating host immune responses, there is growing evidence that microbial HSPs are often one of the major antigens of the pathogen itself. Increased HSP synthesis may result from host infection with pathogenic microorganisms. HSP has been considered to be a prominent antigen that triggers most of the immune system. In particular, HSP60 and HSP70 stimulate high levels of antibody response in many infections. Members of the HSP60 and HSP70 families are the primary targets of antibodies in many helminth, protozoan, and bacterial infections. Hsp70 is a ligand for toll-like receptor (Toll), and shrimp can reduce infection through these receptors (Vabulas et al. 2002b). When combined, HSPs activate TLR and transmit inflammatory signals to cells of innate immune system to promote disease resistance. Studies have shown that exogenous HSP can induce immune cells to express and secrete cytokines, such as TNF-α, IL-1, and IL-6, and adhesion molecules, as well as E-selectin and intercellular cell adhesion molecules, and they can activate the NF-κB and Toll/IL-1 signaling pathways to produce an immune response against infection (Multhoff 2002; Vabulas et al. 2002a). A recombinant HSP70 protein of exogenous protozoa can activate goldfish macrophages; stimulate the production of pro-inflammatory cytokines such as interferon (IFN)-γ, TNF-α, IL-1β, and IL-12; and upregulate the expression of inducible nitric oxide synthase to induce goldfish macrophages to produce a strong nitric oxide response (Oladiran and Belosevic 2009; Vabulas et al. 2002b).

Like other invertebrates, shrimp and bivalve shellfish lack an adaptive immune system and their ability to eliminate pathogens depends on the cooperation of innate cellular and humoral mechanisms. Phagocytosis of blood cells is the primary cellular immune response against pathogens (Bayne 1990). A large number of studies have pointed out that, after bacterial infection, the expression level of HSP protein in blood cells of aquatic invertebrates is increased, and this increase is closely related to the antibacterial peptide and phagocytic activity in hemolymph. However, the direct relationship between the expression of these antimicrobial peptide-related genes and HSP protein is still unclear. HSP is believed to maintain phagocytes by repairing

damage or protecting itself from autolysis and apoptosis caused by auto-oxidation (Vega and De Maio 2005). Under the stimulation of LPS, the expression of HSP70 gene in grass carp is enhanced in 12 kinds of immune-related tissues. The release of HSP70 helps grass carp's immunity and disease resistance (Zhang et al. 2011). In the process of adaptive immunity, HSPs may play an indispensable role in antigen presentation by assembling the major histocompatibility complex (MHC)–peptide complex and activate T cells to destroy or synergistically kill pathogens and infected dysfunctional cells (Houlihan et al. 2009; Panjwani et al. 1999).

Therefore, HSPs are widely involved in the innate immunity and acquired immunity of aquatic animals and play an important role in regulating the immunity of the body. Research on the function of HSPs will also help to reveal strategies for protecting aquatic animals.

17.5 Regulation of HSPs to Enhance the Management of Aquatic Animal Diseases

17.5.1 Promote the Expression of HSPs in Aquatic Animal

From the numerous researches, we can see that promoting the synthesis of HSP proteins in the body can help increase the survival rate of aquatic animals under the condition of pathogenic microorganisms and stress. Moreover, the transient nonfatal heat shock is the common solution to stimulate the expression of HSPs. And it needs to hatch for several hours in nonstress conditions (Sung and MacRae 2011). The nonfatal heat shock can activate the transcription and synthesis of HSPs. HSPs may protect the body to resist the subsequent stressors. However, there is little information about the influence of heat shock or other striking stressors on the drug resistance of subsequent pathogenic stressors and the function of HSPs in the pathogenesis of the disease. It has a potential value in exploring the effective inducing methods of nonstress HSPs for the prevention of aquatic animal disease.

Other studies have shown that it can promote the expression of HSPs in aquatic animals through chemical inducer. The TEX-OE from cactus is an effective nonstress inducer of endogenous HSPs (Parker et al. 2014). Pro-Tex can accelerate the expression of HSPs in fish and shellfish and increase stress tolerance by immersion or as a feed supplement (Baruah et al. 2012; Niu et al. 2014). Pro-TEX can act as an enhancer for inducing the expression of HSPs and enhance the resistivity of *A. persicus* to *Aeromonas hydrophila* ATCC7966 by inducing different immune factors (Baharloei et al. 2020). It can also increase the release and expression of HSP72 protein by adding TEX-OE (Martinod et al. 2007). However, the TEX-OX is more often than not used to reduce the stress during fish transportation and increase fish tolerance. More and more studies have shown that TEX-OX can also enhance the disease immunity of fish (Sung et al. 2011). Therefore, it has an important foundation and application significance for illuminating the immunomodulatory effects of TEX-OE and another bioactive compound.

Paeoniflorin comes from the herbaceous peony. It can strengthen the expression of Hsp70 in cultured mammalian cells, and it is the substitute for TEX-OE. Paeoniflorin can induce heat resistance in HeLa cells. This effect was enhanced by heat shock for 2 h under 42 °C (Dai Yan et al. 2004). Bioactive compound tripterine is a kind of quinone methylated triterpenes from Chinese herbal medicine tripterygium wilfordii. It may increase the synthesis of Hsp70, Hsp40, and Hsp27 in HeLa cells (Westerheide et al. 2004). Schisandrin b (Scheme B) is the most active dibenzocyclooctyne in *Schisandra*. Its treatment increased Hsp70 by quantity-dependent mode in mouse liver (Ip et al. 2001). Curcumin (methane dierucic acid) is the dominant sector of turmeric, and it can induce the expression of Hsp70 in K562 cells (Teiten et al. 2009). Carvacrol comes from the oil of the oregano species, which may lead to the expression of Hsp70 and promote the T cell to recognize the endogenous Hsp70 (Wieten et al. 2007). In addition, some anticancer drugs can cause the rise of HSP60, HSP70, and HSP90 in exosomes and increase the immunocompetence of natural killer cells (Lv et al. 2012). All above are the related substances that have been reported to act as the HSP inducer and use the new tactics of controlling aquatic animal diseases.

17.5.2 Exogenous HSP Administration

Reports have shown that it's a new way to control aquaculture diseases by feeding aquatic organisms through bacteria rich in HSPs. Feeding the *Escherichia coli* can express a large number of DnaK (the equivalent of HSP70) to the artemia. When infected by the *Vibrio campbellii*, the survival rate was significantly higher than the control group (Sung et al. 2009). Then, this may be related to stimulate the phenol oxidase cascade system of artemia through feeding the bacteria containing DnaK (Baruah et al. 2011). By feeding yeast rich in HSP70 to artemia, it can enhance its endurance capacity to *Vibrio campbellii* (Wang et al. 2010). Injected the HSPs, DnaK, and GroEL protein of recombinant bacteria can protect the platyfish *Xiphophorus maculatus* from the effect of the *Yersinia ruckeri* (Ryckaert et al. 2010). However, it has little research about the exogenous administration of HSPs in aquatic animals at present. It's feasible to study and exploit new drug delivery routes and then strengthen the premunition through improving the content of HSP in the body.

17.5.3 HSPs as Potential Vaccine for Aquatic Animals

The use of HSP proteins derived from bacteria as antigenic proteins has been confirmed to be an effective disease-resistant vaccine, providing a certain guarantee for the healthy breeding of aquatic animals. At present, the research and development of HSPs as vaccines has been systematically reported (Hoos and Levey 2003) (Bolhassani and Rafati 2008). The injection of the HSP60 and HSP70 recombinant proteins of the pathogenic *P. salmonis* in *atlantic salmon* significantly reduced the

mortality caused by salmon infection with *P. salmonis* (Wilhelm et al. 2005). HSP60 and HSP70 of *P. salmonis* and recombinant protein of salmon flagellin FlgG can protect salmon from rickettsia septicemia (SRS) (Wilhelm et al. 2006). In *Oncorhynchus mykiss*, the recombinant protein and DNA vaccine of HSP60 and HSP70 of *Flavobacterium psychrophilum* were injected, and it was found that only rHSP70 protein vaccine had better protective effect (Plant et al. 2009). DnaJ as bacterial HSP40 homologs can also be screened as vaccines. In *Lates calcarifer*, the recombinantly expressing four HSP proteins (HSP90, HSP70, HSP33, and DnaJ) of *Photobacterium damselae* subsp. were used as protein vaccines, and their protection efficiencies were 48.28%, 62.07%, 51.72%, and 31.03%, respectively (Pham et al. 2021). Research results show that HSP33 is more suitable as a vaccine protector for *Photobacterium damselae*. The *Edwardsiella* DnaJ recombinant protein protects the *Paralichthys olivaceus* against this gram-negative pathogen, and the survival rate is increased by 62% compared with the non-immunized control (Dang et al. 2011). The c-terminal recombinant protein that stimulates *Cryptocaryon irritans* HSP70 can achieve more than 95% protection efficiency by being wrapped in chitosan nanoparticles as a vaccine (Josepriya et al. 2015). It can be concluded from these studies that pathogen-derived HSP is an ideal vaccine candidate in aquaculture, which can reduce the occurrence of aquatic animal diseases.

HSPs are also considered candidates as vaccine adjuvants. When covalently linked to HSP proteins, synthetic peptides can significantly increase the body's immune response (Suzue and Young 1996). This property has been reported in HSP70, HSP110, and HSP170 (Udono and Srivastava 1994). HSP can interact with antigen presenting cells (APC). The HSP-peptide complex is ingested by the APC and appears on the MHC molecule of the APC, induces macrophages and dendritic cells to secrete inflammatory cytokines, chemokines, and NO, and upregulates the expression of costimulatory molecules on dendritic cells (Basu et al. 2000). These effects involve receptor participation, signaling, and translocation of NF-κB to macrophages and dendritic cell nuclei. The HSP chaperone peptide enters the APC through specific receptors such as Toll-like receptor (TLR), lectin-like oxidized LDL receptor, and CD91 (Binder et al. 2004), and induces T cells by increasing antigen presentation of MHC class I and class II molecules (Srivastava 2002). So far, there has been no research on the use of heat shock proteins as vaccine adjuvants in aquaculture. Nevertheless, these HSP proteins have particular importance in the development of vaccines against viruses or intracellular bacteria.

17.6 Conclusion

Aquatic animals can protect the body from damage caused by various stresses by producing various proteins such as sHSPs, Hsp70, Hsp60, and Hsp90. By exerting the functions of molecular chaperones, it can regulate the folding of new proteins, prevent irreversible protein denaturation, and refold or help eliminate damaged proteins. Where, HSPs can not only be used as a biomarker for some water environmental pollutants, but the accumulation of HSPs is positively correlated

with the tolerance level to environmental stress. HSPs are also widely involved in the immune regulation of aquatic animals, and enhancing the expression of HSPs will help improve the disease resistance of aquatic animals. Exogenous HSPs can also be used as vaccines to increase resistance to pathogens by stimulating the humoral and cellular aspects of the innate immunity of the host. HSPs can also be used as vaccine adjuvant, playing an important role in the development of effective vaccines against aquatic diseases. Therefore, HSPs can be used as an important target site for aquatic animals. By regulating the expression and function of HSPs, it can not only enhance the antistress ability of aquatic animals but also play an important role in disease prevention and control.

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Nanotechnology in Fish Health and Welfare: Recent Advancements and New Perspectives

18

Irfan Ahmad Bhat and Hussna

Abstract

Nanoscience, where objects are measured at one billionth of a meter, enables the particles with the unique properties that function as a unit within the size range. In aquaculture, nanotechnology has a wide range of applications, from the delivery of drugs, nucleic acids, peptides, feed, nutraceuticals, etc., to the water treatment system. In general, nanotechnology has started replacing the antiquated fish production systems especially in breeding, disease management, and postharvest technology. In the fish disease management system, the nanostructured materials are being used as immunomodulatory substances to more efficiently manipulate or deliver immunologically active substances to the target location. Starting from the disease-causing bacteria to the deadly viruses, nanomedicine at a lower dosage has been used to curb all kinds of infections at a faster rate in aquaculture species. In this chapter, the general outline of nanotechnology and its current use in fish production management is discussed. Further the current trends of the use of nanotechnology in fish disease prevention are thoroughly summarized. Successful applications of nanotechnology in the field of fish disease management enable to develop new effective vaccines, adjuvants, as well as immunomodulatory drugs to enhance clinical outcomes in response to a variety of noninfectious and infectious diseases.

Keywords

Nanotechnology · Breeding · Disease · Immunomodulatory substances · Vaccines

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18.1 Introduction

Nanotechnology deals with the science of developing and using particles of nanometer size. In definition, the particles that fall in the size between 1 and 10 nm is categorized under nanoparticles (Nps). Broadly speaking, Nps can be in the form of ultrafine nano-sized particles that are typically found in nature or can be constructed and designed artificially for a specific purpose (Oberdörster et al. 2005). At the nanoscale, the properties (electrical, catalytic, magnetic, and thermal) of the substances change which allows them to have tremendous applications in the biomedical, environmental, and other scientific fields. The specific properties of Nps allow them to have novel applications that otherwise are not possible to harness from a substance when it is present on other measuring scales in nature. Due to unique applications, nanotechnology has become an extensive area of study. Nanotechnology has created a significant impact in almost every field of science. In the biological field particularly in medicine, Nps are being used in diagnostics, vaccines, drug, and gene delivery. This science is concerned with the design and utilization of biomedical uses of Nps and nanodevices (Cavalieri et al. 2014). Since the discovery of the unique properties of the Nps, scientists are trying to include more and more substances into the biological field for specific functions. The available Nps are classified based on their origin, formation, size, shape, and applications in different areas. The small size of the Nps makes them have a reasonably higher surface area to volume ratio than the usual forms. The nanomaterials can be produced as a surface film (one dimension), strand or fibers (two dimensions), or as particles (three dimensions) in various regular and irregular shapes such as rods, tubes, wires, etc. The Nps can be a sphere-shaped in which substances get adsorbed or attached on the surface, nanocapsules that encapsulate the drug, tubes (carbon nanotubes), liposomes, branched dendrimers, and polymeric Nps. The different types of Nps along with their applications are presented in Fig. 18.1.

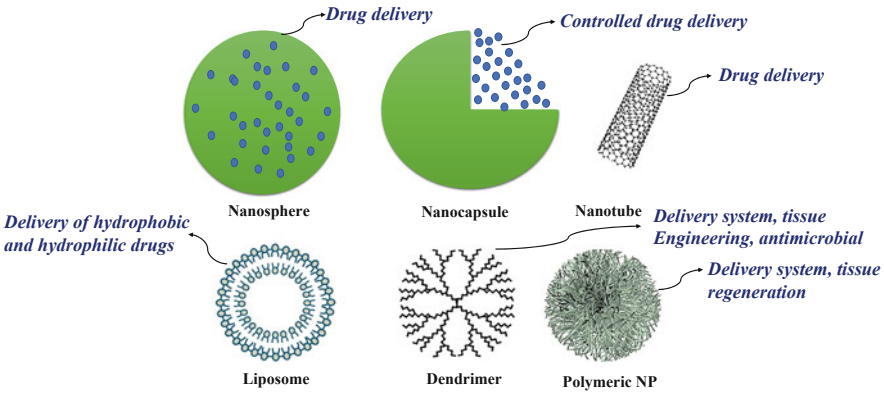

Fig. 18.1 Various types of Nps used in aquaculture and their applications

As far as fishery and aquaculture are concerned, Nps have a wide range of applications including wastewater treatment, sterilization of fishponds, fish packaging, barcoding, and tagging as indirect use and in feeding and healthcare as direct use. The different Nps are continuously used which poses a threat to the environment due to the nanotoxicity associated with them on biological species including plants, humans, fish, and other invertebrates. Many studies have been performed to know about the potentially harmful effects of Nps as well as to determine the dosage that is considered safe for the tissue or cells. It allows the establishment of a safe concentration limit to be applied. In this chapter, we are dealing with the applications of nanotechnology in fish healthcare and other fields related to increasing aquaculture production.

18.2 Synthesis of Nps

The key methods for nanoparticle synthesis are categorized into top-down or bottom-up approaches (Holmes et al. 2003). In the top-down approach, the bulk substance at macro or microscale is ground to transform it into the nanoscale, which is accompanied by the addition of stabilizing agents that protect the Nps from aggregation or instability. In the bottom-up approach, the Nps are created from atoms or molecules by different processes like vapor deposition, sol-gel process, etc., to convert them into nanomaterials (Fig. 18.2).

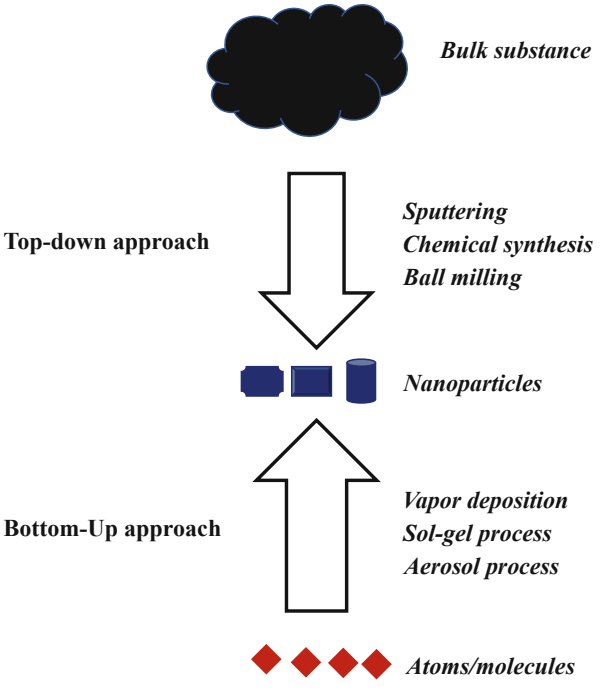

Fig. 18.2 Top-down and bottom-up approaches for the synthesis of Nps

18.2.1 Physical, Chemical, and Biological Synthesis

The physical methods to produce Nps involve the use of mechanical pressure, electrical energy, radiations, thermal energy, etc. These processes bring out the changes in the material like abrasions, evaporation, and melting which results in the Nps formation. The physical methods have the advantage of being fast without the use of toxic substances, but productivity is usually lesser compared to other methods. Physical synthesis using microwaves is being used to produce the silver Nps, and in comparison, with the thermal method, it is faster and produces a higher concentration of Nps at a given temperature and exposure. Laser ablation is one of the simple methods of production of Nps, but the high cost limits its usage. Ball milling is the process of reducing the higher size of the particles and blending them into new phases.

The chemical synthesis involves the bottom-up approaches, and the process involved is called nucleation where the use of water-soluble cations triggers the reduction of macro substances to metal monomers. The metals are allowed to remain in a chemical solution, and after chemical reactions, the Nps are formed (Stepanov et al. 2014).

Synthesis of nonmetallic Nps is on a trend nowadays, and different methods of their preparation have been validated. The chitosan Nps are prepared by the ionotropic gelation, with the addition of polyanion tripolyphosphate (TPP) which acts as a crosslinker between the chitosan molecules. The technique involves the usage of acidic solutions like acetic acid in which chitosan is dissolved, and the approach depends on electrostatic contact between the chitosan amine group and polyanion polymer groups. The TPP is added drop by drop to allow the proper cross-linking between the chitosan molecules (Bhat et al. 2018a). Other methods are the microemulsion method which utilizes the addition of surfactants, with the major disadvantage of the usage of organic solvents, prolonged preparation time, and the difficulty of the washing processes. The most common method for synthesis of Poly D, L-lactide-co-glycolic acid (PLGA) Nps is the precipitation method combined with the double emulsion solvent evaporation method.

The biological method also known as green synthesis is the most eco-friendly approach to produce Nps. Biologically synthesized Nps involve the usage of bacteria, fungi, and plants. This method comes under a bottom-up approach that mostly involves reduction/oxidation reactions (Prabhu and Poulose 2012). The enzymes present in the microbes and chemicals in plants have antioxidant or reducing properties that act on precursor compounds to produce the desired Nps. So, according to Prabhu and Poulose (2012) for the biosynthesis of Nps, there is a requirement of three systems. Viz., a solvent medium for synthesis, a reducing agent, and a stabilizing agent.

Antibacterial activity and cytotoxic effects against a human lung cancer cell line were demonstrated by silver Nps synthesized using *Origanum vulgare* leaf extract. Cashew nutshell liquid was used for the green synthesis of both silver and gold Nps, which showed bactericidal activity against several fish pathogens (Velmurugan et al. 2014). In juvenile *Feneropenaeus indicus*, silver Nps synthesized using tea leaf

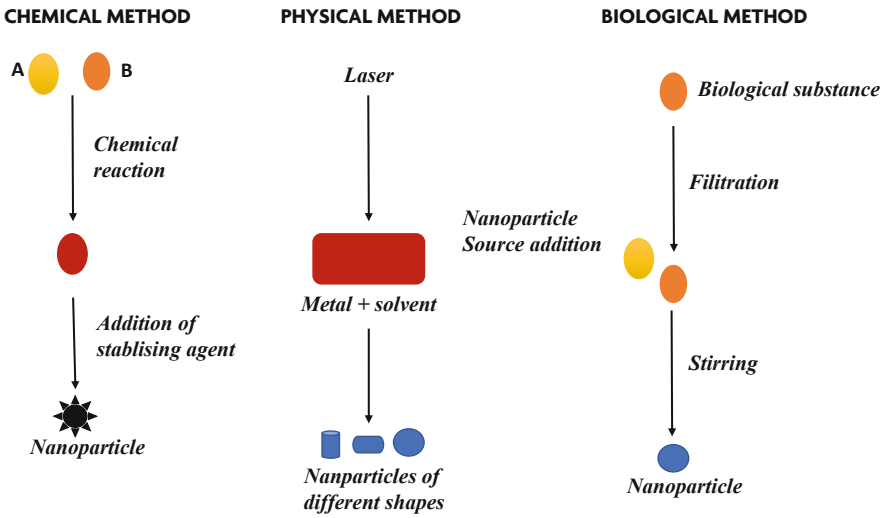

Fig. 18.3 Different methods for synthesis of Nps

extract (*Camellia sinensis*) exhibited bactericidal activity against *Vibrio harveyi* (Vaseeharan et al. 2010). For the green synthesis of zinc oxide Nps (ZnO Nps), a broth of aloe leaf extract was used, which have shown higher bactericidal activity than Nps by standard chemistry (Gunalan et al. 2012). A novel method for the biological synthesis of zinc oxide Nps uses *Aeromonas hydrophila* bacteria as the reducing agent. This method is environment-friendly and economically viable and produces ZnO Nps with antibacterial and antimycotic properties. The illustration of different methods used for NP synthesis are shown in Fig. 18.3.

18.3 Nanotechnology in Aquaculture

18.3.1 Application in Feed Technology

In fish feed technology, nanotechnology is emerging as a fast technology to be used for many processes including delivery, protection, and stability of the enriched feed particles. Also, some of the Nps act as growth promoters as well as immunostimulants and have been used in fish with significant results. Some of the examples include the usage of chitosan-coated calcium alginate to prevent shark liver oil leakage (Peniche et al. 2004). Similarly, chitosan prevented tuna oil from degradation (Klinkesorn and McClements 2009), and usage of carbon nanotubes prevented the leaching of nutrients from feed (Fraser et al. 2011; Bisesi et al. 2015; Ramsden et al. 2009). In Nile tilapia, the nanospheres loaded with nerolidol increased survivability and prevented the fish from oxidative brain damage when infected with *Streptococcus agalactiae* (Baldissera et al. 2020). The use of selenium (Se) NPs has boosted the growth and immunity of many aquaculture species. It

increased the beneficial cellular responses in the crucian carp and promoted the growth in another fish species (Wang et al. 2013; Deng and Cheng 2003). Nano Se-supplemented fish diet prevented the particle dissolution, aggregation, and release of feed and was not toxic to fish compared to Se alone (Monikh et al. 2020). Se NPs along with riboflavin reduced the stress responses caused due to high temperature and arsenic pollution in *P. hypophthalmus* (Kumar et al. 2020). Zinc (Zn) in nano form improved growth and immune response in grass carp (Faiz et al. 2015) and increased growth performance in rainbow trout (Ramsden et al. 2009). Zn NPs supplemented through feed were found to increase the growth rate in fish compared to inorganic Zn (Mondal et al. 2020). Bhattacharyya et al. (2015) investigated the use of nanomaterials to increase the proportion of nutrients passing through the gut tissue.

18.3.2 Application in Fish Reproduction

In fish, the NPs have been used to deliver the peptides, hormones, and genes to sustain their effects for longer durations. Chitosan conjugated with Au NPs were used to deliver salmon GnRH in common carp which resulted in increasing the reproductive parameters (Rather et al. 2013). Similarly, chitosan-conjugated LHRH increased the transcript level of gonad-developing gene, Sox9, in fish and reproductive hormones in *Clarias batrachus* (Bhat et al. 2016). Chitosan NPs sustained the plant extract eurycomanone an extract of *Eurycoma longifolia* which resulted in increasing the gene expression as well as the reproductive parameters like fertilization rate, the hatching percentage, and survival rate in *C. magur* (Bhat et al. 2018, 2019). Chitosan-conjugated aromatase inhibitors were used to augment the gonadal development of male *C. magur* (Wisdom et al. 2018). In male Asian catfish, the chitosan was used to deliver methyltestosterone for increasing reproductive performance (Saha et al. 2018). In one study, the StAR gene construct which is important to start the steroidogenesis inside the cell was delivered through chitosan Nps in *C. batrachus*, and results indicated the increase in gonadal transcript of testes and reproductive hormonal profile (Kumar et al. 2017).

18.3.3 Nanotechnology in Water Treatment of Aquaculture Pond

The water contamination of pond due to excess feed or environmental conditions or intentionally (pesticides, chemicals, antibiotics) creates stress to the aquatic animals which result in their poor growth or mass death. So, the pond water must be either replaced or aerated continuously, which makes the management difficult. Nanotechnology has been applied to purify the water by using nanoengineered substances. The best example to remove harmful substances, heavy metals, and noxious gases from pond water through nanotechnology is the usage of metallic Nps and carbon nanotubes (Ren et al. 2011; Xu et al. 2012; Pradeep 2009; Rather et al. 2011; Chen et al. 2013). La (Lanthanum) oxide Nps are useful in treating water to make it free

from disease-causing bacteria like *Chlorella vulgaris*, *Escherichia coli*, *Penicillium roqueforti*, and *Staphylococcus carnosus* (Gerber et al. 2012).

Nanocheck, a La-based device, is used to get rid of phosphates in pond water (Mohd Ashraf et al. 2011). Nanodevices improve the water quality in shrimp ponds thereby reducing the water exchange rate (Wen et al. 2003). Another problem related to cages, pens, nets, and ships is biofouling which results in the degradation of the surfaces by bacteria (Champ 2003). Antifouling paints rich in metallic Nps are being used, and they have been found more effective than the normal paints used to curb biofouling (Ashraf and Edwin 2016). The bacterial antibiofilm activity of Ag and Au Nps synthesized from *Turbinaria conoides* extracts was evaluated by Vijayan et al. (2008), highlighting that Ag in nanoform was successful in preventing the formation of biofilms.

18.3.4 Advances in Drug and Gene Delivery

Nps are an ideal delivery system for the transfer of drugs, vaccines, and peptides in the living cells or tissues. The Nps used for the delivery should be safe, biocompatible, and biodegradable and should protect the conjugated substance for a longer duration (De Jong and Borm 2008). Lots of Nps are being used in the process, but the polymeric Nps including chitosan and PLGA are mostly safe with least toxicity for the living cells. The chitosan Nps have the property to slowly release the drug and sustain it for longer durations (Bhat et al. 2018). It has been used for the delivery of peptides, plant extracts, vitamins, genes, etc. (Alishahi et al. 2011; Rather et al. 2013; Kumar et al. 2017; Bhat et al. 2018). Chitosan Nps coupled with vitamin C after administration in rainbow trout showed that the release of the vitamin was regulated up to 48 h. Further, the innate immunity, as well as nonspecific defense mechanisms, were upregulated in the treated fish which is mainly because of the synergistic effects of vitamin C and the chitosan polymer (Alishahi et al. 2011). All the studies indicated that the effect of the delivery agent remained for longer durations without any harmful effect in fish. Similarly, PLGA a copolymer of polylactic acid and polyglycolic acid has the same properties as chitosan and is used as a delivery agent in many fish species. In zebrafish, it was used to deliver rifampicin to treat *Mycobacterium marinum* infections, in rohu for the delivery of *A. hydrophila*, and DNA vaccine against lymphocystis in Japanese flounder (Behera et al. 2010; Tian and Yu 2011; Fenaroli et al. 2014). These studies indicate that PLGA can be effectively used in the delivery process in aquaculture species with a lower risk. Furthermore, Nps dependent on silica can be used for drug administration because of its porous structure and capacity to integrate at elevated doses (Strømme et al. 2009).

18.3.5 Advances in Aquaculture Species Health

The intensification of aquaculture has increased the production exponentially. The modern facilities for breeding, rearing, and feeding of aquaculture species have led

to a boom in the aquaculture industry, but at the same time, it has led to the introduction of deadly disease-causing agents (Pulkkinen et al. 2010). Most of the aquaculture farms are either using traditional methods to eradicate harmful substances or using antibiotics to treat the diseases. The use of excess antibiotics has led to an increase in antibiotic-resistant microorganisms which is becoming very difficult to control. To limit the use of excess antibiotics, nanotechnology plays an important role. The use of Nps can decrease the dosage of antibiotics which will be effective for longer durations thereby minimizing the harmful effects. Nps act as a potential antimicrobial substance, and the antibiotic-resistant bacteria conjugated with nanoscale particles can be delivered as vaccines for disease prevention (Shaalan et al. 2016).

Silver Nps possess an antimicrobial property and has been tested for it (Mathur et al. 2014). The ions (Ag+) present in the silver Nps connect with the membranes and proteins of bacterial cells and destruct them which results in cell death (Lara et al. 2010; Huang et al. 2011). The *Azadirachta indica*-constructed Ag Nps resulted in the resistance against *A. hydrophila* in mrigal (Rather et al. 2016). In the study higher survival rate was detected compared to the control group suggesting that Ag Nps have an immunomodulatory and antibacterial role. Chitosan-Ag nanocomposites were tested for their antimicrobial property against fish pathogen *Aliivibrio salmonicida* (Dananjaya et al. 2016). The results indicated that it acts as a potential antibacterial agent against the disease. Antony et al. (2013) constructed Ag Nps using Aspera and suggested that it has an antimicrobial property against *A. hydrophila* infections in *Catla catla*. Ag NPs and Zn oxide Nps inhibited the growth of *A. salmonicida*, *A. invadans,* and *Yersinia ruckeri in fish* (Shaalan et al. 2017). Other than Ag and Zn, the other metallic Nps having antimicrobial activity is copper, and it has been tested in many fish species. Ag Nps have been used to treat fungal infections in rainbow trout eggs, whereas ZnO Nps disrupted the bacterial cell membrane (Johari et al. 2015; Pati et al. 2014).

18.3.6 Nanotechnology in the Diagnostics

Nanotechnology has strengthened its roots in the field of diagnosis in the biomedical field, and now in the aquaculture industry, there has been a shift towards the usage of nano-constructed diagnostic kits for pathogen detection. Gold Nps are the most used ones for the purpose and has been used to detect *A. salmonicida* and *Aphanomyces invadans* in fish (Saleh et al. 2011; Kuan et al. 2013). Guo et al. (2016) in a study used an immunomagnetic Nps-based microfluidic device for the detection of *Staphylococcus aureus* by creating a microfluidic indium tin oxide chip. It was better than the colony counting process, and the detection process took a shorter time without cultivation of the colony. A colorimetric approach using Au Nps and loop-mediated isothermal amplification was developed for the detection of the yellow head virus and white spot syndrome virus (WSSV) in shrimps (Jaroenram et al. 2012; Seetang-Nun et al. 2013). Similarly, a colorimetric approach was used to develop a sensor using Au Nps for detection of spring viremia of carp and DNA herpesvirus 3 in fish

(Saleh et al. 2012; Saleh and El-Matbouli 2015). Furthermore, nervous necrosis virus (NNV) detection was done by using magnetic Nps coated with rabbit anti-NNV antibody, and also the biosensor was developed using Au Nps for detection of the virus (Yang et al. 2012; Toubanaki et al. 2015). The examples of Nps used in diagnostics in fish are presented in Table 18.1.

18.3.7 Nanotechnology-Based Fish Vaccines

Nanotechnology has some exciting applications in delivering of vaccines in aquaculture species. Nps have been efficiently used either as adjuvants or as delivery vehicles for the vaccines used in fish and shellfish. The current trend of the use of nanotechnology in vaccines is continuously rising in world aquaculture including India. Nps prevent the drug, antigen, or other substances from enzymatic attack, guide it to reach the target site, slowly release it so that the effect could be felt for longer durations, as well as due to the smaller size can cross any barrier inside the body.

18.3.7.1 Polymeric Nps

Polymeric Nps are being used in vaccine delivery due to their lesser side effects and easy degradable nature. They also act as immunostimulants to protect the antigen as well as trigger the immune system during the need. Nps conjugated with antigens protect and guide it to reach the targeted immune cells (Zhao et al. 2014). Some other examples of Nps used in the vaccine delivery are particles identical to viruses, immunostimulant complexes, liposomes, metallic Nps, nanoemulsions, etc. (Zhao et al. 2014; Shaalan et al. 2016). Polymeric Nps can be of natural type of synthetically derived ones with examples as chitosan, hyaluronic acid, alginates of the former, and PLGA of the latter.

Chitosan Nps enhanced mucosal immunity when delivered orally in fish. Chitosan conjugated with inactivated virus vaccine against infectious salmon anemia virus (ISAV) increased the survivability up to 77 percent against this pathogen (Rivas-Aravena et al. 2015). The use of chitosan Nps as a delivery vehicle to deliver vaccines against viral hemorrhagic septicemia (VHSV) was successfully performed in zebrafish (Kavaliauskis et al. 2016). The oral DNA vaccine against *V. anguillarum* in Asian sea bass (*Lates calcarifer*) was developed with chitosan as a delivery agent and adjuvant (Vimal et al. 2014). Similarly, in black seabream (*Acanthopagrus schlegelii*), the recombinant nanovaccine containing chitosan elicited a defensive immune response against *V. parahaemolyticus* (Li et al. 2013). A Japanese flounder (*Paralichthys olivaceus*) oral DNA vaccine containing chitosan as a carrier was developed against the lymphocystis disease virus (LCDV) (Tian et al. 2008). Chitosan conjugated with DNA vaccines against *Philasterides dicentrarchi* in *Scophthalmus maximus* (León-Rodríguez et al. 2013), RNA in *Labeo rohita* (Ferosekhan et al. 2014), *V. parahaemolyticus* in *Acanthopagrus schlegelii* (Li et al. 2013), and hematopoietic necrosis virus in rainbow trout (Adomako et al. 2012) has been developed successfully. DNA-based vaccines conjugated with Nps generated the immunological proteins that protect shrimps

Table 18.1 Applications of different Nps in vaccine delivery and diagnostics in fish

Type of nanoparticle used	Delivery against disease	Fish	References
Chitosan	*Salmon anemia virus*	Atlantic salmon	Rivas-Aravena et al. (2015)
	Viral haemorrhagic septicaemia virus (VHSV)	Zebrafish	Kavaliauskis et al. (2016)
	Turbot reddish body iridovirus TRBIV	Turbot	Zheng et al. (2016)
	Nodavirus	Asian seabass	Vimal et al. (2014)
	Vibrio parahaemolyticus	Blackhead seabream	Li et al. (2013)
	Vibrio anguillarum	Asian seabass	Vimal et al. (2012)
	Vibrio anguillarum (Listonella)	Asian seabass	Rajesh et al. (2008)
	Edwardsiella tarda	Rohu	Kole et al. (2018)
	VHSV	Olive flounder	Kole et al. (2019)
Alginate	*Ichthyophthirius multifiliis*	Rainbow trout	Heidarieh et al. (2015)
	Infectious hematopoietic necrosis virus (IHNV)	Brown trout and rainbow rout	Ana et al. (2010)
Alginate + chitosan	*Yersinia ruckeri*	Rainbow trout	Dezfuly et al. (2020)
PLGA	*A. hydrophila*	Rohu	Dubey et al. (2016)
	Streptococcus agalactiae	Tilapia	Zhang et al. (2015)
	A. hydrophila	Rohu	Rauta and Nayak (2015)
	Infectious pancreatic necrosis virus (IPNV)	Atlantic salmon	Munaŋgandu et al. (2012)
	IHNV	Rainbow trout	Adomako et al. (2012)
	Uronema marinum	Flounder	Harikrishnan et al. (2012a)
	Lymphocystis disease	Flounder	Tian and Yu (2011)
PLGA+ chitosan	*Edwardsiella tarda*	Rohu	Leya et al. (2020)
Liposome	*Vibrio harveyi*	Grouper	Harikrishnan et al. (2012a, b)
	A. salmonicida	Common carp	Irie et al. (2005)
	Koi herpes virus	Common carp	Yasumoto et al. (2006a)
Carbon nanotubes	*Grass carp reovirus*	Grass carp	Zhu et al. (2014)
	Grass carp reovirus	Grass carp	Zhu et al. (2015)

(continued)

Table 18.1 (continued)

Type of nanoparticle used	Delivery against disease	Fish	References
	Rhabdovirus	Common carp	Zhang et al. (2020)
	Grass carp reovirus	Grass carp	Zhu et al. (2020)
	Grass carp reovirus	Grass carp	Qiu et al. (2020)
	Infectious spleen and kidney necrosis virus	Mandarin fish	Zhao et al. (2020)
Calcium phosphate	*A. hydrophila*	Rohu	Behera and Swain (2011)
Diagnostics			
Nanoparticle	*Antibody used/method*	*Detection in fish*	
Gold	*A. salmonicida*	Furunculosis	Saleh et al. (2011)
Gold	DNA based	*Aphanomyces invadans*	Kuan et al. (2013)
Gold	Not needed	Nervous necrosis virus (NNV)	Toubanaki et al. (2015)
Gold	Colorimetric assay, probe used	Spring viremia of carp virus (SVCV)	Saleh et al. (2012)
Gold	Colorimetric assay, probe used	Cyprinid herpes virus-3 (CyHV-3)	Saleh and El-Matbouli (2015)
Magnetic nanoparticles	Rabbit anti-NNV antibody	NNV	Yang et al. (2012)

from white spot syndrome virus (WSSV) for application up to 7 weeks (Rajeshkumar et al. 2009). The delivery of antisense nodavirus DNA was encapsulated with chitosan Nps and resulted in a higher survival rate in freshwater prawns (Ramya et al. 2014). Polyanhydride Nps have been used for vaccine encapsulation and release antigens that decide shrimp immunization through immersion or with feed (Ross et al. 2014). Bicistronic DNA vaccine encoding *Edwardsiella tarda* antigen glyceraldehyde-3-phosphate dehydrogenase and immune adjuvant IFN-γ was conjugated with chitosan Nps and delivered to rohu through oral and immersion methods which resulted in immunity against the infection (Kole et al. 2018). The same bicistronic DNA vaccine was conjugated with chitosan and PLGA Nps to develop immunity against the infection in rohu (Leya et al. 2020). Chitosan Nps were encapsulated with inactivated viral hemorrhagic septicemia virus (VHSV) antigen and administered via mucosal routes in olive flounder to develop protective antiviral immunity against the virus (Kole et al. 2018).

Alginate microparticles were used as a delivery vehicle for transferring plasmid DNA orally against IPNV in brown trout and rainbow trout (Ana et al. 2010). Similarly, they were used to deliver the antigens against *Flavobacterium columnare* in Nile tilapia and *Ichthyophthirius multifiliis* in rainbow trout (Leal et al. 2010; Heidarieh et al. 2015). In rainbow trout, the alginate particles produced by the spray

method was conjugated with the antigens of *Lactococcus garvieae*, and an effective response against the disease was found (Ana et al. 2010). Alginate-chitosan micro/Nps conjugated with *Yersinia ruckeri* lipopolysaccharide enhanced the immunogenicity against the infection in rainbow trout (Dezfuly et al. 2020).

PLGA was used to deliver the vaccines against *A. hydrophila*, *Streptococcus agalactiae*, infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus in rohu, tilapia, Atlantic salmon, and rainbow trout, respectively (Dubey et al. 2016; Zhang et al. 2015; Munanġandu et al. 2012; Adomako et al. 2012). Antigen from *Uronema marinum*, which is a protozoic pathogen that infects flounder and grouper, was encapsulated into PLGA and it sucessfully elicited the immune response against the opportunistic pathogen, and the effect remained for a maximum of 4 weeks (Harikrishnan et al. 2012a, b). In another study on flounder, a plasmid that codes for the major capsid protein (MCP) of lymphocystis disease virus was conjugated with PLGA Nps, and the results suggested that antibodies against the virus were peaked after 30 days of treatment and also the encapsulated treatment was more effective in treating the infection compared to naked group (Tian and Yu 2011).

18.3.7.2 Liposomes

Liposomes are composed of a phospholipid bilayer which is considered self-sealing and could deliver lots of hydrophilic and hydrophobic drugs (Ji et al. 2015). Liposomes were used to deliver formalin killed *V. harveyi* antigen in grouper, and results revealed that in the entrapped group, the mortality recorded was lowest compared to the naked group (Harikrishnan et al. 2012b). In common carp, antigens against *A. salmonicida* were administered orally by using liposomes as vectors, and the survival rate up to 83% was calculated in the liposome-treated group (Irie et al. 2005). Lipopolysaccharide extracted from *A. salmonicida* was entrapped into liposomes to enhance the immune response in rainbow trout (Yasumoto et al. 2006b). Similarly, the koi herpesvirus inactivated by formalin treatment was enclosed within liposomes for vaccination of common carp orally (Yasumoto et al. 2006a). Liposomes have been used to deliver cinnamaldehyde (cinnamon extract) and melittin, an antimicrobial peptide against different pathogens and viral infections in fish, and the liposome-entrapped group was found to have an upper edge in eliciting the immune response against the disease-causing agent (Faikoh et al. 2014; Falco et al. 2013).

18.3.7.3 Inorganic Nps

The most efficient inorganic Nps that is currently having a tremendous application is carbon nanotubes. These Nps have been used in every field ranging from physics, engineering, to biomedicine. The main issue associated with the use of carbon nanotubes is the toxicity, so before applying a safe dosage needs to be selected. Carbon nanotubes have two forms, viz., single-walled and multiple-walled, and both are used for drug delivery and have different properties. A single-walled carbon nanotube has been used to deliver plasmid that codes for VP7 protein of grass carp reovirus (GCRV) intramuscularly in grass carp. The immune response against the infection was high in the nanotube-conjugated group compared to naked treatment

(Zhu et al. 2015). In a recent study, single-walled carbon nanotubes were used to deliver GCRV VP7 antigen into fish through immersion against the viral disease, and a good immune response was reported (Zhu et al. 2020). Carbon nanotubes were successfully used to deliver the antigens against rhabdovirus infection in fish (Zhang et al. 2020). Single-walled carbon nanotubes were used to deliver antigen against infectious spleen and kidney necrosis virus (ISKNV) in mandarin fish, and successful results were obtained (Zhao et al. 2020). Qiu et al. (2020) employed carbon nanotubes to deliver antigen against grass carp reovirus, and a higher percentage of immunity was generated compared to the naked treatment.

Calcium phosphate Nps when adsorbed to S-layer protein of *A. hydrophila* resulted in stimulation of both innate and adaptive immune response in fish, and the complete protection against the infection was provided (Behera and Swain 2011). Table 18.1 presents the examples of Nps used in the vaccine delivery against the particular disease in fish.

18.4 Conclusions

In summary, the present chapter deals with the advancement of nanotechnology in the aquaculture sector with more emphasis on its applications in disease prevention and diagnosis. The Nps of all kinds have been proven to be sufficient to enhance the resistance against the disease in fish. Ranging from the nature of Nps, polymeric Nps still occupy the top position in the list in terms of the application in fish medicine. Carbon nanotubes due to their unique properties are now being used in the disease management of aquaculture species.

The awareness about the benefits of Nps in the different fields like biomedicine, food processing industry, as well in the fisheries sector has led to an increase in their usage. At the same time, the harmful effect of the Nps on the surrounding environment is a matter of concern. Even though polymeric Nps are considered biodegradable and safe, still care and attention are needed to use them in a better way. Metallic Nps and carbon nanotubes are considered extremely hazardous for the living tissues, developing embryos, and growth in general when used at high concentrations. So, before their usage, safer concentration needs to be calculated to prevent long-term harmful effects.

Conflict of Interest None of the authors declare any conflict of interest.

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Advances in Management Methods for Argulosis in Aquaculture

19

Saurav Kumar and Pushpa Kumari

Abstract

Argulus are crustacean macro-ectoparasites that create one of the major threats to the aquaculture due to lack of suitable therapy. Argulosis causes a potentially rapid escalation of infection, causing substantial economic loss to the aquaculture industry worldwide. The use of chemotherapeutics/drugs/chemicals is a routine activity to combat argulosis in aquaculture; however, it has numerous unavoidable drawbacks; therefore, treatment would hardly be feasible with the existing methods. The most recent advancement in the control of fish lice, *Argulus*, is the applications of phytotherapy (plant crude extracts and bioactive compounds), vaccinating fish, epidemiological approaches, immunological interventions, vital gene-targeted drug development, etc. Regardless of several managerial attempts to destroy the parasite *Argulus*, it is seldom possible with present knowledge and warrants effective alternative eco-friendly and economically feasible methods to treat *Argulus* infection. In recent years the application of nanoparticles has resulted in a remarkable success to control the fish parasites like ich, monogeneans, *Lernaea*, etc. very efficiently at a considerably low dose; thus, interventions of nanoparticles as a potent argulocidal drug in aquaculture may be possible in the future. The present article highlights the advancement in management methods to combat argulosis in aquaculture system.

Keywords

Argulosis · Advanced treatment measures · Chemotherapeutics · Phytotherapy · Vaccination · Immunological methods · Epidemiology · Nanoparticles

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19.1 Introduction

Aquaculture has emerged as a promising enterprise to retain with the growing demand for high-quality protein food in an ever-growing world population along with generating employment and income for rural people. With the increasing intensiveness of the aquaculture practice to increase the fish production, stress level on fish are also increasing, resulting in compromised immunity of stocked fish which leads to outbreak of infectious diseases (Kumari and Sahoo 2006). Among infectious mediators, the parasites are in general considered opportunistic infectious agent and highly viable under intensified culture conditions. The cultured aquatic animal harbors a diverse group of parasites; however, manifestation with ectoparasites is ubiquitous in the aquatic environment (Tonguthai 1997). The National Surveillance Programme for Aquatic Animal Diseases (NSPAAD) findings from 2014 to 2018 showed that about 74.88% of diseases in freshwater aquaculture systems are caused by parasites alone, in which the incidence of *Argulus* sp. alone accounts for 19.51%, followed by *Dactylogyrus* (18.90%), Myxosporeans (12.80%), *Trichodina* (3.04%), ich (3.04%), other parasites (4.88%), and mixed parasitic infection of 37.80% (Sahoo et al. 2020). *Argulus*, locally called fish lice, is one of the most dreadful crustacean macro-ectoparasites that cause the disease argulosis, a severe growing concern to the aquaculture industry worldwide. Argulosis is responsible for epizootic outbreaks and is a serious economic concern in all phases of the aquaculture sector, from production to marketing (Sahoo et al. 2013a, b, c). *Argulus sp*. is diverse; about 129 species of *Argulus* have been reported worldwide, with 20 species that have been reported from culture and wild fishes in India (Valarmathi 2017; Bari 2018; Sahoo et al. 2020). The fish lice is a prolific breeder with a direct life cycle but comprising several metamorphic stages in their life such as eggs, metanauplius, copepodid, juvenile, and adult stages, and all stages except eggs are infective to fish (Shafir and Van As 1986). The infection leads to reduced appetite, weight loss, and anemia in fish resulting in morbidity and mortality in the chronic stage of heavily infested fish (Mousavi et al. 2011). The intensity of fish lice is very low with less of a threat in the wild fish than to those in captive conditions at all the stages of fish and exerts massive financial insecurity (Walker et al. 2004; Sahoo et al. 2013b). Therefore, the management of argulosis must be given top priority to save the aquaculture industry from this massive loss.

Several managerial measures to treat the infected fish with the *Argulus* parasite have been practiced to achieve better fish health (Hemaprasanth et al. 2012; Hakalahti et al. 2008; Banerjee and Saha 2013; Kumar et al. 2017; Das et al. 2018; Kumari et al. 2020) and to enhance the gross aquaculture production. The use of chemotherapeutics/drugs/chemicals is routine activity to combat argulosis in the aquaculture; however, the main criticisms surrounding the use of these drugs are the residue formation in water, sediment, and treated fish; toxicity in non-targeted organisms; carcinogenic potential for handlers/consumers; and the developing resistance in parasites (Burridge et al. 2010; Rico et al. 2013; Rico and Van den Brink 2014). Besides, the producers are using highly toxic substances, including molecules that have not been registered and approved by authority for treating fish diseases in

aquaculture, which is worrying. Hence, there is an urgent need for novel approach and approved drugs to develop appropriate interventions with lower risks to control argulosis. However, a few methods of physical removal, biological control, integrated pest management, and epidemiological approaches to eradicate the *Argulus* parasite (Hakalahti et al. 2008) are documented, practiced, and available. In addition to these, a progressive step towards vaccine development against *Argulus siamensis* has shown a marginal success (Kar et al. 2017; Das et al. 2018). Recently, phytotherapy is an emerging viable option and cheaper alternative measure for treating diseases in aquaculture (Citarasu 2010; Raman 2017). Remarkable success has been achieved in treating *Argulus* parasite by employing herbal-based medicine under in vitro and in vivo conditions suggested by several workers (Kumar et al. 2012a, b; Mamadou et al. 2013; Banerjee and Saha 2013; Banerjee et al. 2014; Kumari et al. 2019) though its mechanism of action and its pharmacokinetics in host need to be studied thoroughly. The health security against argulosis can be assured by encouraging farmers in intensive aquaculture to meet high production and more significant income. The search for more effective methods and drugs to combat argulosis is a never-ending process. The purpose of this paper is to bring together advancements made so far in the use of new drugs and the methods of application to overcome argulosis in aquaculture.

19.2 Diversity and Host Range of *Argulus* in the Indian Aquaculture System

Argulus sp. are cosmopolitan in distribution, found in all the continents except Antarctica (William 2008), and common ectoparasites of freshwater, estuarine, and marine fishes. About 18 species of *Argulus* is reported from different parts of India (Valarmathi 2017; Saravanan et al. 2017; Sahoo et al. 2020). The *Argulus* species reported in Indian fishes include the following:

1. *Argulus japonicus* (Thiele).
2. *Argulus foliaceus* (Linnaeus).
3. *A. indicus* (Weber).
4. *A. giganteus* (Ramakrishna).
5. *A. mangalorensis* (Natarajan 1982).
6. *A. cauveriensis* (Thomas and Devaraj).
7. *A. krishnagiriensis* (Omprakasam and Manohar).
8. *A. fluviatilis* Thomas & Devaraj 1975.
9. *A. boli*.
10. *A. puthenveliensis*.
11. *A. monody*.
12. *A. quadristriatus* (Marine).
13. *A. parsi* (Tripathi).
14. *A. schoutedeni*.
15. *A. siamensis* (Wilson 1926).

16. *A. siamensis peninsularis*;
17. *A. vittatus* (Marine), and.
18. *Argulus maharashtrians* (Bari 2018).

Among these species, *A. siamensis*, *A. japonicus*, *A. foliaceus*, *and A. bengalensis* are the most commonly encountered in aquaculture farm and wild fishes (Sahoo et al. 2019). In wild and farmed condition, cyprinids are most vulnerable fish species infested with this parasite, and the economic loss caused by *Argulus* sp. is estimated Rs. 300 crores/annum in Indian aquaculture (Sahoo et al. 2013a, b, c). The Indian major carps (*Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*) including exotic carp, *Cyprinus carpio* and varieties, *Hypophthalmichthys molitrix*, and *Ctenopharyngodon idella* are prone to argulosis (Sahoo et al. 2013a, b, c; Das et al. 2016; Alom et al. 2019). Several studies confirm that rohu (*L. rohita*) is the most susceptible species of fish in the freshwater aquaculture system (Kar et al. 2016; Alom et al. 2019). The other susceptible freshwater fishes are minnows, *Tilapia* sp. (Sriwongpuk 2020), salmons, trouts and mahaseer (Mallik et al. 2010), *Channa striata* (snakehead), pangasius (Parvez et al. 2013), *Clarias batrachus*, and *Wallago attu* (Bari 2018); among ornamental fish species are goldfish (*Carassius auratus* and var.), black moor, koi carp (*Cyprinus carpio* var. koi), oscar, dwarf gourami (*Trichogaster lalius*), platy, guppy, tench, and betta (Thilakaratne et al. 2003; Tokşen 2006; Shahraki and Asgari 2014; Mirzaei and Khovand 2015; Iqbal and Haroon 2014; Kumari et al. 2018); and sport fish include *Schizothorax richardsonii*, *Tor putitora*, etc. The estuarine and marine fishes show the incidence of infection with *Argulus* are sunfish, common roach, pike, horse mackerel, sea breams, cobia, gulf sturgeon, flathead catfish, and stingrays (Subburaj et al. 2019; Andres et al. 2019).

19.3 Scenario of Argulosis in Aquaculture

Modern fish culture practices generally operate on high stocking densities and creates a favorable environment for infectious diseases and transmission and reproduction of parasites. *Argulus* parasitizes the skin, fins, and gills of host fish (Yıldız and Kumantas 2002) and feed on mucus, epidermal cells, and blood leading to dermal ulceration, physiological stress, and immune suppression (Saurabh et al. 2011; Sahoo et al. 2013b; Kar et al. 2017). The lesions caused by parasites due to bloodsucking result in secondary infection with opportunistic pathogens like bacteria and fungus (Walker et al. 2004). Besides, *Argulus* acts as a vehicle for other fish pathogens, including rhabdovirus carpio, spring viremia of carp (SVCV), larval nematodes, and the fungus *Saprolegnia* (Woo et al. 2002; Ahne et al. 2002). Ambuali et al. (2020) reported that the parasite secrets certain secretory/excretory proteins (SEP), which help the parasites in attachment with host, tissue digestion, extraction of nutrients, and establishing an infection. Heavy infestations cause severe skin damage, pinpoint hemorrhages, anemia, fin and scale loss, increased mucus production, lethargy, erratic swimming, and poor body condition and mortality

(Woo et al. 2002; Dulaimi 2010; Mousavi et al. 2011; Saha and Bandyopadhyay 2015; Wafer et al. 2015). In a severe infection, fish skin, fins, belly flaps, and head loaded with fish lice make the fish unpleasant or obnoxious, reducing consumer preference and marketability. This situation thus demands efficient antiparasitic drug or managerial measures to tackle argulosis for aquaculture sustainability.

19.4 Considerations for the Use of Antiparasitic Drugs in Aquaculture

The management and control of parasitic diseases in aquaculture are a constant challenge, highly complicated by the current limited availability of efficacious licensed products. Besides, parasite control in aquaculture requires a keen awareness of an environment, water quality, and host parameters. An effective antiparasitic agents development against *Argulus* is facing two great challenges: (1) the diversity of the parasites itself shows high adaptive potential to acquired resistance against exposed stable chemicals, and (2) the capability of the parasites to infect a wide range of fish species means for developing a commercial drug; its efficacy and toxicity must be tested on a number of *Argulus* and fish species to get the safe dose. Some of the critical factors considered for general decision-making to drug treatment include true parasite identification, selection of appropriate chemical, the delivery strategy employed, dosage and mechanism of action, and its impact on aquatic organisms and humans and the environment (Shinn and Bron 2012). Ideal antiparasitic drugs must fulfill the above all characteristics for its sustainable use; unfortunately, none of the methods has so far developed as an ideal parasiticide and still awaiting.

19.5 Various Methods of Combating Argulosis

19.5.1 Chemotherapy

The regular initial attempts to control parasitic infections in aquaculture tend to use chemical pesticides or insecticides (Padmavathi and Prasad 1998), either as oral medications or bath treatments (Costello 2006). The easy availability, lower dose, effectivity at single application mostly, and quick result appreciate the use of chemical-based drugs by the aquaculturists (Pathak et al. 2000; Dewi et al. 2018).

Organophosphates like malathion, parathion, cypermethrin, gammexane, avermectin, doramectin, ivermectin, sumithion, trichlorfon, emamectin benzoate, nuvan, DDVP (dichlorvos, 0,0-dimethyl-0-2,2-dichloro vinyl phosphate), etc. are suggested to be most active in encountering argulosis (Padmavathi and Prasad 1998; Toovey and Lyndon 2000). It has been observed that weekly 6 h immersion with the milbemycin oxime and lufenuron at 0.015 mg L^{-1} and 0.30 mg L^{-1} could effectively eradicate *Argulus* sp. from stingrays (Tang 2018). Another study suggested that the early infective stages of *Argulus* were killed on fish immersed in 0.01 mg L^{-1}

glacial acetic acid for 5 min (Singhal et al. 1986). Further, an in vitro assay showed 100% killing of *Argulus* sp. when treated with potassium permanganate, Neguvon, and formaldehyde solutions with 15 minutes of exposure to the experiment (Cardoso et al. 2020). Various chemicals like potassium permanganate, formalin, hydrogen peroxide, and sodium chloride are also used against *Argulus* infection (Singhal et al. 1986; Roth et al. 1993; Hakalahti et al. 2008). The drugs (organophosphates) generally block and inactivate acetylcholinesterase enzyme (neurotransmitter) (Table 19.1).

The treatment with avermectins increases the permeability of chloride ions in the neurons, inhibits synapses, and causes paralysis and subsequent death to the parasites (Arena et al. 1995). Organochlorines affect Na + ion channel activation

Table 19.1 Showing different chemicals and drugs used against the *Argulus* parasite along with its dose and mode of action

Sl. No.	Drugs/chemicals	Dosage	Mode of action	References
1	Trichlorfon	0.25 to 0.4 mg L^{-1}	Anticholinesterase act on the neuromuscular transmission	Tavares et al. (1999)
2	Emamectin benzoate	0–50 μg kg^{-1}	A GABA receptor binding cl^{-} channel activator	Hanson et al. (2011)
3	Dichlorvos	1.0 mg L^{-1}	Anticholinesterase act on the neuromuscular transmission	Tokşen (2006), Walker et al. (2004)
4	Gammexane	0.1–0.2 mg L^{-1}	Direct absorption of the chemical into the parasite and ova	Singhal et al. (1986)
5	Malathion	0.15–0.25 mg L^{-1}	Anticholinesterase act on the neuromuscular transmission	Rao et al. (1992)
6	Cypermethrin	0.005 mg L^{-1}	Na + ion channel modulator	Roth et al. (1993)
7	Pyrethrum	20–100 mg L^{-1}	Na^{+}-ion channel activators, neurotoxic	Kabata (1985)
8	Avermectin, doramectin and ivermectin	500–750 μg kg^{-1}	GABA receptor binding cl^{-} channel activator	Hemaprasanth et al. (2012)
9	Sumithion	0.1 mg L^{-1}	Neurotoxin (cholinesterase inhibitor)	Chowdhury et al. (2006)
10	$KmNO_4$	0.6 ml L^{-1}	Strong oxidizing action	Hakalahti et al. (2008)
	Formalin	0.01 g L^{-1}	Depletion of oxygen in water	
11	Sodium chloride (NaCl)	20–80 g L^{-1}	Osmoregulatory problem	Hakalahti et al. (2008)
				Dewi et al. (2018)

Table 19.2 Commercially available drugs against fish lice used in aquaculture industry

Drug/medicine	Parasite	Dose	Mode of action	References
Lufenuron and diflubenzuron	*Argulus* sp.	15 mg L^{-1}	Chitin inhibitors	Wolfe et al. (2001), Mayer et al. (2013)
SaniKoi Paratex	*Argulus* sp.	250 ml for 5000 liters of water	Parasiticidal effects	https://www.velda.com/pond-fish/diseases/argulus/
Taktic®amitraz (12.5% EC)	*Argulus* sp.	(0.0035 mg L^{-1})	Endocrine disrupters, reproductive failure	Pradesh (1999), Filazi and Yurdakok-Dikmen (2018)

and subsequent synaptic hyperactivity, but the organophosphates are acetylcholinesterase (AChE) inhibitors causing paralysis (Niesink et al. 1996). The synthetic pyrethroids, such as deltamethrin and cypermethrin, interact with the gamma-aminobutyric acid (GABA) receptors, affecting the sodium channels and nerve cells (Blagburn and Lindsay 1995). The rapid growth of resistance to the insecticide among *Argulus*-exposed populations has already been demonstrated (Lahav et al. 1962). These pesticides remain residues in the aquatic environment upon release and are subjected to dispersion and hydrolysis (Kumar et al. 2017); hence, their use is generally critically unsigned and discouraged (Piasecki and Avenant-Oldewage 2008). The other argulocidal drugs like diflubenzuron and lufenuron are less toxic to fish than the organophosphates and are 25,000 times more toxic to aquatic invertebrates than fish (Plumb 1999). The diflubenzuron and lufenuron act by inhibiting the synthesis, polymerization, and deposition of chitin and impairing the parasites developing from larvae to adults (Wolfe et al. 2001). Generally, *Argulus* infestation could be controlled by insecticides/chemical pesticides. Still, the long-term use may cause residue formation; create a potential for resistance development, potential threat to the aquatic invertebrate, and toxic effect on the aquatic animals and human health; and produce ecological disturbance and deleterious impact on the environment (Kabata 1985; Hakalahti et al. 2008; Hemaprasanth et al. 2012; Bahmani et al. 2014, Table 19.2).

Therefore, there is a call for new approaches with higher efficacy against parasites and safe, eco-friendly, and reliable treatment measures to replace chemical pesticides.

19.5.2 Nonchemical Approaches to Combat Argulosis

Though the use of chemotherapeutic drugs is invariably the first preference for the control of parasitic infections, a series of nonchemical approaches are implemented to minimize the probability of establishing or controlling the already established

disease. Aquaculturists had tended to use nonchemical methods only when chemotherapeutics were inadequate, unsafe for the host, or unavailable.

19.6 Physical Methods for Removing Parasite off the Host

The fish lice is a macro-ectoparasite comprised of egg, copepodid, juvenile, and adult stages, and all these life stages are well visible to the naked eye. Thus physical/mechanical methods of the removal can be applied. This method is the most common, easy, and comparatively cheap practice in dropping a load of *Argulus* parasite from the host and ponds and tanks. Techniques are adapted from the conventional handpicking of individual *Argulus* from the host fish body to interrupt the egg-laying mechanism which is probably one of the most effective, indigenous, and environmentally sound control measures. For example, farmers install bamboo poles/sticks and gunny bags (submerged) in ponds where fish can get rid of the parasite by rubbing their bodies against the substratum, and gravid female *Argulus* deposits the eggs on it. Hence, periodic removal of these poles and gunny bags and subsequent drying will kill the attached *Argulus* and the eggs deposited over them (Goswami et al. 2006; Harrison et al. 2006; Kumar et al. 2017). To control *Argulus*, remove the submerged vegetation; a wooden lattice placed in the pond will serve as an artificial substrate to deposit its eggs, which can be withdrawn at intervals to kill the eggs. It has been observed that the *Argulus* parasites prefer silt-free wooden surface over plastic, fine gravel and dark-colored over light-colored substratum (Hakalahti et al. 2004; Taylor et al. 2009; Sahoo et al. 2013a, b, c).

Furthermore, the egg-laying strategies of lice are influenced by the habitat usage of the host fish (Taylor et al. 2009) and were found to be maximum in the pond's middle zone, followed by the upper and lower zone (Sahoo et al. 2013a, b, c). This knowledge can be exploited for the parasite's control by providing the most preferred artificial substratum and strategically placing them in the host's suitable habitat zone to ultimately remove the laid eggs. Even though these controlling measures are eco-friendly and effective, it is laborious and dependent on parasite willingness to lay eggs on the substratum provided. The physical method may also include filtration, matting or ozonization, and reducing the temperature, and good fish husbandry practices result in significant removal of *Argulus* with minimal stress to the fish (Walker et al. 2004; Bandilla et al. 2005). Hoffman (1977) suggests that covering pond inlets with a 3.2-mm mesh could prevent the introduction of adult lice, although this may not be practical since filters may get blocked and will certainly require high maintenance.

Another effective physical method to get rid of the *Argulus* in which there is a complete drying out of the pond/tanks to destroy off deposited eggs is in most cases undefended. Ponds showing severe argulosis when drained, dried, and treated with lime at a concentration of 0.2 g L^{-1} for 2 days before refilling and stocking with fish could effectively reduce the prevalence and intensity of infestation with the parasites (Singhal et al. 1986). It has been observed that the mechanical removal of epibionts, which form a symbiotic relationship with the eggs of *Argulus* as a feeding ground,

and help develop the embryo of *Argulus* inside the egg to hatch (Banerjee et al. 2016) if removed, can directly affect the hatching percentage of eggs. An earlier worker suggests the mechanical treatment by shaking infected fish in a hand net was an effective means of detaching parasites from the fish and resulted in >80% decreases in parasite numbers. Applications of light traps to capture the phototactic stages of *Argulus* (newly hatched metanauplius), which lure towards the light, could be easily removed with a plankton net. Besides these all, just a handful of gravid females in the fish pond may represent enough reproductive ability to restart the parasite infection in the system, and the bet hedging strategies of the parasites ensure an extended infection period (Hakalahti and Valtonen 2003; Bandilla et al. 2005; Mikheev et al. 2007). Thus, relying only on physical remedial measures is not sufficient. There is a high risk of getting reinfection, so a combined physical and chemical approach is called for, provided cautious consideration to the environmental impact to get rid of argulosis is substantially needed (Kumar et al. 2017).

19.7 Effects of Manipulation in Water Quality Parameters on *Argulus* Load

Argulus is distributed in any part of the world and ecosystem because of their ability to adapt to different aquatic ecosystems, like extremes of temperature, pressure, salinity, and even anoxia. The quality of water used for pisciculture is the main factor used as a theoretical basis for the prevalence of *Argulus*. Thus argulosis prevention and control can be done to treat fish well by paying attention to the water quality suitable for fish. The previous study explained the existing correlations between high burdens of *Argulus at* low water clarity and slow stock turnover rates, but the high/low pH, ammonia, and DO level do not affect the prevalence (Taylor et al. 2009; Yunikasari and Mahasri 2020).

Several studies reveal the positive correlation between water quality and the prevalence of *Argulus*, especially temperature, which, when raised, will also increase the *Argulus* prevalence (Taylor et al. 2009; Yunikasari and Mahasri 2020). Bakshi et al. (2006) stated that June, July, and August were the most potential months for *Argulus* infestation. Water temperature has a direct influence on parasite maturation, egg-laying, embryo development, hatching rate, survival, their growth, and parasitizing the host fish, which gets fastened at increased temperature (>24 ° C) compared to the lower water temperature (Taylor et al. 2009; Hunt and cable 2020). At lower temperatures, there was a significant delay in egg hatching (>20 days), and a reduced egg hatching percentage (<30%) was obtained (Sahoo et al. 2013a). At high temperatures, besides the other developmental strategies, the parasites are likely to molt more frequently and complete their life more rapidly and ultimately; such results contribute to the prediction of population dynamics (Hunt and cable 2020), aiding the development of effective control by bringing down the temperature (optimal level) to improve animal health and reduce loss to the industry.

19.7.1 Immunoprophylaxis Method

Prevention is in the first line of disease management, and control of argulosis through vaccination seems an efficient and effective alternative. A successful development of immunoprophylaxis against fish lice depends on the proper identification of potential protective antigens. An approach towards developing a vaccine against *Argulus* using potential ribosomal protein P0 has resulted in only partial protection against *Argulus siamensis* in *Labeo rohita* (Kar et al. 2017). Further, intraperitoneal immunization of rohu with whole antigens of *A. siamensis* (50 μg protein/fish) and adjuvant (FCA) showed 35.42% of low-grade infection and 22.92% of high-grade infection as compared to 14.58% and 41.67%, respectively, in control fish. Additionally the immunized fish showed reduced hemorrhages on the body surface and higher antibody response than controls. The result indicates the possibility of vaccine development against this parasite for long-term protection (Das et al. 2018). The *Argulus* is an invertebrate that exhibits neuropeptides and peptide receptors for neuromuscular function and can serve as potential drug targets, not reported in vertebrates (Greenberg 2005).

The *Argulus* invasion could induce the high-level expressions of immunoglobulin types IgZ and IgD in skin and mucus of infected *L. rohita* to provide systemic as well as local protection in the host, which probably acts as an effective antigen for vaccine development against *A. siamensis*, which feeds on those tissues (Kar et al. 2015). Interestingly the researchers have identified and characterized salivary secretory/excretory proteins (SEPs) of *Argulus* parasite that are vital in establishing its biological function like attachment, feeding, and pathogenesis. In this regard, Ambuali et al. (2020) studied the SEPs of *A. foliaceous* comprised of transporters, peroxidases, metalloproteases, proteases, and serine protease inhibitor that participate in parasite immune evasion/induction (e.g., astacin), immunomodulation (e.g., serpin), and digestion (e.g., trypsin). This novel information will pave the way in identifying potential vaccine candidates or drug targets for the control strategies of argulosis more sustainably. Further, Nolan et al. (2000) found that the administration of cortisol reduced parasite establishment; this could be due to the vesicle synthesis in the epidermis, but it is unlikely that this could be the basis of a successful control method. Even so, vaccination is a promising alternative, but its cost of production, the parasite size and its complexity, multiple sites of attachment of parasite onto the host, identification of promising candidate antigens for vaccine development, and limited protection restrain its successful application.

19.7.2 Biological Approach

In the absence of effective chemical control methods and vaccines against this parasite, biological control could be a viable solution. The introduction of living organisms into the environment to control a target parasite to reduce clinical problems and economic losses is the key to biological control measures. Ni et al. (2010) found that the introduction of a predatory fish species Thai silver barb (TSB)

(*Pontius gonionotus)* could reduce *Argulus load* up to 96% in 24 h from goldfish. Similarly, the cleaner fish Ballan wrasse (*Labrus bergylta*) and lumpfish (*Cyclopterus lumpus*) are the most widely used sea lice control strategies in Atlantic salmon aquaculture (Brooker et al. 2018). Unfortunately, the explosive increase in parasitic infection could not be compensated by less fecund biological control agents (Pirali-Kheirabadi 2012). Biological control has always been a popular concept; still, its success has never lived up to the expectation. It needs a deep understanding of parasite biology, host-parasite interaction, and related consequences, thus requiring a long time, patience, and effort with comparatively less accessibility to the farmers. In this regard the epidemiological studies have made a considerable contribution to the health of the wild and farmed aquatic animals through improved biosecurity and surveillance in control of diseases (Peeler and Taylor 2011; Kumar et al. 2017). It can be anticipated that the extensive usage of such studies may render reduced incidence and impact of argulosis in the aquaculture industry.

19.7.3 Immunological Interventions in Combating Argulosis

It has been found that the parasite *Argulus* significantly stimulates the immunological parameters of the host by acting as foreign antigenic agents. For example, the mucus of *A. siamensis*-infected fish showed significant upregulation of interleukins (IL 6, IL 15, and IL 1β), toll-like receptor 22, pathogen presentation, and β2 microglobulin, with the increased expression of lysozyme G than the control fish (Parida et al. 2018). A study conducted by Devi et al. (2020) demonstrated that the rohu immune system was strongly affected by *A. siamensis* infection. But, oral administration of the madecassic acid (a triterpenoid bioactive compound) enriched diet at 5 mg kg^{-1} could significantly modulate in both innate-adaptive immune response and immune cytokine genes expression and reduce/prevent the prevalence and intensity of infection. The madecassic acid is comparatively cheaper and proven more beneficial and economical for preventing argulosis in aquaculture (Table 19.3).

19.8 Phytotherapeutics against *Argulus*

19.8.1 Herbal Crude Extracts

To overcome the issues which arose from using chemotherapeutics/drugs and chemicals, physical removal, (Integrated pest management) IPM, biological control, and vaccination methods, we are forced to think of a viable alternative option. Medicinal plants are among the most interesting options as it is a cheaper, safer, and eco-friendly replacement for chemotherapeutic products (Pereira et al. 2020). Additionally, some plant-based natural pesticides are more effective, and the degradation rate for herbal drugs is much faster than synthetic pesticides (Prakash and Rao 1996; Duso et al. 2008). The plants are rich sources of biochemical compounds like

Table 19.3 Various plant extracts used for the treatment of *Argulus* parasites

Sl. No.	Plant species	Solvents	Host fish and stage of *Argulus* parasite	Dosage g/L	Phytoconstituents and its mode of action	References
1	*Morinda citrifolia* (noni fruits)	Aqueous	*C. auratus auratus (Argulus sp.)*	3.5% of the volume of water for 15 min/ per day	Scopoletin a phytochemical inhibits the smooth muscles and nerve functions, increases serotonin production and its accumulation, and causes vasoconstriction of the heart and brain membranes that inhibits the nutritional intake of *Argulus* which loses its grip on the host	Setyaningsih and Subekti (2019)
2	*Azadirachta indica* leaf	Aqueous	*C. auratus* (*A. japonicus*)	3–3.5 g L^{-1}	Terpenoids have argulocidal effects	Kumari et al. (2019)
3	*A. indica* leaf	Aqueous	In vitro: Eggs and adult *Argulus* sp.	250 mg L^{-1}	Phytoconstituents penetrate inside and impair with the embryo development and kill the parasite	Banerjee et al. (2014)
4	Azadirachtin	Bioactive compound of *A. indica*	*C. auratus* adult *Argulus*	15 ppm	Affect molting, reproductive hormones, and acetylcholine esterase enzyme and interfere with the dividing cells to block microtubule formation	Kumar et al. (2012a, b), Sharma (2016) (thesis)
5	*Moringa oleifera* leaf	Aqueous	*C. auratus* (*A. japonicus eggs*)	8% concentration	Phytochemicals (tannins, saponins, alkaloids, and flavonoids) scrape mucus, cause wrinkled surface and fluid leakage, interfere with the egg permeability, cause imbalanced hydromineral content, impair embryo development, and decay the eggs	Idris and Mahasri (2020)
6	*Carica papaya seeds*	Aqueous	*Eggs of A. japonicus*	100 g L^{-1}	Papaya seeds contain saponins, tannins, flavonoids, and alkaloids which act as a natural insecticide	Kismiyati et al. (2015)

7	*Carica papaya* leaf	96% ethanol solution	Adult *Argulus sp.*	6 g L^{-1}	Karpain binds Na + ions to the nerves and suppresses the central nervous system. *Argulus* nerves relate to a sucker and when pressed cannot deliver nerve impulses to the sucker, resulting in *Argulus* being unable to infest host	Nur (2002), Azizah and Fasya (2019)
8	*Ocimum gratissimum*	Aqueous	*Oreochromis niloticus* adult *Argulus*	In vivo: 1271 mg L^{-1}	Eugenol, thymol, and geraniol have an argulocidal effects	Mamadou et al. (2013)
9	Banana plant stems	Aqueous	*Cyprinus carpio* (*Argulus* sp.)	In vivo 1500 mg L^{-1}	Alkaloids of the extract can weaken the *Argulus* nervous system	Pricilia et al. (2017)
10	*Coffea canephora*	Aqueous	*Cyprinus carpio Adult A. japonicus*	1600 mg L^{-1} for 30 min	Caffeine (alkaloids) can inhibit the working system of the enzyme acetylcholinesterase, damage the CNS, and cause organ paralysis	Afifah and Kenconojati (2020)
11	*Curcuma longa* Kunyit	–	*Cyprinus carpio* (*Argulus indicus*)	0.7 g L^{-1}	Phenol and curcuminoid (bioactive compound) have antiparasitic effects	Rahmi (2003)
12	*Nerium oleander*	Ethyl alcohol	Ornamental fish (adult *Argulus foliaceus*)	In vitro test: 0.5 g/ 5 mL for 62 min	Neriin, oleandrin, and cardenolides (flavonoids and alkaloids) interfere with insect metabolism and reduce digestibility	Al-zayyadi (2019)
13	*Cymbopogon citratus*	Essential oil	*Schizodon fasciatus* (*Argulus* sp.)	140 μg/L for 24 h	Geranial and neral monoterpenes have antiparasitic activity	Pereira et al. (2020)
14	Tobacco leaf	Aqueous	Eggs and adult of *Argulus* sp.	8 mg/L for 18 day	Nicotine penetrates the egg membrane, and a synaptic transmission blocker affects the ganglia functioning and kills the parasite	Banerjee and Saha (2013)
15	Piperine	Commercial	*C. auratus (adult Argulus)*	9.0 mgL^{-1}	Piperine an amide group can exert argulocidal activities	Kumar et al. (2012a, b)

(continued)

Table 19.3 (continued)

Sl. No.	Plant species	Solvents	Host fish and stage of *Argulus* parasite	Dosage g/L	Phytoconstituents and its mode of action	References
16	Nootkatone from citrus fruits	Commercial	Adult *Argulus*	300 mgL^{-1}	Inhibition of acetylcholinesterase enzyme activity	Anderson and Coats (2012), Goldsmith (2017)
17	*Allium sativum* (garlic)	Aqueous	*Cyprinus carpio* (*Argulus* sp.)	At 12% volume of water for 8.12 minutes	Allicin organosulfur compound is a chitin inhibitor	Wijayanto et al. (2013)

alkaloids, flavonoids, terpenoids, tannins, and saponins, known as the underlying reasons for the broad mechanism of action against microbes and parasites (Wink 2015). Alkaloids are polar compounds that can weaken the parasitic nervous system by affecting the acetylcholinesterase enzyme and cause organ paralysis (Patil et al. 2020) and also disrupt the peptidoglycan constituent of the parasitic cells (Idris and Mahasri 2020). Saponins, triterpenoid, and flavonoids have been suggested to be more potent in disrupting the egg membrane's permeability that causes cell dehydration, ultimately leading to parasitic death (Sari 2014; Kismiyati et al. 2015). Flavonoid compounds could attack nerve parts of many vital organs, leading to nerve weakness. The maxilla, which helps in firm attachment of *Argulus* with the host upon flavonoid contact, can cause paralysis in the maxilla, thereby lose their inherent ability to attach with the host so that they couldn't stick in the fish body. The terpenoids cause the deficiency of dissolved oxygen in water, which may cause the death of eggs, larvae, and adult parasites (Mordue and Nisbet 2000). Recently, phytotherapeutants have been documented as most efficient method in combating with *Argulus* infections. Earlier researchers have reported that the crude aqueous extract of neem leaf (Kumari et al. 2019), and basil extract (Mamadou et al. 2013) is very useful in killing the *Argulus* parasite. Thus, the central focus should be on phytotherapeutic agents to treat different life stages of *Argulus* under in vitro and in vivo conditions. It has been observed that treatment with lime, neem, and copaiba extract could result in 100%, 100%, and 80% killing of *Argulus*, respectively, at 3 h exposure (Cardoso et al. 2020).

In another study, it was found that the exposure of *A. bengalensis* to *Azadirachta indica* extract could reduce the number of eggs per oviposition, declined egg hatching percentage, and markedly affected embryonic development (Banerjee et al. 2014). Sahoo et al. 2019 revealed that the neem leaf extracts could significantly upregulate the expression of ion channel genes such as GABA, ICA-3 (ion channel activator protein 1–4), and NTR (neurotransmitters proteins) of *Argulus siamensis* than the other herbal and antiparasitic drugs thus can be targeted to develop a drug against *Argulus*. It is clear from the studies that the herbal extracts also act on the parasite either by activation of GABA or ion channels, as is the case in ivermectin or a macrocyclic lactone, thus leading to an influx of chloride ions into cells and paralytic death of parasites by hyperpolarization of nerve endings (Sahoo et al. 2019). Since, the bioactive metabolites produced by the medicinal plants may vary in quantity, quality, and composition according to the climate, soil composition, time of collection, part of the plant collected, age, and stage of the plant cycle (Gonzales et al. 2020; Santos et al. 2009) yet researchers are trying to overcome these problem to formulate a commercial product. Although, the broad mechanism of action herbal crude extracts against adults and larval stages of *Argulus sp.*, is anticipated the low purity, varying composition, lack of standardized extraction protocols and purification methods, large dose requirement limits its wide usage and thus need a pervasive study for commercialization and popularization of the plant product (Kumar et al. 2012a, b; Mosihuzzaman and Choudhary 2008). Thus, the antiparasitic efficacy of

the crude extracts of the plant is not constant all around the year of the experimentation. Therefore, it warrants the use of purified bioactive compounds which are more stable in its consistency and efficiency.

19.8.2 The Bioactive Compound of Plants and its Argulocidal Effects

In the current situation where most of the management strategies are not reaching up to the desired level for effective eradication of argulosis from aquaculture, the intervention of the application of pure bioactive compounds of plants must be a suitable solution to aquaculturists (Wunderlich et al. 2017). The work in this area has already been started; for example, active herbal biomolecules, such as azadirachtin (15 ppm) and piperine (9 ppm), has shown argulocidal effects in *Carassius auratus* (Kumar et al. 2012a, b, 2013). Similarly, tobacco leaf's rotenone and nicotine are bioactive compounds used as biphasic control agents to combat with argulosis (Banerjee and Saha 2013). Also, the nootkatone, a bioactive compound of citrus fruits when directly sprinkled or poured at 300 ppm showed the effective killing of *Argulus* (Goldsmith 2017) by inhibiting the acetylcholinesterase enzyme activity (Anderson and Coats 2012). In another study, it was observed that the treatments with the *Cymbopogon citratus* essential oil at 140 μg/L for 24 h could notably reduce the oviposition and egg hatching and uplift the killing of *Argulus* parasite. This treatment could cause extensive structural alterations in the eyes (ommatidium and rhabdomeres) that affect the appearance of visual and nervous problems in *Argulus* sp., subsequently resulting in death (Pereira et al. 2020). An attempt to treat the *Argulus* parasite using chlorophyllin photodynamic substances exposed to darkness and simulated solar radiation did not show the remarkable result but showed 100% killing of other parasites (Hader et al. 2016). Hence, it is understood that plant bioactive compounds are promising antiparasitic agents that kill the parasite by modulating several mechanisms of action; however, the method of purification is very tedious and expensive (Kumar et al. 2012a, b). It also requires large quantity of raw materials to produce a meager quantity of the pure product and therefore not affordable by the big aquaculturists and again seeking for an economically feasible and effective methods to combat with the disease argulosis.

19.8.3 Integrated Pest (Parasite) Management (IPM) Method

The central goal of IPM is to combine all the available preventive and curative methods to minimize the impact of pathogens in the production system and on the environment and avoid future side effects (Sitja-Bobadilla and Oidtmann 2017). IPM strategies do not discard some chemicals when necessary but seek to find alternative approaches to minimize their use and its application. Hakalahti et al. (2008) employed IPM strategy either to minimize the probability of *Argulus* establishment or to control the established argulosis which involved prevention of infections, monitoring infection levels, using thresholds for action, and finally

implementing multiple management tactics (chemical, physical, and biological control) for complete eradication of parasite. These methods may include the aquaculture production site, the source and water flow through the system, the quality stock and feed, husbandry practices, implementation of a veterinary health plan, adherence to biosecurity codes of practice, and the use of physical, biological, and mechanical interventions, where appropriate.

The earlier authors note that "diversity is the spice" and suggested that we farmers should exploit the maximum diversity of control measures available for control of sea louse in the aquaculture industry (Brooks 2009). If followed in the control of argulosis, it will probably bring a remarkable breakthrough in mitigating the *Argulus* in the fish farms.

- Monitor fish lice on farmed fish.
- Mechanical removal of lice from the fish body by shaking them in a net if mild infection.
- Avoid unnecessary use of anti-*Argulus* agents.
- Feed the fishes with balanced diets to build their healthy immunity and thereby increase their capability to resist the infection with the parasites.
- Keep nets clean to increase water flow and minimize retention of lice larvae.
- Remove moribund fish, slow swimmers, and runts on a frequent basis.
- Use cleaner fish, such as wrasse when they are available.
- Use chemotherapeutants at appropriate times and in accordance with the label.
- Synchronize control strategies through the use of area-wide management plans.
- Rotate the use of chemicals, drugs, vaccination, and herbal-based drugs having different modes of action.
- Monitor the efficacy of treatments.

Though the IPMS methods are economically viable and sustainable management methods, the strategy is multidisciplinary and demands specific knowledge of the environment, the parasites, and the hosts (Brooks 2009).

19.8.4 How to Prevent Fish Lice Reinfection

First and foremost, improving water quality is perhaps crucial to prevent fish lice from being established in the fish pond or tank. By performing regular water changes, keep the water well filtered (fish lice prefer water with high organic load) and aerate properly so that DO, pH levels, temperature, and all other water quality parameters are maintained at optimum daily. Also, provide quality feed so that fish immune systems are healthy and better able to fend off parasites and secondary infections. New stock of fish must be quarantined first for several days to ensure fish is healthy or not as well to confer a carrier of any infectious agent; in the case of infected fish, before releasing them to the pond/tank, they must be treated with disinfectants like $KMnO_4$ and sodium chloride to kill the fungus or parasites and

prevent its spreading to the entire pond. Isolating the infected fish as soon as possible from the healthy fish may prevent the infection from breaking out.

19.9 Conclusions and Future Prospective

Argulosis causes a potentially rapid escalation of infection, causing substantial economic loss to the aquaculture industry worldwide. Current approaches to control this parasite using chemicals and pesticides have numerous drawbacks and necessitate an effective alternative eco-friendly and economically feasible methods to treat *Argulus* infection. In this aspect, plant-based products are regarded as novel candidates to reverse this negative trend and assure better fish health management. Still, the concerning issues innervate its substantial application in the aquaculture system. Despite several studies, argulosis remains a major concern in aquaculture sector due to the non-availability of any permanent control measures that drive the researcher's mind to think of some novel advanced control strategies against *Argulus*. Since control of fish parasites in nature is seldom possible, present knowledge warrants the development of an effective drug based on the potent targets identified in recent years, employing an epidemiological approach. Interventions of nanoparticles as a potent argulocidal drug in aquaculture may be possible in the future, but still, at present, there appears to be little progress towards such a measure.

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Metagenomic Approaches to Identify Fish Gut Microbiome and the Effect of Prebiotic Supplements on Gut Microbes and Health Management

20

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Abstract

Aquaculture is one of the important food-producing sectors, growing rapidly at a pace in terms of production and productivity. However, incidence of disease outbreak decides the fate of production and productivity of the farm produce. In addition to this, many intrinsic and extrinsic factors also influence the aquaculture production. Feed and feed supplements used in the aqua feed industry are ecologically safe and viable alternatives that could reduce the immunosuppression and disease outbreaks. Prebiotics, probiotics, and synbiotics are commonly used as immunomodulatory agents for the improvement of health of aquatic organisms. Probiotics are the microbes that has beneficial effect on health, and prebiotics are the saccharides which enhances the gut microbiota and resulting in the health of the host. Knowledge on usage of prebiotics is in infancy stage, and different types of prebiotics like inulin, mannanoligosaccharides (MOS), fructooligosaccharides (FOS), and other oligosaccharides are known to improve the immune response of the fish and shellfish. It also has positive effect on the improvement of microbiome and results in the growth and disease resistance. In this chapter, attention has been focused on the metagenomic approaches to decipher gut microbiome in relation to the different prebiotics in fish and shellfish.

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20.1 Introduction

The microbial assembly in the gastrointestinal tract of fish is termed as gut microbiota. Gut microbiota is an indispensable part of the host that enhances the growth, affects the physiology, increases immunity, and partly protects the host against pathogens (Nie et al. 2017). As gut microbiota plays a pivotal role in the fitness of the host, it is necessary to understand the diversity of these microorganisms. Until recently, investigations carried out to recognize the microbiota of the GI tract of fish utilized the conventional culture-based approaches. But the culture-based method presents a number of disadvantages particularly for the cultivation of fastidious and obligate anaerobes since the growth of microorganisms depends on the culture media and culture conditions used (Hiergeist et al. 2015). Moreover, these methods are time-consuming and require expertise for the identification of the microorganisms. The lack of cultivability of the bulk of the commensal microbiota necessitated the search for culture-independent and accurate methods. The use of modern molecular approaches has provided an edge over the conventional methods in appreciating the fish gut microbiota that provides a positive benefit to the host. Metagenomics is one such molecular technique that has helped in the identification of gut microorganisms and also has facilitated the establishment of the phylogeny of the community members (Tarnecki et al. 2017). The field of metagenomics has revolutionized the investigations involving gut microbiota wherein both qualitative and quantitative constructs of the microbiota representing cultivable and non-cultivable ones can be obtained. By employing metagenomic approaches, a plethora of studies has reported the diversity of gut microflora in fish. Eventually, the objective of these studies is to contribute towards the scientific knowledge for evolving effective strategies for modulating the gut microbiota to encourage animal health and enhance productivity.

A drastic rise in the demand for fish food had warranted the use of antibiotics to increase the growth rate in fish. The excessive use of antibiotics amplified the problem of drug resistance in bacteria, caused suppression of the immune system and destruction of microbial populations in aquatic animals, and increased the presence of antibiotic residues in food. Thus, the use of antibiotics in aquaculture has been criticized during the last two decades. As an alternative approach to minimize the use of antibiotics, to ensure the growth of fish farming industry, and to satisfy the consumers' needs, prebiotics has received considerable attention from the scientific community in recent years. Prebiotics, used as dietary supplements, manipulate the conditions that favour the growth of certain gut microbiota which can further enhance the feed efficiency, abate disease susceptibility, and also modulate the immune responses in the host (Chen et al. 2020).

In this chapter, we provide insights on the gut microbiota of fish with an emphasis on their functions in the host, factors responsible for their fluctuation, metagenomic approaches for identification, and prebiotics as a strategy for modulation of gut microflora in enhancing the growth efficiency and improving immune system of the host.

20.2 Fish Gut Microbiota

Microbiota is defined as the assembly of microorganisms that reside in a particular environment (Burokas et al. 2015). The microbiota includes commensals, symbionts, and also pathogens (Sandrini et al. 2015). Of the different niches occupied by these microorganisms, the digestive tract ranks first with respect to the number and types of microorganisms. Gut microbiota encompasses those microorganisms that are positioned in the intestine. The symbiotic microbiota that lives in the gut provides protective, metabolic, and structural functions to the host, and the host, in turn, provides a nutrient-rich environment for the microbiota (Tarnecki et al. 2017). The microbiota of the gut plays a vital role in altering the physiology of the host (Feng et al. 2018). As the gut microbiota portrays an important role in the intestinal development and physiology along with an influence on the growth and health of the host, it is often referred to as an "extra organ" (Gonçalves and Gallardo-Escarate 2017).

The microbiota in the gut can be transient (allochthonous) or resident (autochthonous) depending on the length of their stay (Banerjee and Ray 2017a). Resident microorganisms are lifelong members found in the intestine where they share a symbiotic relationship with the host. Transient ones enter the gastrointestinal tract along with food where they cannot stay for a longer period of time due to the presence of the resident microflora (Zhang et al. 2016). Various studies have been conducted to understand the gut microbiota of fish (Table 20.1).

Table 20.1 Studies on fish gut microbiota

Fish species	Reference
Zebrafish (*Danio rerio*)	Cantas et al. (2012), Russo et al. (2015)
Atlantic salmon (*Salmo salar*)	Cantas et al. (2011), Green et al. (2013)
Atlantic cod (*Gadus morhua*)	Ringo et al. (2006a), Star et al. (2013)
Common carp (*Cyprinus carpio*)	Kuhlwein et al. (2013), Ye et al. (2014)
Rainbow trout (*Oncorhynchus mykiss*)	Mansfield et al. (2010), Navarrete et al. (2012)
Reef fish (*A. nigricans*, *Lutjanus bohar*)	Smriga et al. (2010)
Guppy (*Poecilia reticulata*)	Sullam et al. (2015)
African cichlid (*A. burtoni*)	Baldo et al. (2011)
River trout (*Salmo trutta fario*)	Skrodenyte-Arbaciauskiene et al. (2006)
Catfish (*Silurus asotus*)	Di Maiuta et al. (2013)

Table 20.2 Diversity of bacteria found in fish gut

Fish species	Bacteria	References
Atlantic cod (*Gadus morhua*)	*Clostridium perfringens*, *vibrio* sp.	Star et al. (2013)
Cardinalfish (Apogonidae) Damselfish (Pomacentridae)	*Pseudomonas*, *Alteromonas*, *Psychrobacter*, *Shewanella* sp., *Endozoicomonas* sp., *Vibrionaceae*, *Pasteurellaceae*, *Vibrio harveyi*	Parris et al. (2016)
Parrotfish (*Chlorurus sordidus*) Surgeonfish (*Acanthurus* sp.)	*Proteobacteria*, *vibrio* sp., *photobacterium*, *Faecalibacterium*, *Bacteroidetes*, *Enterovibrio*, *Desulfovibrio*, non-vibrio *firmicutes*, *clostridium* sp.	Miyake et al. (2015)
Atlantic halibut (*Hippoglossus Hippoglossus*) Atlantic salmon (*Salmo salar*)	*Lactobacillus* sp., *vibrio* sp., *A. junii*, *mycoplasma*, *Lactococcus* sp., *photobacterium*, *bacillus* sp.	Verner-Jeffreys et al. (2003) Holben et al. (2002)
Butterfish (*Odax pullus*) Marblefish (*A. arctidens*)	*Papillibacter*, *Cinnaminovorans*, *clostridium* sp., *Eubacterium desmolans*	Clements et al. (2007)
Sea trout (*Salmo trutta trutta*)	*Pseudomonas*, *A. sobria*	Skrodenyte-Arbaciauskiene et al. (2008)
Pinfish (*Lagodon rhomboides*)	*Corynebacterium*, *clostridium*, *pseudomonas*, *mycoplasma*, *photobacterium*, *Staphylococcus*, *Propionibacterium*	Givens et al. (2015)
Southern flounder (*Paralichthys lethostigma*)	*Clostridium*, *photobacterium*	Ransom (2008)

Microorganisms found in the fish gut are diverse, including bacteria, archaea, yeasts, and viruses (Merrifield and Rodiles 2015). Although there is diversity in the fish gut microbiota found in various species, several phyla of bacteria predominate the gut. Ninety percent of the fish intestinal microflora is dominated by members belonging to phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* (Eichmiller et al. 2016). Bacteria the predominating group of microorganisms found in the fish intestine (Rombout et al. 2011) is the primary focus of research thus far (Table 20.2).

20.3 Functions of Gut Microbiota

Commensal microorganisms of the gut are generally recognized to serve a multitude of functions. They aid in digestion, protect against diseases, and help in the development of the mucosal system (Ringo et al. 2007). A study by Rawls et al. (2004)

illustrated the role of gut microbiota in the regulation of 212 genes related to nutrient metabolism, immune response, and epithelial proliferation.

20.3.1 Digestion

Various studies have indicated the key role of GI microbiota in digestion. Cellulase, amylase, chitinase, protease, and phytase produced by gut bacteria might play a significant role in digestion (Ray et al. 2012). It has been proved that any change in the composition of microbiota alters the metabolic pathways of carbohydrates, amino acids, and lipids (Ni et al. 2014). The isolation or 16S rRNA characterization of cellulolytic bacteria from the gut of herbivorous fish signifies the role of these bacteria in the digestion of plant material (Wu et al. 2012). In vitro studies on the loricariid catfishes have also shown the gastrointestinal cellulolysis by gut bacteria (Nonogaki et al. 2007). Gut microbiota has been shown to regulate fat storage. Gut bacteria have been demonstrated to be responsible for the stimulation of fatty acid uptake in the intestinal epithelium (Semova et al. 2012). The mechanism of intestinal microorganisms regulating fat storage has been established in the zebrafish model (Camp et al. 2012). The suppression of the inhibitor of lipoprotein lipase, angiopoietin-like 4, in the intestinal epithelium was observed upon colonization with microorganisms. The wood-eating catfish *Panaque nigrolineatus* assimilates the biofilm associated with submerged wood. The organisms responsible for the cellulolytic activity are derived from the surface of wood which becomes a part of the microbiota (German and Bittong 2009). Marine herbivorous fish species with high intestinal short-chain fatty acid concentrations depend on gut microorganisms to assimilate algal components such as mannitol (White et al. 2010). In freshwater omnivorous tilapia and herbivorous marine turtles, gut microbiota has been suggested to contribute to host protein metabolism (Newsome et al. 2011; Arthur et al. 2014). In grass carp (*Ctenopharyngodon idella*), many biosyntheses and metabolism pathways of carbohydrates, amino acids, and lipids change as the composition of microbiota changes.

20.3.2 Anti-Pathogenic Effect

The indigenous microbiota of gut produces various inhibitory substances that inhibit or kill the pathogens (Teplitski et al. 2009). Lactic acid bacteria belonging to *Firmicutes* are one of the predominant bacterial genera colonizing the fish gut. They produce growth inhibitory substances such as bacteriocins, diacyls, and hydrogen peroxide that prevent the proliferation of pathogenic species in the gut (Li et al. 2018). The genera of *Lactobacillus*, *Streptococcus*, *Carnobacterium*, *Oenococcus*, *Leuconostoc*, and *Lactococcus* produce a variety of bacteriocins (Ringø et al. 2016). There are several reports of lactic acid bacteria isolated from fish gut possessing antagonistic activity against fish pathogens causing streptococcosis, peduncle disease, furunculosis, and columnaris (Sahoo et al. 2016; Banerjee and Ray 2017b). A

strain known as BTSS-3 was isolated from the gut of deep sea shark which possessed antibacterial activity against various pathogenic bacteria. The antagonistic activity of BTSS-3 was attributed to the elaboration of bacteriocins (Bindiya et al. 2015). *Lactococcus lactis* TW34, a bacteriocin-producing bacteria, was isolated from a marine fish gut that possessed antagonistic potential against *Lactococcus garvieae* (Sequeiros et al. 2015).

20.3.3 Immunity

Although the mechanism for colonization resistance is not clear, there are speculations that the commensal bacterial community competes with the invaders for niche and the antimicrobial peptides that they secrete provide protection against pathogens (Kim et al. 2017). The gut microflora plays a pivotal role in the development and maturation of gut-associated lymphoid tissue that mediates several host immune functions (Wang et al. 2018). Any disturbance in the gut microbiota makes the host prone to infections. Researchers have shown the influence of gut microbiota in increasing immunity in the host. Colonization of the germ-free zebrafish with commensal microflora induces transcriptional activation of NF kappaB (Kanther et al. 2011). Commensals in the newly hatched zebrafish boost the production of neutrophils and activate genes encoding proinflammatory and antiviral mediators thus increasing the resistance of larvae to viral infections (Galindo-Villegas et al. 2012).

20.3.4 Epithelial Renewal

Stimulation of intestinal cell proliferation by the gut microbiota has been demonstrated in the gnotobiotic zebrafish (Rawls et al. 2004). The activation and stability of β-catenin in the intestinal epithelial cells by the gut microflora promotes cell proliferation (Cheesman et al. 2011). The gnotobiotic zebrafish exhibited incomplete development with impaired functions, which was reversed with the inoculation of bacteria (Lescak and Milligan-Myhre 2017).

20.3.5 Reproduction

Gut microbiota contributes to the development of gonads and successive reproduction of the fish. *Lactobacillus rhamnosus* when administered continuously from birth to sexual maturation changes the gut microbiota and increases the larval development of zebrafish and also aids in sex differentiation (Carnevali et al. 2013).

20.3.6 Stress Response

Gut microbiota counteracts with stress response, anxiety, in particular, that can in turn affect the feeding habit and energy homeostasis. The mechanism for this behaviour may be attributed to the lowering of corticotrophin-releasing hormone and cortisol by the gut microflora. To substantiate this, studies were carried out in *Carassius auratus* (de Pedro et al. 1997) and *Oncorhynchus mykiss* (Ortega et al. 2013) by disrupting the gut microbiota. The studies revealed an increase in the level of stress hormones that resulted in a decrease in the feeding ability.

20.4 Factors Affecting the Gut Microbiota

Both intrinsic (gender, age, nutritional status, genetics, immunity, etc.) and extrinsic factors (environmental) factors affect the gut microbiota (Stephens et al. 2016). These factors dictate the composition as well as the functions of microorganisms. Various factors that influence the diversity of microorganisms in the fish gut have been studied in detail by researchers: diet (Cordero et al. 2015), habitat (Bano et al. 2007), trophic level (Clements et al. 2007), life stage (Hansen and Olafsen 1999), season (Hovda et al. 2012), phylogeny (Miyake et al. 2015), and sex (Dhanasiri et al. 2011). Of the several factors, habitat, trophic level, and phylogeny of the host most likely influence the diversity of microorganisms (Sullam et al. 2012).

20.4.1 Extrinsic Factors

Fish embryos (within the egg or the mother) develop in a microbe-free environment and thus are free from microorganisms at the time of hatching. It acquires microbes from its surroundings after hatching (Li et al. 2017). Thus, environmental factors significantly influence the acquisition of microorganisms which in turn reflect on the gut microflora.

20.4.1.1 Influence of Water

Water influences the gut microflora of freshwater and marine fish. A study by Navarrete et al. (2009) and Vatsos (2016) reported the predominance of *Pseudomonas* and *Aeromonas* in the gut of freshwater fish. Vibrios were the most common genus to be found in marine fish (Vatsos 2016). Water temperature and salinity are the two important factors that affect the gut microbiota. Water temperature has been shown to influence the intestinal lactic acid bacteria composition (Hagi et al. 2004). An increase in temperature generally increases microbial growth (Neuman et al. 2016). Schmidt et al. (2015) revealed the variation in the diversity of bacteria with an increase in salinity. The fluctuation in the microbiota in different seasons is due to variation in the nutrient content in water and alters the food consumption (Al-Harbi et al. 2004).

20.4.1.2 Rearing Condition

The composition of the gut microbiota also depends on the rearing conditions. A study by Dehler et al. (2017) has revealed a significant difference in the gut microbial community of Atlantic salmon fish held in two different conditions (indoor recirculating aquarium and cage culture in an open freshwater).

20.4.1.3 Pollutants and Toxins

Environmental pollutants and toxins also have a profound effect on the gut microbiota. A disturbance in the intestinal microflora was observed in zebrafish exposed to polystyrene microparticles (Jin et al. 2018) and common carp exposed to waterborne copper (Meng et al. 2018). A change in the gut microflora increases the susceptibility of the fish to pathogens. Furthermore, other pollutants such as heavy metals, antibiotics, and pesticides also foster inflammation of the intestinal layer, thereby decreasing the efficiency to absorb nutrients (Giri et al. 2018; Zhou et al. 2018; Kan et al. 2015).

20.4.2 Intrinsic Factors

20.4.2.1 Genetics

Based on the genetic variations, both intra- and interspecific variations in gut microbiota are found in fish. Although related species are exposed to the same environment, differences between species are observed in relation to their gut microbiota. Intraspecies differences in the gut microbial communities are also noticed (Li et al. 2012).

20.4.2.2 Sex

Difference between the gut microflora has been observed in fish of different sex. For example, variation in the gut microbiota has been found in Eurasian perch and three spine stickleback (Bolnick et al. 2014). Contrary to this there exists no difference in the gut microorganisms in zebrafish (Liu et al. 2016). The reason behind such differences is yet to be understood.

20.4.2.3 Age

Differences in gut microbiota have been observed in young and adult fish. Zebrafish adults have lower bacterial richness than the juveniles. This decrease could be due to the increase in the sex hormone and the development of gut-associated lymphoid tissue (Cantas et al. 2012). In Atlantic salmon, the gut composition varies between embryonic stages and hatchlings (Lokesh et al. 2018). Modifications in the diet could also be one of the possible reasons for the difference between juvenile and mature fish.

20.4.2.4 Diet

A growing body of evidence has demonstrated a strong influence of diet on the fish gut microbiota (Sullam et al. 2012; Ye et al. 2014). Although the fish gut is colonized

by microorganisms at an early stage, the diet type can lead the way in a different direction. The gut microbiota of rainbow trout was dominated by *Firmicutes* when fed with plant source oils, and *Proteobacteria* were prevalent when fed with fish oil (Desai et al. 2012; Ingerslev et al. 2014; Gatesoupe et al. 2018). Nutrient depletion in the environment also induces change in the gut microbiota which favours those bacterial species that utilize diverse energy source and are capable of surviving under limited nutrient conditions (Vatsos 2016).

20.4.2.5 Feeding Habit

Gut microbiota is also influenced by the feeding habit of the fish. It has been demonstrated that the microbial diversity increases in the order of carnivores, omnivores, and herbivores (Larsen et al. 2014; Miyake et al. 2015). In the same rearing environment, herbivorous grass carp harboured a greater number of bacterial species than the omnivorous gibel carp and carnivorous black carp (He et al. 2013). Herbivorous fish were found to be with more of cellulose-degrading bacteria such as *Citrobacter*, *Clostridium*, and *Leptotrichia*, and carnivorous fish were found to be with more of *Halomonas* and *Cetobacterium* (Liu et al. 2016). This trend is similar in both freshwater and marine fish (Egerton et al. 2018).

20.5 Prebiotics in Aquaculture

The demand for food fish has increased exponentially worldwide. According to the United Nations Food and Agriculture Organization, by 2030, two-thirds of the world's seafood intake will depend on fish raised in farms (Thorpe et al. 2018). To suffice the market needs, antibiotics were used excessively to treat the diseases and to increase the stocking density of fish. This resulted in an increase in antibiotic resistance in bacteria, the presence of antibiotic residues in food, and suppression of the immune system in aquatic animals (Sapkota et al. 2008). In most of the countries, the antibiotics were either restricted or banned in aquaculture as a preventive measure to stop the spread of antibiotic resistance. Banning antibiotics for fish production and increased awareness among the public for healthy practises in fish production led to a rise in the use of potential functional feeds as health promoters. Prebiotics are the dietary supplements that help to ameliorate growth and increase gut microbiota and their activities. Prebiotics are non-digestible components that are metabolized by specific gut bacteria (Bozkurt et al. 2014). Basically, prebiotics are carbohydrates, classified according to their molecular size and polymerization. They are either monosaccharides, oligosaccharides, or polysaccharides (Ringø et al. 2010). Numerous prebiotic studies have investigated modulation of the intestinal microbiota of finfish and crustacean by prebiotics (Ringø et al. 2010; Daniels and Hoseinifar 2014). They are used with an intention of reinforcing the immune system in fish (Dawood and Koshio 2016; Hutkins et al. 2016). Contrary to probiotics, prebiotics do not introduce new microorganisms to the gut but act as substrates for the host gut microorganisms providing health benefits to the host (Gibson et al. 2017). Prebiotics are considered to be an eco-friendly feed additive used in the

aquaculture industry. Fermentable carbohydrates are recognized to have a positive influence in terms of composition and activity of the indigenous gut microbiota (Bauer et al. 2006). The fish gut bacteria ferment prebiotics, and the by-products of the fermentation are used in enhancing the fish health. Thus, prebiotics by benefiting the existing bacterial species increases the growth efficiency and resistance in the host (Reverter et al. 2014).

The desirable prebiotics, to have a better performance and efficacy, should have the following properties: non-carcinogenic, easily isolated from polysaccharides, easily digestible, incorporation in feed, maintain viscosity in intestine, should activate beneficial microbes and also supress the pathogenic microbiota, and should be effective at low concentration (Ganguly et al. 2010).

20.5.1 Functions of Prebiotics

A variety of common food ingredients have been developed as possible prebiotics for humans and various species. However, few compounds, including inulin, oligofructose, lactulose, and galactooligosaccharides, are developed for direct application to humans (Gibson et al. 2004; Yousefian and Amiri 2009). The compounds are characterized as carbohydrates, mostly short-chain oligosaccharides composed of units of 3–10 carbohydrates. The most influential prebiotic compounds that have been tested to a lesser degree in terrestrial and aquatic animals are listed below.

Inulin-type fructans are fructose polymers that usually have prebiotic-like terminal glucose (Teitelbaum and Walker 2002; Denev et al. 2009). From the partial enzymatic hydrolysis of inulin polymers or enzymatic synthesis of fructooligosaccharides, various oligofructose compounds of different chain lengths can be made. Some prebiotic oligosaccharides include mannanoligosaccharides (MOS) (White et al. 2002), which are glucomannoprotein complexes originating from yeast cell walls (*Saccharomyces cerevisiae*), galactoglucomannans (Zhou et al. 2010), and lactose-mannans (Zhou et al. 2010). A combination of oligosaccharides known as trans-galactooligosaccharides causes enzymatic transglycosylation of lactose (TOS). GroBiotic®-A, a mixture of partly autolyzed materials, is another feed additive shown to contain prebiotic properties in many different aquatic organisms.

It has been found that certain carbohydrates such as polysaccharides or oligosaccharides act as prebiotics to boost growth, digestibility, feed conversion ratio, and fish immunity (Burr et al. 2008; Gatlin and Burr 2009; Merrifield et al. 2010). Different oligosaccharides have been well known as practical prebiotics, such as fructooligosaccharides (FOS), mannan oligosaccharides (MOS), xylo-oligosaccharides (XOS), and inulin and polysaccharides such as ß-glucan (Denev et al. 2009; Xu et al. 2009). However, research in this field is still in its infancy, and more systematic study will be required to clarify the associated mechanism.

20.5.2 Different Types and Effect of Prebiotics on Gut Microbiota of Fish Health

Several carbohydrates, especially fructooligosaccharides (FOS), were first researched, particularly fermented by bacteria and having a major influence on mammalian growth in Japan during the 1970s (Yazawa et al. 1978). Prebiotics consist predominantly of oligosaccharides, which have been shown to encourage beneficial bacterial growth within the GI tract (Gibson et al. 2003; Cerezuela et al. 2014). Many classes of bacteria extract energy from oligosaccharides and generate lactic, acetate, and other short-chain organic acids as the final product (Fig. 20.1). These oligosaccharides are primarily mannan oligosaccharides (MOS), FOS, and trans-galactooligosaccharides (including short-chain fructooligosaccharides, scFOS) (TOS, including galactooligosaccharides, GOS). The reported prebiotic-related positive effects in fish and fewer difficulties during administration have resulted in a plethora of studies focusing on prebiotics in aquaculture. The studies address the influence of different prebiotics on survival efficiency, feed efficiency, immune responses, gut microbiota, and growth in the host. Common prebiotics established in fish are inulin, fructooligosaccharides (FOS), mannanoligosaccharides (MOS), galactooligosaccharides (GOS), arabinoxylooligosaccharides (AXOS), levan, and GroBiotic-A (Ringø et al. 2016).

20.5.2.1 Inulin-Type Prebiotics

Naturally, inulin is extracted from the plant sources and belongs to the members of larger group called "fructans". It represents the plant oligo and polysaccharides and having glycosidic bonds. Enzymes can easily digest the molecule in the small intestine epithelial villi, in the first bond between the glucose and carbon (fructose).

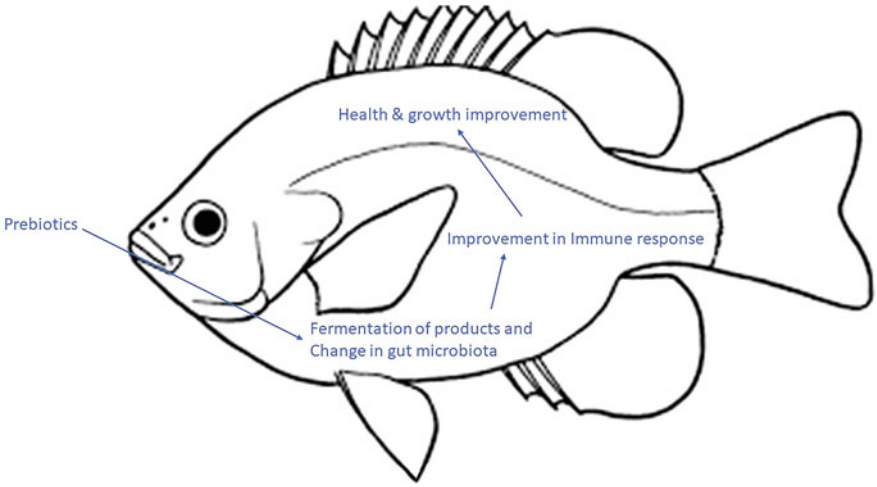

Fig. 20.1 An overview of prebiotics in host

However, this type of formation is not necessary, but the structurally the fructans can either be linear or branched polymers (Kelly 2008). The nomenclature of inulin is defined as the hot water extracts that result in inulin fructans that have not undergone further processing (Kelly 2008). Fructans can be described by degree of polymerization and also referring the fructose which is available in the form of fructan and also be designated as either Fn or Fm which represent the fructose. All linear fructans with β (2-1) fructosyl-fructose glycosidic bonds (Roberfroid 2007). This structure allows the molecule as unique structural and physiological properties. The inulin is naturally extracted from the chicory root, Jerusalem artichoke, garlic, raw asparagus, raw onion pulp, wheat, raw barley, banana, as well as fruit and vegetables (Ritsema and Smeekens 2003). However, chicory root is preferred since it contains high amount of inulin (Kelly 2008). This prebiotic has a beneficial effect on many animals' intestinal microbiota and may therefore be an effective aquaculture compound, promoting beneficial intestinal bacteria that suppress pathogens and enhancing the immune response of farm animals indirectly. Inulin-type fructans are widely researched in human and animal studies with reported beneficial impacts on the *Bifidobacterium* and *Lactobacillus* populations. This can also be utilized by some *Eubacterium* and *Roseburia* strains, which generate high levels of SCFA and an increased molar ratio of butyric acid to total SCFA compared with other fibres (Tremaroli and Backhed 2012). Although inulin-type fructans are not natural fibres in fish diets, they may have interesting applications in aquaculture to stimulate colonization of the "good" gut bacteria, suppress pathogens, and enhance immune response (Ringø et al. 2010). Usage of 0.5% probiotic inulin (*Weissella cibaria*) in the feed of the hybrid surubin (*Pseudoplatystoma corruscans* x *P. fasciatum*) controlled for just 15 consecutive days resulted in a rise in the concentration of probiotic bacteria and a consequent decrease in the number of pathogens, in addition to an increase in the concentration of overall surubin immunoglobulins resulting in a positive immunoglobulin concentration (Mourino et al. 2012). Akrami et al. (2009) examined the impact of three inclusion amounts in a sample using juvenile beluga (~16.1 g): 1 percent, 2 percent, and 3 percent of inulin on the overall cultivable allochthonous bacteria and total presumptive allochthonous LAB level in the entire intestinal tract. Absolute numbers of bacteria measured by tryptic soy agar (TSA) after 4 and 8 weeks of feeding were not greatly affected by the diet. However, a substantial decrease in average bacterial counts was found in fish fed with 3 percent inulin relative to the basal and 2 percent inulin classes using nutrient agar. The presumptive LAB standard did not follow a similar trend, in comparison to the results found in the overall bacterial count. Intestinal LAB dramatically improved after 4 and 8 weeks of feeding in fish fed 2 percent inulin and in fish fed 1 percent inulin, respectively. Both basal and prebiotic feeding fish were dominated by plantarum in the intestine.

Inulin seems to have a beneficial effect on the gut microorganisms of the endothermic animals (Possemiers et al. 2009). Although inulin is not a natural fibre in fish diets, it has shown beneficial effects on the gut microflora. Wang and Wang (1997) carried out the first study on grass carp and tilapia administered with inulin intraperitoneally. Inulin increased the survival rate against *A. hydrophila*

although not significantly as compared to the control fish. Mahious et al. (2006) demonstrated a significant increase in the production of short-chain fatty acids (SCFA) in sturgeon fed with inulin. The increase in SCFA was attributed to the fermentable abilities of gut microbiota which used inulin as a substrate. The produced SCFA was used as an energy source by the intestinal cells that resulted in higher growth rate. Refstie et al. (2006) evaluated the effect of 7.5% dietary inulin in Atlantic salmon. The study demonstrated stimulation of intestinal growth without causing any damage to the intestine. But there was no increase in the absorption capacity of the salmon GI tract. Similarly, 35 g/Kg of inulin administered to leopard grouper increased the lysozyme activity (Reyes-Becerril et al. 2014). The addition of 0.5% inulin to hybrid surubim increased the concentration of lactic acid bacteria and reduced the number of pathogenic ones (Mpurino et al. 2012). Contradictory results have been observed in studies by Ringo et al. (2006a, b) and Bakke-McKellep et al. (2007). The studies evaluated the inclusion of inulin in the diet of Arctic charr. Both the investigations showed a notable reduction in the diversity of gut microbiota.

20.5.2.2 Oligofructose and Fructooligosaccharides (FOS)

Oligofructose is used to describe inulin-type fructan mixes with a maximum degree of polymerization (DPmax) which should be less than 10 that have been produced by partial hydrolysis of inulin and then undergone physical separation to remove all long-chain (DP $\geq$10) inulin-type fructans (Kelly 2008). Fructooligosaccharides (FOS) are used to describe short-chain, inulin-type fructan mixes synthesized from sucrose (Kelly 2008). The fructosyl units are bound and linked to a terminal glucose unit by β-(2-1) glycosidic bonds. FOS is one of the most common prebiotics studied in humans and terrestrial animals. Humans can't digest the FOS, since they lack the enzyme β-fructosidases. However, FOS can be fermented by certain gut bacteria (lactobacilli and bifidobacteria) expressing the enzyme β-fructosidases (Manning and Gibson 2004). Thus, FOS selectively support the growth and survival of certain species of bacteria found in the gut. Dietary supplementation of FOS has been shown to increase the growth rate and innate immune defences in some of the aquatic animals. Augmentation of FOS in hybrid tilapia enhanced the activity of innate defences such as the lysosomal and alternative complement activity (He et al. 2003). An 8-week feeding of short-chain FOS to tilapia increased the growth rate, feed intake, and population of gut microbiota (Hui-Yuan et al. 2007). In a 4-month study, Atlantic salmon were fed with 10 g/Kg of FOS. At the end of the trial, there was an elevation of 5% and 6% in the feed efficiency and energy retention, respectively (Grisdale-Helland et al. 2008). Zhou et al. (2009) investigated the resident gut bacteria in hybrid tilapia fed with short-chain FOS. They observed an increase in some of the uncultured bacteria. The beneficial activity of these bacteria needs to be investigated. Ye et al. (2011) studied the application of 5 g/Kg of FOS in Japanese flounder for a period of 56 days and found an increase in the lysozyme activity in the host. Similarly, another study by Song et al. (2014) revealed the enhancement of lysozyme activity and a difference in the phagocytic index in the FOS fed host. Investigations on Caspian roach and ovate pompano has also demonstrated a rise in the lysozyme activity and increased immunological levels (Soleimani et al. 2012;

Hoseinifar et al. 2015; Zhang et al. 2016). Studies on red swamp cray fish served with FOS at a concentration of 8 and 10 g/kg showed substantial improvement in the activities of phenoloxidase and superoxide dismutase along with an increase in the expression of immune genes such as lysozyme and crustin 1. This increase in the immune responses enhanced the survival of the host in response to infection with *A. hydrophila* (Dong and Wang 2013; Akhter et al. 2015). FOS showed little change in development, but improved survival and provided an improvement in the function of the innate immune system by feeding tilapia hybrids. The use of FOS improved the growth rate and feed consumption of white shrimp (*Litopenaeus vannamei*) and reduced feed conversion.

20.5.2.3 Mannan Oligosaccharides (MOS)

Mannan oligosaccharides (MOS) are glucomannoprotein derived from the yeast, *Saccharomyces cerevisiae*, cell wall and one of the frequently assessed prebiotics in fish (Merrifield et al. 2010). MOS at a concentration of 2 g/Kg was evaluated in channel catfish. Although the supplementation did not affect growth performance and immune functions in catfish, it definitely increased the survival ability against *Edwardsiella ictaluri* infection (Welker et al. 2007). In some trials, this yeast is commonly used as an immunostimulant and prebiotic food supplement, as it has around 40 percent β-glucans, 40 percent alpha-mannans, and 28 percent protein. Mannans induce bacteria and improves the development of macrophages and lymphocytes that secrete antimicrobial substances. European sea bass fed with 4% MOS for a period of 21 days decreased the rate of infection in fish with *Vibrio alginolyticus* (Torrecillas et al. 2007). Increased growth performance was observed in rainbow trout fed with 1.5 g/Kg of MOS (Yilmaz et al. 2007). The effect of 4 g/Kg of MOS was investigated in rainbow trout. MOS elevated the phagocytic activity, stimulated growth, and increased survival when challenged with *Vibrio anguillarum* (Dimitroglou et al. 2009). Live feed enriched with MOS fed to cobia larvae resulted in a greater survival following exposure to hypersaline water by increasing the stress response (Salze et al. 2008). The supplementation of MOS improved the lysozyme activity in red drum (Zhou et al. 2010) and snakehead (Talpur et al. 2014). The use of MOS as a feed additive improved the diet efficiency and growth of young common carp (Dawood and Koshio 2016). A study conducted by Rodriguez-Estrada et al. (2013) evaluated the effect of MOS and inactivated cells of *E. faecalis* on rainbow trout. The investigation used different concentrations of MOS (2.5–5 g/kg), *E. faecalis* (2.5–5 g/kg), and combination of both (2.5–5 g/Kg each) in the diet. The results revealed an increase in the weight of fish fed with MOS. In all the experimental groups, the phagocytic activity was significantly higher than that in the control group. A challenge test with *Aeromonas salmonicida* significantly decreased the mortality in all the experimental groups. The study concluded that single or combined supplementation of MOS and/or inactivated *E. faecalis* affects the fish performance. Inclusion of MOS improves the development of macrophages and lymphocytes that secrete antimicrobial substances.

20.5.2.4 Galactooligosaccharides (GOS)

Galactooligosaccharides (GOS) are produced through enzymatic reactions using β-galactosidases from *Lactobacillus reuteri*, *Aspergillus oryzae*, or *Kluyveromyces lactis* of lactose and composed of galactose and glucose which consist of 2 to 20 molecules. It is one of the widely used prebiotics in endothermic animals (Vos et al. 2007; Patel and Goyal 2011). There are very few studies which have been carried out in fish. Red drum administered with 10 g/Kg of GOS for 4 weeks decreased the lipid ADC and increased protein ADC (Burr et al. 2008). Improved lysozyme activity was found in red drum administered with 10 g/Kg of GOS for 8 weeks (Zhou et al. 2010). Contradictory observations were reported by Grisdale-Helland et al. (2008). They showed no effect of GOS on growth and feed intake in Atlantic salmon. Ten g/Kg of GOS decreased lysozyme production. Ziolkowska et al. (2020) studied the effect of GOS on blood parameters, growth, and intestine morphometry of common carp. GOS was fed for a period of 60 days at a concentration of 1% and 2% to two experimental groups. The GOS feed had a beneficial effect on the development of the intestine and growth of the fish. The study recommended a concentration of 1% of GOS to produce a satisfactory result in comparison to 2%. An investigation by Hoseinifar et al. (2019) investigated the effects of GOS on the gut microbiota of Caspian roach and Caspian white fish fingerlings. The diet comprised of 1% and 2% of GOS fed for a period of 6 weeks. At the end of the experimental period, the 2% prebiotic-treated group had a significantly higher levels of lactic acid bacteria than the control group. The authors concluded that the prebiotic supplementation of GOS is favourable towards the development of beneficial bacterial communities in Caspian roach and Caspian white fish fingerlings.

20.5.2.5 Arabinoxylooligosaccharides (AXOS)

Arabinoxylooligosaccharides (AXOS) are a newly discovered class of candidate prebiotic carbohydrates with promising health-promoting properties especially bifidobacterial, which stimulate the action of colon-specific bacteria (Rivière et al. 2014). AXOS are the non-starch polysaccharides found in cereal grains (Grootaert et al. 2007). Rurangwa et al. (2008) reported a rise in the total SCFA production in Siberian sturgeon fed for 10 weeks with AXOS at a concentration of 1 and 2%. Siberian sturgeon gut microbiota was monitored after the supplementation of AXOS for 18 weeks. The study showed a direct influence of AXOS on gut microbiota with an increase in their diversity (Delaedt et al. 2008). Serum peroxidase activity and enhanced ACH50 were elevated in Siberian sturgeon when administered with AXOS (Geraylou et al. 2013).

20.5.2.6 Levan

Levan is a fructose polymer used as a prebiotic in aquaculture. Incorporation of levan in the diet of rohu (Gupta et al. 2008), common carp (Rairakhwada et al. 2007), and orange spotted grouper (Huang et al. 2014) increased the lysozyme activity at a concentration of 1–50 g/Kg for a period of 12 weeks, 75 days, and 45 days respectively. Levan at a concentration of 0.1–1% was fed to common carp juveniles to check their effect on the immunomodulatory system. The group fed with 0.5%

levan displayed an increase in the total leucocyte count and blood phagocytes along with an increased lysozyme activity. The relative survival percentage against *A. hydrophila* was also highest in the group fed with 0.5% levan. The study suggested the use of 0.5% levan as an immunostimulant for common carp juveniles (Rairakhwada et al. 2007). Microbial levan, an ideal prebiotic and immune nutrient in fish diet, has demonstrated to augment growth performance, non-specific immune response, haematological response, and thermal tolerance and to maintain liver homeostasis under fipronil toxicity in fishes (Gupta et al. 2008, 2010, 2011, 2014, 2013, 2015). Gupta et al. have decoded the molecular mechanism of disease protection in fish and revealed significant upregulation of immune responsive cytokine genes (IL-1β, TNF-α, IL-12p40, TLR22, β-2M, and IFN-γ); however, downregulation of regulatory gene (IL-10 and TGF-β) was observed in pathogen-aggravated rohu (Gupta et al. 2018, 2020). Of late, Gupta (2021) have reported noticeable improvement in glycogen level, serum cholesterol, triglyceride, LPO, HSP-70, myeloperoxidase content, as well as total immunoglobulin (Ig) level of the *C. carpio* fed with 0.75% of levan.

20.5.2.7 Grobiotic-a

Grobiotic-A is a commercial prebiotic with a mixture of dried fermentation components, autolysed brewer's yeast, and dairy ingredients. Juvenile hybrid striped bass was fed with 10–20 g/Kg of Grobiotic-A diet for 4 weeks. The prebiotic supplement enhanced survival efficiency of the host against *Streptococcus iniae* and *Mycobacterium marinum* and also increased growth performance and feed efficiency (Li and Gatlin III 2004, 2005). Sink et al. (2007) revealed increased survival of golden shiners against *Flavobacterium columnare* when supplemented with Grobiotic-A. Increased protein and organic matter levels were observed in red drum treated with Grobiotic-A (500 g/Kg) for 10 days. The increased nutrient digestibility was speculated to be because of the enzymes produced by the gut microbial community (Burr et al. 2008).

20.5.3 Metagenomics

Metagenomics is a rising tool in the aquaculture sector that facilitates a better understanding. Regarding the interaction among the host microbiota in addition to the surroundings, in turn underlying ailment outbreaks monitoring the dynamics of microbial range in farmed animals specific to environmental situations or perturbations. Metagenomics is also referred to as community genomics, environmental genomics, and population genomics (Schaechter 2009). Anciently this technique looked at the non-cultured microflora; however, this era shifted its view towards the observation of microbial diversities in deep sea, soil, and gastrointestinal tract ecosystems of animals and human. The use of metagenomic approach towards the interaction of the microbiome inside the intestine and the gastrointestinal tract of the animals and humans is dynamic. Indeed, in the recent present, the metagenomic approach is used to increase and improve probiotic applicants for the fish. The

combination of metagenomics with microbial genomics and chemical ecology may additionally allow and apprehend the characteristic uncultivable microorganisms. Metagenomics can provide a deeper insight into the ones ties by using associating with host or surroundings particular host species the facts revealed by the extracted DNA (Wood-Charlson et al. 2015; Gianoulis et al. 2009). New sequencing and bioinformatics technologies have facilitated a variety of intracellular bacteria to be tested as well as be elucidated. The field of metagenomics has shed light to investigate the diversity and quantity of various microbes and genes in the spatially temporal sample and has identified stronger associations between positive microbial communities and the host genotype (Delhaes et al. 2012; Fierer et al. 2012). Metagenomics offers more evidence for better understanding of the microbial diversities in the aquaculture facilities by means of studying hypervariable areas of 16S rDNA for prokaryotes and 18S for eukaryotes and thus enabling to apprehend the extensive diversity of microorganisms (Hugerth et al. 2014). Metagenomics can be used to assess antibiotic resistance in the bacterial groups according to the target of analysis in two ways: functional metagenomics and series preliminary-based metagenomics (Sukumar et al. 2016). Functional metagenomics consists of the cloning and transmission of biologically derived DNA right into a bacterial host to perceive the roles of genes that won't be elucidated by analysing their sequences. Series-based metagenomics encompasses the random sampling of environmental samples. Shotgun metagenomics of medical or random environmental samples is a promising alternative that circumvents traditional strategies' boundaries. At the same time as this technique has usually been used to examine genomic variety, it could additionally be useful in clinical detection of viral pathogens (Rosario et al. 2009; Bibby et al. 2011). Virus research of metagenomic methods has been these days promoted due to the quality and amount of genomic facts received with subsequent generation sequencing, whilst the Sanger collection also can be offered as an identity tool (Svraka et al. 2010; Yadav et al. 2019). In comparison to different methods together with PCR or microarrays, metagenomics has proven higher efficiency and accuracy of detection of a couple of genomes (Yozwiak et al. 2012). Metagenomics libraries for unique biodegradation genes may be constructed, amplified, and screened. This method examines by cloning genes into microorganism and incubating them in tremendously poisonous compound medium (Brown et al. 2011). New molecules can be supplied with exceptional functions through metagenomics, consisting of new enzymes or bioactive molecules, that could offer therapeutic programmes or monetary techniques (Lorenz and Eck 2005).

Metagenomic studies of fish intestine microbiome provided numerous records on the physiological and immunological capabilities of the intestine microbiota of diverse fish. Gut microbiota influences the fish metabolism and modulates the fish immunogenicity with recognition to pathogenic microbes. Fish gut microbial composition differed relying on habitat, species, and feeding behaviour. The intestine microbes which have been proven to have a superb effect at the fitness of the fish can be used as a probiotic candidate looking ahead to further examine. This technique also helpful in figuring out the fucntional capacity of a microbiome. 16S rRNA

analyses to genes along with metagenomes have been shown to be useful in figuring out the functional capacity of a microbiome.

20.6 Conclusion

Importance of prebiotics in improving the health management of aquatic organisms is gradually progressing. In aquaculture, the impact of prebiotics on various aspects like growth, muscle development, feed conversion ratio, gut microbiota, and health improvement has been investigated to a limited extent and variably in different organisms. Many extrinsic and intrinsic factors influence the microbiome of the organisms. Development of culture-independent techniques has brought new dimensions for better understanding and identification of new and novel groups species. In addition to this, high priority should be given to research on prebiotics, and molecular review should be used as standard criterion for determining their impact on fish health and nutrition. Future studies on prebiotic results should require transcriptome and proteome analysis using high-throughput assays, following the various genome-sequencing methods that are currently used. In addition, in order to know the various modes of action of different probiotic species, transcriptome and proteome profiling of intestinal microbiota should be extensively recorded. Therefore, in the future, high priority should be given to research on prebiotics, and molecular review should be used as standard criterion for determining their impact on fish health and nutrition.

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Microbiome Interventions for the Prevention and Control of Disease Outbreaks in Shrimp Aquaculture

21

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Abstract

The extensive use of antimicrobial agents in shrimp aquaculture has led to the emergence of antimicrobial-resistant bacteria, which poses a huge threat to environmental and human health. Therefore, an eco-friendly and economic alternative treatment strategy has been devised these days, which involves the microbiome-based treatment. The role of gut microbiota in regulating the health, growth, and survival of aquaculture species has been a subject of interest in the research community. Researchers have found various biological agents such as probiotics, prebiotics, and synbiotics capable of manipulating microbiome to control diseases and improve survival of shrimp. Moreover, the use of biofloc technology and recirculating aquaculture systems aids in sustainable aquaculture. This chapter highlights the importance of microbiome-based and environment friendly treatment strategies for the prevention and control of disease outbreaks in shrimp aquaculture.

Keywords

Biofloc technology · Shrimp diseases · Microbiome · Probiotics · Prebiotics · Synbiotics

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21.1 Introduction

Aquaculture is the practice of rearing aquatic species for food, ornamental species, commercial stock augmentation, recreational fishing, bait production, and recovery of endangered species (Stickney 2000). The practice of aquaculture originated in China (Nakajima et al. 2019) and is now one among the most emerging animal-based food supplies globally (Subasinghe et al. 2009; De Bruijn et al. 2018). Aquaculture is quite different from the land animal farming due to its enhanced diversity (Sicuro 2021). Limitations in expanding the wild capture fishery and increased demand for marine products has led to the development of marine aquaculture to meet the seafood demand (Gentry et al. 2017). Globally, as well as in Asia, aquaculture dominates the aquatic food supply (FAO 2020). Since 1970, the shrimp aquaculture became lucrative and spread across the tropic countries (Boyd and Clay 1998). During the 1970–1980s, the shrimp aquaculture began with *Penaeus monodon* as the mainly cultured shrimp. While in the 1990s, a specific pathogen-free (SPF) genetically upgraded shrimp, *Penaeus vannamei*, became the choice of interest to the farmers (Flegel et al. 2008; Govindaraju et al. 2019). *P. vannamei* has its origin from tropical Pacific Coast of the America and Australia, while *P. monodon* originated from East Africa, South East Asia, and South Asia (Cock et al. 2017). The enhanced production of *P. vannamei* has increased the global shrimp production (mainly in Asia), despite the fall in *P. monodon* production (Cock et al. 2017). The main intention of farming shrimp is for the purpose of food, but apart from that, chitosan, a by-product obtained during shrimp shell processing, has many other benefits in various industries such as cosmetics, agrochemicals, food and beverages, pharmaceuticals, etc. (Roy et al. 2020).

Due to the cultivation away from its natural habitat, the shrimp experiences various stresses which make them susceptible to pathogens due to their weakened immune response (Mohan et al. 2019). Susceptibility of shrimp to various bacterial, viral, fungal, and parasitic diseases has led to high mortality rate, which in turn causes an economic loss to the aquaculture sector (Mohan et al. 2019; Roy et al. 2020). Use of vaccines and antibiotics as treatment options has led to the emergence of drug-resistant pathogens. Therefore, it is very important to devise newer and efficient strategies to prevent the spread of antibiotic-resistant microbes (Mohan et al. 2019). This has led to the development of microbiome-based therapies which are the most trending treatment strategy these days, due to their cost-effective and eco-friendly nature. The use of probiotics, prebiotics, and a combination of pro- and prebiotics, known as synbiotics, has gained great attention, recently. Moreover, improved farming techniques such as recirculating aquaculture systems (RAS) and biofloc technology (BFT) are emerging strategies to enhance the shrimp aquaculture.

21.2 Shrimp Aquaculture and Infectious Diseases: A Global Perspective

Around 60% of shrimp diseases is caused by virus, 20% by bacteria, and 20% by other microbes such as fungus and parasites (Govindaraju et al. 2019; Flegel et al. 2008; Flegel 2006; Chaivisuthangkura et al. 2014). The major shrimp diseases include viral infections such as white spot disease (WSD) caused by white spot syndrome virus (WSSV) (Lo et al. 2011), yellow head disease (YHD) caused by yellow head virus (YHV) (Senapin et al. 2010), covert mortality disease (CMD) caused by covert mortality nodavirus (CMNV) (Zhang et al. 2014), Taura syndrome virus disease (Tang et al. 2017), infectious myonecrosis virus disease (Prasad et al. 2017), and bacterial infections such as acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio parahaemolyticus* (Tran et al. 2013), luminous vibriosis caused by *Vibrio harveyi* (Zhang et al. 2020), hepatopancreatic microsporidiosis (HPM) (Aranguren et al. 2017), etc. AHPND has caused a loss of about US $43 billion in Mexico and across Asian countries like China, Thailand, Vietnam, Malaysia, etc. (Leung and Bates 2013; FAO 2014; Kumar et al. 2018; Shinn et al. 2018; Flegel 2019). In India, *Enterocytozoon hepatopenaei* (EHP) and WSSV disease in *P. vannamei* caused huge loss in production. Loss due to WSSV was more when compared to EHP (Patil et al. 2021).

The government agencies highlight the disease exclusion and eradication, while the farmers try to improve the management strategies (Cock et al. 2017). Due to the strict import etiquettes, various issues have come into picture, such as illegal import of aquatic species, emergence of new diseases, a narrow genetic base making it more difficult to develop genetically improved disease-resistant varieties, reliance on brood stock which are not properly adapted to the local environment, etc. (Cock et al. 2017). The recent pandemic (COVID-19) has lowered the shrimp production, consumption, and trade due to the strict restrictions imposed (FAO 2020; Rajeev et al. 2021). The high commercial value and short production cycle of shrimp makes its large-scale production possible despite the infectious diseases and other constrains in the rearing process (Asche et al. 2020). The major shrimp disease treatment option is the use of antimicrobials (Uddin and Kader 2006; Thornber et al. 2020). ARGs are predominant in commercial shrimps (Liu et al. 2019). Antibiotic resistance genes (ARGs) are serious environmental contaminants (Pruden et al. 2006) because of the hazard it causes to the public health (WHO 2014; Zhu et al. 2013; Su et al. 2020) and are predominant in commercial shrimps (Liu et al. 2019). The shrimp cultured in marine aquaculture and the sediment are found to be main causes of dissemination of ARGs (Su et al. 2020). Therefore, use of antibiotics should be replaced by other eco-friendly alternatives.

21.3 Importance of Microbiome in Shrimp Aquaculture

Shrimp aquaculture is one of the important sources of income for Asian and Latin American countries (Hernández-Rodríguez et al. 2001). The role of gut microbiota in regulating the health, growth, and survival of aquaculture species has been a subject of interest in the research community. This is due to the availability of cheap and effective high-throughput sequencing facilities because of which a great deal of knowledge regarding the unculturable microorganisms was revealed (Cornejo-Granados et al. 2017). The term microbiome has been reframed as "a characteristic microbial community occupying a reasonable well-defined habitat which has distinct physiochemical properties" (Berg et al. 2020). The microbial consortia of shrimp gut vary within species; for example, the proportion of *Cyanobacteria* in the gut of *Litopenaeus vannamei* is substantially higher than that compared to *Penaeus monodon* (Cornejo-Granados et al. 2017). The most reported members of the gut microbiota of shrimp are *Vibrio* and *Photobacterium* spp. followed by bacteria belonging to the taxa: Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria (Rungrassamee et al. 2014).

The gut microbial community improves the growth of shrimp by enhancing the biosynthesis of digestive enzymes such as lipase, amylase, and pepsin (Xiong et al. 2017). The beneficial microbes present in the shrimp gut resist infection by secreting antimicrobial compounds directed towards pathogenic bacteria or compete with them for space and nutrition (Kim et al. 2017). Metabolites derived from the commensalic bacteria of the gut (e.g., short-chain fatty acids) play a pivotal role in immunomodulation which includes the regulation of T-cell differentiation, apoptosis, gut cell proliferation, and mucin production (Kim et al. 2014; Montalban-Arques et al. 2015).

The composition of gut microbiota is under the influence of different factors such as environmental stress, diet, physiological conditions, starvation, and the developmental stage (Butt and Volkoff 2019). The intestinal flora of shrimp gut is also influenced by the rearing water and sediment microbiome (Wu et al. 2012; Rungrassamee et al. 2013; Wang et al. 2014; Fan et al. 2019). The dominant members of microbial population in water, sediment, and intestine differ, and they perform ecological functions specific to their habitat. For example, the gut microbiome consists of microbes that aid in the digestion of food, whereas the sediment microbial consortia consist of sulfur-reducing bacteria, and rearing water microbiome comprises autotrophic bacteria (Sun et al. 2019). With the increased use of antibiotics in aquaculture, the gut microbiota of farmed shrimp is found to harbor antibiotic-resistant bacteria which can challenge human health. Researchers have found various biological agents such as probiotics, prebiotics, and synbiotics capable of manipulating microbiome to control diseases and improve survival of shrimp (Seethalakshmi et al. 2021).

21.4 Microbiome Interventions for Successful Shrimp Farming

The gut microbiome has a crucial role in maintaining the health status of shrimp and resistance to pathogens. An imbalance in the compositional microbiota of gut can result in dysbiosis which progresses to disease conditions (Rajeev et al. 2021). Dysbiosis is proven to be a major factor contributing to infectious diseases in shrimp such as APHND (Hossain et al. 2021) and white feces syndrome (WFS) (Huang et al. 2020). Hence, microbiome modulation can be implemented as an alternative to antibiotic therapy in preventing infectious diseases without harming the environment. Although much focus has been given for the manipulation of gut microbiome using probiotics, prebiotics, and synbiotics through dietary supplementation, recent studies have reported the advantages of probiotic applications in manipulating rearing water microbiome to alter the gut microbiota of shrimp.

21.4.1 Probiotics

Probiotics are microorganisms that can confer health benefits to host by immune stimulation, enhancement of digestive enzyme production, or competitive elimination of pathogens (Kumar et al. 2016; Hoseinifar et al. 2018; Li et al. 2018a, b). The first report elucidating the benefits of supplementing probiotic bacteria in feed additives dates back to late 1980s, since then research in this area have progressed continuously (Verschuere et al. 2000). Probiotics are administered to shrimp either orally (Wang 2007) or are directly incorporated into the rearing system (Ringø 2020). *Lactobacillus plantarum* has been extensively used as a probiotic bacterium for shrimp aquaculture (Chauhan and Singh 2019) because of the versatile antimicrobial compounds it secretes which includes organic acids, hydrogen peroxides, and antimicrobial peptides (Fooks and Gibson 2002; Vieira et al. 2008; Abdel-Latif et al. 2020). However, the molecular mechanisms involved in regulating adaptive immune response of shrimp upon receiving *Lactobacillus* spp. need further investigation (Naiel et al. 2021). *Bacillus* strains are ideal probiotic candidates for shrimp as they produce hydrolytic enzymes for the digestion of lipids, carbohydrates, proteins, and lipids (Ochoa-Solano and Olmos-Soto 2006). In certain studies, it has been suggested that the mixed probiotics consisting of *Bacillus* strains and yeasts are more beneficial for shrimp health than the individual counterparts (Nimrat et al. 2019; Wang et al. 2019).

21.4.2 Commercial Probiotics

Commercial probiotics are presumed to be more effective than indigenous probiotic strains as they are refined to possess many desirable qualities like improved enzyme activity and survival in the shrimp gut, etc. A comparative study of commercially available probiotic strains with indigenous strains pointed out that the former demonstrated a better performance in improving the immunophysiology and growth

Table 21.1 Commercially available probiotics and their functional role in shrimp aquaculture

Name of the commercial probiotic	Probiotic species	Functional role	References
Protexin probiotics international ltd	*B. subtilis* and *Bacillus licheniformis*	Improved growth and immunophysiological characters in shrimp	Abdollahi-Arpanahi et al. (2018)
Protexin Aquatech	*B. subtilis*, *B. licheniformis*, *Bacillus polymyxa*, *Bacillus laterosporus*, *and Bacillus circulans*	Improved specific growth rate and feed conversion ratio of shrimp	Ziaei-Nejad et al. (2006)
Efinol PT	*Bacillus* spp., lactic acid bacteria, *Lactobacillus* spp., *Saccharomyces* spp.	Increased the load of viable heterotrophic bacterial count in rearing water	Arias-Moscoso et al. (2018)
Epicin ponds-Epicin hatcheries	Unspecified		
Mix laboratory robles			
HUOJUNWANG (P1)	10 species of spore-forming bacteria of *Bacillus*, *Streptococcus faecalis*, actinomycetes, yeast, lactic acid bacteria, and *Pediococcus*	Prevented the growth of harmful cyanobacteria and removed toxic nitrogenous compounds	Lukwambe et al. (2019)

than the latter (Abdollahi-Arpanahi et al. 2018). In contradiction to several reports suggesting the beneficial role of probiotics in improving rearing water quality and growth performance (Cai et al. 2019; Nimrat et al. 2019; Lukwambe et al. 2019), Arias-Moscoso et al. (2018) found that commercial probiotics did not show any significant improvement of water quality or productive parameters of shrimp. It will be worthy of investigating the changes in the microbiome of rearing water upon addition of commercial probiotics to rearing systems. Commercially available probiotics and their functional role in shrimp aquaculture are enlisted in Table 21.1.

21.4.3 Prebiotics

The gut microbiota of shrimp can be modified by the intake of prebiotic orally or prebiotic-supplemented feedstuff (Zhou et al. 2020). Prebiotics are known to upregulate immune-related genes which include signal transducer and activator of transcription (STAT); anti-lipopolysaccharide factor (ALF); crustin; toll-like receptors 1, 2, and 3; and prophenoloxidase (Li et al. 2018a, b). The effects of prebiotics derived from plants and algae are very commonly studied in shrimp; however, recent studies have suggested prebiotic components of bacterial origin to be effective immunomodulators (Kiran et al. 2020; Prathiviraj et al. 2021). The immune regulation property of prebiotics can be boosted with the use of a

combination of prebiotic components rather than single components. This is evident when mannan oligosaccharides (MOS) derived from coprameal did not show any significant upregulation of genes: penaeidin, crustin, and anti-lipopolysaccharide factor in shrimp (Rungrassamee et al. 2021), but a combination of MOS with inulin resulted in upregulation of these immune genes (Li et al. 2018a, b).

21.4.4 Synbiotics

Synbiotics are redefined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" by the panel of International Scientific Association for Probiotics and Prebiotics in May 2019 (Swanson et al. 2020). Synbiotic diets are found to have synergistic effect of probiotic and prebiotic components on stimulation of immune genes and resistance to pathogens (Huynh et al. 2018; Muharrama et al. 2021). Moreover, synbiotic diets are found to improve morphometry of intestine and gut microbiota of shrimp by enhancing lactic acid bacterial population with decrease in *Vibrio* spp. (Boonanuntanasarn et al. 2016). Shrimp fed with synbiotic diets have higher short-chain fatty acid (SCFA) content in the intestine and better resistance to ammonia (Chen et al. 2020). Synbiotic diets have been proven to be effective in eliminating many bacterial pathogens of shrimp such as *V. parahaemolyticus* (Muharrama et al. 2021) and *Vibrio alginolyticus* (Zhang et al. 2011). But the effect of synbiotic diets in controlling viral and parasitic infections is yet to be explored.

21.4.4.1 Biofloc Technology and Recirculating Aquaculture Systems (RAS) for Shrimp Culture

The shortage of land and water has demanded for improvement in aquaculture technologies to improve the production in an eco-friendly manner (El-Sayed 2021). The closed aquaculture system, due to its various advantages such as low water exchange rate, highly controlled input, and smaller space requirement than the traditional ponds, plays a huge role increasing biosecurity, decreasing water consumption, and producing marine species far away from the coastline (Browdy and Moss 2005). Closed aquaculture system mainly consists of recirculating aquaculture systems (RAS) and biofloc technology (BFT) systems.

BFT is a trending technology used for sustainable aquaculture (El-Sayed 2021) and the amplification of *P. vannamei* shrimp production (Samocha et al. 2012), which could help to achieve FAO Sustainable Development Goals (SDGs) for food security (El-Sayed 2021). The use of minimum/zero water exchange (Ahmad et al. 2017) and high stocking densities to lower the surface area for culturing makes it a better farming option than the semi-intensive systems (Samocha et al. 2012). In the BFT, high amount of carbon is supplemented to the culture system (Crab et al. 2012), which alters C/N ratio causing a spike in heterotrophic bacteria. The heterotrophic bacteria convert the nitrogenous waste into microbial proteins (Avnimelech 1999). The microbes and the detritus phytoplankton can be consumed by the shrimp; hence BFT also aids in in situ feed production and waste management (Hargreaves

2006; Crab et al. 2012; Ahmad et al. 2017; Rajeev et al. 2021). BFT systems also reduces the pathogen load and improves the growth and performance of shrimp (Luis-Villaseñor et al. 2016).

Clear water RAS is equipped with external bio filter for better surface area, solid filter to eliminate solids from the water, and an aerobic milieu for nitrifying bacteria. Certain systems also contain UV lamps for sterilization (Timmons and Ebeling 2007; Ray et al. (2017). RAS uses minimum amount of water and land (Badiola et al. 2018); hence they are water-efficient, eco-friendly, and extremely productive intensive farming system. Culturing aquatic species in RAS safeguards from habitat destruction, eutrophication and water pollution, ecological ill effects on biodiversity, and infectious outbreak (Ahmed and Turchini 2021). Even though RAS has many benefits, certain drawbacks such as high operational cost and energy requirement, along with the use of fossil fuels, also exist (Badiola et al. 2018). The use of a hybrid zero water discharge RAS helps in optimum shrimp growth at the stocking density of 500 post larvae/m^3, especially at low salinity levels (Suantika et al. 2018). For indoor marine culturing of shrimp, RAS is a better option as compared to BFT (Ray et al. 2017). *P. vannamei* cultured in hybrid biofloc-based recirculating aquaculture system (biofloc-RAS) revealed better total ammonia nitrogen (TAN) removal and higher abundance of nitrifying microbes, indicating high nitrification activity (Xu et al. 2020).

21.5 Conclusion

Shrimp aquaculture being one of the major aquatic-based food supply sectors demands more attention for better productivity. The rearing of shrimp away from its natural habitat renders it susceptible to various external stresses and pathogens. The conventional disease treatment strategy includes the use of antimicrobials. The extensive use of antimicrobial agents has led to the emergence of antimicrobial-resistant bacteria which poses a huge threat to environmental and human health. Therefore, an eco-friendly treatment strategy has been devised these days, which involves the microbiome-based treatment. The use of probiotics, prebiotics, and synbiotics has proven to be highly beneficial in improving the gut health, growth, and overall performance of the reared shrimp. The use of closed aquaculture system such as RAS and BFT has improved the shrimp aquaculture by judicial use of natural resources. Therefore, the aquaculture-based production of shrimp can be boosted by the effective execution of such environment-friendly techniques.

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Reinventing the Micronutrients beyond Nutrition: Functions in Immune Modulation and Stress Mitigation of Fish

22

Tincy Varghese, Amrutha Gopan, and VJ Rejish Kumar

Abstract

The vitamins and minerals are an essential part of fish nutrition, although their requirements are in low doses. Their functions, dosages, and toxicities are yet to be defined entirely for many important commercial species. However, in altered dosages, they have different functional significance than providing nutrition. The roles of these micronutrients are strongly implicated in enhancing innate and adaptive immune responses and alleviating stress responses. Stress and immunity are deeply associated, while both are significant aspects of fish welfare. Exposure to stress weakens the innate immunity of the aquatic species through diverse crosstalk between endocrine and paracrine pathways. The concept of complete diet in recent times are more intriguingly about the correct incorporation of micronutrients, the consumption of which protects the animal from any ailments due to weak immunity.

Keywords

Vitamins · Minerals · Micronutrients · Nutrition · Health

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22.1 Introduction

As a general assumption, stress will weaken the immune system, resulting in increased susceptibility to diseases (Yada and Tort 2016). The loss of immunocompetence will lead to the outbreak of diseases and mortality rates (Mateus et al. 2017). Thus, stress mitigation and immunity enhancement become complementary at many levels. Stress leads to the release of corticosteroids which are primarily immunesuppressive. Stress affects the immune system through a fundamental interaction between stress responses and immune responses at molecular and systemic levels. Among the stress responses, chronic stress affects more severely the immune system than acute stress responses (Ashley 2007). Immune status is a significant indicator of fish welfare and tertiary stress responses (Braithwaite and Ebbesson 2014; Huntingford and Kadri 2014). In aquatic animals, the effects of stressors on immunity vary depending upon species, age, sex, maturity stage, and magnitude and duration of the stress exposure (Tort 2011). The interaction of stress and immune function is manifested at the endocrine level, mainly the interaction between cytokines and hormones, causing the disruptions in the actions of both (Mateus et al. 2017). This interaction will lead to alterations in metabolism, inflammatory response, and impairment in the functions of immune cells (Dhabhar 2014). Cortisol suppresses many critical elements of the fish immune system, such as antibody production, phagocytosis, cytokine expression, and circulating leukocyte numbers (Tort 2011; Yada and Tort 2016), thus causing the suppression of the adaptive immunity of the fish.

22.2 Vitamins

Vitamins are organic molecules that act as cofactors or substrates in several metabolic reactions. They are generally required in relatively small amounts along with the diet, and their deficiency may result in disease or poor growth. They are increasingly used to alleviate different stress responses in fishes. Among different vitamins, L-ascorbic acid (vitamin C) and α-tocopherol (vitamin E) are widely studied and the most commonly used vitamins in aquatic animals, including fish. In addition to their normal physiological function, they also participate in stress mitigation and immune enhancement when supplied in additional amounts (Table 22.1).

22.2.1 Vitamin C

Vitamin C is a water-soluble vitamin, which acts as a potent reducing agent in several metabolic reactions. As vitamin C is involved in hydroxylation reaction, it acts as a cofactor for many enzymes implicated in the biosynthesis of collagen, carnitine, and neurotransmitters, particularly dopamine β-monooxygenase involved in the transformation of dopamine to norepinephrine (Yousef 2004). Vitamin C

Table 22.1 Vitamins in immunity/stress mitigation in fish

Vitamins and dose	Species	Response	References
L-ascorbic acid (vitamin C)	Nile tilapia (*Oreochromis niloticus*)	Reduced crowding stress	Hayat (2013)
L-ascorbic acid (vitamin C)	Turbot larvae (*Scophthalmus maximus*)	Lower salinity stress	Merchie et al. (1996)
L-ascorbic acid (vitamin C)	Rockfish (*Sebastes schlegelii*)	Prepare fish for coping with lead intoxication	Kim and Kang (2017)
L-ascorbic acid (vitamin C)	Puffer fish (*Takifugu obscurus*)	Prepare fish for coping with cold stress	Cheng et al. (2018a)
Vitamin C supplemented (1000 mg/kg) feed	Nile tilapia, *Oreochromis niloticus*	Reduced crowding stress, lowered plasma cortisol, blood glucose	Mustafa et al. (2013)
L-ascorbic acid (vitamin C)	Sea bream (*Pagrus major*)	Reduced crowding, chasing, and low salinity stresses	Dawood et al. (2017)
Diet supplemented by a high level of ascorbic acid (100 mg/100 g)	Freshwater catfish (*Clarias gariepinus*)	Pesticide-associated stress reduced	Datta and Kaviraj (2003)
L-ascorbic acid (vitamin C)	Parrot fish (*Oplegnathus fasciatus*)	Intermittent hypoxic stress	Ishibashi et al. (1992)
Vitamin C levels 600 mg/kg diet	Tilapia (*Oreochromis niloticus*)	Prepare fish for coping with low temperature stress	Falcon et al. (2007)
L-ascorbic acid (vitamin C)	Guppy	Ameliorating adverse effect of osmotic shock	Lim et al. (2002)
Dietary vitamin C	Puffer fish, *Takifugu obscurus*	Reduce reactive oxygen species level, apoptosis, and DNA damage, under low temperature stress	Cheng et al. (2018a)
Vitamin C	Nile tilapia (*Oreochromis niloticus*)	Reduced microcystin and lead toxicity	Prieto et al. 2008; Tanekhy and Khalil (2014)
Vitamin C	Rohu (*Labeo rohita*)	Improved nitrite stress resistance	Ciji et al. (2013a, b, 2014); Ciji and Akhtar (2020)
Vitamin C	Puffer fish and dark barbel catfish	Ameliorating adverse effect of ammonia stress	Cheng et al. (2018b); Li et al. (2014)
Vitamin C	Wuchang bream (*Megalobrama amblycephala*)	Ameliorating adverse effect of crowding stress	Liu et al. (2014)

(continued)

Table 22.1 (continued)

Vitamins and dose	Species	Response	References
α-Tocopherol (vitamin E)	Rohu (*Labeo rohita*)	Nitrite-induced inhibition of steroidogenesis was overcome	Ciji et al. (2013b)
Vitamin E supplementation at 50 and 100 mg/kg	Channel catfish, (*Ictalurus punctatus*)	Improved the growth performance as well as intestinal structure and function	Cheng et al. (2018b)
150 mg/kg vitamin E	Rohu (*Labeo rohita*)	Increase the survival of stressed fish during the bacterial challenge	Ciji et al. (2013c)
α-Tocopherol (vitamin E)	Channel catfish, (*Ictalurus punctatus*)	Potent antioxidant function protective effect through its modulating HSPs expression	Cheng et al. (2018b)
Vit E diet (150 mg/kg)	Pacu (*Piaractus mesopotamicus*)	Higher weight gain and specific growth; haematocrit, erythroblast number, and total plasma protein increased	Sado et al. (2013)
Pyridoxine (vitamin B_6) Dietary administration of pyridoxine (45 days)	*Labeo rohita* and *Chanos chanos*	Improved growth performance and stress tolerance	Akhtar et al. (2010, 2011, 2012); Kumar et al. (2017a, b).
Synergistic effect of dietary selenium nanoparticles (5 mg/kg diet) and riboflavin (0.5 mg/kg diet)	*Pangasioanodon hypophthalmus*	Enhanced thermal efficiency and stress tolerance (high temperature and arsenic stress)	Kumar et al. (2020)
Dietary folic acid (0.68–0.82 mg/kg)	Blount snout bream, *Megalobrama amblycephala*	Improved growth performance, and feed efficiency increased digestive enzyme activity	Sesay et al. (2016).
Dietary folic acid (more than 1.63 and 2.71 mg/kg)	Siberian sturgeon, *A. baerii*	Higher lysozyme activity and immunoglobulin M (IgM)	Falah et al. (2020)
Dietary folic acid 1.82–4.41 mg/ kg	Siberian sturgeon, *A. baerii*	Improved growth performance, immuno-physiological response, and antioxidant status	Falah et al. (2020)
Dietary folic acid supplementation of basal diet from 1.0, 2.0, and 5.0 mg/kg	Blount snout bream, *Megalobrama amblycephala*	Improved high-temperature tolerance via regulating serum cortisol and blood glucose levels, immune responses, antioxidative capacities, and expression levels of HSPs	Sesay et al. (2016)

(continued)

Table 22.1 (continued)

Vitamins and dose	Species	Response	References
Dietary folic acid (2.0 mg/kg)	*M. rosenbergii*	Enhanced survival, growth, and antioxidant defence system and production	Asaikkutti et al. (2016)
Dietary incorporation of choline	Kuruma shrimp (*Marsupenaeus japonicus*)	Improved tolerance against salinity and formalin stress	Michael and Koshio (2013)
Dietary incorporation of choline	Giant grouper (*Epinephelus lanceolatus*)	Improved tolerance against ammonia stress	Yeh et al. (2015)

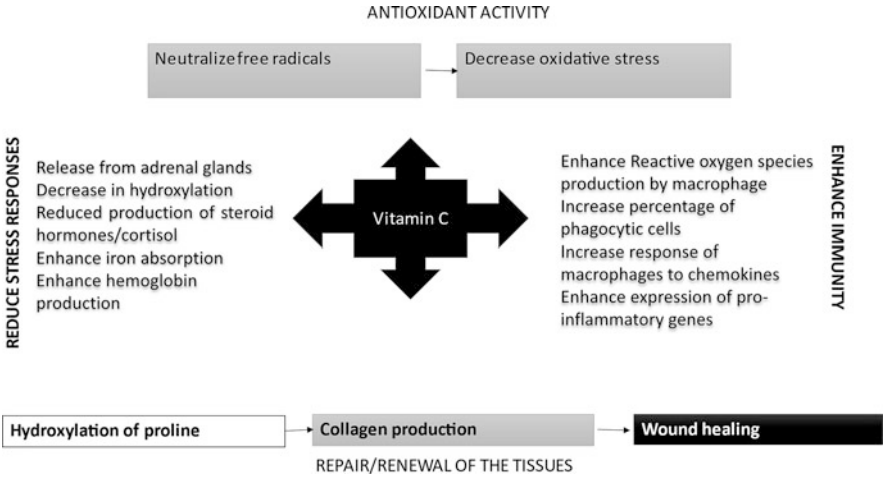

Fig. 22.1 Multiple roles of vitamin C. It act as a stress mitigating agent by reducing cortisol production in adrenal glands. Vitamin C reduces iron, i.e. ferric to ferrous form, which increase iron absorption, leading to enhanced heme synthesis and haemoglobin production. The immune enhancing functions of vitamin C is more direct on the immune cells by increasing the proportion of phagocytic cells, enhancing ROS production from phagocytes, and upregulating the inflammatory genes

inactivates the damage due to free radicals formed due to regular cellular activity from various stressors (Chew 1995). Fish and crustaceans are incapable of biosynthesis of vitamin C since they lack L-gulonolactone oxidase, which is the enzyme responsible for synthesising vitamin C, and rely on dietary supplementation (Mæland and Waagbø 1998). Hence, they require a dietary supply in adequate quantity to prevent the incidence of deficiency symptoms such as reduced growth, poor wound healing, melanised lesions throughout the collagenous tissue underlying the exoskeleton, and eventually death. Thus, the roles of ascorbic acid are implicated as an antioxidant, stress reducer, immune nutrient, and wound healing (Fig. 22.1). Thus, they are often supplemented in mega-doses in fish diets.

Generally, vitamin C requirements increase under stressful husbandry conditions as evidenced by reduced ascorbic acid content in serum, liver, and kidney (Chien et al. 1999; Datta and Kaviraj 2003). On the contrary, few other studies reported no change in the ascorbic acid content in fishes subjected to stressful conditions (Li et al. 1998; Henrique et al. 2002). In fish tissues, the anterior kidney, which contains adrenal tissue, functions as a storage site for vitamin C. Under stress, the ascorbic acid content of this tissue gets reduced rapidly with the production of adrenal steroids. Vitamin C inhibits steroidogenesis by preventing the conversion of unsaturated lipids into cholesterol esters, the parent compound for cortisol biosynthesis, which ultimately hinders the formation of cortisol (Dawood et al. 2017). Thus, it helps regulate cortisol and prevents blood pressure from spiking in response to stress (Wan et al. 2014). Furthermore, dietary vitamin C reduces the mobilisation of glycogen under stressful environments (Trenzado et al. 2008), resulting in low blood glucose levels in stressed fish (Peng et al. 2013). Vitamin C also facilitates iron absorption by the intestine and its redistribution to different tissues, thus enhancing haemoglobin production and efficient oxygen delivery during hypoxic stress. Vitamin C was demonstrated to enhance tolerance to different stressors such as hypoxic stress in parrotfish (Ishibashi et al. 1992), copper toxicity in tilapia (Ghazaly 1994), salinity stress in turbot larvae (Merchie et al. 1996), osmotic shock in the guppy (Lim et al. 2002), pesticide toxicity in freshwater catfish (Datta and Kaviraj 2003), stress due to claw ablation in *Macrobrachium rosenbergii* (Manush et al. 2005), cold stress in tilapia (Falcon et al. 2007), crowding and low salinity stresses in sea bream (Hayat 2013; Dawood et al. 2017), crowding stress in Nile tilapia (Hayat 2013), alkalinity stress in Wuchang bream (Wan et al. 2014), lead toxicity in rockfish (Kim and Kang 2017), and crowding stress in puffer fish (Cheng et al. 2018a, b).

Vitamin C modulated the antioxidant capacity and immune reactions, including the proliferation of immune cells, natural killer cell and macrophage activities, bactericidal activity, and complement and lysozyme levels in Japanese eel (Shahkar et al. 2015). The antioxidant property of vitamin C aids to scavenge free radicals generated through regular cellular activity and in response to various stressors. Thus, it helps maintain the structural and functional integrity of various immune cells, erythrocytes, and biomolecules such as lipids, protein, carbohydrates, and nucleic acids. The antioxidant property of vitamin C is also strengthened by its capability to regenerate other antioxidants such as vitamin E and glutathione (Yousef 2004). Vitamin C supplementation also regulated the expression of heat shock proteins (HSPs) (Wan et al. 2014) and antioxidant defence (Vieira et al. 2018) during stress exposure in fish. Cheng et al. (2018a, b) demonstrated that dietary vitamin C supplementation reduces reactive oxygen species (ROS) level, apoptosis, and DNA damage in puffer fish, *Takifugu obscurus*, under low temperature stress. A combination of both the vitamin C and E at a dose double than the usual dose effectively maintained the different physiological, biochemical, and haematological profiles of *Labeo rohita* when exposed to fenvalerate (Prusty et al. 2011).

22.2.2 B-Complex Vitamins

The B-complex vitamins have been commonly being utilised for the normal metabolic functions in the body as they are the co-enzymes for many critical metabolic enzymes. In addition to their function of catalysing most anabolic pathways, they act as anti-stress vitamins. Many of the B vitamins are involved in maintaining the normal function and metabolism of the immune system (Alpert 2017). The B vitamins are also required for steroid biosynthesis in the adrenal cortex. Several studies have shown that adrenal malfunction occurs with riboflavin nicotinamide, pantothenic acid, pyridoxine, and biotin deficiencies (Hendler and Rorvik 2001).

22.2.2.1 Vitamin B1 (Thiamine)

The level of thiamine affects immune function of lake trout (Ottinger et al. 2014), as deficiencies disrupt regulation of inflammation response. Dietary thiamine supplementation modulated oxidative defence and inhibited lipid peroxidation and protein oxidation in Jian carp (Li et al. 2014). Additionally, thiamine (vitamin B_1) protects against lead-induced oxidative damage in common carp (Mirmazloomi et al. 2015) and modulates immune functions in fish (Xun et al. 2019). Wen et al. (2015) evaluated the effect of thiamine on intestinal immunity of *Ctenopharyngodon idella*. They found that thiamine is positively associated with the production of non-specific immune markers of the head, kidney, spleen, and liver.

22.2.2.2 Vitamin B2 (Riboflavin)

Kumar et al. (2020) demonstrated the combined effect of riboflavin (0.5 mg/kg diet) and nano-selenium (5 mg/kg) on enhancing thermal efficiency and stress tolerance (high temperature and arsenic stress) in *Pangasioanodon hypophthalmus.* Riboflavin (vitamin B_2) is known to possess potent antioxidant properties and act as a component of the glutathione redox cycle (Marashly and Bohlega 2017).

22.2.2.3 Vitamin B3 (Niacin)

Although studies in fish are scarce, dietary administration of niacin ameliorated methyl mercury-induced oxidative stress and genotoxicity in rats (Pereira et al. 2020). Niacin is also part of the glutathione redox cycle and possesses remarkable antioxidant properties (Ilkhani et al. 2016). Thus, the inclusion of this vitamin can be attempted in the diet of fish as a stress mitigation strategy.

22.2.2.4 Vitamin B6 (Pyridoxine)

Dietary pyridoxine can boost the production of serotonin and GABA (gamma-aminobutyric acid), which are crucial for stress response. Dietary pyridoxine plays a role in stress mitigation, immunomodulation, and enhancing thermal tolerance. Dietary supplementation of pyridoxine 100 mg/Kg diet resulted in immunomodulation and mitigation of stress due to endosulfan (Akhtar et al. 2010) in *L. rohita*. The thermal tolerance of *L. rohita* could be enhanced by dietary supplementation of pyridoxine at 100 mg kg^{-1} (Akhtar et al. 2011). Dietary supplementation of pyridoxine at 100 mg kg^{-1} diet may reverse the adverse effects caused

by elevated temperature and protect the haemato-immunological status of *L. rohita* reared at higher water temperature. The inclusion of dietary pyridoxine in the above studies had a significant effect on growth performance and the activities of various antioxidative enzymes and stress parameters. The pyridoxine-treated groups exhibited higher specific growth rate (SGR), lower activity levels of liver catalase, superoxide dismutase (SOD) of liver and gills, and acetylcholinesterase of brain tissue and lower levels of blood glucose and cortisol. Haemato-immunological parameters such as erythrocyte count, leukocyte count (WBC), haemoglobin content, serum albumin, globulin, and lysozyme activity were significantly higher in the treatment groups as compared to the control. Dietary administration of pyridoxine (45 days) improved growth performance and stress tolerance in *Chanos chanos* (Kumar et al. 2017a). This 'anti-stress' potential of pyridoxine can partially be attributed to its ability to increase the production of neurotransmitters, viz. serotonin and GABA (Hartvig et al. 1995), which are crucial for control of depression, pain perception, and anxiety (McCarty 2000). Studies showed that modulation of GABAergic and/or serotonergic signalling reduces/mitigates stress-related behavioural responses in adult zebrafish (Davis et al. 2016) and rainbow trout (Schjolden et al. 2006). Further, several enzymes engaged in the kynurenine metabolic pathway of tryptophan that produces kynurenines require pyridoxine as a cofactor, and many of the kynurenines have neuroprotective and immunomodulatory effects (Ueland et al. 2017). Thus, through the increased synthesis of immunomodulatory kynurenines, pyridoxine is suggested to bring an enhanced immune response. Pyridoxine also has a role in erythropoiesis, as it is a co-enzyme for the enzyme for the rate-limiting step and final step in heme synthesis.

22.2.2.5 Vitamin B5 (Pantothenic Acid)

The administration of pantethine improved the adrenal cortex function in several experimental animals. The administration of pantethine downregulated the hypersecretion of cortisol during stress (Kosaka et al. 1973). Though Lieberman and Bruning (1997) described pantothenic acid (vitamin B_5) as an 'anti-stress vitamin', its stress-relieving potential has not been studied in fish. Pantothenic acid and its derivatives have been reported to protect the cell membrane against oxidative damage (Slyshenkov et al. 2004) and dietary pantothenic acid deficiency-induced oxidative stress in juvenile blunt snout bream (Qian et al. 2015).

22.2.2.6 Vitamin B9 (Folic Acid)

Folic acid/vitamin B_9 (active form as tetrahydrofolate) are essential in one-carbon pathways involved in amino acid and nucleotide metabolism (National Research Council 1993). Dietary folic acid supplementation imparted thermal tolerance to blunt snout bream, *Megalobrama amblycephala*. Along with thermal tolerance, folic acid helped to enhance non-specific immune responses in blunt snout bream. Besides, folic acid stimulates immunity, antioxidant activity, and resistance against bacterial infection (Sesay et al. 2016). Earlier studies in animal models showed the ameliorative effect of folic acid against oxidative stress and chemical/metal toxicity (Réus et al. 2018).

22.2.2.7 Vitamin B12 (Cyanocobalamin)

Cortisol has a role in controlling circadian rhythm in the body which act in association with melatonin. Although methylcobalamin does not impact total levels of cortisol, evidence suggests that it might help shift the cortisol secretion peak, which aids in secretion at normal levels (Tomoda et al. 1995). On the other hand, studies in higher animals, including humans, showed that cobalamin (vitamin B_{12}) reacts with superoxide anion as efficiently as SOD and helps to maintain cellular redox status (Suarez-Moreira et al. 2011). This superoxide anion scavenging ability of cobalamin is likely related to its core corrin ring structure (Chan et al. 2018). Recently, intramuscular injection of butaphosphan (a phosphoric acid compound) and cyanocobalamin combination drug has been reported to attenuate stress effects in olive flounder *Paralichthys olivaceus* (Seo et al. 2020).

22.2.3 Vitamin E (Tocopherol)

Several studies have shown that acute or chronic exposure to stress increases free radical formation throughout the body but specifically in the adrenal cortex. In response to stress, vitamin E has been shown to protect the adrenal cortex from free radical damage and reduce cortisol production (Wilson et al. 2006). As an antioxidant, vitamin E has been shown to protect the body from a wide range of effects of free radicals (Hendler and Rorvik 2001). Vitamin E (α-tocopherol) is another potential vitamin advocated for stress management in aquaculture. Dietary administration of vitamin E helped to protect several fishes from the deleterious effect of various stressors, including heat stress in golden shiner (Trenzado et al. 2008. 2004), microcystin and lead toxicity in Nile tilapia (Tanekhy and Khalil 2014), nitrite stress in rohu (Ciji et al. 2014), ammonia stress in puffer fish and darkbarbel catfish (Cheng et al. 2018a, b), and crowding stress in Wuchang bream (Liu et al. 2014). Feeding vitamin E helped increase the immunity, thereby survival of nitrite-stressed fish after the bacterial challenges (Ciji et al. 2014). Like ascorbic acid, vitamin E exerts this protective effect through its potent antioxidant function (Cheng et al. 2018a, b), modulating HSPs expression (Cheng et al. 2018a, b) and reducing cortisol (Montero et al. 2001) and glucose levels (Liu et al. 2014). It further improved immune responses via increased complement, lysozyme, and respiratory burst activity and increased phagocytic index, total immunoglobulin, and total serum protein content (Li et al. 2014; Liu et al. 2014; Ortuño et al. 2003).

Additionally, vitamin E protects red blood corpuscles against haemolysis, and its deficiency is reported to increase erythrocyte fragility and reduce stress resistance (Montero et al. 2001). A study by Ciji et al. (2014) revealed that nitrite stress in *L. rohita* juveniles could be effectively mitigated by supplementing vitamin E and diet. In this study, dietary supplementation of vitamin E ameliorates the ill effects of nitrate-nitrogen such as nitrite accumulation, effects on growth, haematological variables, and ionic balance. Antioxidant enzymes such as catalase and SOD were significantly lower in the experimental group having the maximum effect of vitamin E. The level of blood glucose was significantly lower in the groups supplemented

with vitamin E, which indicates the role of this vitamin in the mitigation of stress. Dietary supplementation of additional amounts of vitamin E enhanced the detoxification of nitrite by the met-haemoglobin reductase system. Dietary vitamin E was also found to be effective in mitigating hypoxia-mediated oxidative injury in *Cirrhinus mrigala* (Varghese et al. 2017).

22.2.4 Vitamin A (Retinol)

Retinoic acid/retinol and carotenoid precursors are powerful antioxidants essential in the production of steroid hormones. When a diet deficient in retinoic acid was fed to rats, they showed significant stunting of the adrenal cortex and could not produce average amounts of corticosteroids involved in stress response. Vitamin A (a fat-soluble vitamin) is essential to maintain healthy blood count and immune response of vertebrates, including fish. Vitamin A is also essential for converting pregnenolone into cortisol, and even mild deficiency of vitamin A causes significant blunting of cortisol production. Researches show retinoic acid or retinol to have a positive impact on adrenal health. Retinoic acid has a role in both innate and adaptive immune responses. The deficiency of it may affect the ability of this metabolite to control tolerance and immunity. Vitamin A plays a role in maintaining normal mucosal immunity by regulating T-cell function (Hall et al. 2011).

22.2.5 Choline and Inositol

These vitamins are known as macro-vitamins as they are added in high quantities during the formulation of aquafeed. Dietary incorporation of choline, another water-soluble vitamin, improved tolerance of kuruma shrimp (*Marsupenaeus japonicas*) against salinity and formalin stress (Michael and Koshio 2013) and giant grouper (*Epinephelus lanceolatus*) against ammonia stress (Yeh et al. 2015). Yeh et al. (2015) hypothesised a possible regulatory role of choline on corticotrophin-releasing factors and hypothalamus-pituitary-interrenal axis against environmental stress. Myo-inositol, another water-soluble macro vitamin, being a major intracellular osmolyte and a constituent of the cell, defends cells from different stressors (Michell 2008). Dietary myo-inositol administration reported augmenting salinity tolerance by regulating various physiological functions in several fish such as turbot (Cui et al. 2020) and pacific white shrimp (Chen et al. 2018a, b). Further, myo-inositol inhibited oxidative damage and improved immune responses of stress-exposed aquatic animals such as Jian carp subjected to copper toxicity, *P. vannamei* exposed to hypoxia (Chen et al. 2020), and Wuchang bream exposed to ammonia stress (Ciji et al. 2014).

22.3 Minerals

Animals require minerals/inorganic elements to maintain many of their metabolic functions and provide major structural elements. The effects of the minerals are dependent upon the availability and deficiency of other minerals. The majority of the studies on mineral nutrition in fish are primarily concentrated on determining the minimum/essential requirement for supporting maximum growth and health (Izquierdo et al. 2017). Some of the minerals act as stress mitigating agents as well as immune nutrients (Table 22.2).

Among these, selenium (Se) is the most widely studied trace mineral for stress management, and the research data clearly showed its positive effects in several vertebrates, including teleosts. Earlier studies in Chinook salmon and rainbow trout

Table 22.2 Minerals in immunity/stress mitigation in fish

Minerals and dose	Species	Response	References
Dietary administration of optimal selenium (organic/inorganic/nano-selenium); dose (0.3 and 0.6 mg/kg nano-selenium)	Grass carp *Ctenopharyngodon idella*	Shown to enhance antioxidant capacity and hypoxia tolerance	Yu et al. (2020a, b)
Dietary supplementation with selenium nanoparticles (1 to 2 mg/kg level)	Red Sea bream (*Pagrus major*)	Increased stress resistance against low salinity and higher serum and mucosal immune responses	Dawood et al. (2017)
Diet supplemented with 0.2 mg/kg nano-selenium	Chinese mitten crab (*Eriocheir sinensis*)	Alleviated hypoxia stress and enhanced immune response	Qin et al. (2016)
Diet supplemented with nano-selenium (0.3 and 0.6 mg/kg)	Grouper, *Epinephelus malabaricus*	Reduced oxidative stress and improved the immune response of the fish	Rider et al. (2009a, b).
Diets supplemented with mineral zinc (ZnSO4), zinc nanoparticles (ZnO-NPs), organic zinc (Zn-proteinate, bioplex Zn®) of 40 mg/kg each	Rainbow trout (*Oncorhynchus mykiss*)	Improved sperm and seminal plasma quality indices and reproductive performance in broodstock males	Kazemi et al. (2020)
Iron (82.51–86.05 mg/kg feed)	Stinging catfish (*Heteropneustes fossilis*)	Improved the growth performance, conversion efficiencies, red blood cell counts, and haemoglobin, haematocrit, mean cell haemoglobin, and mean corpuscular volume	(Zafar and Khan 2020)
Dietary micronutrient supplementation (zinc, selenium, ascorbic acid, and niacin, levels higher than recommended)	European seabass, (*Dicentrarchus labrax*)	Promotor of skin health and prevent from fin erosions, potentially through an increase in the antioxidant defence system	Kokou et al. (2020)

documented that physical stressors (transportation, handling, and confinement) caused higher utilisation of Se, resulting in loss of carcass Se. Hence, Se supplementation to commercial diets may be required to cover the requirements under stressful husbandry situations (Rider et al. 2009a, b). Dietary administration of optimal Se (organic/inorganic/nano-selenium) has shown to reduce the impact of stress associated with poor husbandry conditions in several fishes, including hypoxia stress in grass carp (Yu et al. 2020a, b), hypoxia and nitrite stress in Chinese mitten crab (Wang et al. 2019), low salinity stress in red sea bream (Dawood et al. 2017), nitrite and ammonia stress in Wuchang bream (Guo et al. 2020), confinement stress in gilthead seabream (Mechlaoui et al. 2019a, b), and crowding stress in trout (Küçükbay et al. 2009). Elevated utilisation of Se was noticed during stress, and hence supplementation of Se in commercial diets may improve stress responses. Dietary Se supplementation in grouper, *Epinephelus malabaricus*, reduced oxidative stress and improved the immune response of the fish. Se status was more effectively maintained by Se-yeast than selenite (Rider et al. 2009a, b) under stressful conditions. The protective effect of Se on heavy metal and pesticide-induced oxidative stress in fish is well documented (Xie et al. 2016).

On the contrary, dietary inclusion of Se (1 mg Se kg^{-1} diet) increased nitrite toxicity *in Penaeus vannamei* (Wang et al. 2006). The protective role of Se to overcome the adverse impact of stress is ascribed to its antioxidative properties (Yu et al. 2020a, b). Selenium is crucial for the activity of different proteins (selenoproteins), including antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase (Pacitti et al. 2015), as well as selenoprotein P implicated in the metal detoxification (Pacitti et al. 2015). Selenium/selenoprotein is also involved in several immune system components (Pacitti et al. 2015). Additionally, recent studies demonstrated the inhibitory effect of Se on cortisol secretion in adrenocortical cells of several fishes (Mechlaoui et al. 2019a, b). Although selenium is beneficial at low dietary levels, it is potentially toxic in higher quantities (Lee et al. 2016). Therefore, the dietary inclusion level of Se must be cautiously determined in every species and life stages to avoid its detrimental effects.

Zinc (Zn), copper (Cu), and manganese (Mn) are the other potential trace minerals implicated in stress alleviation as they function as cofactors of several enzymes, including antioxidative enzymes such as superoxide dismutase (Zn/Cu/Mn-SOD). Küçükbay et al. (2009) studied the effect of zinc picolinate (ZnPic) supplementation on growth, feed intake, feed efficiency, and concentrations of malondialdehyde (MDA), Zn, Cu, and Mn and serum alkaline phosphatase (ALP) activity of rainbow trout. The results indicated a significant reduction of oxidative stress as a result of ZnPic supplementation. Zinc also acts as a neuromodulator at excitatory synapses, playing an essential role in stimulating ATPase, particularly Na^+/K^+ ATPase activity contributing to stress tolerance (Frederickson et al. 2005) and also acts as a component of metallothioneins (Kumar et al. 2017c). Further, in vitro studies in common carp head kidney showed that zinc stimulates hemopoietic cell proliferation, resulting in an increased number of red blood cells (Chen et al. 2013). Recently, dietary Zn supplementation at 10–20 mg/kg diet improved

tolerance to lead toxicity and higher temperature stress in *Pangasius hypophthalmus* (Kumar et al. 2017c).

Dietary copper and manganese inclusion has been demonstrated to modulate immune reaction and antioxidant defence in some fishes (Nie et al. 2016). However, studies evaluating the stress-relieving potential of copper and manganese are scarce in aquatic animals. Like selenium, higher inclusion levels of Zn and Cu are potentially toxic in aquatic animals (Bielmyer et al. 2012).

Dietary calcium and sodium supplementation reported protecting several fishes such as rainbow trout, Coho salmon, and Nile tilapia against metal (particularly cadmium, zinc, lead, and copper) toxicity by downregulating the rate of calcium and sodium uptake across the gills, thereby decreasing the entry and accumulation of toxic metals (Kosai et al. 2011). Dietary sodium protected yellow perch *Perca flavescens* from copper-induced olfactory impairment (Azizishirazi et al. 2015). Furthermore, incorporation of sodium chloride, either as a dietary or water additive, was found to improve the resistance of several fishes to nitrite toxicity (Welker et al. 2012), *Oncorhynchus mykiss* to acid (low pH) stress (D'Cruz and Wood 1998), and *L. rohita* to transportation stress (Biswal et al. 2021).

Dietary phosphorus levels have been found to influence the immune function in fish (Chen et al. 2017). However, the authors reported that phosphorus-deficient diet-fed fish exhibited a lower upper lethal temperature (0.6 °C) than fish fed with adequate phosphorus levels. Dietary inclusion of magnesium has shown to augment antioxidant status in fish (Dezfouli et al. 2019). Deficiencies of magnesium will cause negative changes throughout the HPA axis (Ismail and Ismail 2016). Magnesium is an essential cofactor for the activation of many processes and is specifically needed to activate the transport of pyridoxine (Eby III and Eby 2010), which mitigate stress. The essential micro-minerals, such as zinc, copper, manganese, selenium, molybdenum, chromium, and iodine, act against oxidative stress and are also essential cofactors of enzymes involved in adrenal cortex function (Guo et al. 2013).

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Health-Promoting Effects of Amino Acids in Fish

23

Seyyed Morteza Hoseini and Miriam Reverter

Abstract

Tryptophan, arginine, sulfur amino acids, histidine, branched-chain amino acids, glutamate, and glutamine are functional amino acids in fish. Functional amino acids have important roles in fish health by modulating various functional physiological pathways. Numerous researches have been conducted on the functions of these amino acids in fish. Some of them, such as tryptophan, arginine, and sulfur amino acids, have been studied frequently; but others such as histidine and branched-chain amino acids have been less studied. Among the topics, assessment of immune and antioxidant responses to these amino acids have been extensively studied, as modulation of antioxidant and immune systems is a shared property of these amino acids; however, they have some unique properties, as well. Tryptophan is a modulator of serotonergic and melatonergic system and affects fish stress axis and antagonistic behaviors. Arginine has somatotropic and ureagenic effects in fish and affects fish growth and ammonia metabolism. Histidine is precursor of imidazoles and N-acetyl histidine; thus this amino acid helps fish to maintain buffering capacity during burst swimming and prevents cataract. Glutamine and glutamate are involved in fish intestinal development and health that, in turn, modulate the fish health and growth. Moreover, glutamine and glutamate involve in ammonia detoxification processes that help the fish ammonia resistance. In this chapter, the aforementioned roles of the functional amino

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acids have been discussed to conclude the current knowledge about the roles of these molecules in fish health.

Keywords

Amino acids · Immune responses · Antioxidant responses · Welfare · Nutrition

23.1 Introduction

Fishes do not need dietary protein, but a balanced mixture of different amino acids that cover various biological processes (Wilson 2002). Amino acids are mostly used to construct skeletal muscle; thus, they affect fish growth rate. As such, dietary needs for certain amino acids have been often determined by monitoring fish growth responses (Wilson 2002). However, some amino acids are versatile and multifunctional and are vital in multiple physiological pathways. These are sometimes called "functional amino acids" and include tryptophan, arginine, histidine, methionine, taurine, glutamate, glutamine, valine, leucine, and isoleucine (Andersen et al. 2016). This means the need for these amino acids is context-dependent; i.e., change in rearing conditions and dietary composition may affect the requirement for these amino acids. Among them, tryptophan is found at a low concentration in fish muscular protein structure, whereas, taurine is totally absent in protein structure (El-Sayed 2014; Hoseini et al. 2019b). They, however, participate in numerous physiological and biological pathways such as the brain neurotransmitter function, fish behavior, immune function, corticosteroid responses, bile salt formation, osmotic homeostasis, the liver health, and antioxidant capacity (El-Sayed 2014; Hoseini et al. 2019b). Thus, fish growth response might not be a suitable tool to determine the requirement for functional amino acids and other physiological responses should be considered in order to determine the fish requirements for the functional amino acids. In this chapter, we have focused on the health-boosting effects of these amino acids under normal and stressful conditions and discuss the knowledge on their dietary requirements.

23.2 Tryptophan

Tryptophan is one of the essential amino acids in animals and should be supplied by daily diet. The fish requirement for tryptophan has been determined in different species using growth responses, being 0.31–3% of the dietary protein levels. Scoliosis, lordosis, cataract, and shortening of operculum are clinical signs of dietary tryptophan shortage in fish. Anxiety and aggression are behavioral signs of dietary tryptophan deficiency (Hoseini et al. 2019b).

Tryptophan is the precursor of serotonin (5-HT) and melatonin, both seem to be responsible for most of tryptophan effects in fish. 5-HT and melatonin are found in the fish gut and brain, but most of their effects on physiological pathways relate to

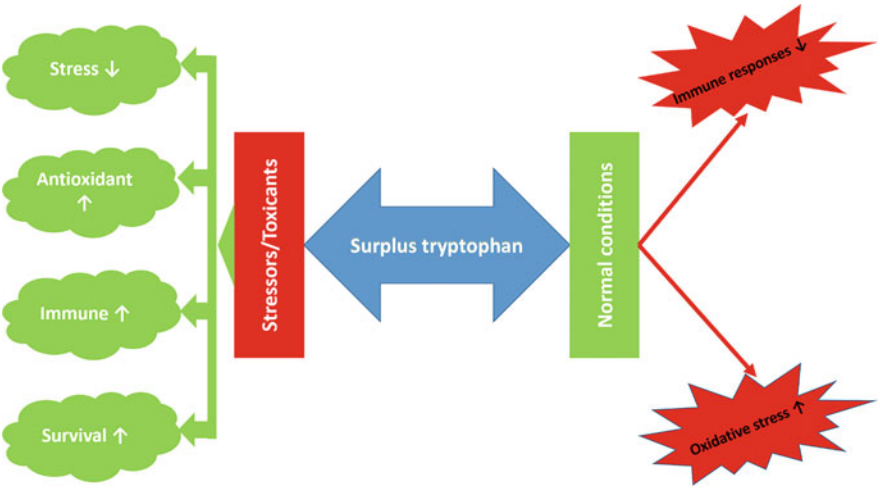

Fig. 23.1 Effects of dietary surplus tryptophan supplementation on fish under normal and non-normal (stressors and toxicants)

their levels in the fish brain (Hoseini et al. 2019b). However, tryptophan effects on fish health is situation-dependent and surplus levels of dietary tryptophan negatively affect fish health (Fig. 23.1).

23.2.1 Modulation of Agonistic Behavior by Dietary Tryptophan

5-HT is a monoamine that acts as a neurotransmitter in the brain and plays a paracrine or endocrine role in the blood and intestine (Lepage et al. 2005a). Small amount of tryptophan is used in the synthesis of 5-HT, which is essential for the proper functioning of certain organs. Increased tryptophan absorption in the gastrointestinal tract causes an increase in tryptophan level in the blood, which in turn, increases the available tryptophan for absorption in the brain (Lepage et al. 2005a). Tryptophan is converted to 5-HT by the activity of tryptophan hydroxylase and aromatic L-amino acid decarboxylase in the presence of vitamin B_6. The activity of tryptophan hydroxylase is not saturated by the substrate (tryptophan), so increasing the tryptophan intake leads to a steady increase in the amount of 5-HT and also the main metabolite of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), in the brain (Lepage et al. 2003). 5-HT is important in gastrointestinal tract function, dilation and contraction of blood vessels, and accumulation of neutrophils; however, these aspects were rarely studied in fish. On the other hand, the brain 5-HT has various roles in fish welfare, which were studied extensively in the fishes (Hoseini et al. 2019b).

Some fish species have agonistic behavior characterized by biting, cannibalism, and social hierarchy. These behaviors are clearly detrimental for the fish health.

Cannibalism causes direct loss of fish in a population that is economically important. Biting causes stress in the fish that decreases feeding rate (causing growth depression) and susceptibility to pathogens (increasing disease-associated mortality). Hierarchical behavior leads to dividing a fish population into two groups, ordinates and subordinates (Øverli et al. 2000). The subordinates exhibit avoidance behavior and eat less food. This leads to size heterogeneity in a fish population (that is not a good factor in aquaculture) and deteriorated welfare of the subordinates (Øverli et al. 2000; Hseu et al. 2003). Tryptophan administration stimulates the synthesis of 5-HT in the brain, which, according to studies on rainbow trout, *Oncorhynchus mykiss*, stimulates the hypothalamic-pituitary-interrenal (HPI) axis, thereby controlling the fish aggressive behavior and appetite (Lepage et al. 2003). Tryptophan administration leads to an elevation in the brain concentration of 5-HIAA and 5-HIAA:5-HT ratio, thereby suppressing aggressive behaviors in the fish. For example, elevation in dietary tryptophan from 0.47 to 0.94% led to lower aggressive behavior in matrinxã, *Brycon amazonicus* (Wolkers et al. 2012). Likewise, supplementation of a basal diet (containing 0.7% tryptophan) with 0.5% tryptophan significantly increased the brain 5-HT and decreased size heterogeneity and cannibalism in grouper, *Epinephelus coioides* (Hseu et al. 2003). Such results were reported in other fish species such as cichlid *Cichlasoma dimerus*, rainbow trout, and Atlantic cod, *Gadus morhua*, proving dietary tryptophan supplementation as a practical tool to suppress the adverse effects of agonistic behavior in fish (Winberg et al. 2001; Höglund et al. 2005; Morandini et al. 2019).

23.2.2 Modulation of Stress Responses by Dietary Tryptophan

Serotonergic activity plays an important role in the HPI axis, and this activity is usually measured by the rate of production of 5-HIAA (Lepage et al. 2003). Such an increase in the serotonergic activity must be supported by tryptophan availability. The amount of tryptophan required in stressful conditions increases because it has been observed that the plasma tryptophan concentration in farmed fish increases under stress compared to non-stressed fish (Aragao et al. 2008; Costas et al. 2008). Therefore, oral administration of tryptophan can be a good nutritional strategy for managing fish health in aquaculture. Most of the researches in this field have focused on short-term effects of dietary tryptophan administration. For example, a 7-day period of tryptophan administration was found to mitigate a stress-induced cortisol elevation in rainbow trout and European seabass, *Dicentrarchus labrax*, by enhancing the serotonergic activity in the fish brain (Lepage et al. 2003; Herrero et al. 2007). Sometimes, tryptophan administration produced an anxiolytic effect in fish; for example, a 7-day period of tryptophan administration significantly subsidized a stress-induced anorexia in brown trout, *Salmo trutta* (Höglund et al. 2007). Stress-mitigating effects of tryptophan administration may lead to higher chance of fish to resist under non-desirable conditions. For example, dietary tryptophan administration for 15 days mitigated cortisol response of common carp, *Cyprinus carpio*, to a lethal osmotic stress. Such mitigation in cortisol response was accompanied by

higher survival rate during the stress (Hoseini and Hosseini 2010). In ruho, *Labeo rohita*, and mrigal, *Cirrhinus cirrhosis*, oral administration of tryptophan for 45–90 days under chronic stress conditions (high stocking density and nitrite exposure) inhibited the blood glucose and cortisol elevation and increased the fish growth rate, indicating that under stressful conditions the need for tryptophan increased to maximize the fish growth (Tejpal et al. 2009; Akhtar et al. 2013a; Kumar et al. 2014). However, such effects were not always observed in different studies. For example, tryptophan supplementation significantly decreased blood cortisol and glucose and increased growth performance of rainbow trout at a low, but not a high stocking density (Hoseini et al. 2020c). In meagre, *A. regius*, tryptophan administration increased blood cortisol at a low fish density, but had no effects at a high density (Herrera et al. 2020). Interestingly, when totoaba, *Totoaba macdonaldi*, was subjected to handling and hypoxia, diet containing 1.1% tryptophan intensified cortisol response compared to a basal diet (0.5% tryptophan). A diet containing 1.65% tryptophan intensified cortisol response to hypoxia not handling, and a diet containing 2.9% tryptophan exhibited no significant difference, compared to the basal diet. However, only the diet containing 1.65% tryptophan inhibited hyperglycemia after the stresses (Cabanillas-Gámez et al. 2018). A subsequent study by the same authors showed that, under a high stocking density, dietary tryptophan supplementation at low level (0.99%) significantly increased cortisol, compared to the fish fed a control diet (0.42%); however, blood glucose levels showed an opposite trend. The fish fed high levels of tryptophan (2.19%) exhibited no significant elevation in blood cortisol and glucose, but had lower weight gain under a high stocking density (Cabanillas-Gámez et al. 2020). Thus, the anti-stress effects of tryptophan depend on species, nature of stress, and experimental conditions that force one to measure other supportive parameters than cortisol and glucose levels in blood. As mentioned above, due to the time-dependent nature of cortisol and serotonergic responses to stress, time is an important factor in assessing the effects of tryptophan on the fish stress responses. When evaluating the effects of tryptophan on an acute stress, the amount of tryptophan administration and sampling time should be considered. It is also necessary to consider the amount of tryptophan in the control diet; because in the studies mentioned above, the control diets contained different amounts of tryptophan (0.3–4.45%). The issue may become more complicated when species differences are added up.

23.2.3 Modulation of Fish Immune Responses by Dietary Tryptophan Supplementation

Fish metabolism and immunity are affected by stressful conditions, and if the diet is not well-balanced in terms of nitrogenous content, it may deteriorate the fish health. A balanced diet provides adequate health and immune power for the fish. Recent evidence suggests that stress affects amino acid metabolism in fish, and in some stressful situations, the need for specific amino acids increases (Aragao et al. 2008; Costas et al. 2008). Amino acids act as messengers to regulate the immune responses

and the activity of natural killer cells and macrophages, cell regenerative conditions, gene expression, lymphocyte proliferation, and production of antibody, cytokines, nitric oxide, and superoxide (Wu 2013). In addition, the innate and acquired immune responses depend on the presence of sufficient amino acids to synthesize antigen-presenting molecules, immunoglobulins, and cytokines (Sakkas et al. 2013).

Tryptophan plays an important role in the neuroendocrine and the immune systems. Stress suppresses the fish immune response, and tryptophan-containing diets have been shown necessary to counteract stress. In addition, tryptophan metabolites such as 5-HT and melatonin may improve fish health by decreasing the free radicals and pro-inflammatory cytokines (Perianayagam et al. 2005). However, the dose of tryptophan and ambient conditions are determinant in this case. For example, prolonged administration of tryptophan has reduced the negative effects of water nitrite or salinity and thermal stress on the immune system in rohu (Akhtar et al. 2013a, b; Ciji et al. 2015). Enrichment of the diet with tryptophan (twice the required level) has increased the immune strength and disease resistance of Senegal sole, *Solea senegalensis*, at either low (12.5 kg/m^2) or high (31 kg/m^2) stocking density. In the same study, it was found that higher levels of tryptophan in the diet (4 times the required level) was beneficial under the high density culture, but decreased immunity and reduced disease resistance at the low culture density (Azeredo et al. 2016). In rainbow trout, administration of 0.5% tryptophan for 70 days increased plasma lysozyme and bactericidal activity at two stocking densities (15 and 25 kg/m^3), but administration of 1% tryptophan had no positive effects on the fish immune parameters (Hoseini et al. 2020a). These studies clearly indicate that dietary requirement for tryptophan tightly depends on culture conditions. In fact, tryptophan supplementation above the required levels might be beneficial under stressful conditions, during which, fish need more tryptophan to counteract the stress by increasing the serotonergic and melatonergic activities. However, surplus tryptophan under normal conditions left excess tryptophan available in the fish body and may mimic the effects of an imbalanced diet. In this case, it has been reported that in perch, *Sander lucioperca*, tryptophan administration reduced plasma lysozyme activity and growth performance after 91 days of feeding (Mandiki et al. 2016). Such results have been reported in grass carp, *Ctenopharyngodon idella*, where, both low and excess levels of dietary tryptophan induced pro-inflammatory cytokines in the fish gill and intestine, accompanied by a lower growth rate, that indicate physiological disturbances (Wen et al. 2014).

23.2.4 Antioxidant Effects of Tryptophan in Fish

Tryptophan has antioxidant activity because it reacts with highly reactive free radicals (such as hydroxyl radicals) and also regulates the activity of antioxidant enzymes. However, the mechanisms of the antioxidant effects of tryptophan are not well-understood. Many of the antioxidant properties of tryptophan have been attributed to its metabolites. 5-HT and melatonin, the major molecules synthesized from tryptophan, are important antioxidants; although compared to melatonin, the

antioxidant capacity of 5-HT has been less studied. Numerous studies have shown that 5-HT can act as a direct scavenger of free radicals and reduce the peroxidation of fats in tissues. In addition, 5-HT has been shown to be able to chelate iron ions, and its direct metabolite, N-acetyl serotonin, is known as an antioxidant molecule (Hoseini et al. 2019b).

The effects of tryptophan-enriched diets on farmed fish under oxidative stress have rarely been studied. The effects of dietary enrichment with tryptophan (0.56–3.5%) in ruho under chronic stress (thermal and osmotic stress, and nitrite toxicity) have been studied, and it has been observed that tryptophan increases antioxidant power and reduces oxidative stress in the fish (Ciji et al. 2012; Akhtar et al. 2013a; Kumar et al. 2014). In this study, surplus tryptophan levels were used, which shows that the tryptophan requirement increases under oxidative conditions due to environmental stress and pollutants. In grass carp exposed to water copper, oral administration of tryptophan (0.07, 0.17, 0.31, 0.4, 0.52, and 0.61%) improved the antioxidant parameters at both protein and transcription levels. Moreover, middle levels of tryptophan (0.31 and 0.4%) decreased the products of oxidative stress (malondialdehyde and protein carbonyl) in the fish intestine. However, high levels of tryptophan caused oxidative stress, which again indicates that the optimal amount of tryptophan administration varies under different conditions (Wen et al. 2014). Oral administration of tryptophan in rainbow trout had no effect on plasma superoxide dismutase activity but altered catalase activity, depending on dose and the fish rearing density. However, administration of 0.5% tryptophan to this species reduced oxidative stress (lower malondialdehyde content) under both low and high stocking densities (Hoseini et al. 2020a).

23.2.5 Tryptophan Administration in Fish Exposed to Toxicants

Toxicant exposure generally induces oxidative stress and immunosuppression in fish; thus, considering the immunomodulating and antioxidant capacity of tryptophan, some researchers tried to use the amino acid to subsidize the adverse effects of toxicants in different fish species. Dietary tryptophan administration (30 and 60 days) significantly decreased mortality of Caspian roach, *Rutilus caspicus*, following an acute copper exposure (Fatahi and Hoseini 2013). A long-term (60 days) tryptophan administration significantly mitigated adverse effects of chronic nitrite toxicity on growth performance, immune status, and antioxidant parameters of ruho (Ciji et al. 2015). Moreover, 8 weeks administration of tryptophan to grass carp significantly mitigated immunosuppression, oxidative stress, and intestinal inflammation following a 4-day exposure to water copper (Wen et al. 2014). On the other hand, short-term (2 weeks) dietary tryptophan administration significantly decreased mortality, stress responses, and hepatic damages of common carp, during a 7-day exposure to water copper (Hoseini et al. 2012).

Although the above studies show that supplementation of the diet with tryptophan is beneficial for fish during exposure to toxicants, its main underlying mechanisms have not been yet identified. Some of these toxicants may interfere with the brain's

serotonergic activity and disrupt 5-HT synthesis, as was observed in common carp exposed to copper (De Boeck et al. 1995), and snakehead, *Channa punctatus*, exposed to carbofuran (Gopal and Ram 1995). Therefore, by increasing serotonergic activity, tryptophan can help counteract the negative effects of these toxicants on fish. This hypothesis has not yet been tested in fish. Another possible explanation for the positive effects of tryptophan on fish exposed to toxicants may be the reduction of oxidative stress through its antioxidant properties, as the toxicant agents used in these studies lead to oxidative damage in fish. These topics deserve further investigations.

23.2.6 Involvement of Melatonin in Tryptophan-Mediated Effects on Fish

Melatonin is produced from 5-HT, which coordinates daily processes in which the pineal gland and retina play major roles (Hoseini et al. 2019b). In melatonin-producing cells, tryptophan is first converted to 5-HT and subsequently to melatonin in two enzymatic steps: conversion of 5-HT to n-acetyl serotonin, which is catalyzed by arylalkylamine N-acetyltransferase, and then conversion to melatonin by hydroxyindole-O-methyltransferase. Thus, tryptophan is an indirect precursor to the biosynthesis of melatonin, so that, melatonin may be responsible for some of the effects of tryptophan administration on vertebrates.

Different studies in fish have reported variable results from tryptophan administration on melatonin concentrations. Similar to mammals and what happens to 5-HT, tryptophan administration increases melatonin production in rainbow trout enterochromaffin cells (Lepage et al. 2005b). Evidence shows that in teleost, melatonin has an anti-stress effect at the level of HPI axis. In this regard, melatonin inhibits or delays the glucocorticoid response to stress in fish, which is similar to the effects of tryptophan-supplemented diets (Hoseini et al. 2019b). The mechanism of action of melatonin on the HPI axis is not well-understood. There is some evidence in mammals suggesting that melatonin has a direct effect on the adrenal glands, but there is also evidence that melatonin acts directly on the brain and regulates the central pathways that control the hypothalamic-pituitary-adrenal and HPI axes.

Melatonin is the most important antioxidant molecule derived from tryptophan metabolism. Unlike other antioxidants, this substance and its metabolites can scavenge up to 10 free radicals (Tan et al. 2015). In addition, melatonin is not only an active antioxidant that has radical scavenging property, but also has the ability to activate major antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Studies have shown that melatonin administration can improve antioxidant capacity and decrease oxidative stress in fish and shellfish under normal conditions or during toxicant exposure and social challenge (Gülçin et al. 2009; Jung et al. 2016).

Melatonin has been reported to modulate immune system in aquatic animals. It involves in the development of the thymus and spleen and also regulates innate and acquired immune responses (Carrillo-Vico et al. 2005). However, information about

the role of melatonin in the immune function of teleost is limited. Intraperitoneal injection of melatonin (1 or 10 mg melatonin/kg body weight) to gilthead sea bream, *Sparus aurata*, increased peroxidase activity, phagocytosis, production of reactive oxygen molecules, and expression of interleukin 1 beta, factor 1 regulating interferon and Mx, and immune markers of lymphocytes in the fish head kidney (Cuesta et al. 2008). Moreover, a positive correlation was found between the plasma melatonin level and immune parameters in perch (Baekelandt et al. 2020). Melatonin administration significantly increased immune responses of a shellfish, *Eriocheir sinensis*, to bacterial challenge and hypoxia (Yang et al. 2020b; Song et al. 2021).

The above information clearly suggests that the beneficial effects of tryptophan on fish might be mediated, at least in part, by increasing rate of melatonin synthesis. Assessment of melatonin responses to stress and toxicant exposure can be helpful to increase the knowledge about this topic.

23.3 Arginine

Arginine is an essential amino acid in fish with various physiological roles in growth performance and health. Diet is the main source of arginine for different species of fish, but there is a little evidence supporting arginine biosynthesis from citrulline in certain species of fish, although the rate of synthesis is not as high as that of the higher vertebrates. The arginine content among the various feedstuffs is in the range of 1.12–11.23% of the protein. Fish meal, salmon processing waste, gelatin, hydrolyzed feather meal, rice protein concentrate, pea protein concentrate, soybean protein concentrate, slaughterhouse waste, rice bran, wheat flour, and meat and bone meal contain high levels of arginine. Oats, wheat bran, dry whey, wheat, and algae powder have lower levels of arginine (Hoseini et al. 2020b).

23.3.1 Arginine Synthesis in Fish

Arginine can be synthesized in animals. The urea cycle includes the enzymes carbamoyl phosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate lyase, and arginase. Despite the excretion of most nitrogenous wastes as ammonia, some fish have the urea cycle and related enzymes. This system is likely to be used by the fish to combat ammonia toxicity, which converts ammonia to urea using bicarbonate and ATP. Ammonia is converted to carbamoyl phosphate, L-citrulline, arginine succinate, L-arginine, urea, and L-ornithine. L-ornithine then restarts the cycle (Hoseini et al. 2020b). In mammalian liver, the urea cycle is the most important route of urea production, but not for arginine biosynthesis, because arginase is highly active in the liver. Thus, the synthesis of arginine in the kidney is the main source of arginine production in the body, which is called the renal-intestinal axis, in which L-glutamine, L-glutamate, and L-proline are converted to L-citrulline in the mitochondria of the enterocytes through the action of pyrrole-5-carboxylate synthase, ornithine aminotransferase, and ornithine carbamoyl

transferase. L-citrulline then enters the bloodstream and is absorbed by the kidneys for the synthesis of arginine through the activity of argininosuccinate synthetase and argininosuccinate lyase (Hoseini et al. 2020b).

In teleost, existing evidence suggests that some species are able to synthesize arginine in the body, but the exact mechanisms and responsible organs are unclear. In rainbow trout, the addition of L-citrulline to the fish diet has been shown to increase plasma arginine level, compared to the fish fed a diet supplemented by ornithine or glutamate, and the growth rate in this group is similar to that of fish fed the control diet (containing a sufficient amount of arginine). In addition, L-citrulline-labeled carbon was detected in the arginine retained in the fish body, so that, rainbow trout can utilize citrulline to produce arginine. However, the addition of glutamine to the diet had no effect on plasma arginine level or the fish growth rate, indicating that this compound has no role in arginine biosynthesis in this species (Chiu et al. 1986). In channel catfish, *Ictalurus punctatus*, the addition of glutamine to an arginine-deficient diet has been shown to increase feed efficiency, survival rate, plasma arginine, citrulline, and ornithine levels (Buentello and Gatlin III 2000). Thus, glutamine is involved in arginine synthesis, but no information is available on the responsible organs and pathways. Growth rate and blood arginine level of common carp fed an arginine-deficient diet containing N-carbamoyl glutamate was equal to the fish fed a diet containing sufficient level of arginine (Wang et al. 2019). The addition of N-carbamylglutamate to diet significantly increased the growth rate and blood arginine levels of in Nile tilapia, *Oreochromis niloticus* (Li et al. 2015). On the contrary, the addition of glutamine to the diet had no effect on plasma-free arginine level in Nile tilapia, but reduced the fish growth and protein retention (Pereira et al. 2017). Such a discrepancy might be due to the optimal level of arginine in the basal diet, which masked the potential benefits of glutamine supplementation.

23.3.2 Dietary Arginine and Fish Growth

The addition of arginine to fish diets should be based on the amount required by each species and taking into account the arginine supplied by the feedstuffs. If dietary arginine is not enough, it can lead to various problems in fish, including decreased growth rate, anorexia, and mortality (Hoseini et al. 2020b). Increased plasma arginase activity, primarily due to hemolysis of red blood cells or liver damage, causes arginine deficiency (Hoseini et al. 2020b); however, this issue has not yet been studied in fish and deserves more attentions.

Dietary arginine requirements in different fish show high variation and contradictions. For example, the arginine requirement of the fingerlings of catla, *Catla catla*, (5.06% of dietary protein) is higher than that reported for the fry stage (4.80% of dietary protein) (Ravi and Devaraj 1991; Zehra and Khan 2013). Also, various studies have reported that the arginine required for optimal growth of ruho is 5.75 and 3.47% of dietary protein (Murthy and Varghese 1995; Abidi and Khan 2009). Arginine requirement for Atlantic salmon, *Salmo salar*, has been reported equivalent to both 4.1% and 4.8% of dietary protein (Lall et al. 1994;

Berge et al. 1997). These differences are probably due to differences in fish size and age, ration size, quality of diet used, laboratory conditions, and species differences.

The positive effects of arginine on fish growth are not only due to the optimization of arginine levels for protein synthesis (structural tissues), but also due to stimulation of the somatotropic axis (i.e., growth hormone and insulin), stimulation of the mTOR signaling pathway, and improvement of antioxidant system and promotion of the overall health of fish (Hoseini et al. 2020b). Therefore, other components of the diet (somatotropic agents and antioxidants) may alter the arginine requirement in fish. The somatotropic axis plays an important role in fish growth, and GH and IGF-1 are the two main players in this axis, which are reduced during catabolism or malnutrition (Hoseini et al. 2020b). Studies on fish have shown that dietary arginine administration can increase gene expression and levels of GH and IGF-1, which are usually associated with increased growth performance in fish (Hoseini et al. 2020b). The mechanisms by which arginine stimulates the somatotropic axis are not known in fish, and further studies are needed to demonstrate the controlling factors in arginine-induced activation of the somatotropic axis. Stimulation of the somatotropic axis modulates the fish immune function (Yada 2007) along with growth promotion. There is a body of evidence showing that GH and IGF-1 have strong immunostimulation effects in fish (Franz et al. 2016). Moreover, GH is necessary for osmoregulation (McCormick 2001); thus, arginine treatment might be a useful tool to augment fish resistance against osmotic shock.

Arginine was also found to increase insulin levels in animal, which might explain the growth-promoting effects of arginine. Arginine administration to fish leads to several folds increase in circulating insulin levels that last for hours (Hoseini et al. 2020b). But the effects of arginine on insulin levels and anabolic function have not been fully studied in fish, and there are conflicting results. For example, a diet supplemented by arginine increased blood insulin level in rainbow trout, but failed to lower circulating level of glucose. In addition, arginine administration increases the levels of glucagon-like peptides and glucagon, which have the opposite effects to insulin (Mommsen et al. 2001). Despite the above information, it is not yet clear whether increased insulin levels and its anabolic effects (i.e., increased tissue uptake of glucose and amino acids) are responsible for increased fish growth rate, when fed diets are supplemented with arginine or not.

The nutritional and metabolic interactions of arginine with lysine and glutamate have been reported in various organisms. Arginine and lysine are diamino monocarboxylic acids, therefore, they have a shared transporter through the intestinal barrier. In addition, lysine competes with arginine for reabsorption in the renal tubules and reduces arginine reabsorption, which can have negative growth consequences (Hoseini et al. 2020b). Current information on the interaction of lysine and arginine in different species of fish is contradictory. According to researches, the concentration of arginine in the blood of rainbow trout decreases as dietary lysine level elevates (Kaushik et al. 1988). Also, with increasing arginine in the diet, the concentration of lysine in the blood and muscles of Atlantic salmon decreases significantly; but increasing dietary lysine did not have such effects on muscle and blood arginine levels (Berge et al. 1997). Seemingly, these studies indicate the need

for further researches on this topic. Glutamine is an important amino acid and a major source of nitrogen and carbon in the metabolism of amino acids between different organs. The synthesis of citrulline from glutamine, as well as the sparing effects of glutamate on dietary arginine requirements, has been reported in channel catfish (Buentello and Gatlin III 2000). Also, the addition of glutamine to the diet increased blood arginine levels in common carp and Nile tilapia (Li et al. 2015; Wang et al. 2019). But the data are linited and further studies are needed in this field.

23.3.3 Effects of Arginine on Stress Markers

Arginine has exhibited promising characteristics as an anti-stress amino acid in fish. Arginine administration affects NF-E2-related nuclear factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) transcription, which affect antioxidant responses (Wang et al. 2015). It has been demonstrated that an arginine-deficient diet leads to decrease in antioxidant power of fish and increases lipid peroxidation and protein carbonyl formation. However, it has been demonstrated that surplus arginine levels in fish diet, also, induce antioxidant power depression (Wang et al. 2015). Under oxidative conditions, such as toxicant exposure, arginine administration was found to improve the antioxidant system at the transcription level in common carp (Yousefi et al. 2021). The abovementioned studies suggest arginine can be applied to protect fish against oxidative conditions.

It has been reported that blood arginine is depleted under both acute and chronic stressful conditions in fish (Costas et al. 2008). Arginine administration was found capabling to reduce the blood cortisol levels in mammals and fish (Hoseini et al. 2020b). Studies on fish have demonstrated that arginine administration is capable to help the animal under chronic stressful conditions by subsidizing the effects of the stress on different health aspects of the fish. For example, certain levels of dietary arginine demoted immunosuppression in common carp reared at a high stocking density. Such effects were accompanied by lower blood cortisol levels in the fish; however, higher and lower levels of the amino acid did not exhibit such effects (Hoseini et al. 2019a). Likewise, in Indian major carp, *Cirrhinus mrigala*, dietary arginine supplementation significantly improved the fish growth under normal and hypoxic conditions. Moreover, hypoxia led to immunosuppression and higher susceptibility to bacterial disease, the effects that were neutralized by arginine supplementation (Varghese et al. 2020). These studies demonstrate arginine is a functional anti-stress amino acid.

23.3.4 Arginine as a Strong Immunomodulator

Arginine is an excellent immunostimulant in vertebrates and has recently been considered as an immunostimulant in fish (Hoseini et al. 2020b). Arginine deficiency leads to complications in the immune system, including decreased leukocyte count, lysozyme, complement and phagocytic activity, and the production of superoxide

anion, nitric oxide, and immunoglobulin in various species of fish, as well as susceptibility to pathogens (Hoseini et al. 2020b). Thus, several researches have focused on this topic, although there is a lack of information about some aspects.

23.3.4.1 Nitric Oxide Production

Nitric oxide is produced from arginine by various types of nitric oxide synthase (NOS). Nitric oxide produced by inducible NOS (iNOS) plays an important role in immune function and elimination of pathogens. However, excessive iNOS activity may lead to overproduction of nitric oxide, which is harmful to the host cells (Hoseini et al. 2020b). In fish, arginine can stimulate the production of nitric oxide in macrophages, playing an important role in the innate immune mechanisms of the host (Buentello and Gatlin III 1999; Costas et al. 2011, 2013). Interestingly, a number of studies on fish have shown that the effect of dietary arginine levels on NOS and growth performance was uniform and depends on dietary arginine levels (Ren et al. 2013; Rahimnejad and Lee 2014; Zhou et al. 2015; Wang et al. 2017b); however, these responses may be species-specific, because other studies have not reported such relations (Costas et al. 2013; Chen et al. 2015; Lin et al. 2015). There is no information about the effects of dietary arginine on NOS activity and production of nitric oxide by macrophages of fish, when stimulated by pathogens. These types of studies can show the role of dietary arginine in the nitric oxide response of diseased fish. This is important because it has been shown that arginine may not have any effects on the immune responses under normal conditions, but it does increase the immune responses of the fish exposed to pathogens (Yue et al. 2015).

23.3.4.2 Polyamine Synthesis and Leukocyte Proliferation

Polyamines are products of arginine metabolism and are essential for cell growth and proliferation, stimulation of macrophages to secrete cytokines, and leukocyte immune responses (Hoseini et al. 2020b). Thus, activation of the immune system after arginine administration may be associated, at least in part, to increased polyamine production. There is a few studies on this subject in fish, showing that administration of polyamines to gilthead sea bream leukocytes enhances immune responses (at the cellular and transcription levels), depending on the dose and type of polyamine (Reyes-Becerril et al. 2011). Also, administration of arginine to Atlantic salmon cell culture medium has increased the expression of genes involved in the production of polyamines by reducing an inflammatory marker (interleukin 8 gene expression) in response to lipopolysaccharide stimulation (Holen et al. 2014). Therefore, arginine administration appears to increase the biosynthesis of polyamines, and these compounds can increase the number and function of leukocytes. This hypothesis has been supported by other studies, showing that arginine intake leads to an increase in the number of leukocytes and changes their types and functions (Costas et al. 2013; Rahimnejad and Lee 2014; Chen et al. 2015; Li et al. 2015; Zhou et al. 2015). On the other hand, limited studies have shown that arginine intake leads to an increase in immunoglobulins, which may be due to an increase in the number of B lymphocytes due to an increase in polyamine synthesis (Pohlenz et al. 2012a, c). Clearly, further studies are needed to evaluate the effects of

arginine administration on fish polyamine production and its association with the immune responses under normal or disease conditions.

23.3.4.3 Modulation of Inflammation by Arginine

There is evidence showing that arginine regulates fish inflammatory responses at the transcriptional level. For example, arginine increases the expression of pro-inflammatory (interleukin 1 beta and tumor necrosis factor alpha) and anti-inflammatory (transforming growth factor beta and interleukin 10) genes in the anterior kidney and spleen of common carp, under normal rearing conditions. Interestingly, the expression of the anti-inflammatory cytokine gene increased only in the fish fed high levels of dietary arginine (more than the species requirement), which may be due to negative feedback to counteract the adverse effects of acute inflammation (Chen et al. 2015). Other studies have shown that arginine has anti-inflammatory properties to control the negative effects of inflammation caused by lipopolysaccharide in the tissues of different species (Holen et al. 2014; Jiang et al. 2015a; Martins et al. 2019). In addition, both the low and high levels of dietary arginine reduce the expression of pro-inflammatory cytokine genes in common carp head kidney, under normal or stressful rearing conditions (Hoseini et al. 2019a). On the contrary, there is evidence indicating that, under normal rearing conditions, addition of arginine to the diet suppresses the pro-inflammatory response in European seabass and reduces the resistance of the fish to a pathogenic bacterium (Azeredo et al. 2015). Therefore, the available data suggest that the effects of arginine on cytokines can be variable and that this amino acid has strong effects on inflammatory responses.

23.3.5 Counteraction to Ammonia Toxicity

As mentioned above, arginine is involved in the urea cycle and the conversion of ammonia to urea in fish; thus, it can reduce ammonia toxicity (Fig. 23.2). In this context, it has been shown that a diet rich in arginine (i.e., 6.6 g of arginine per 100 g of protein) leads to lower mortality of yellow catfish, *Pelteobagrus fulvidraco*, after 72 h of exposure to water ammonia compared to fish fed a control diet (5.7 g arginine per 100 g of protein) (Chen et al. 2016). The positive effect of arginine on ammonia toxicity in fish can be explained in two ways. The first is the activation of the urea cycle by arginine, which leads to increased ammonia detoxification. Supporting this, arginine administration has activated the urea cycle and increased ureagenesis in common carp exposed to water ammonia (Hoseini et al. 2019c). The second mechanism is mitigating the negative effects of ammonia toxicity such as physiological stress and oxidative stress. For example, dietary arginine mitigated a downregulation in hepatic expression of interleukin 10 and upregulation of heat shock protein 70 in common carp exposed to ammonia, suggesting that arginine was capable to mitigate oxidative stress and inflammation (Yousefi et al. 2021).

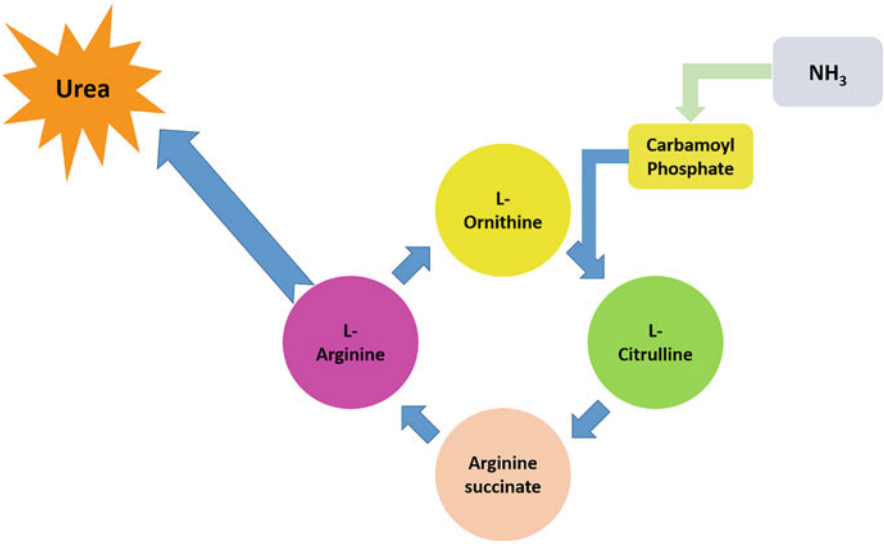

Fig. 23.2 Urea cycle as an arginine-dependent pathway for ammonia detoxification

23.3.6 Arginine Roles in Wound Healing

In aquaculture, fish may have skin wounds due to handling, trauma, and disease. Wound healing is an important process that protects fish against further contamination of the deeper tissues. Moreover, a study on gilthead sea bream has indicated that skin wounds lead to intestinal dysfunction including inflammation and tight junction disruption (Chen et al. 2020b). These effects may lead to problems in nutrient absorption and fish health. Healing process in fish differs slightly relative to that of the mammals, as the fish lack the blood clot phase, but have the rest phases including inflammation, re-epithelialization, new tissue formation, and remodeling (Chen et al. 2020b). Arginine has been found to be beneficial during wound healing, as it stimulates the production of nitric oxide that is necessary for early stages of the healing. Nitric oxide production prevents bacterial infection, increases blood flow, and stimulates collagen formation. After this stage, nitric oxide formation declines, and arginine is converted to ornithine that regulates collagen protein synthesis and cell proliferation and differentiation (Chen et al. 2020a). Study on gilthead sea bream has indicated that dietary arginine administration helps skin wound healing by mitigation of inflammation, stimulation of transforming growth factor beta gene expression, antioxidant power, re-epithelialization, and accelerating whole body homeostasis (Chen et al. 2020a). Moreover, arginine had positive effects on the fish intestinal health during skin healing, as evidenced by suppressing inflammatory responses and increasing tricellulin and occludin gene expressions (Chen et al. 2020b).

23.4 Sulfur Amino Acids

Functional sulfur amino acids include taurine and methionine. In addition to its high importance in fish growth, methionine plays an important role in the physiological pathways of fish (Wilson 2002). Taurine is a nitrogenous substance similar to amino acids that has sulfinic acid, instead of the carboxyl group, in its structure. This substance does not participate in structure of the proteins but is found to a large extent in the intercellular fluids of animals (El-Sayed 2014). The taurine amount required by the living organisms can be supplied through food or internal synthesis (from methionine and cysteine); therefore, there is an interaction between dietary taurine and methionine in fish, and the amounts of each affect the performance of the other (Salze and Davis 2015). Plant sources contain lower amounts of methionine than animal sources, and their taurine content is particularly low, compared to animal sources (El-Sayed 2014). In the living organisms, taurine can be made from methionine and cysteine. Methionine is converted to homocysteine, and this substance is converted to cystathionine by cystathionine synthase and contribution of serine. Cystathionine is converted to cysteine under the influence of cystathioninase. Cysteine is converted to cysteine sulfinate by the enzyme cysteine dioxygenase, which is converted to hypotaurine and eventually to taurine by cysteinesulfinate decarboxylase (Salze and Davis 2015). Some fish, such as common carp, can use methionine to produce the required amount of taurine, but most marine carnivorous species do not (Kim et al. 2008). Therefore, when high amounts of plant protein are used in the diet of fish, in addition to methionine, sufficient amounts of taurine must be added to the diet to support the fish growth and health.

23.4.1 Growth Promotion by Sulfur Amino Acids

Methionine participates in the skeletal muscle structure, so its deficiency directly reduces fish growth rate. According to researches, the amount of methionine required by different fish is 1.6–4% of dietary protein (Wilson 2002). Also, due to the large amount of taurine in the body of fish, it is inferred that this substance is essential for them. Many studies show that the addition of taurine to the diets of different fish (1.5–6% of dietary protein) has resulted in elevations in their growth rate (El-Sayed 2014; Salze and Davis 2015). However, a few studies have shown that adding taurine to the diet of some fish does not affect their growth performance, and some studies exhibited contradictory results. For example, a preliminary study on carp has shown that this species is able to synthesize taurine from mthionine and cysteine (Kim et al. 2008); therefore, the addition of taurine to the diet of this species has no effect on the fish growth. But the next study on the same species shows that the highest growth occurs when the diet contains 4.3% taurine (of dietary protein), regardless of the dietary methionine and cysteine (Abdel-Tawwab and Monier 2018). Some studies suggest that there may be an interaction between dietary taurine and methionine. It has been found that when rainbow trout is fed a diet based on plant protein, increasing dietary methionine from 0.7% to 1.6%

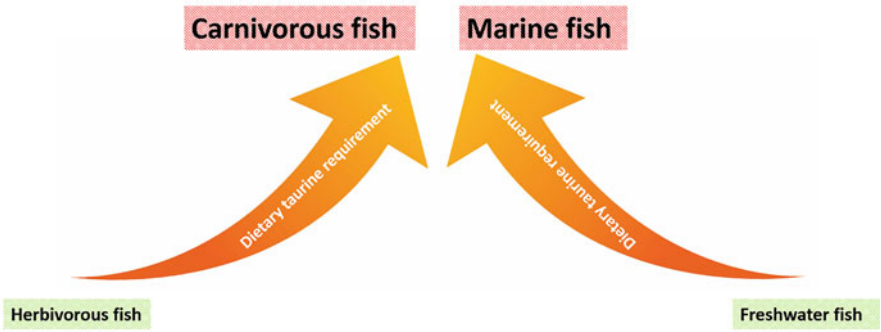

Fig. 23.3 Importance of dietary taurine in fish from different environments and with different nutritional habituates

reduces the fish growth. Also, when methionine is 0.7%, taurine (0.5% of diet) increases growth (Gaylord et al. 2007). In meagre, *A. regius*, dietary methionine alone is not sufficient for synthesizing the required taurine, and the addition of taurine to the diet is mandatory to support best performance (de Moura et al. 2018). It should be noted that growth-promoting effects of sulfur amino acids might be as a result of elevation in the digestive and brush border enzymes' activity (Tang et al. 2009; Zhang et al. 2018a; Dehghani et al. 2020) that probably augment digestion and absorption capacity. Growth retardation due to taurine deficiency might be related to interfered osomoregulation that disturbes fish physiological homeostasis, as taurine is an important osmolytes in fish body. As a result, carnivorous and marine fish needs higher taurine than herbivorous and freshwater fish, due to both insufficient indigenous synthesis and osmoregulatory mechanisms (Fig. 23.3).

23.4.2 Role of Taurine during Stress

Stress in fish is associated with a cortisol response that, in addition to increasing energy consumption, reduces the fish immune function. It has recently been shown that administering taurine to fish can reduce the cortisol response to stress and reduce anxiety (Mezzomo et al. 2019). In fact, taurine interacts with $GABA_A$, glycerinergic, and glutaminergic receptors; therefore influences fish behavior and anxiety under stressful situations (Mezzomo et al. 2019). Recent studies on zebrafish have shown that placing fish for 1 hour in water containing taurine reduces the response of cortisol to an acute stress and subsidizes subsequent anxietic behaviors. Also, taurine administration alone has had no effect on these components (Mezzomo et al. 2019). Another study found that enriching the diet of common carp with taurine increased the fish resistance against a lethal osmotic stress (Abdel-Tawwab and Monier 2018). Taurine has been found beneficial in pufferfish, *Takifugu obscurus*, during low-temperature stress by improving the antioxidant parameters and decreasing

oxidative stress and apoptosis. In addition, taurine helps the fish to regain cytoplasmic calcium ion homeostasis and reactive oxygen species (ROS) generation during the thermal stress (Cheng et al. 2018). However, more studies are needed to increase the current knowledge, because the effects of oral taurine administration on stress responses over short and long periods of time need to be determined.

23.4.3 Immunostimulation by Dietary Sulfur Amino Acids

Sulfur amino acids have positive effects on fish health, and dietary shortage of these amino acids reduces the health and immune function of fish. For example, a lack of taurine in fish diets causes anemia and a decrease in the number of red blood cells (Takagi et al. 2005). This is because taurine is one of the most important fish osmolytes that plays a role in regulating the osmotic pressure of the body fluids. Lack of taurine lowers osmotic pressure of blood plasma and leads to swelling and bursting of the red blood cells (Maita et al. 2006). This condition is very evident in carnivorous fish such as yellowtail, *Seriola quinqueradiata*, when fed plant-based diets without taurine supplementation (Takagi et al. 2005; Maita et al. 2006).

Taurine alone contains half of the free amino acids of lymphocytes. These cells have mechanisms for the active transport of taurine into the cytoplasm, indicating the importance of taurine for these cells. Taurine seems to be necessary for well-functioning of the fish immune system (El-Sayed 2014). Dietary taurine deficiency in yellowtail causes hemolysis and reduces the fish resistance to bacterial infection (Maita et al. 2006). In yellowfin sea bream, *Acanthopagrus latus*, the addition of taurine to diet increases the non-specific immune parameters of the skin mucus, but this increase has been observed solely at the optimal level of dietary taurine (Dehghani et al. 2020). Importance of taurine in immune function and disease resistance of fish was recently more pronounced. It seems that taurine is an important component mediating the immunostimulatory effects of other immunostimulants. In zebrafish, dietary malate administration was found to increase the fish disease resistance; subsequent analyses have indicated that malate increases taurine content in the fish body by stimulating taurine synthesis. Supporting this, dietary administration of taurine also improved the fish resistance to the bacterial infection (Yang et al. 2020a). The finding of this study demonstrated that malate and taurine changed immunological responses of the fish to suppress inflammation after the bacterial infection and, thus, improved the fish survival (Yang et al. 2020a). Moreover, taurine helps fish to regain the normal population of the intestinal microbiota, under unfavorable conditions. Fish gut microbiota have an obvious effect on the immune function and disease resistance; thus, immunomodulatory effects of taurine in fish might be associated with the gut microbiota and health. For example, oxidized fish oil induces inflammation and dysbiosis of the intestinal microbiota in rice field eel, *Monopterus albus*; but taurine administration significantly prevented such effects (Peng et al. 2019). Overall, taurine is a promising immunostimulant in fish and this topic needs more attention.

Methionine is also an important immunostimulant in fish, and dietary methionine deficiency reduces immunity and disease resistance. For example, in yellow catfish, *Pelteobagrus fulvidraco*, methionine deficiency reduces lysozyme, complement, phagocytosis, and respiratory burst activities and increased the fish susceptibility to a bacterial infection (Elmada et al. 2016). Methionine supplementation significantly improved the innate immune parameters and decreased mortality of ruho, following aeromonas septicemia (Mir et al. 2017). Methionine induces cell proliferation by increasing S-Adenosyl methionine levels in the body. So, increase in the number of leukocytes has been observed in the fish fed methionine-supplemented diets. This may help the fish to have an early response to pathogens by expressing higher phagocytic activity. A 4-week administration of methionine decreases pro-inflammatory genes' expression and increases polyamine-related genes' expression along with neutrophil counts in rainbow trout (Machado et al. 2021). Dietary methionine administration increases leukocyte count, neutrophil count, plasma complement, and bactericidal activity in European seabass after 15 days (Machado et al. 2015). Such effects were more pronounced when the fish were fed a plant-based diet for a long time (12 weeks), where methionine supplementation was necessary to increase neutrophil proliferation and apoptosis mitigation. The results also indicated that the fish requires higher dietary methionine levels, when they were fed a plant-based diet compared to a fishmeal-based diet (Machado et al. 2020).

23.4.4 Antioxidant Effects of Sulfur Amino Acids

Taurine stimulates the antioxidant system in fish by increasing the activity of the antioxidant enzymes and decreasing lipid peroxidation. In Totoaba, *Totoaba macdonaldi*, and common carp addition of taurine to diet increased superoxide dismutase and catalase activities and decreased lipid peroxidation (Bañuelos-Vargas et al. 2014; Abdel-Tawwab and Monier 2018). Such antioxidant effects of taurine make it a suitable and crucial dietary supplement for fish under oxidative stress. For example, dietary taurine supplementation was found beneficial in European seabass fed a plant-based diet, during forced swimming. Taurine supplementation led to elongation of fatigue time after the forced swimming and decreased ROS production (Ceccotti et al. 2019). Taurine mitigated oxidative stress in pufferfish during thermal stress by improving antioxidant enzymes' gene expression and decreasing ROS formation (Cheng et al. 2018). Moreover, taurine administration has been found to suppress oxidative stress in several species during exposure to different toxicants (Xing et al. 2016; Hano et al. 2017b; Zhang et al. 2018b).

Various studies have shown that methionine improves the antioxidant system of fish, but it is not clear whether this effect is direct or due to taurine synthesis elevation. Methionine, in the liver, is converted to cysteine and finally glutathione; thus, its shortage may decrease the production of this important antioxidant molecules (Salze and Davis 2015). For example, deficiency of methionine leads to growth reduction due to an increase in mitophagy in rainbow trout. Moreover, the fish have lower glutathione and higher malondialdehyde and protein carbonyl in the

liver, indicating methionine deficiency induces oxidative stress, probably due to lower production of glutathione (Séité et al. 2018). High levels of dietary fat induced hepatic oxidative stress and structural damages in yellow croaker, *Larimichthys crocea*; however, dietary methionine supplementation significantly increased antioxidant capacity and decreased malondialdehyde in the fish liver and decreased circulating levels of alanine aminotransferase and aspartate aminotransferase, indicating hepatoprotective effects of methionine by improving antioxidant power (Li et al. 2021). Likewise, methionine deficiency significantly induced oxidative stress in the hepatopancreas and intestine of grass carp (Wu et al. 2017b). Further studies are needed to determine the direct effect of methionine on the antioxidant responses of fish cells in vitro.

23.4.5 Taurine as an Anti-Toxicant Agent

A few studies have evaluated the role of taurine in reducing the effects of toxicants in fish. These studies have indicated the positive roles of taurine in increasing resistance to toxicants and reducing the accumulation of pollutants in the fish body. Taurine administration has been proven as an efficient tool to reduce ammonia toxicity in fish. Injection of taurine to grass carp has reduced the acute ammonia toxicity caused by the injection of ammonium acetate, which has been associated with a decrease in the concentration of ammonia in the fish brain and the prevention of oxidative stress (Xing et al. 2016). Similar results were obtained in yellow catfish, as taurine injection reduced ammonia concentration in the fish liver and brain after ammonia acetate injection. Moreover, taurine improved antioxidant and immune parameters and decreased apoptosis in the fish injected by ammonia acetate (Zhang et al. 2018b). In crucian carp, *Carassius auratus*, treated in the same way, similar results were obtained in addition to higher survival of the fish treated with taurine and injected with ammonium acetate (Ren et al. 2016). Interestingly, a long-term feeding with plant-based diets containing graded levels of taurine showed that yellow catfish had improved antioxidant, innate immune strength, and liver health. Injection of ammonium acetate to the fish revealed that dietary taurine supplementation at 2.55% lowered mortality and the fish brain ammonia, glutamine, and glutamate content by half, indicating taurine as a functional amino acid in plant-based diets (Li et al. 2016). The antioxidant and immunostimulant nature of taurine would be helpful during oxidative and immunosuppressive conditions, such as ammonia toxicity.

There are limited information about the effects of taurine on accumulation of toxicants in the fish body. In red sea bream, feeding a diet supplemented by taurine for 55 days increased the fish resistance to acute cadmium toxicity and reduced the metal accumulation in the liver and muscle after chronic exposure (Hano et al. 2017b). However, a subsequent study on this species has shown that although enrichment of the diet with taurine increases fish resistance to phenanthrene toxicity, it does not have a positive effect on its accumulation in fish tissues (Hano et al.

2017a). More researches are necessary to determine the mechanisms involved in lower toxicant accumulation in the fish body by taurine administration.

23.5 Histidine

Histidine is one of the essential amino acids in fish that has important physiological roles in the body. Chemically, histidine has an imidazole functional group in its structure. Histidine acts as a catalytic component in the structure of many proteins and is a precursor to histamine; for this reason, it plays an important role in cellular signaling (Andersen et al. 2016). On the other hand, histidine is a precursor to useful imidazole compounds such as the dipeptides carnosine and anserine; therefore, is effective in the intracellular fluid buffering system and the antioxidant capacity of animals (Li et al. 2009). This amino acid is partly responsible for the fish flesh texture and taste, and high histidine abundances ensure the quality of fish carcass after death. However, little is known about the physiological role of histidine in fish (Andersen et al. 2016). Here, the fish requirements for histidine and its roles in growth, cataract, antioxidant and buffering capacity, and osmoregulation of the intracellular fluids will be reviewed.

23.5.1 Dietary Histidine and Fish Growth

Since histidine is an essential amino acid, it should be provided through fish diets. Therefore, it is necessary to control the amount of histidine in fish diets. Rich sources of histidine in fish diets include fish meal and blood meal. Based on the available information, around 1.5–3.5% histidine (based on dietary protein) should be provided for fish via dietary route (Wilson 2002). This information is mostly based on the response of fish growth to graded levels of dietary histidine. Histidine intake elevates histidine concentration in the fish plasma; therefore plasma histidine concentrations can be used to determine the need for histidine in fish. However, once the histidine dietary need has been met, supplementary histidine intake will not elevate plasma histidine concentrations (Wilson 2002). Since histidine is a functional amino acid, fish will require different quantities of histidine depending on their metabolism and development stages, and histidine deficiency will have multiple health impacts. For example, Asian stinging catfish, *Heteropneustes fossilis*, needs more histidine at a younger age, when the fish grow faster (Khan 2013; Khan and Abidi 2014); moreover, triploid salmon needs higher histidine to prevent cataract than diploids (Taylor et al. 2015).

Various studies have shown that when dietary histidine is higher or lower than the optimal levels, it has a negative effect on fish growth (Feng et al. 2013; Khan 2013; Gao et al. 2016; Peachey et al. 2018). Beside the roles in skeletal muscle composition, histidine regulates the size of intestinal villi, the activity of digestive enzymes, and the population of beneficial intestinal bacteria. In common carp, an optimal histidine level results in the highest intestinal villi size, the highest activity of

digestive enzymes, and beneficial intestinal bacterial communities, but sub- and super-optimal histidine levels negatively impacts the aforementioned parameters as well as the fish growth performance (Zhao et al. 2012a). On the other hand, histidine is abundant in fish red blood cells and specifically in the structure of hemoglobin. Therefore, optimizing the amount of histidine in the diet increases hemoglobin and increases the body's oxygen supply capacity, resulting in increased fish growth (Andersen et al. 2016). It should be noted, however, that a study on Asian stinging catfish has shown that high levels of dietary histidine, although increasing hemoglobin, decrease erythrocyte membrane stability (Khan and Abidi 2014). Therefore, optimal intake of histidine is extremely important to maintain fish health.

23.5.2 Histidine Deficiency and Cataract

Histidine deficiency, which is often related to suboptimal histidine dosing in the diet, is one of the causes of fish cataracts. Cataract is a complication that results in loss or weakness of fish vision ability, affecting their behavior and leading to fish malnutrition as the affected fish have difficulty to see food (Peachey et al. 2018). Histidine protects the ocular lens from oxidation and regulates its osmotic pressure, thus ensuring its normal function (Andersen et al. 2016). In Atlantic salmon, this phenomenon is more common in smolts and in sea water, and it has been found that high water temperature (16 ° C) increases the incidence of this complication. On the other hand, the use of blood meal or histidine in the fish diet reduces the incidence of cataract (Breck et al. 2003). Histidine deficiency has also been observed to cause cataract in red drum, *Sciaenop ocellatus*, fry. Histological studies in this species have shown that histidine deficiency in the diet causes degeneration of ocular lens fibers and formation of Morgagnian globules in the lens (Peachey et al. 2018). N-acetyl histidine is the important osmolytes in the fish lens that maintains the lens osmotic pressure and well-functioning. The amount of N-acetyl histidine in the lens depends on the amount of histidine in the diet, and the low concentration of N-acetyl histidine in the lens is directly related to the severity of cataract in Atlantic salmon (Breck et al. 2005). Some studies have also shown relationships between fish metabolism response to abiotic environmental conditions such as temperature, histidine deficiency, and cataract incidence, highlighting the need to consider environmental conditions when calculating optimal histidine intake. For example, studies on Atlantic salmon have shown that a temperature increase from 12 to 18.5 °C results in increased production of structural proteins, which in turn reduced the concentration of N-acetyl histidine and impaired the osmoregulation of the lens, resulting in higher cataract incidence (Breck et al. 2003).

23.5.3 Modulation of the Antioxidant System by Histidine

Histidine is also renowned for its antioxidant and anti-inflammatory properties (Wade and Tucker 1998). Laboratory studies have previously shown that

L-histidine anti-inflammatory properties might be related to its capacity of scavenging toxic oxygen species (Wade and Tucker 1998). However, although the antioxidant and anti-inflammatory properties of histidine have been extensively studied in terrestrial organisms and in vitro assays, only a few studies have been performed to evaluate the antioxidant effect of histidine in fish. In common carp, the addition of histidine to diet increases the antioxidant capacity and activity of antioxidant enzymes in various tissues and prevents oxidative stress (Feng et al. 2013). Addition of histidine to diet of grass carp also improves the function of the antioxidant system after hypoxia stress (Gao et al. 2016). In the same species, the highest expression of Nrf2 gene is observed at the optimal level of dietary histidine, while the expression of Keap1 gene is decreased. Moreover, optimal intake of histidine reduced oxidative stress, which consecutively decreased apoptosis of the gill cells and increases their integrity (Jiang et al. 2016b). However, both higher and lower levels of dietary histidine has negative effects on the gill tissue, further highlighting the importance of right histidine administration.

23.5.4 Osmoregulation and Buffering Capacity of Histidine

Histidine involves in maintenance of fish buffering capacity and osmotic pressure (Fig. 23.4). Imidazole compounds, which contain both free L-histidine amino acids and histidine-containing dipeptides, display strong proton buffering activities. Several metabolic activities such as prolonged anaerobic exercise or anoxia can lead to proton accumulation and the fluctuation of intracellular pH (pH_i), which in turn, can affect several metabolic functions (Abe 2000). Due to their chemical structure, imidazole compounds have pH values that are close to pH_i; therefore, the nitrogen of the imidazole ring can be protonated within the physiological range of pH, conferring them buffering properties (Abe 2000). Histidine and anserine (i.e., dipeptide containing β-alanine and 1-methylhistidine) are important buffering agents in fish muscle and are especially important to withstand anaerobic metabolism during burst swimming activity (Ogata and Murai 1994). For example, the requirement of histidine, anserine, and the buffering capacity in the muscle of Masu salmon, *Oncorhynchus masou*, vary in the parr and smolt stages and depend on the fish ability to swim explosively (Ogata et al. 1998). In young yellow tail, the tissue buffering capacity is also related to the available amounts of histidine and anserine (Ogata 2002). Changes in environmental conditions such as temperature and oxygen saturation can impact the buffering capacity of imidazole compounds (Somero 1985; Abe and Okuma 1991). For example, histidine buffering capacity decreases with increasing temperature, although anserine buffering capacity does not seem to be sensitive to temperature within the physiological pH range (6.5–7.5) (Abe and Okuma 1991). Recent research suggests that the metabolic responses to high temperatures are species-specific and depend on the muscular concentration of imidazoles (Geda et al. 2017). Osmoregulatory properties of N-acetyl histidine are not restricted to the fish lens. Researches have shown that after transfer of Atlantic salmon to the sea, the concentration of N-acetyl histidine in the heart tissue

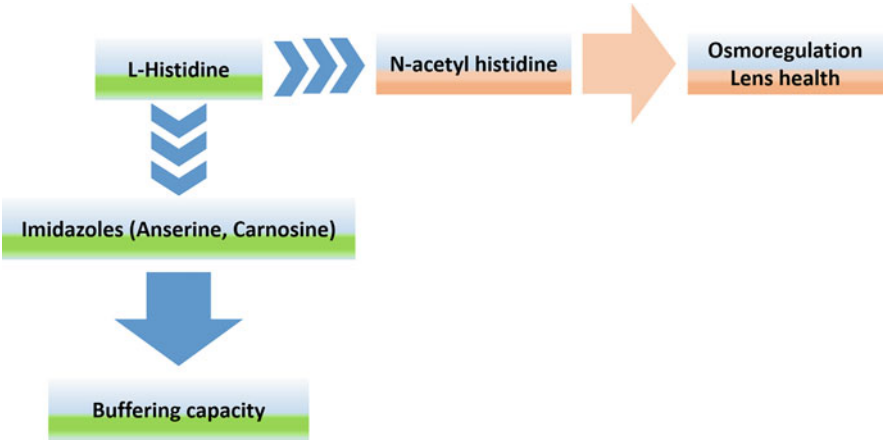

Fig. 23.4 Histidine maintains buffering capacity and osmotic pressure in fish

increases, as does the concentration of anserine in muscle, when the histidine content is at a moderate level (Remø et al. 2014). Anserine and carnosine are not found in the heart of Atlantic salmon, whereas N-acetyl histidine is present in this tissue and has a buffering function similar to that of anserine and carnosine (Remø et al. 2011, 2014).

23.6 Branched-Chain Amino Acids

Leucine, isoleucine, and valine are branched-chain essential amino acids in fish and mammals (Wilson 2002). Branched-chain amino acids are essential for protein synthesis and also regulation of protein breakdown. Unlike other amino acids, the breakdown of branched-chain amino acids begins outside the liver before being transferred to the liver, which then produces glutamate and branched-chain ketoacids (Andersen et al. 2016).

These essential amino acids and their metabolites protect the body muscle mass during prolonged activity. For example, in salmon, branched-chain amino acids are involved in maintaining muscle mass during activities such as forced swimming (Grisdale-Helland et al. 2013). In humans, malnutrition due to deficiency of branched-chain amino acids is associated with the liver disease. In chronic liver disease such as cirrhosis, the concentration of branched-chain amino acids in the blood decreases, and the concentration of essential aromatic amino acids is elevated (Andersen et al. 2016). In mammals, administration of branched-chain amino acids has an effect on longevity, mitochondrial synthesis, and defense against ROS due to the activation of sirtuins (Andersen et al. 2016). Sirtuins are a family of proteins that regulate key metabolic pathways by modulating PGC1-α, FoxO, p53, AMPK, eNOS, and several other enzymes (Houtkooper et al. 2012).

Branched-chain amino acids, primarily leucine, increase muscle protein synthesis pathways through both insulin-dependent and insulin-independent pathways.

Leucine directly activates the mTOR pathway in the skeletal muscles and stimulates p70S6 kinase phosphorylation, 4E-BP1 ribonucleic acid translation, and protein synthesis, although this signaling is transient (Manders et al. 2012). In addition, branched-chain amino acids reduce the muscle wasting by interfering with the proteasome-ubiquitin pathway (Andersen et al. 2016).

A recent research has shown that leucine is involved in activating the mTOR pathway in rainbow trout liver cells (Lansard et al. 2011). At present, however, little is known about the effect of branched-chain amino acids on protein metabolism in fish. Because branched-chain amino acids, especially leucine, are associated with fish muscle growth, future studies may provide a better perspective on the possibility of maximizing fish muscle growth via dietary supplementation with branched-chain amino acids.

Beta-hydroxy beta-methylbutyrate (HMB) is a metabolite derived from leucine catabolism known to increase the immune response and survival rate in fish during bacterial infections (Siwicki et al. 2011). The beneficial effects of consuming branched-chain amino acids on fish nutrition have not been studied in detail, as most articles on the nutrition of salmonids and branched-chain amino acids specify only the amounts required for the species and have not evaluated the functional properties of the amino acids (Andersen et al. 2016). Given that branched-chain amino acids significantly affect mammalian growth, health, and metabolism, such researches are likely to increase in fish in the future.

23.6.1 Branched-Chain Amino Acids and Fish Growth

Deficiency of branched-chain amino acids, like other essential amino acids, reduces growth and increases mortality in fish (Wilson 2002). Suboptimal dietary levels of these amino acids are known to induce immunosuppression, oxidative stress, inflammatory reactions in the intestine and gills, decreased expression of tight junction protein genes in the intestine and gills, reduced intestinal villi size, decreased activity of intestinal digestive enzymes, decreased Na/K-ATPase activity in the gut, and apoptosis in the gill (Zhao et al. 2012b, 2014; Feng et al. 2015; Jiang et al. 2015c).

Although dietary requirements for branched-chain amino acids are often species-specific, estimates suggest that 3.3–5.2% of leucine (based on dietary protein level) is required, whereas, the need for dietary isoleucine and valine is 1.2–4% and 2.5–4.8% of dietary protein, respectively. However, higher amounts of 4.5 and 4.8% of valine have been reported in Nile tilapia and grass carp (Wilson 2002).

Studies have shown that different branched-chain amino acids interact between themselves, suggesting an interdependency in their required quantities and their functions. For example, in Chinook salmon, *Oncorhynchus tshawytscha*, the need for dietary isoleucine increases with increasing dietary leucine levels; however, such results have not been observed in common carp and channel catfish (Wilson 2002). Also, in Japanese flounder, *Paralichthys olivaceus*, adding valine to a diet containing 2% leucine increased growth, but adding valine to a diet containing 5% leucine decreased growth and increased feed conversion ratio (Han et al. 2014). Also

in this species, when the amount of dietary leucine is 2.6% of dietary protein, increasing dietary isoleucine from 1.44 to 2.21–4.44% of dietary protein increases growth of the fish. However, when dietary leucine is 5.1% of dietary protein, increasing dietary isoleucine reduces the fish growth (Wang et al. 2017a). The mechanism of such interactions in aquatic animals has not been determined, and more researches are needed to provide balanced diets for different species.

23.6.2 Branched-Chain Amino Acids and Immune Function

Branched-chain amino acids are involved in the fish immune function, and optimal amounts of each of them are essential for the proper functioning of the fish immune system. Administration of leucine to tilapia increases resistance to streptococcal infection (Ma et al. 2015). Studies on different fish have also shown that suboptimal levels of dietary leucine (both higher and lower) have negative effects on the fish immune indicators. For example, in black carp, *Mylopharyngodon piceus*, suboptimal levels of dietary leucine decreases lysozyme and complement activities and increases alanine aminotransferase activity, which is a sign of hepatic damage. These effects are also visible at the transcription level, and the expression of lysozyme, interferon, and complement genes increases in the fish blood (Wu et al. 2017a). In blunt snout bream, *Megalobrama amblycephala*, dietary leucine imbalance also reduces serum complement activity and immunoglobulin and increases the expression of inflammatory genes in the liver (Liang et al. 2018). In grass carp, dietary leucine imbalance increases the expression of inflammatory genes and decreases the expression of anti-inflammatory genes in fish intestine (Jiang et al. 2015c).

Studies in common carp have shown that dietary isoleucine at the optimal level (i.e., the level that produces the highest growth) enhances the number of leukocytes. Exposure of these fish to *Aeromonas hydrophila* also results in higher immunoglobulin M, anti-*A. hydrophila* antibodies, complement proteins, lysozyme activity, phagocytosis, hemagglutination, and the fish survival. In addition, the expression of the pro-inflammatory genes in the head kidney of these fish was low, which indicates that the optimal level of isoleucine in the diet of common carp is necessary to increase immunity and disease resistance and reduces inflammation following the bacterial infection (Zhao et al. 2013). Studies have shown that providing optimal amounts of valine in the diet of grass carp is effective in the intestinal health of fish. Dietary valine imbalance in this fish causes inflammation and structural damage (Luo et al. 2014). In red sea bream, *Pagrus major*, optimal dietary valine levels increase lysozyme, respiratory burst, myeloperoxidase activities, and immunoglobulin concentration (Rahimnejad and Lee 2013).

23.6.3 Modulation of Antioxidant Responses by Branched-Chain Amino Acids

Various studies have shown that branched-chain amino acids stimulate the antioxidant system, and an imbalance of these amino acids in the diet weakens the antioxidant power and causes oxidative stress. Some studies have also shown that the effect of branched-chain amino acids occurs at the transcription levels. In blunt snout bream, dietary leucine imbalance decreases the activity of superoxide dismutase, catalase, and glutathione peroxidase and decreases serum antioxidant capacity, which was associated with increased malondialdehyde and oxidative stress. This decrease is also observed at the transcription of antioxidant enzymes' genes and the signaling pathway of the antioxidant system (Nrf2) in the fish liver (Jiang et al. 2015c). Similar results were observed in grass carp, in which leucine levels affect the genes regulating the antioxidant system in the intestine (Nrf2 and Keap1) and optimal levels of dietary leucine are required for the proper function of the antioxidant system in the fish intestine (Deng et al. 2014).

In vitro tests have shown that supplementation of a culture medium with leucine results in suppressing oxidative stress (i.e., decrease in malondialdehyde and carbonyl protein contents) and damage in the common carp enterocytes exposed to copper (Zhao et al. 2017). Research on grass carp has also shown that optimal dietary isoleucine levels reduce inflammation, apoptosis, and oxidative stress in the gills of grass carp, but isoleucine imbalance has several negative effects on the fish gills (Feng et al. 2017). In common carp, dietary isoleucine imbalance causes oxidative stress in the kidney and intestine and results in increases in malondialdehyde and carbonyl protein contents and decreases in the activity of the antioxidant enzymes (Zhao et al. 2013, 2014).

A few studies have been executed on the effect of valine on the fish antioxidant system. An imbalance in the amount of valine in the diet causes a variety of adverse effects such as oxidative stress, decreased activity of antioxidant enzymes, inflammation, and apoptosis in the gills of grass carp. The authors also found valine affected the signaling pathway of the antioxidant system, and imbalance in the amount of valine in the diet increases Keap1 gene expression and decreases Nrf2 expression in the gills of this fish (Feng et al. 2015).

23.7 Glutamine and Glutamate

Glutamine and glutamate are vital amino acids that contribute to the maintenance of the fish intestinal health, protection of cells from oxidative stress, also acting as a major energy supply for a variety of tissues and organs (gut, small intestine, liver, skeletal muscle, and kidney) (Li et al. 2020). Since both glutamine and glutamate can be synthesized de novo from other α-amino acids and ammonia in different fish organs (e.g., skeletal muscle, liver, intestines, kidneys, and brain), they are considered as nutritionally nonessential (Li et al. 2020). However, most animals, including fish, have high physiological requirements for glutamine and glutamate, and

therefore they must be adequately incorporated into the diet in order to support the animal's health and the optimal growth (Li et al. 2020). Glutamine and glutamate are found at high concentrations in fish feedstuffs such as fish meal, soybean meal, and cottonseed meal (Li et al. 2011). Researches have shown that fish need different amounts of glutamine and glutamate at different ages. For example, in grass carp, the need for these amino acids is higher in the early stages of life; as when the diet is low in protein or fish meal contents, the expression of glutamine synthetase gene is upregulated to increase glutamine production in the body, so glutamine and glutamate play important roles in the survival and development of fish at the early stages of life (Hu et al. 2017).

Researches have now showed that both glutamine and glutamate play major roles in the fish health, growth, and homeostasis. They are involved in glucose, fat, and glutathione production, improvement of immune system, and mitigation of ammonia toxicity in the fish (Li et al. 2020). Glutamate is the major glycogenic compound in the fish liver, and both glutamate and glutamine are also involved in the production of fatty acids in the liver (Zhao et al. 2019). Oxidation of these amino acids causes the production of acetyl coenzyme A, and this compound is used to synthesize long-chain fatty acids (Li et al. 2020).

23.7.1 Gut Health and Growth

Glutamine and glutamate play major roles in the fish growth and gut health through the promotion of cell proliferation and provision of energy supplies (Cheng et al. 2011; Jiang et al. 2015b; Yoshida et al. 2016). Several studies have shown that dietary glutamate intake enhances growth of several fish species such as common carp, grass carp, and Atlantic salmon (Oehme et al. 2010; Zhao et al. 2015, 2020). Similarly, dietary intake of glutamine was also shown to improve feeding efficiency and growth of many fish species such as hybrid striped bass, *Morone chrysops* x *Morone saxatilis*, red drum, turbot, *Scophthalmus maximus*, and tongue sole, *Cynoglossus semilaevis* (Cheng et al. 2011, 2012; Liu et al. 2015, 2018).

Dietary glutamine and glutamate affect digestion and gut health through multiple mechanisms in fish. Glutamine intake, for example, is known to improve the intestinal morphology and functionality (Cheng et al. 2011; Pohlenz et al. 2012a). Glutamine dietary intake has also protective effects against enteritis produced by high levels of dietary soybean meal in turbot, which was associated with increased expression of MUC-2 and PPAR-γ and inhibition of NF-κB-MLCK signaling pathway, which involves in anti-inflammatory responses (Gu et al. 2017; Liu et al. 2018). Glutamate dietary intake also improved body weight, microvillus thickness, and promoted nucleotide synthesis in the gut of rainbow trout fed with a soybean meal-based diet (Yoshida et al. 2016). Furthermore, glutamate supplementation improved hepatic glucose metabolism of gilthead seabream, altered the lipid profile, and upregulated genes related to myogenesis in common carp, while glutamine promoted higher protein retention in gilthead bream (Caballero-Solares et al. 2015; Zhao et al. 2019). Altogether, the previous results suggest that diet supplementation

with these amino acids could provide novel opportunities to reduce dietary protein quantities and replace them by carbohydrates. Finally, both dietary glutamate and glutamine are known to increase the fish intestinal antioxidant capacity (Liu et al. 2015; Zhao et al. 2015, 2019), which in turn, reduces hypoxia stress related in tongue sole larvae (Liu et al. 2015) and protected the intestinal cells against copper toxicity (Jiang et al. 2016a).

23.7.2 Immune Defenses

Dietary intake of glutamine and glutamate has also been shown to stimulate the innate and acquired immune responses. For example, dietary supplementation with 1–2% of glutamine increases lysozyme activity in hybrid striped bass, common carp, red drum, and Nile tilapia (Cheng et al. 2011, 2012; Hu et al. 2015; Pereira et al. 2017). Glutamine supplementation also stimulates serum complement in Jian carp and respiratory burst activity of phagocytes in red drum (Cheng et al. 2011; Hu et al. 2015). In vitro tests have shown that enrichment of glutamine in a culture media significantly increases the secretion of IgM and IgT in rainbow trout leukocytes, which leads to increase in C3 expression (Li et al. 2019). Glutamine enrichment leads to the proliferation of naive T and B lymphocytes in channel catfish leukocytes upon mitogenic exposure (Pohlenz et al. 2012b). Furthermore, these authors also showed that glutamine dietary supplementation in channel catfish enhanced the acquired immunity in the fish vaccinated against *Edwardsiella ictaluri*, by elevating antibody titers, increasing B-cell proportions in the head kidney, and enhancing the responsiveness of the head kidney lymphocytes against *E. ictaluri* (Pohlenz et al. 2012c).

23.7.3 Ammonia Detoxification

Glutamine and glutamate play major roles in removing ammonia from different fish organs (Fig. 23.5). Catabolism of amino acids is the major source of ammonia in fish. While in ureogenic (i.e., able to biosynthesize urea) fish the most common mechanism of ammonia detoxification is ammonia conversion to urea in the liver, glutamine formation plays a major role in detoxifying ammonia in other tissues and in non-ureogenic fish (Veauvy et al. 2005; Ip and Chew 2010). For example, a few studies report increased glutamine levels in the brain, muscle, and liver tissues after feeding or exposure to high environmental ammonia concentrations (Wicks and Randall 2002; Wright et al. 2007).

Glutamine is produced from glutamate and ammonia, through a reaction catalyzed by the glutamine synthase enzyme. Glutamate is also produced from ammonia and α-ketoglutarate (α-KG) by the aminatic activity of the glutamate dehydrogenase (GDH) (Ip and Chew 2010). In the hepatocytes, glutamate reacts with ammonia to produce glutamine; the resultant glutamine may be used for anabolic processes such as purine and pyrimidine synthesis or may be converted

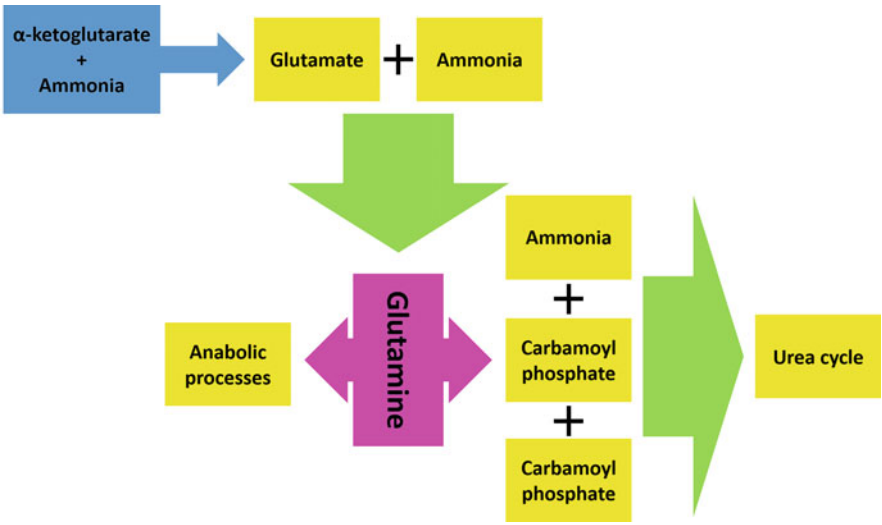

Fig. 23.5 Ammonia detoxification by glutamate and glutamine

to carbamoyl phosphate in mitochondria, which is used for ammonia detoxification in the urea cycle (Ip and Chew 2010). However, whether administration of glutamate to fish increases the rate of ammonia detoxification when the fish is exposed to high water ammonia is not clear and deserves more studies.

23.8 Concluding Remarks

In conclusion, functional amino acids display numerous functions in fish. They do not only contribute to form proteins to build the skeletal muscles, but also play important roles in the immune system, antioxidant defenses, osmoregulation, and even detoxification of toxicants. Despite some of these amino acids can be synthesized by the fish (i.e., taurine, glutamine and glutamate), dietary supplementation has been shown to be key to maintain their optimal levels. Furthermore, an increasing amount of evidence now shows how deficiency of these amino acids has serious health consequences for the fish, highlighting the importance of optimal supplementation.

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Application of Indian Pennywort *Centella asiatica* in Carp Aquaculture against *Flavobacterium columnare* Infection

24

Sudeshna Sarker and Thangapalam Jawahar Abraham

Abstract

With the increase in intensification in aquaculture practices, there has been an increase in the incidence of diseases. Though the Indian major carp aquaculture has witnessed tremendous growth rates in the recent past, still it suffers production loss due to diseases and management issues. Globally, the fish farmers rely on aggressive use of chemotherapeutics to combat infections and infestations. Though they give positive effects, their use is limited due to their residues and other side effects on the host and consumers. It necessitated the search for alternative approaches in aquacultural operations, which have the characteristics of promoting the growth and immune system. Herbal plants are the storehouses and sources of safer and cheaper novel compounds. Herbal preparations are used to prevent various diseases, as they contain growth-promoting, antioxidant, antimicrobial, anti-stress, immunostimulating and other activities. Indian pennywort *Centella asiatica* is one of the potent tropical medicinal plants that have various medicinal properties. This medicinal plant and its preparations have been in use since ancient times in the traditional medical system of India and other countries. This chapter describes the potentiality of dietary *Centella asiatica* extract as a growth promoter, anti-stressor and immunoprotective agent against *Flavobacterium columnare* infection in *Labeo rohita*.

Keywords

Centella asiatica · Carp aquaculture · *Labeo rohita* · Immunomodulation · *Flavobacterium columnare*

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24.1 Introduction

Indian pennywort or Asiatic pennywort *Centella asiatica* is one of the potent tropical medicinal plants that has been claimed to have various medicinal effects (Kartnig 1988). This medicinal plant and its preparations have been in use since ancient times especially in the Ayurvedic medical system of India and in the folk medicine of China and Madagascar (Jaganath and Teik 2000). The phytochemical investigations on *C. asiatica* had led to the isolation of biologically active triterpenoids and glycosides (Mamtha et al. 2004), phenolic compounds (Zainol et al. 2003; Zainol 2004), free acids, volatile oils and flavonoids (Mamtha et al. 2004), tannins and reducing sugars (Arumugam et al. 2011) and other novel chemical constituents (Al Laham and Al Fadel 2014; Agme-Ghodke et al. 2016). The chemical composition of *C. asiatica* has a very important role in medicinal and nutraceutical applications, most likely due to its biologically active constituents of triterpenes, namely, asiatic acid and asiaticoside (Inamdar et al. 1996). The phenolic compounds present were highly responsible for the antioxidant activity in *C. asiatica*, and the activity was found to be as good as that of α-tocopherol at the same concentration tested (Zainol et al. 2003). The bioactive compounds of *C. asiatica* have antibacterial properties against *Helicobacter pylori*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Norzaharaini et al. 2011) and *Klebsiella pneumoniae*, *Streptococcus pyogenes* and others (Sarumathi et al. 2013). Besides the antibacterial principles, *C. asiatica* reportedly possessed antifungal, anticancer, wound healing, neuroprotective, immunomodulatory, anti-inflammatory, hepatoprotective, insecticidal and antioxidant activities (Jagtap et al. 2009; Citarasu 2010; Vaishali et al. 2016), which can be exploited for use in aquaculture as immunomodulators or as an alternative to antibiotics. Purkait et al. (2018) demonstrated that the chloroform extract of *C. asiatica* has the highest antibacterial activity against *Edwardsiella tarda* in vitro, which can be applied as an alternative to the commercial antibiotic to control *E. tarda* infection in aquaculture.

Indian aquaculture has witnessed tremendous growth rates over the last two decades, with freshwater aquaculture contributing over 95% of the total aquaculture production (Jayasankar 2018). The fishes in any aquaculture system are continually exposed to a range of stressors such as handling, crowding, inappropriate water quality, improper feed management and other unscientific management practices, causing synergistic or antagonistic physiological effects (Petitjean et al. 2019), ultimately leading to stress and diseases. Stress mitigation is one of the most challenging tasks in aquaculture nutritional manipulation, which is considered to be one of the most promising ways to prevent diseases. *Flavobacterium columnare*, the causative agent of columnaris disease, is usually of low pathogenicity and infects fish under stressful conditions. In aquaculture, the risk for the columnaris is associated with environmental stress (Declercq et al. 2013). Although several antibiotics are used as curative measures for columnaris disease (Declercq et al. 2013), with the upcoming antimicrobial resistance focus should be laid on the search for preventive measures. The development of effective preventive measures using herbal products is, therefore, of prime importance. Sharma et al. (2014)

demonstrated that dietary administration of *C. asiatica* showed better growth promotion, feed utilization and antibacterial properties compared with other experimental diets. *Centella asiatica* has already been successfully used in controlling columnaris disease caused by *F. columnare* without any inverse impact on fish (Rattanachaikunsopon and Phumkhachorn 2010a). They reported that out of six tested herbs, the aqueous extract of *C. asiatica* exhibited the strongest antimicrobial activity against *F. columnare*. The immunostimulating activity of *C. asiatica* extract regarding both non-specific cellular immune responses and humoral immune responses was also documented (Srichaiyo et al. 2020). *Centella asiatica* is also known for its reputation as a wound healing agent and brain stimulant (Oyedeji and Afolayan 2005). In this chapter, the effects of dietary supplementation of crude chloroform extract of *Centella asiatica* on the growth, serum biomarkers of stress and kidney and liver functions, immunity and wound healing of carp *Labeo rohita* against *Flavobacterium columnare* infection are reported.

24.2 Materials and Methods

24.2.1 Preparation of *Centella asiatica* Powder and Extract

Fresh leaves (Fig. 24.1a) of Indian pennywort *Centella asiatica* were collected from the farm complex of the Faculty of Fishery Sciences (FFSc), West Bengal University of Animal and Fishery Sciences, Kolkata (Lat. 22°47'N; Long. 88°40′E). The leaves were thoroughly washed in running water and air-dried. The leaves were then dried

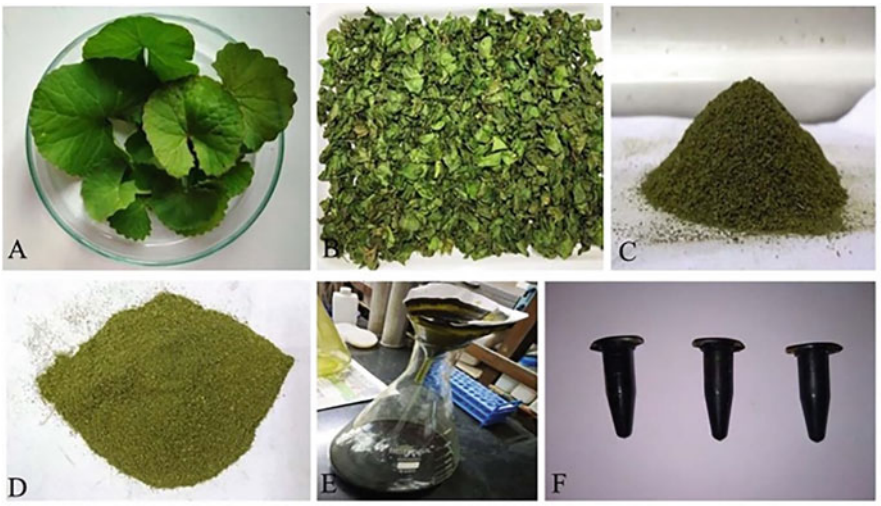

Fig. 24.1 Preparation of *Centella asiatica* powder and crude chloroform extract. (**a**) fresh leaves; (**b**) air-dried leaves; (**c**) grounded leaves; (**d**) sieved fine powder; (**e**) filtration of chloroform soaked *C. asiatica*; (**f**) concentrated crude chloroform extract of *C. asiatica*

(Fig. 24.1b) in the hot air oven at 50 °C for 24 h, ground (Fig. 24.1c) and sieved (ϕ: 0.9 mm) to a fine powder (Fig. 24.1d). The sieved fine powder was subsequently soaked in chloroform (1:4 ratio), selected based on the previous study (Purkait et al. 2018), for 2 days with continuous shaking at 200 rpm at 30 °C. The extract was filtered twice using Whatman No. 1 filter paper (Fig. 24.1e). The filtrates were concentrated, filter sterilized through a 0.45 μm membrane filter and stored at −20 °C until further use (Fig. 24.1f).

24.2.2 Assessment of Anti-*Flavobacterium columnare* Activity of *Centella asiatica* Extract

A fish pathogenic bacterial strain *Flavobacterium columnare* SGM4 (NCBI accession number KU851952) from the collections of FFSc, Kolkata, was used (Sarker and Abraham 2019). The bacterial strain maintained on Cytophaga agar (CA) slants were streaked onto CA plates separately and incubated at 30 ± 2 °C for 24 h to get young culture. The agar disc diffusion assay was performed using a 24 h culture of *F. columnare* SGM4 at 30 °C. The bacterial lawn was prepared by spreading the cell suspension using a sterile cotton swab on CA plates to get a uniform bacterial growth (Wayne 2010). Sterile discs (HiMedia, India) of 6 mm diameter were placed onto the seeded CA and loaded with 20 μL of a sterile crude chloroform extract (CCE) of *C. asiatica* and chloroform as control (negative). An oxytetracycline disc (30 μg/disc; HiMedia, India) was used as a positive control. The plates were incubated at 30 °C for 24 h, observed for the zones of inhibition and measured in mm. The soft agar overlay well diffusion assay was also performed as per Hockett and Baltrus (2017). Briefly, the wells of 5 mm diameter were made aseptically on deep CA using a sterile well borer. The bottom of the wells was sealed-off using molten soft CA (CB + 0.7% agar). The filter sterilized CCE of *C. asiatica* in different volumes (50 μL, 100 μL and 200 μL) and chloroform (100 μL) as control were added into the respective wells. The extracts were allowed to diffuse into the medium for 1 h. The plates were then overlaid with 10 mL molten soft CA seeded with 10 μL of 24-h-old culture of *F. columnare* SGM4. The plates were incubated for 24 h at 30 °C and observed for the zones of inhibition.

24.2.3 Molecular Docking of *Flavobacterium columnare* Virulent Genes

The virulence gene proteins, namely, Gliding Motility Protein (*gldB*) and Chaperone Protein (*dnaJ*), of the fish pathogenic *F. columnare* were considered as a drug target. The naturally available antivirulent herbal compounds were used as the ligands. The main objective of molecular docking is to attain ligand-receptor complex with optimized conformation and to possess less binding free energy. Four antibacterial compounds, viz. *C. asiatica* (Indian pennywort), *Allium sativum* (garlic), *Curcuma longa* (turmeric) and *Zingiber officinale* (ginger), were taken as ligands to dock

against the virulence gene proteins *gldB* and *dnaJ* of *F. columnare*. The chemical structures of the virulence gene proteins were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/), the chemical database. The three-dimensional chemical structures were downloaded in Structure Data File (SDF) format and converted to Protein Data Bank (PDB) format using the software Open Babel (O'Boyle et al. 2011). Docking studies of the PDB structure of the receptor and ligands were carried out using HEX 8.0.0.0 Cuda (Macindoe et al. 2010). The net binding free energy between the target virulence gene proteins from SWISS-MODEL and the ligands from Pubchem were predicted and tabulated. The lesser the net binding free energy, the stronger the docking and considered to be the best ligand for the target protein.

24.2.4 *Centella asiatica* Crude Chloroform Extract Dose Standardization

The experimental fish *Labeo rohita* of weight 27.25 ± 3.51 g and length 13.84 ± 1.18 cm were procured from commercial fish breeders of Sonarpur, South 24 Parganas district, West Bengal, India, and transported to the laboratory in oxygen-filled bags. On reaching the laboratory, the fish were disinfected by immersion in 5 ppm potassium permanganate solution and acclimated for 15 days in circular fibreglass-reinforced plastic (FRP) tanks containing 300 L water at 50 numbers/tank. The fish were fed with commercial pellet feed of 3.0 mm dia (CP Private Limited, India) on demand. The fish without any gross abnormalities and infections from the acclimatized population were then collected and randomly allocated among 12 FRP tanks with 10 fish each. The experimental fish were allotted into 4 groups, viz. group 1, control; group 2, 10 mg CCE/kg feed; group 3, 100 mg CCE/kg feed; and group 4, 1000 mg CCE/kg feed, in triplicate. About 50% of the water was replaced twice weekly to evade the buildup of wastes. The water quality parameters, viz. water temperature, 25.17–29.17 °C; pH, 7.30–7.97; dissolved oxygen, 5.24–6.40 mg/L; nitrite, 0.23–0.66 mg/L; and nitrate, 0.25–0.60 mg/L, were maintained optimally during the experimental period.

24.2.5 Preparation of *Centella asiatica* Crude Chloroform Extract-Supplemented Diets

Four different types of feeds were prepared with or without the CCE of *C. asiatica*. The CCE in 3 doses, i.e. 10 mg, 100 mg and 1000 mg, were added separately into 5 mL vegetable oil, mixed thoroughly and then admixed with 1 kg of basal pellet feed. The control feed without CCE was prepared by mixing 5 mL vegetable oil with 1 kg basal pellet feed. The feeds were mixed thoroughly by closing the respective airtight plastic containers and vigorously shaking the contents for uniform mixing. After proper mixing, the feeds containing varied levels of CCE of *C. asiatica* were uniformly spread separately, dried under the fan for 24 h and stored in airtight plastic containers at room temperature.

24.2.6 Feeding Schedule for the Assessment of Growth Indices and Serum Biomarkers

During the 30 days feeding regime for dose standardization, the control *L. rohita* (group 1) was administered the control diet. The treatment groups (groups 2–4) were fed with respective experimental diets at 3% of the bodyweight (BW) in two equal portions per day. The wastes and faeces were removed twice weekly followed by an exchange of 50% water. Five numbers of fish were carefully caught randomly from each tank during sampling on day 0 and day 30. The total weight and length of sampled fish was recorded on each sampling day to the nearest gram using a digital balance to evaluate the specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) following standard formulas (Karnatak et al. 2021).

The blood and serum were collected on day 0 and day 30 of feeding. Before the blood collection, two fish from each tank of the respective groups were anaesthetized using clove oil (40 μL/L). The blood was collected by caudal vein puncture (Roberts 2012) using a 2 mL sterile plastic syringe. An aliquot of blood was heparinized using 2.7% EDTA and processed for measurement within an hour of collection. The blood in the syringe was then allowed to clot by keeping the syringe in a slanting position and then incubated at 4 °C overnight. The serum was collected by centrifugation at 1500 rpm for 15 min and transferred to Eppendorf tubes. The serum samples of two fish from each of the three replicate were pooled separately, labelled and stored at −20 °C until use.

24.2.7 Experimental Design for the Immunomodulation of *Centella asiatica*

The experimental fish *L. rohita* of 30–33 g size without any gross abnormalities and infections from the acclimatized population were then collected and randomly allocated among 6 FRP tanks with 20 fish each. The fish were allotted into 2 groups, viz. group 1, control, and group 2, 10 mg CCE/kg feed, in triplicate. About 50% of the water was replaced twice weekly to evade the buildup of wastes. The water quality parameters were maintained optimally during the experimentation period. During the 30 days feeding regime, the control group was offered the control diet. Group 2 was fed with an experimental diet containing CCE of *C. asiatica* at 10 mg / kg feed at 3% BW in two equal portions per day. The wastes and faeces were removed twice weekly followed by an exchange of 50% water.

24.2.8 Experimental Challenge Trials in *Labeo rohita* with *Flavobacterium columnare* SGM4

Thirty days post-feeding (dpf), *L. rohita* of the control group was divided into two sub-groups, CS (control fish injected with saline) and CFC (control fish injected with *F. columnare* SGM4). The test group was also divided into two sub-groups, viz. TS

(*C. asiatica* extract-fed fish injected with saline) and TFC (*C. asiatica* extract-fed fish injected with *F. columnare* SGM4). The bacterial cell suspension was prepared by first aseptically inoculating one young discrete colony of *F. columnare* SGM4 from CA plate into 10 mL Cytophaga broth (CB) and incubating at 30 ± 2 °C for 24 h. Mass culture was done in 500 mL CB at 30 ± 2 °C for 48 h and centrifuged at 7500 rpm at 20 °C for 20 min. The cell pellet, thus, obtained was washed thrice with physiological saline and finally resuspended in 10 mL sterile saline. The number of cells in the suspension was determined by spread plating on CA.

The fish of the groups CFC and TFC were intramuscularly administered with 0.1 mL of diluted *F. columnare* SGM4 cell suspension adjacent to the dorsal fin to get a sublethal dose of approximately 10^7 cells/fish and maintained in their respective tanks. Similarly, the CS and TS groups received 0.1 mL of sterile physiological saline. Mortality, external signs of infection and behavioural abnormalities were recorded daily. The experiments were carried out in triplicate. The blood and serum were collected on day 0 and 30 of feeding and on day 1, 7, 15, 21 and 28 post-challenge (dpc) with *F. columnare* SGM4 as described elsewhere in this chapter. An aliquot of blood was heparinized using 2.7% EDTA and processed for measurement within an hour of collection. The serum samples of two fish from each of the three replicate were pooled separately, labelled and stored at −20 °C until use.

24.2.9 Assessment of Serum Biomarkers

Stress indicator serum glucose was determined by using a glucose test kit (Span Diagnostics Ltd., India) following the GOD-POD method. The serum creatinine level was determined by using a creatinine test kit (DiaSys Diagnostics Systems, GmbH, Germany) following the modified kinetic test without deproteinization according to Jaffe's reaction. Serum C-reactive protein (CRP) was determined by using the CRP FS kit (DiaSys Diagnostic Systems, GmbH, Germany) by following the immunoturbidimetric test method. Total protein test kit (DiaSys Diagnostics Pvt. Ltd., GmbH, Germany) was used to determine serum total protein content by following the Biuret method. As liver function representative, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined by using ALT and AST test kits (Span Diagnostics Ltd., India) following the modified UV (IFCC) and kinetic assay methods, respectively. All measurements were performed in a photometer (Model: 5010 v5+, Robert Riele KG, Berlin) as per the manufacturer's instructions.

24.2.10 Assessment of Immunological Parameters

The effects of different doses of CCE of *C. asiatica* in feeds were tested by assessing the non-specific immune parameter respiratory oxidative burst (ROB) activity (Sahoo et al. 2011) using the heparinized blood collected on day 0 and 30. In the immunomodulatory experiment, the effects of dietary supplementation of CCE of

C. asiatica were tested by assessing the non-specific immune parameters like serum myeloperoxidase activity (Sahoo et al. 2011), serum phagocytic activity using *Staphylococcus aureus* (Anderson et al. 1995), in vitro nitrite production assay (Devi et al. 2012) and ROB activity (Sahoo et al. 2011). The specific immune parameter lymphocyte proliferation assay was performed as described in Adikesavalu et al. (2016).

24.2.11 Effect of Dietary *Centella asiatica* Crude Chloroform Extract on the Wound Healing in *Labeo rohita*

The effect of dietary CCE of *C. asiatica* on the wound healing in challenged *L. rohita* of 30–33 g size was assessed by intramuscular (IM) and abrasion-immersion (AI) assays. The experimental design on the feeding trial with dietary *C. asiatica* containing 10 mg CCE/kg feed was as described in Sect. 24.2.7. The fish without any gross abnormalities and infections from the acclimatized population were then collected and randomly allocated among 12 FRP tanks with 20 fish each. A total of six tanks each were allocated for IM and AI challenge. Each challenge assay group had 3 tanks each for the control and CCE diets. The fish were fed with control and CCE diets at 3% BW in two divided doses for 30 days. Following 30 dpf, the control groups of the IM and AI challenge assays were divided into two sub-groups, i.e. CS and CFC, with 10 fish each. Likewise, the CCE diet groups were divided into two sub-groups, i.e. TS and TFC, with 10 fish each.

The intramuscular (IM) injection challenge was carried out for the CFC and TFC groups, in triplicate, by administering 0.1 mL of diluted *F. columnare* SGM4 cell suspension adjacent to the dorsal fin as described in Sect. 24.2.8 to get a sublethal dose of approximately 10^7 cells/fish. Similarly, the CS and TS groups received 0.1 mL of sterile physiological saline, in triplicate. For the abrasion-immersion (AI) challenge assay, the scales of all 10 fish from each tank were scraped off gently with a scalpel from the caudal peduncle to the anal fin, i.e. in the opposite direction (abrasion). All the abraded fish of the CFC and TFC groups, in triplicate, from each tank were then immersed in bacterial cell suspensions containing *F. columnare* SGM4 at 2.40×10^5 cfu/mL for 30 min. Likewise, the abraded fish of the CS and TS groups were immersed in physiological saline for 30 min. Following the 30 min immersion, all fish groups were transferred to their respective tanks. The fish of all the groups were maintained in the respective tanks for a month for observations. The challenged fish were fed with a control diet on demand during the observation period. Mortality, external signs of infection and behavioural abnormalities were recorded daily.

The wounds at the site of IM injection or abrasion were digitally photographed weekly during the treatment regime and on the last day of the experiment to assess the wound progression and healing. The images were transferred to a computer, and the tissue damages were assessed from 6 digital images of each group on each sampling days using a score ranging from 0 to 6, depending on the degree and extent of damage based on the scale proposed by Bernet et al. (1999). The extent of wound

progression and healing was qualitatively classified as 0, no damage or undamaged with no pathological importance; 0.5, very mild damage with little or no pathological importance; 1, very mild damage with minimal pathological importance; 2, mild damage with minimal pathological importance; 4, moderate damage with moderate pathological importance; and 6, severe damage with marked pathological importance. Intermediate values were also considered.

24.2.12 Statistical Analyses

The results of the different experiments were expressed as the mean ± standard deviation and analysed using the Statistical Package for Social Sciences (IBM-SPSS) version 22.0, considering a probability level of $P < 0.05$. The results of the immune parameters and serum biomarkers of *L. rohita* were analysed by one way ANOVA (analysis of variance) followed by Tukey's post-hoc tests for pairwise comparisons among different treatments. Repeated measures ANOVA with Greenhouse-Geisser correction and Bonferroni correction for pairwise comparison were performed among different days within treatments. The qualitative scores of wound progression and healing within and/or among the challenge groups were analysed by related samples Friedman ANOVA and the independent samples by Mann-Whitney U test.

24.3 Results and Discussion

24.3.1 Anti-*Flavobacterium columnare* Activity of *Centella asiatica* Extract

The results of the in vitro inhibition of *F. columnare* SGM4 by the CCE of *C. asiatica* through agar disc diffusion and agar overlay well diffusion assays are presented in Table 24.1. In agar disc diffusion assay, the CCE of *C. asiatica* at a level

Table 24.1 Antibacterial activity of *Centella asiatica* against *Flavobacterium columnare* SGM4 by agar disc diffusion assay and agar well diffusion assay

Assay	Compound	Concentration (μL)	Zone size (mm)
Agar disc diffusion	Chloroform	20	6.00
	Oxytetracycline	30 μg/disc	26.00
	Crude chloroform extract (CCE) of *Centella asiatica*	20	6.00
Agar well diffusion	Chloroform	100	6.00
	Crude chloroform extract (CCE)of *Centella asiatica*	50	6.00
		100	29.30
		200	38.00

1 μL of CCE of *C. asiatica* is equivalent to 4.5 mg of powdered *C. asiatica* dry leaves

of 20 μL/disc did not inhibit the *F. columnare* SGM4. Oxytetracycline (30 μg/disc), an approved antibiotic for aquaculture use, inhibited the bacterium with a zone size of 26 mm. However, Rattanachaikunsopon and Phumkhachorn (2010a) demonstrated an in vitro inhibition of *F. columnare* by agar disc diffusion assay with a zone size of 25.2 mm using the aqueous extract of *C. asiatica* at 40 μL/disc. According to them, out of six tested herbs – *Andrographis paniculata*, *Cassia alata*, *C. asiatica*, *Garcinia mangostana*, *Punica granatum* and *Psidium guajava* – the aqueous extract of *C. asiatica* exhibited the strongest antimicrobial activity against *F. columnare*. Nevertheless, in agar overlay well diffusion assays, the zones of inhibition were recorded as 6.00 mm, 29.30 mm and 38.00 mm against 50 μL, 100 μL and 200 μL of CCE of *C. asiatica*, respectively. These results indicated that the anti-*F. columnare* activity of *C. asiatica* against is dose-dependent. Likewise, Purkait et al. (2018) demonstrated in vitro inhibition of *Aeromonas hydrophila* and *E. tarda* by the crude extracts of *C. asiatica* by agar disc diffusion and agar overlay well diffusion assays. They found that among aqueous, methanol and chloroform extracts, only the CCE of *C. asiatica* exhibited the maximum inhibitory activity against *E. tarda* with the zone size of 11.25 ± 0.35 mm and 30.50 ± 6.40 mm in agar disc diffusion (20 μL/disc) and agar overlay well diffusion assays (50 μL/well), respectively. In contrast, the ethanolic extract of *C. asiatica* against *Proteus vulgaris*, *S. aureus* and *E. coli* (Jagtap et al. 2009) and water extract of *C. asiatica* against *E. coli*, *K. pneumoniae*, *S. pyogenes* and *S. aureus* (Sarumathi et al. 2013) gave the best results. The antibacterial properties of *C. asiatica* have also been demonstrated in other bacterial species like *H. pylori*, *S. aureus*, *E. coli* and *P. aeruginosa* (Norzaharaini et al. 2011); *Bacillus subtilis*, *B. cereus*, *E. coli*, *K. aerogenes*, *P. vulgaris*, *P. mirabilis*, *P. aeruginosa*, *S. aureus* and *Salmonella typhi* (Samy and Chow 2011); and *E. coli*, *Vibrio parahaemolyticus*, *V. cholerae*, *P. aeruginosa*, *S. typhimurium*, *A. hydrophila*, *S. aureus* and *Shigella* sp. (Mamtha et al. 2004). Triterpene asiaticosides were found to be responsible for the antimicrobial activity of *C. asiatica* by weakening the membrane structure, which results in dissolving the cell walls of the microorganisms (Bisignano et al. 1999).

24.3.2 Molecular Docking of *Flavobacterium columnare* Virulent Genes

In recent years, the use of plants in the management and treatment of diseases has gained considerable importance. Plants are considered one of the main sources of biologically active compounds. An estimate of the World Health Organization (WHO) stated that around 85–90% of the world's population consumes traditional herbal medicines (WHO 2002). Molecular docking plays an important role in the rational design of drugs (Ruyck et al. 2016; Sethi et al. 2019). The main objective of molecular docking is to attain ligand-receptor complex with optimized conformation and to possess less binding free energy in the form of E-value or ΔG_{bind} (Dar and Mir 2017). In this study, Hex docking was carried out to compare the phytochemical compounds like asiatic acid, allicin, crucumin and gingerol as ligands against

Table 24.2 Molecular docking of *Flavobacterium columnare* virulent genes Gliding Motility Protein (*gldB*) and Chaperone Protein (*dnaJ*) against various antivirulent herbal compounds using HEX tool

Herbs	Antivirulent compound	E-value (ΔG_{bind})[a]	
		gldB Gliding Motility Protein	*dnaJ* Chaperone Protein
Centella asiatica (Indian pennywort)	Asiatic acid	−288.91	−234.49
Allium sativum (garlic)	Allicin	−201.66	−180.66
Curcuma longa (turmeric)	Crucumin	−293.53	−267.31
Zingiber officinale (ginger)	Gingerol	−272.25	−265.35

[a]ΔG_{bind}: Net binding free energy

receptors like virulent genes (*gldB* and *dnaJ*) of *F. columnare*. These ligands were used to target the receptors and to inhibit their functions. The bioactive compound asiatic acid from *C. asiatica* with an E-value of −288.91 and − 234.49 against *gldB* and *dnaJ* genes, respectively, indicated that the asiatic acid can be a potent drug (Table 24.2). These results suggested that its use as a herbal drug may control the columnaris disease and supported the work of Rattanachaikunsopon and Phumkhachorn (2010a). Besides *C. asiatica*, other three antibacterial herbs, viz. *A. sativum* (allicin), *C. longa* (crucumin) and *Z. officinale* (gingerol), were also found effective against virulent genes of *F. columnare*. Similarly, several other phytocompounds from *Eichhornia crassipes* against *P. fluorescens* (Kumar et al. 2018), caulerpin against *V. anguillarum* (Subramani et al. 2016), *Sargassum polycystem* against *P. aeruginosa* (Rajkumar et al. 2018) and eugenol and its derivatives against pseudomonads (Dhurga et al. 2016) showed a notable docking simulation. *Centella asiatica* also contained compounds like flavonoids with antibacterial activity against *S. aureus* and *B. cereus* and beneficial effects on the host (Pisano et al. 2019). Overall, the findings of the docking study through the bioinformatics tools supported the concept of testing the efficacy of herbal compounds as a drug of choice to control bacterial infection in fish.

24.3.3 Immunomodulatory Effects of *Centella asiatica* in *Labeo rohita*

24.3.3.1 Dose Standardization

The effects of dietary supplementation of CCE of *C. asiatica* at different levels on the growth indices, select serum biomarkers and non-specific immune parameters of *L. rohita* were studied up to 30 days and the results are presented in Figs. 24.2 and 24.3.

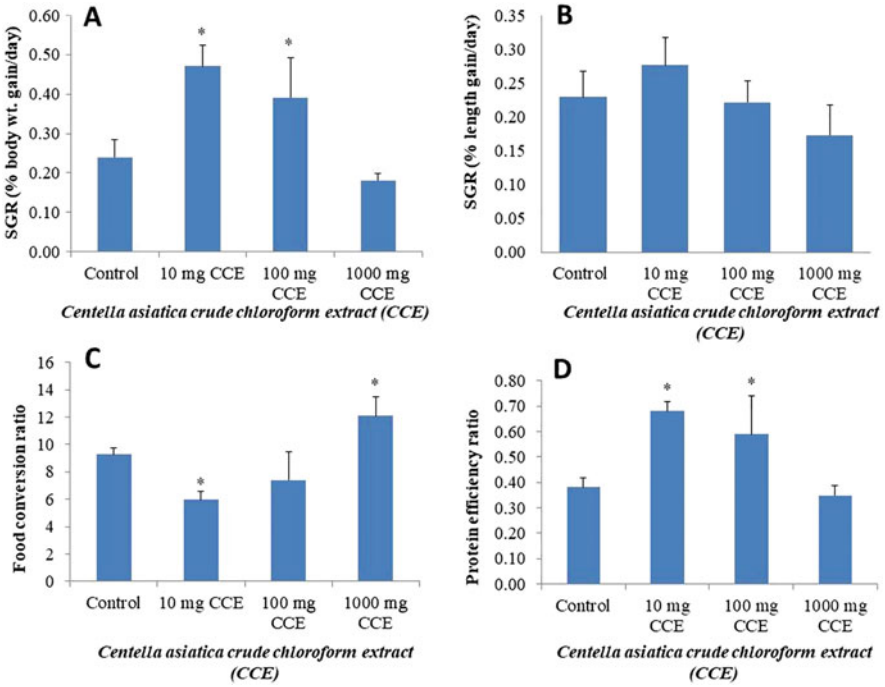

Fig. 24.2 Effect of dietary supplementation of crude chloroform extracts (CCE) of *Centella asiatica* on the (**a**) specific growth rate (SGR) (% body weight gain/day), (**b**) specific growth rate (% length gain/day), (**c**) food conversion ratio and (**d**) protein efficiency ratio of *Labeo rohita* when fed for 30 consecutive days. Values are expressed as mean ± SD. Bars with an asterisk (*) differed significantly compared to control ($P < 0.05$)

Growth Indices

Growth is the change in size (length and weight) either of an organisms' whole body or of its various tissues, and it is a good indicator of the health of an individual (Moyle and Cech 1996). In the present study, control *L. rohita* fed on the feed containing 28% crude protein gave an SGR of 0.24 ± 0.04% in 30 dpf (Fig. 24.2a). The confined environment in the experimental tanks may cause a loss of appetite in fish (Hussain and Shah 2016), which could be the reason for the low SGR in *L. rohita*. Likewise, Ali et al. (2006) also documented an SGR of 0.13 ± 0.83% in healthy *L. rohita* when maintained in fibreglass fish aquaria. The SGR was in the range of 0.16–0.41% in pond-raised Indian major carps (IMC) (Mamun and Mahmud 2014), 0.62 ± 0.02–1.12 ± 0.1% in IMCs from a polyculture system (Hosen et al. 2019), 0.184 ± 0.038% in healthy *L. rohita* juveniles (Gandotra et al. 2017) and 1.15 ± 0.03–1.78 ± 0.03% (Khan et al. 2004) in *L. rohita*. In contrast, the *L. rohita* fed the CCE of *C. asiatica* diets at 10 mg, 100 mg and 1000 mg/kg feed documented higher but varied levels of SGR(W). As shown in Fig. 24.2a, among all diets, the CCE at 10 mg/kg feed (0.47 ± 0.05%) and 100 mg/kg feed (0.39 ± 0.10%) fed groups showed significantly high SGR(W) compared to control (0.24 ± 0.04%).

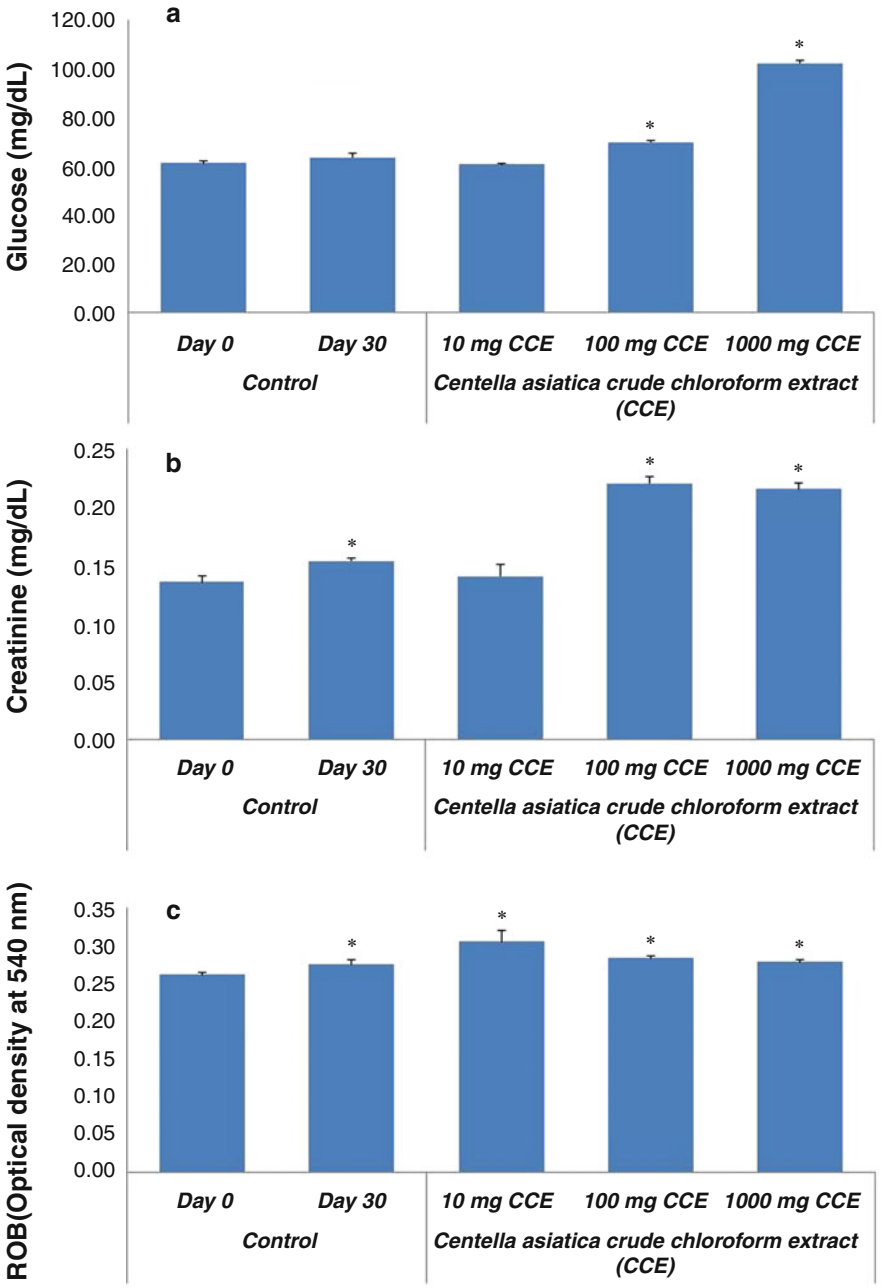

Fig. 24.3 Effect of dietary supplementation of crude chloroform extracts (CCE) of *Centella asiatica* on the (**a**) serum glucose, (**b**) serum creatinine levels and (**c**) respiratory oxidative burst (ROB) activity of *Labeo rohita* when fed for 30 consecutive days. Values are expressed as mean ± SD. Bars with an asterisk (*) differed significantly compared to control 0 ($P < 0.05$)

On the other hand, the SGR(W) of *L. rohita* fed the CCE diet containing 1000 mg/kg feed was lower than the control. These results indicated that the growth-promoting effect of CCE is dose-dependent with CCE of *C. asiatica* at 10 mg/kg feed offering a better growth promotion effect than the other doses. *Centella asiatica* is considered as nutraceutical due to its biologically active constituents like triterpenes, namely, asiatic acid and asiaticoside (Inamdar et al. 1996). Possibly, the higher levels of CCE may make the feed unacceptable for *L. rohita* by changing the texture and taste. Likewise, the administration of methanol extract of *C. asiatica* improved the growth and feed utilization in *Macrobrachium rosenbergii* (Sharma et al. 2014) and *Penaeus monodon* (Mohtar et al. 2017) compared to other experimental diets. However, the lengthwise SGR(L) data didn't show any significant differences among the experimental groups (Fig. 24.2b).

The FCR, in its simplest form a comparison of the amount of feed used per unit weight gain of the species being grown, offers a measure of aquaculture production efficiency. The variations in the FCR of *L. rohita* fed with CCE of *C. asiatica* and control diets are presented in Fig. 24.2c. The FCR of control diet-fed *L. rohita* was 9.27 $\pm$ 0.50 in 30 dpf, although high, this corroborate the reports (10.70 $\pm$ 0.51–14.02 $\pm$ 3.33) of Gandotra et al. (2017). Earlier studies reported varied levels of FCR, viz. 3.28 $\pm$ 0.15 (Khan et al. 2004), 3.30 $\pm$ 0.10 (Erfanullah and Jafri, 1998) and 4.12 $\pm$ 0.40 (Ahmed et al. 2012), in normal diet-fed healthy *L. rohita* depending on the composition of feed and habitat. The plant extracts have been shown to improve the digestibility and availability of nutrients thereby increasing feed conversion (Citarasu 2010). The fish fed the 10 mg CCE-supplemented diet gave a significantly lower FCR (5.97 $\pm$ 0.58) compared to other diets, thus establishing a significant improvement in the feed conversion. The results, more or less, were in conformity with Sharma et al. (2014), where the lower doses of *C. asiatica* powder (500 mg/kg feed) offered the best FCR in *M. rosenbergii*. Also, Nuwansi et al. (2019) found high SGR and low FCR in *Cyprinus carpio* when cultured with *C. asiatica* plant in aquaponics. In *L. rohita* offered the CCE of *C. asiatica* at 1000 mg/kg feed, significantly higher ($P < 0.05$) FCR (12.09 $\pm$ 1.36) was noted, which was almost double the FCR values of the lowest dose.

The PER is a measurement of how the protein sources in a diet could provide the essential amino acid requirement of the fish feed. Varying reports are available on the PER range, as it depends on the food composition and digestibility of food. The PER of control fish was 0.38 $\pm$ 0.04 (Fig. 24.2d), which was within the range of the earlier studies, such as 0.246 $\pm$ 0.002 (Gandotra et al. 2017), 0.80 $\pm$ 0.03 (Erfanullah and Jafri, 1998) and 1.02 $\pm$ 0.07, (Khan et al. 2004) on *L. rohita*. Compared to control, the CCE of *C. asiatica* at 10 mg/kg feed (0.68 $\pm$ 0.04) and CCE at 100 mg/kg feed (0.59 $\pm$ 0.15) diets fed to *L. rohita* showed significantly ($P < 0.05$) high PER. The PER was insignificantly ($P > 0.05$) lower (0.35 $\pm$ 0.04) in *L. rohita* fed the CCE at 1000 mg/kg feed. These results indicated that the feed containing CCE of *C. asiatica* at 10 mg/kg feed was able to provide better protein efficiency.

Assessment of Serum Biomarkers of Stress and Kidney Functions

The mean serum glucose level of the control diet-fed *L. rohita* was 61.00 ± 1.00 and 63.50 ± 1.50 mg/dL on day 0 and day 30 of feeding, respectively (Fig. 24.3a), which corroborate the findings of Kandeepan (2014) recorded in healthy *L. rohita* (60.0 ± 0.05 mg/dL). Unchanged or lower glucose levels compared to the control group is always desirable for the good health condition of fish. *Labeo rohita* fed the CCE diet at 10 mg/kg feed had almost similar glucose levels (60.50 ± 0.50 mg/dL) as that of control (day 0), thereby indicating that the CCE at this level did not pose stress to fish. On the other hand, the glucose levels were significantly high in fish fed the CCE of *C. asiatica* at 100 mg/kg feed and 1000 mg/kg feed, suggesting that these levels may trigger stress in fish. In support of the present study, multiple reports are available on the use of different herbal extracts in lowering the glucose levels in carps (Harikrishnan et al. 2010; Mallik et al. 2019).

The mean serum creatinine levels of control *L. rohita* ranged between 0.135 ± 0.005 mg/dL and 0.143 ± 0.0075 mg/dL during the 30 days feeding regime (Fig. 24.3b), which corroborate the observations of Tiwari and Pandey (2014) reported in healthy *L. rohita* (0.14 mg/dL). The fish fed the CCE of *C. asiatica* at 10 mg/kg feed had creatinine levels (0.14 ± 0.01 mg/dL) close to control, thus indicating that the functioning of the kidney was normal in this group. The results of the present study corroborate results of using herbal extracts in *Clarias gariepinus* (Emeish et al. 2018) and chicken (Kumar and Mandal 2018) on serum creatinine. In contrast, the creatinine levels were significantly high in *L. rohita* fed CCE of *C. asiatica* at 100 mg/kg feed (0.22 ± 0.005 mg/dL) and 1000 mg/kg feed (0.215 ± 0.005 mg/dL) compared to the control ($P < 0.05$). The present data proved that the functioning of the fish kidney was not affected by CCE at a level of 10 mg/kg feed, while the CCE at the higher doses had a negative role on the functioning of the fish kidney.

Assessment of Respiratory Oxidative Burst (ROB) Activity

The enhancement of the ROB activity of HK leucocytes is a widely used indicator of immune competence, especially when provoked by stimulators and is related to the secretion of cytokines and inflammatory responses (Reverter et al. 2014). The ROB activities of the control diet-fed *L. rohita* were 0.261 ± 0.004 and 0.274 ± 0.007 OD on day 0 and day 30 of feeding, respectively (Fig. 24.3c), which validate the observations made earlier in *L. rohita* (Sen et al. 2014; Yadav et al. 2014). The immunostimulating activity of *C. asiatica* extracts on the non-specific cellular immune responses and humoral immune responses has been documented (Mukherjee et al. 2014; Srichaiyo et al. 2020). In the present study, a significant elevation ($P < 0.05$) in the ROB activity was noticed in 30 dpf with CCE at 10 mg/kg feed (0.305 ± 0.015 OD), 100 mg/kg feed (0.278 ± 0.003 OD) and 1000 mg/kg feed (0.278 ± 0.003 OD) compared to the control on day 0 (Fig. 24.3c), indicating an improved immune competence in *L. rohita*. Alike, Srichaiyo et al. (2020) recorded a significant increase in ROB activities in *Oreochromis niloticus* when fed with *C. asiatica* powder (5 and 10 g/kg feed)-supplemented feed compared to the control. Likewise, several other herbal products reportedly increased the ROB activities in

fish (Bulfon et al. 2018; Bilen et al. 2019; Doan et al. 2019; Sarhadi et al. 2020). In general, the results of the growth indices, serum biomarkers and ROB activity indicated that the feed supplemented with CCE of *C. asiatica* at 10 mg /kg feed was the best as it gave the higher SGR, PER and ROB activity, lower FCR and no negative effect on the serum biomarkers of stress and kidney functioning when fed to *L. rohita*. Based on these results, a diet containing CCE of *C. asiatica* 10 mg/kg feed was selected as an optimal dose for further studies on immunoprotective effect against *Flavobacterium columnare* infection.

24.3.3.2 Immunoprotective Effect of Dietary *Centella asiatica* against *Flavobacterium columnare* Infection in *Labeo rohita*

Flavobacterium columnare SGM4 Challenge

A wide range of plant products has been attributed with health benefits, including protection against bacterial disease and immunomodulation when administered orally to fish (Harikrishnan et al. 2011; Vaseeharan and Thaya 2014). In the present study, following 30 days of feeding with CCE of *C. asiatica* at 10 mg/kg feed and control diets, *L. rohita* were injected intramuscularly with a sublethal dose of *F. columnare* SGM4 at 2.07×10^7 cfu/fish. The challenged fish had clinical symptoms like lethargy, abnormal and erratic swimming behaviour. In the control group, 3 fish died (30%) on 15 dpc with *F. columnare* SGM4 infection. No mortalities were observed in CCE diet-fed and challenged *L. rohita* till 28 dpc. Similarly, no mortalities in the treated group after 2 weeks of challenge were observed by Rattanachaikunsopon and Phumkhachorn (2010a) in *O. niloticus* treated with *C. asiatica* at 100 mg/L and subsequently challenged with *F. columnare* at 2.37×10^5 cfu/mL. In contrast, the control group showed 50% mortality at the same challenge dose (Rattanachaikunsopon and Phumkhachorn 2010a).

Assessment of Serum Biomarkers

There was no significant increment in the serum glucose level in 30 dpf ($P > 0.05$) compared to day 0 (Fig. 24.4a), which proved no adverse effect of the dietary CCE at 10 mg/kg feed. The glucose levels of the CFC group increased significantly on 1 dpc to 88.725 ± 1.525 mg/dL, which, though reduced, remained significantly higher till 28 dpc (68.435 ± 3.935 mg/dL) compared to control. Similarly, Zaki et al. (2016) reported glucose level as 60.30 ± 0.34 mg/dL in healthy *Clarias lazera*, which rose to 80.00 ± 0.73 mg/dL on 7 dpi with *F. columnare*. However, the glucose levels in the TFC group increased significantly during the initial phase of post-challenge ($P < 0.05$). Gradually, it became normal as that of control on 28 dpc (61.65 ± 1.65 mg/dL). In contrast, the glucose levels of CFC group were significantly higher on 21 dpc (75.83 ± 0.76 mg/dL) and 28 dpc (68.43 ± 3.93 mg/dL) compared to TFC group. These results revealed the efficacious outcome of the dietary *C. asiatica* on fish physiology. Possibly, it could be attributed to the anti-hyperglycemic or anti-diabetic effects of *C. asiatica* on animals (Kabir et al. 2014; Supkamonseni et al. 2014). The madecassic acid, a pentacyclic triterpenoid bioactive compound, from *C. asiatica* was found to be anti-diabetic (Barcroft and Myskja

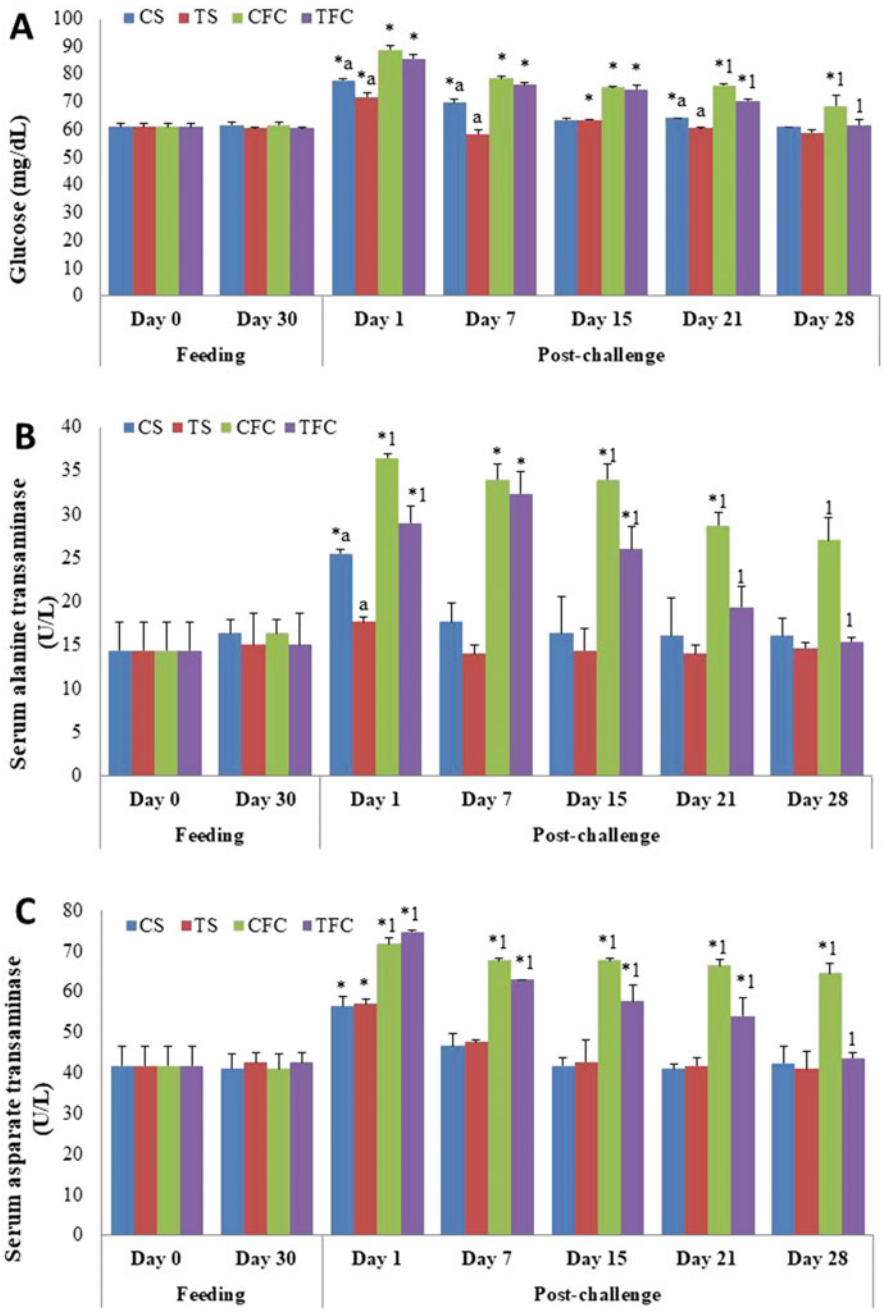

Fig. 24.4 Effect of crude chloroform extract of *Centella asiatica* diet-fed *Labeo rohita* at 10 mg/kg feed during the different feeding and post-challenge (*Flavobacterium columnare* SGM4) periods on the (**a**) serum glucose, (**b**) serum alanine transaminase (ALT) and (**c**) serum aspartate transaminase (AST) levels. Values are expressed as mean ± SD. Bars with an asterisk (*) differed significantly ($P < 0.05$) compared to respective controls on day 0. Bar sharing an alphabet "a" or numeral "1"

2003). Likewise, Harikrishnan et al. (2018) demonstrated that *Agaricus bisporus*-enriched diet had an effective role to control fish mortality caused by *F. columnare*. The CS (77.61 ± 0.86 mg/dL) and TS (71.67 ± 1.48 mg/dL) groups also showed a significant increase in glucose levels on 1 dpc possibly due to handling stress during IM injection. Yet, the glucose levels of the TS group were significantly lower compared to the control. On 7 dpc, the glucose levels reached the normal range in the TS group (58.27 ± 1.61 mg/dL), while they remained high (69.56 ± 1.56 mg/dL) in the CS group. Nevertheless, the glucose levels became normal in both CS and TS groups on 28 dpc. The role of different herbal extract-supplemented feeds to reduce serum glucose levels of carps has been discussed in several studies (Abdel-Tawwab et al. 2018; Acar et al. 2018; Mallik et al. 2019) as agents of anti-stressor even after infection with pathogens.

Hepatoprotective activity is mainly determined by the levels of AST and ALT (Cui et al. 2014). Multiple research trials were successful in demonstrating the efficiency of *C. asiatica*-supplemented feeds in rat models to reduce the serum AST and ALT levels (Choi et al. 2016; Ghosh et al. 2017; Oyenihi et al. 2019). Similarly, the present study demonstrated the hepatoprotective activity of CCE diet-fed *L. rohita* at different feeding and post-challenge (*F. columnare* SGM4) periods in terms of serum ALT and AST levels (Figs. 24.4b, c). The ALT levels didn't differ much between day 0 (14.33 ± 3.21 U/L) and 30 dpf (15.00 ± 3.60 U/L) with CCE diet at 10 mg/kg feed (Fig. 24.4b). The results on the stable ALT levels conform with the results recorded on different fish species when fed with varied herbal extracts (Shehata et al. 2013; Adel et al. 2015). On 1 dpc, the CS group (25.50 ± 0.50 U/L) showed significantly high ALT levels compared to day 0. However, the saline injection didn't affect the ALT levels of the TS group (14.67 ± 0.58 U/L). Within 24 h of the challenge with *F. columnare* SGM4, both CFC (36.50 ± 0.50 U/L) and TFC (29.00 ± 2.00 U/L) groups showed a significant hike in ALT levels ($P < 0.05$). Yet, the increment in ALT levels in the TFC group was significantly low ($P < 0.05$) compared to the control (Fig. 24.4b). Also, on 21 dpc, the ALT levels reached the normal range in the TFC group (19.33 ± 2.31 U/L), while in the CFC group, the ALT levels were significantly ($P < 0.05$) high (27.00 ± 2.65 U/L) even on 28 dpc. Likewise, the AST levels reached significantly high in the CS (56.33 ± 2.31 U/L) and TS (57.00 ± 1.00 U/L) groups compared to day 0 (41.67 ± 4.73 U/L) within 24 h (Fig. 24.4c). On and after 7 dpc, the AST levels started to reduce, but it was still high until 21 dpc and reached the normal level on 28 dpc in the TFC group. But in the CFC group, the AST levels (64.50 ± 2.50 U/L) remained significantly high even on 28 dpc ($P < 0.05$). A similar kind of increase in ALT and AST levels was noticed by Tripathi et al. (2005) in *C. carpio* and Zaki et al. (2016) in *C. lazera* challenged with *F. columnare*. These observations on serum ALT and AST levels reassured the

Fig. 24.4 (continued) within the particular day of treatment differed significantly ($P < 0.05$). *CS* control fish injected with saline, *TS Centella asiatica* extract-fed fish injected with saline, *CFC* control fish injected with *F. columnare* SGM4, *TFC Centella asiatica* extract-fed fish injected with *F. columnare* SGM4

fact that dietary *C. asiatica* has hepatoprotective properties. These results are quite similar to those recorded with other herbal plant products with hepatoprotective activity in fish (Shehata et al. 2013; Acar et al. 2018).

The serum total protein (TP) is an important non-specific immune biochemical variable (Magnadóttir 2006). The 30 days of CCE diet feeding increased the serum TP levels significantly ($P < 0.05$) to 3.30 ± 0.10 g/dL from 3.03 ± 0.06 g/dL (Fig. 24.5a). The results conform with the observations of other dietary herbal products in different fish (Sahu et al. 2007a; Kaleeswaran et al. 2012; Abdel-Tawwab et al. 2018). There was a drop in TP levels in 24 hours of *F. columnare* challenge. On and after 7 dpc, the TP levels of the TS group restored to normal. The TP levels of the CFC group were significantly low till 21 dpc (2.75 ± 0.05 g/dL) compared to control on day 0, while in the TFC group, lower TP levels were noted till 15 dpc (2.72 ± 0.08 g/dL). Likewise, a decline in TP levels was recorded in *F. columnare*-challenged *L. rohita* (Tiwari and Pandey 2014) and *Salvelinus fontinalis* (Řehulka and Minařík 2007) and *Flavobacterium*-challenged *Catla catla* (Ravindra et al. 2019a). The TP levels of the TFC group, though reduced to 2.87 ± 0.06 g/dL on 1 dpc, were always within the normal range till the last day of the experiment (3.15 ± 0.13 g/dL). These results suggested that despite the challenge posed by *F. columnare* infection, *C. asiatica* was able to manage this non-specific immune biochemical variable balanced in *L. rohita*.

The serum creatinine levels of the control and CCE diet-fed *L. rohita* did not vary (0.135 ± 0.005–0.14 ± 0.01 mg/dL) during the feeding trial (Fig. 24.5b), thereby exhibiting no adverse effect of CCE of *C. asiatica* at the tested dose on the kidney functioning of *L. rohita*. The CS (0.285 ± 0.005 mg/dL) and TS (0.265 ± 0.005 mg/dL) groups showed significant rise in creatinine levels even on 1 dpc ($P < 0.01$). Its levels were almost constant in TS and CS groups till 28 dpc. A similar observation was documented in earlier studies where the serum creatinine levels increased significantly in *L. rohita* (Tripathi et al. 2005), *C. carpio* (Tiwari and Pandey, 2014) and *C. lazera* (Zaki et al. 2016) after experimental challenge with *F. columnare*. The creatinine levels of the TFC and CFC groups differed significantly from each other ($P < 0.05$) on and after 21 dpc with the lower creatinine levels (0.25 ± 0.01 mg/dL) in the TFC group. On 28 dpc, the creatinine levels of the TFC group (0.185 ± 0.015 mg/dL) declined and reached a near-normal level, while it was still high in the CFC group (0.26 ± 0.00 mg/dL). The results on this kidney biomarker corroborate the observations made with other herbal products in different fish species (Abdel-Tawwab and Ahmad 2009; Adel et al. 2015; Emeish et al. 2018). The results of the present study demonstrated the usefulness of *C. asiatica* to minimize kidney damage as it maintained the creatinine levels in a reasonable range despite the bacterial challenge.

The mean serum CRP level of *L. rohita* fed with CCE of *C. asiatica* at 10 mg/kg feed on 30 dpf was 2.74 ± 0.32 mg/L (Fig. 24.5c). The CS (3.69 ± 0.23 mg/L) and TS (2.93 ± 0.07 mg/L) groups showed a significant increment in CRP even on 1 dpc ($P < 0.05$), possibly due to the inflammatory responses of intramuscular injection and the associated acute phase reaction. In both CS and TS groups, the CRP levels became normal on 7 dpc. In contrast, the CRP levels of the CFC (7.545 ± 0.035 mg/

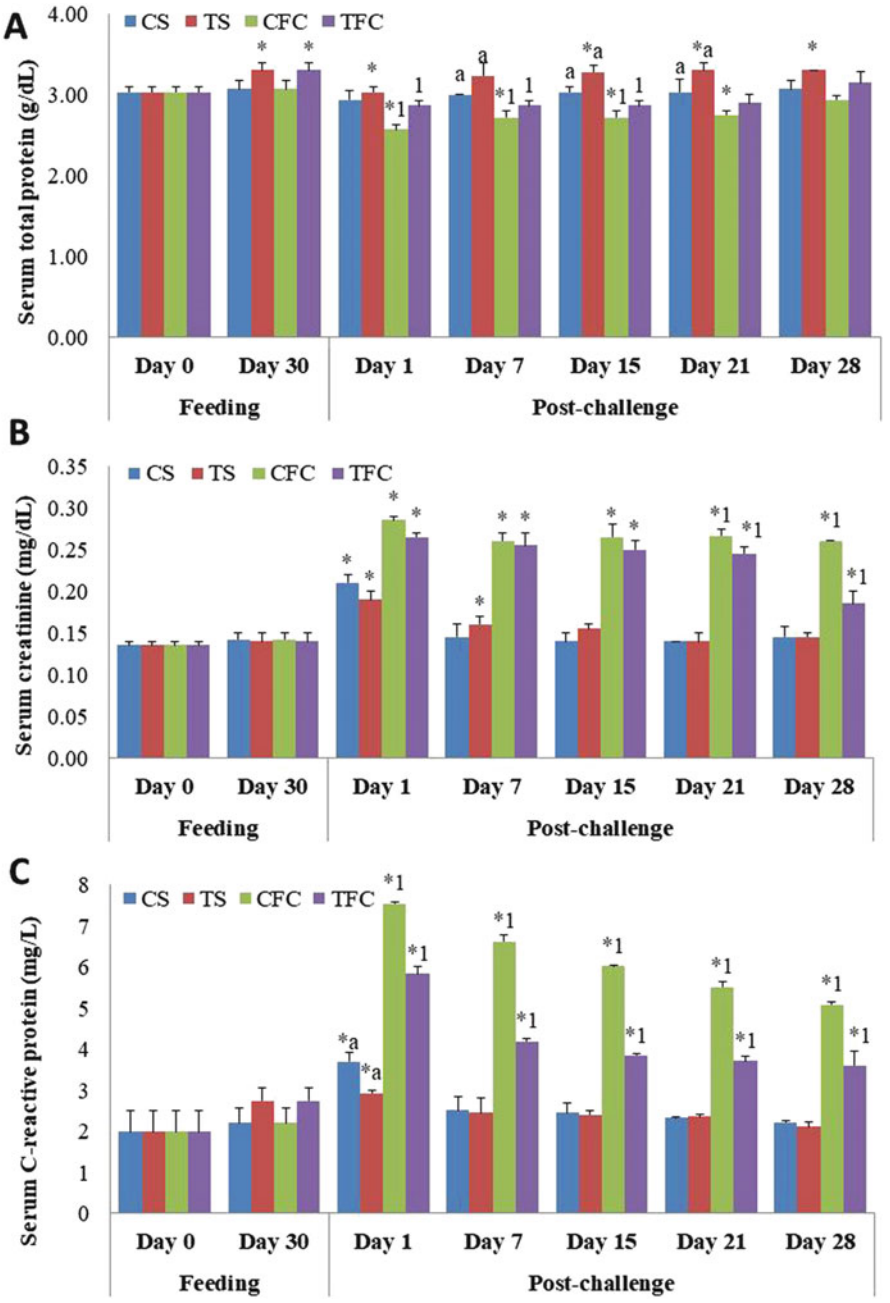

Fig. 24.5 Effect of crude chloroform extract of *Centella asiatica* diet-fed *Labeo rohita* at 10 mg/kg feed during the different feeding and post-challenge (*Flavobacterium columnare* SGM4) periods on the (**a**) serum total protein, (**b**) serum creatinine and (**c**) serum C-reactive protein levels. Values are expressed as mean ± SD. Bars with an asterisk (*) differed significantly ($P < 0.05$) compared to respective controls on day 0. Bar sharing an alphabet "a" or numeral "1" within the particular day of treatment differed significantly ($P < 0.05$). *CS* control fish injected with saline, *TS Centella asiatica*

L) and TFC (5.833 ± 0.185 mg/L) groups increased drastically and significantly on 1 dpc ($P < 0.05$). Both these groups had significantly high CRP levels until 28 dpc ($P < 0.05$), indicating persuasive inflammatory responses of *F. columnare* infection in *L. rohita*. Though there was a reduction in CRP levels in both groups with time, the reduction was significantly high in the TFC group ($P < 0.05$). It indicated the positive effect of dietary *C. asiatica* on the inflammatory responses due to *F. columnare* infection.

Assessment of Non-specific and Specific Immune Responses

Myeloperoxidase (MPO) is a peculiar and specific haemoprotein released by neutrophils and plays an important role in the defence of an organism. It utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid (Gobi et al. 2016). As seen in Fig. 24.6a, the MPO activity of *L. rohita* increased significantly from 0.237 ± 0.003 to 0.399 ± 0.045 OD in 30 dpf with CCE of *C. asiatica* at 10 mg/kg feed. This indicated the potentiating effect of the CCE diet in facilitating the release of MPO by neutrophils. Similarly, the results of earlier studies indicated an increase in MPO activities of fish fed with dietary herbal products (Kaleeswaran et al. 2011; Acar et al. 2015; Gobi et al. 2016). In the CFC group, the MPO activity increased significantly on 1 dpc to 0.2985 ± 0.0005 OD ($P < 0.05$) compared to the control and then gradually reduced to near-normal level (0.2406 ± 0.0349 OD) on 28 dpc. In the TFC group also, the MPO levels increased significantly (0.298 ± 0.002 OD) within 24 h ($P < 0.05$) compared to the control. From 7 dpc onwards, it increased gradually and significantly ($P < 0.05$) to reach a level of 0.4186 ± 0.0178 OD on 28 dpc, which indicated the increased protective effect of dietary *C. asiatica*. Likewise, Kong et al. (2019) demonstrated the increased level of MPO in *C. asiatica*-fed diseased rats, which proved its immune-modulating effect. Also, significantly higher MPO levels were noted in the CFC group ($P < 0.05$), but the levels were lower than those of the TFC group. In contrast, the IMC challenged with *F. columnare* had higher MPO activity up to 12 hpi, which became normal after 24 hpi (Ravindra et al. 2019a). Likewise, enhanced MPO levels in different fish species upon dietary supplementation of other herbal extracts and bacterial challenge have been demonstrated (Sahu et al. 2007b; Gobi et al. 2016; Yilmaz 2019). Though the *C. asiatica* protected the *L. rohita* against *F. columnare* challenge in this study, some researchers documented the attenuating effect of asiatic acid on myeloperoxidase activation (Xiao et al. 2017; Ugur et al. 2017; Nagoor et al. 2018).

The phagocytic activity by neutrophils and macrophages is one of the crucial mechanisms of the non-specific immune system in fish (Esteban et al. 2015). The antimicrobial defence mechanisms in teleosts mostly rely on reactive oxygen species (ROS) produced by the phagocytes (Das et al. 2011). As depicted in Fig. 24.6b, the serum phagocytic activity of *L. rohita* increased significantly ($P < 0.05$) from

Fig. 24.5 (continued) extract-fed fish injected with saline, *CFC* control fish injected with *F. columnare* SGM4, *TFC Centella asiatica* extract-fed fish injected with *F. columnare* SGM4

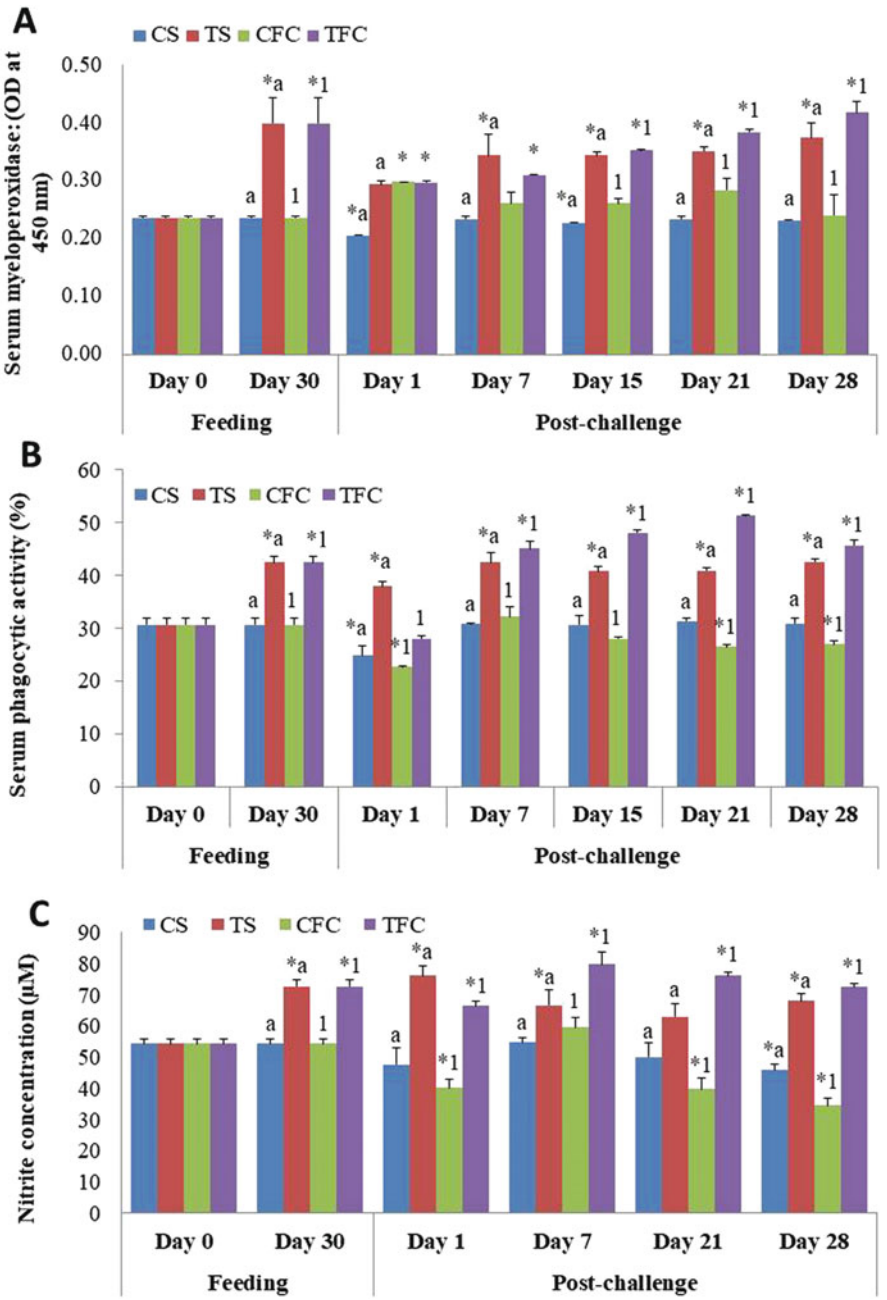

Fig. 24.6 Effect of crude chloroform extract of *Centella asiatica* diet-fed *Labeo rohita* at 10 mg/kg feed during the different feeding and post-challenge (*Flavobacterium columnare* SGM4) periods on the (**a**) serum myeloperoxidase activity, (**b**) serum phagocytic activity and (**c**) in vitro nitrite production. Values are expressed as mean ± SD. Bars with an asterisk (*) differed significantly ($P < 0.05$) compared to respective controls on day 0. Bar sharing an alphabet "a" or numeral "1" within the particular day of treatment differed significantly ($P < 0.05$). *CS* control fish injected with

30.63 ± 1.28% to 42.37 ± 1.28% in 30 dpf with CCE diet. These results confirm the observations of Srichaiyo et al. (2020), who recorded the stimulated phagocytic ability of *O. niloticus* when fed the dietary *C. asiatica*. Likewise, *C. asiatica* was reported as a phagocytic activity enhancer in the human study (Mali and Hatapakki 2008). These results suggested that dietary *C. asiatica* can stimulate phagocytic activity similar to those recorded in other herbal plant products (Bilen et al. 2019; Doan et al. 2019). A significant reduction in the serum phagocytic activity was noticed on 1 dpc in both the TFC and CFC groups ($P < 0.05$). However, the phagocytic activity in the TFC group then rose to a maximum (51.264 ± 0.236%) on 21 dpc ($P < 0.05$). In contrast, the CFC group had significantly low phagocytic activity compared to the TFC group on all dpc ($P < 0.05$). Likewise, the CS group had significantly low phagocytic activity compared to the TS group on all dpc ($P < 0.05$). The TFC group on all dpc had significantly high phagocytic activity compared to other groups ($P < 0.05$). These results, thus, affirmed the improved stimulation in the phagocytic activity by neutrophils and macrophages in CCE diet-fed *L. rohita*. Similarly, Harikrishnan et al. (2018) documented a highly significant enhancement of phagocytic activity in *Flavobacterium*-infected fish when fed with *Agaricus bisporus*-enriched diet. Several other studies on dietary medicinal plant extracts also demonstrated increased phagocytic activity in different fish species against various bacterial challenges (Zhang et al. 2009; Rattanachaikunsopon and Phumkhachorn 2010b).

The nitric oxide (NO), produced by activated phagocytic cells, i.e. monocytes and macrophages, plays an important role in innate immunity as it has antimicrobial activities (Adikesavalu et al. 2016). The feeding trial with CCE diet at 10 mg/kg feed for 30 days significantly increased the NO activity ($P < 0.05$) in *L. rohita* from 32.67 ± 0.00 μM to 73.00 ± 4.35 μM (Fig. 24.6c). These results suggested that the CCE of *C. asiatica* can activate the phagocytic cells like monocytes and macrophages in *L. rohita* to exert stronger antimicrobial activities. The role of NO as a vasodilator and more specifically as a stress reliever in fish was documented (Singh 2018). The results indirectly suggested that the CCE diet could act as a stress reliever by elevating the NO level. After 24 h of *F. columnare* SGM4 challenge, the NO production in the CFC group (38.33 ± 2.52 μM) was observed to be significantly ($P < 0.05$) lower than the TFC group (89.33 ± 3.21 μM). On 7 dpc, though there was an increase in NO production in both CFC and TFC groups, the increase was significantly high in the TFC group ($P < 0.05$). In the CFC group, the NO production reduced from 15 dpc onwards (30.00 ± 1.73 μM). In contrast, the TFC group always maintained significantly high NO levels ($P < 0.05$) on all dpc. From these results, it can be inferred that *L. rohita* has responded to *F. columnare* infection by enhancing the production of NO as an immediate protective mechanism similar to those observed in earlier studies (Sun et al. 2012; Ravindra et al. 2019a). In contrast,

Fig. 24.6 (continued) saline, *TS Centella asiatica* extract-fed fish injected with saline, *CFC* control fish injected with *F. columnare* SGM4, *TFC Centella asiatica* extract-fed fish injected with *F. columnare* SGM4

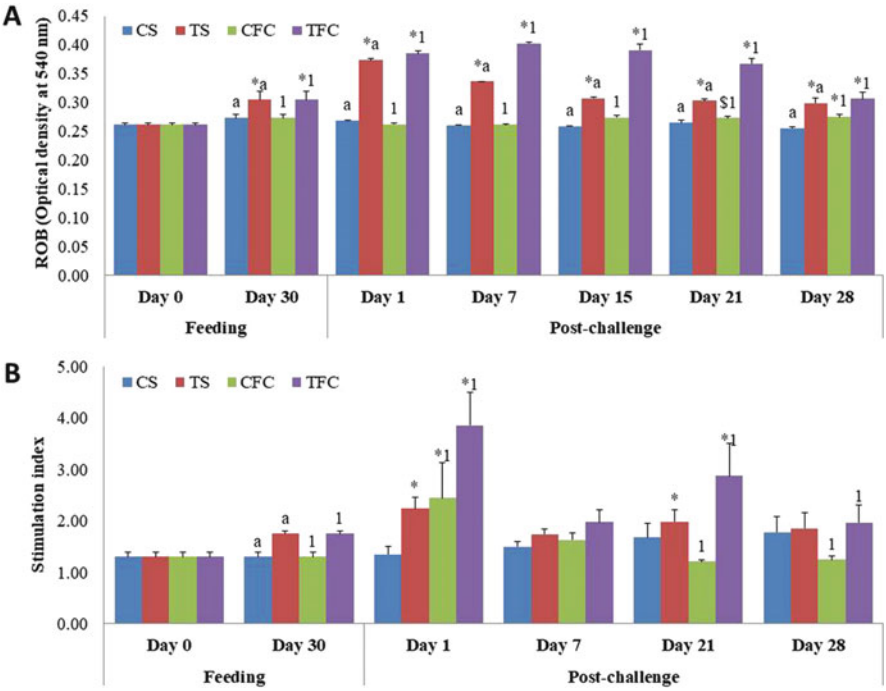

Fig. 24.7 Effect of crude chloroform extract of *Centella asiatica* diet-fed *Labeo rohita* at 10 mg/kg feed during the different feeding and post-challenge (*Flavobacterium columnare* SGM4) periods on the (**a**) respiratory oxidative burst (ROB) activity and (**b**) stimulation index. Values are expressed as mean ± SD. Bars with an asterisk (*) differed significantly ($P < 0.05$) compared to respective controls on day 0. Bar sharing an alphabet "a" or numeral "1" within the particular day of treatment differed significantly ($P < 0.05$). *CS* control fish injected with saline, *TS Centella asiatica* extract-fed fish injected with saline, *CFC* control fish injected with *F. columnare* SGM4, *TFC Centella asiatica* extract-fed fish injected with *F. columnare* SGM4

Zahran et al. (2014) documented inhibition of NO production in fish against *F. columnare* infection. Overall, the *L. rohita* fed the dietary CCE of *C. asiatica* offered extended protection against pathogenic *F. columnare* challenge. The results of the present study corroborate the observations made on the NO activity with other herbal-supplemented diets in carps (Wu et al. 2007; Yuan et al. 2007) and increased resistance to pathogens (Yin et al. 2004).

The ROB activity by neutrophils was determined by the reduction of nitroblue tetrazolium (NBT) to formazan. The enhancement of the ROB activity of HK leucocytes is a widely used indicator of immune competence, especially when provoked by stimulators (Adikesavalu et al. 2016). The 30 days of CCE diet feeding increased the ROB activity significantly ($P < 0.05$) from 0.2615 ± 0.0035 to 0.305 ± 0.015 OD (Fig. 24.7a). In the TS group, the ROB activity was quite high even on 1 dpi (0.374 ± 0.003 OD), which remained significantly higher till 28 dpi (0.29 ± 0.01 OD) than on day 0 ($P < 0.05$). On the other hand, there were no notable

changes in the ROB activity in the CS group until 28 dpc ($P > 0.05$). These results indicated that the dietary CCE of *C. asiatica* has the potency to improve the ROB activity of *L. rohita* and prime the immune system. These results support observations of Srichaiyo et al. (2020), who recorded a significant increase in ROB activities in *O. niloticus* fed with *C. asiatica*-supplemented feeds (5 and 10 g/kg feed) compared to the control. Alike, an improved ROB activity was found in other herbal extract-supplemented diet-fed fish (Bilen et al. 2013; Haghighi et al. 2014), most probably due to the high antioxidant and polyphenolic contents in the herbs. Compounds such as triterpenoids, glycosides, free acids, volatile oils and flavonoids (Mamtha et al. 2004), phenolic compounds (Zainol 2004), tannins and reducing sugars (Arumugam et al. 2011) and antioxidants, polyphenolic and other novel compounds (Srichaiyo et al. 2020) were documented in *C. asiatica*, of which pectin and triterpenoid saponins and methanol extracts have immuno-potentiating effects (Jayathirtha and Mishra 2004; Singh et al. 2010). The ROB activities of the TFC group increased significantly on 1 dpc to 0.386 ± 0.004 OD and remained significantly high even on 28 dpc (0.3066 ± 0.0115 OD) compared to control and/or the CFC group on all dpc ($P < 0.05$). Alike, the earlier studies also demonstrated significantly increased ROB activity in fish fed the herbal extract and challenged with *F. columnare* (Harikrishnan et al. 2018) and in the use of plant products in the vaccine preparation against *F. columnare* (Guz et al. 2014).

Herbal extracts have immunostimulating properties, which help to proliferate the number of neutrophils, macrophages and lymphocytes in fish (Bulfon et al. 2018). In 30 dpf the CCE diet increased the simulation index (SI) of *L. rohita* from 1.29 ± 0.09 to 1.75 ± 0.06 (Fig. 24.7b), which corroborate the report of Yin et al. (2004) recorded in *C. carpio* fed the *Asiasari radix* extract. As presented in Fig. 24.7b, the challenged fish had a significant increase in lymphocytes ($P < 0.05$) similar to those of Sebastião et al. (2011) observed in fish infected with *Flavobacterium.* The CFC group showed a significant increment ($P < 0.05$) in SI on 1 dpc (2.44 ± 0.69) followed by a gradual reduction. The SI of the TFC group rose to a maximum of 3.85 ± 0.64 within 24 h of the challenge. The CFC group, however, had significantly lower lymphocyte proliferation (SI) than the TFC group ($P < 0.05$) on 1, 21 and 28 dpc. This increased number of lymphocytes during CCE feeding, and *F. columnare* infection is an indication of stimulation of the defence mechanism in fish. Similar studies with other herbal supplements also stimulated the proliferation of lymphocytes and post-injection with pathogens in rats (Gomez-Flores et al. 2008).

24.3.4 Effect of Dietary *Centella asiatica* on Wound Progression and Healing in *Labeo rohita*

The wound healing process on fish muscle and its repair mechanism is a natural process. It is achieved through four phases, namely, haemostasis, inflammation, proliferation and remodelling (Vinoth et al. 2018). From the digital images, as shown in Figs. 24.8, 24.9, 24.10 and 24.11, the qualitative ratings (Bernet et al. 1999) of the disease progression and healing process in the control or CCE diet-fed

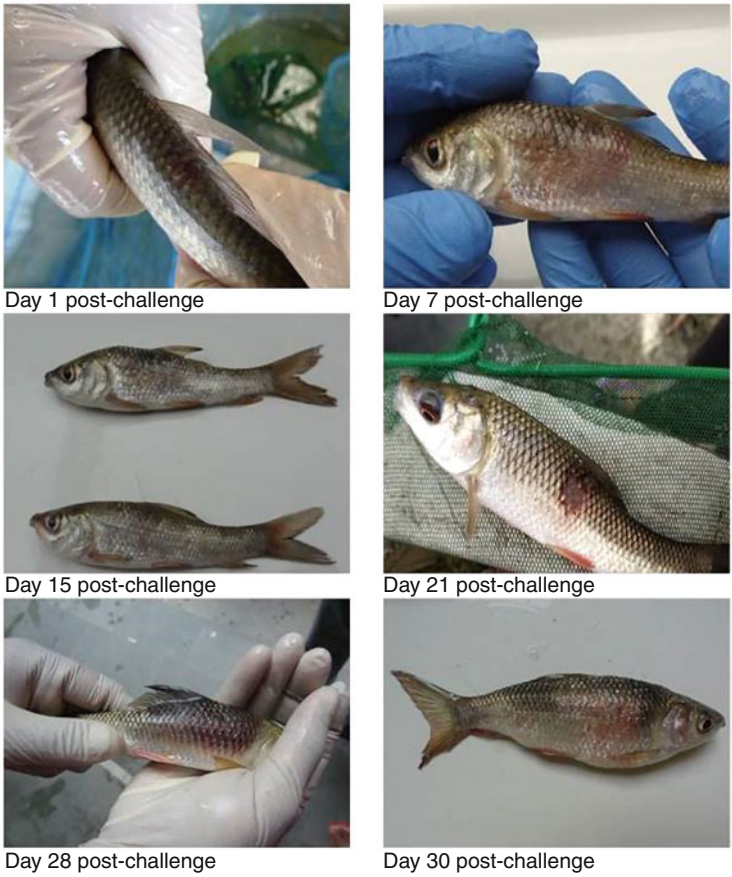

Fig. 24.8 Digital images showing the wound progression and healing in control diet-fed *Labeo rohita* and challenged intramuscularly with *Flavobacterium columnare* SGM4 on different days of post-challenge

L. rohita at different days of IM and AI challenge with *F. columnare* SGM4 were made, and the results are presented in Table 24.3.

24.3.4.1 Intramuscular (IM) Challenge

Intramuscularly injected *Flavobacterium* sp. can elicit cutaneous lesions as well as systemic alterations (Sarker et al. 2017). In the present study, the control diet-fed *L. rohita* challenged with *F. columnare* SGM4 exhibited reddening and mild inflammation at the site of injection on 1 dpc with a wound progression score of 1.67 ± 0.26, which gradually increased ($P < 0.05$) and reached the score of 4.17 ± 0.26 on 28 dpc with skin peeling, scale loss, lesion formation, haemorrhages, etc. (Fig. 24.8). Several researchers also observed similar clinical signs and wound type in *Flavobacterium*-infected fish (Sarker et al. 2017, 2018). On 30 dpc

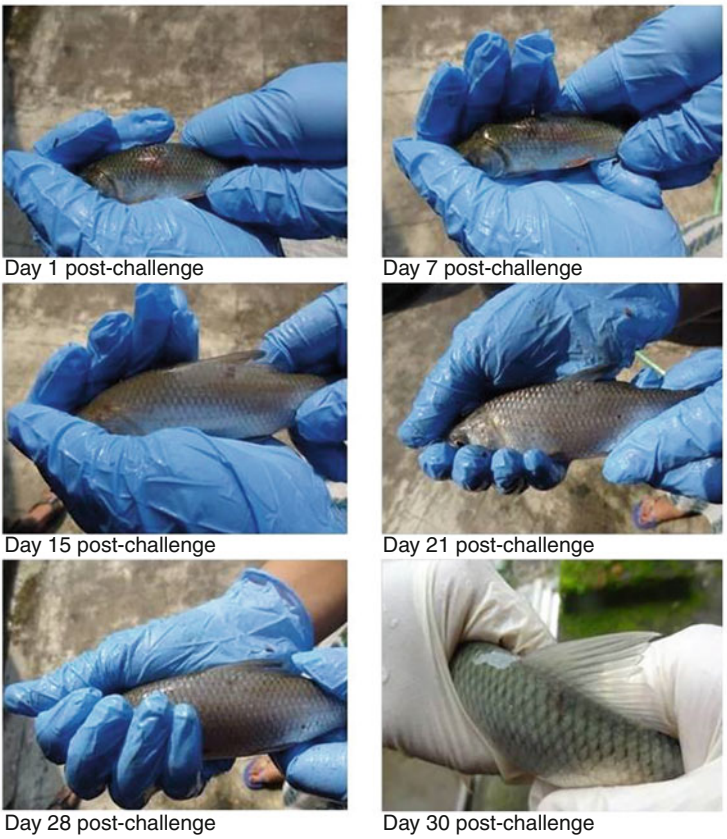

Fig. 24.9 Digital images showing the wound progression and healing in crude chloroform extract of *Centella asiatica* diet-fed *Labeo rohita* at 10 mg/kg feed and challenged intramuscularly with *Flavobacterium columnare* SGM4 on different days of post-challenge

(4.42 ± 0.38), the infection became more severe with fluid accumulation under scale pockets and body cavity, which led to secondary infection with other bacterial flora. The results suggested a progressive increase in *F. columnare* infection with time, which possibly exposed the fish to an increased risk of infection with opportunistic pathogens. Likewise, Julinta et al. (2017) also mentioned slow wound healing in untreated *A. hydrophila*-challenged *O. niloticus.* Significant differences in the scores were noted on all dpc ($P < 0.05$), except for the 7 dpc. The CCE diet-fed and *F. columnare* SGM4-injected *L. rohita* showed an intense inflammation and ulceration on 7 dpc with a wound progression score of 2.67 ± 0.26, which then subsided by the formation of a black scar on 15 dpc (1.17 ± 0.26) (Fig. 24.9). The dark appearance of the skin may be due to the increased number of melanocytes and their activities after the injury (Guerra et al. 2008). The black scar disappearance, the onset of dermal fibrous tissue regrowth and development of skin at the ulcerated scar region were seen on 21 dpc (1.17 ± 0.26). Complete healing was noticed in the

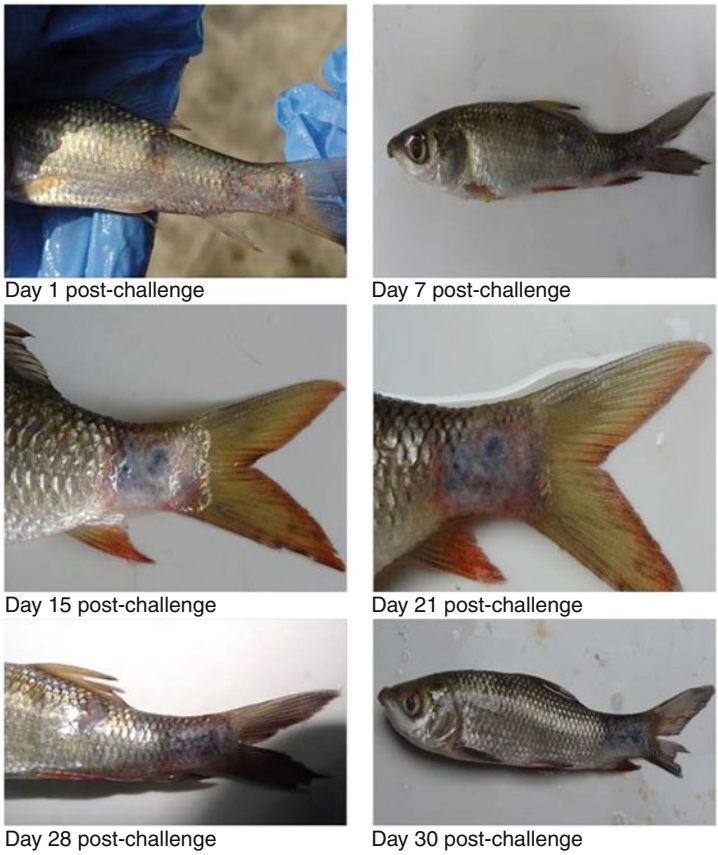

Fig. 24.10 Digital images showing the wound progression and healing in control diet-fed *Labeo rohita* and subsequently abrasion-immersion challenged with *Flavobacterium columnare* SGM4 on different days of post-challenge

wound area within 28–30 dpc (0.67 ± 0.26). The observations on the wound darkening corroborate Řehulka (2002), who observed discolouration of skin in *Oncorhynchus mykiss* artificially infected with *A. caviae*. In some other studies, in incisional wounds in *Clarias batrachus*, the epidermis became normal by 32 days (Dutta and Rai 1994), while in the *A. hydrophila*-induced wounds in *O. niloticus* by IM challenge, the epidermis became normal by 30 days (Julinta et al. 2017). Analogous research of Roy et al. (2019) found wound progression score as 1.00 ± 0.00 on 15 dpc with *A. caviae* in OTC-treated *O. niloticus.* It revealed that *C. asiatica* played almost a similar role of healing against *F. columnare* as that of OTC provided against *A. caviae* (Roy et al. 2019).

Fig. 24.11 Digital images showing the wound progression and healing in crude chloroform extract of *Centella asiatica* diet-fed *Labeo rohita* at 10 mg/kg feed and subsequently abrasion-immersion challenged with *Flavobacterium columnare* SGM4 on different days of post-challenge

24.3.4.2 Abrasion-Immersion (AI) Challenge

Fish and other aquatic animals have developed unique but essential integumentary adaptations that permit intimate contact with the physical, biologic and chemical properties of their aqueous habitat. Any failure or disruption of these anatomical and physiologic characteristics often results in the development of cutaneous disease (Fontenot and Neiffer 2004). Several researchers demonstrated AI challenge on fish with *Flavobacterium* strains and observed significant cutaneous wound development (Olivares-Fuster et al. 2011; Sarker et al. 2017; Ravindra et al. 2019a,b). According to Dutta and Rai (1994), freshwater fish could recover from wound completely within 30–35 days. In AI-challenged (*F. columnare* SGM4) control fish, the wound progressed till 21 dpc (3.83 ± 0.26) with the typical ulcer and white patches on the abraded area (Fig. 24.10). On and after 28 dpc, it started to heal, but mild damages persisted until 30 dpc (1.83 ± 0.26). As the wound did not recover till

Table 24.3 The rate of wound progression and healing in crude chloroform extract (CCE) of *Centella asiatica* diet-fed *Labeo rohita* at 10 mg/kg feed and subsequently challenged with *Flavobacterium columnare* SGM4

	Wound progression and healing score[#]			
	Intramuscular (IM) challenge[$]		Abrasion-immersion (AI) challenge[$]	
Experimental period	Control	CCE diet	Control	CCE diet
Day 0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1 dpc	1.17 ± 0.26^{1a}	1.67 ± 0.26^{1a}	1.33 ± 0.26^{1a}	0.67 ± 0.26^{2ad}
7 dpc	2.75 ± 0.27^{1b}	2.67 ± 0.26^{1b}	1.33 ± 0.52^{2a}	1.17 ± 0.41^{2b}
15 dpc	3.17 ± 0.26^{1b}	1.17 ± 0.26^{1c}	3.83 ± 0.26^{2b}	1.67 ± 0.26^{2c}
21 dpc	3.17 ± 0.26^{1b}	1.17 ± 0.26^{1c}	3.83 ± 0.26^{2b}	1.08 ± 0.20^{2ab}
28 dpc	4.17 ± 0.26^{1c}	0.67 ± 0.26^{1d}	2.17 ± 0.26^{2c}	0.83 ± 0.26^{2abd}
30 dpc	4.42 ± 0.38^{1c}	0.67 ± 0.26^{1d}	1.83 ± 0.26^{2ac}	0.58 ± 0.20^{2d}

[#]As per the scale proposed by Bernet et al. (1999); dpc, day post-challenge. 1–2, Values sharing uncommon numerical within a row between the two challenge routes of the same group (control or CCE diet-fed) differed significantly ($P < 0.05$); a–d, values sharing uncommon alphabetical superscript within a column among different post-challenge days of the respective group differed significantly ($P < 0.05$)

[$]The scores between the control and CCE diet group of the particular challenge group on all dpc, except for 7 dpc, differed significantly ($P < 0.05$)

30 dpc, it is assumed that it could provoke the secondary bacterial infection in control. When CCE diet-fed *L. rohita* was AI challenged with *F. columnare* SGM4, inflammation, skin peeling and scale loss were observed at the site of abrasion initially (Fig. 24.11). It further led to reddening till 15 dpc with a wound score of 1.67 ± 0.26. From 21 dpc onwards (1.08 ± 0.20), the wound started healing followed by black scar formation at the abraded site on 30 dpc (0.58 ± 0.20). A similar kind of observation was noted by Roy et al. (2019), where the closure of wounds with the development of normal tissue colour and the skin layer was eminent within 6 days of post-wounding in *A. caviae*-infected and OTC-treated *O. niloticus*. They recorded a full recovery of normal skin architecture within 29 dpc. The results presented here demonstrated that the degree of wound healing promoted by dietary CCE of *C. asiatica* was similar to that of OTC-medicated feed (Julinta et al. 2017; Roy et al. 2019). The comparative evaluation of the results of the two challenge modes indicated that the IM challenge route is more effective to elicit the pathogenic potency of *F. columnare* SGM4 (Table 24.3).

In support of this study, 2.4% methanolic extract of *C. asiatica* (Azis et al. 2017) and hydrogel formulation of *C. asiatica* (Ahmed et al. 2019) reportedly exerted significant healing percentages in rabbits or other animal models (Ansell et al. 2014). Asiatic acid (asiaticoside) and madecassic acid are recognized as the main active constituents of *C. asiatica* responsible for wound healing (Zainol et al. 2003; Mukherjee et al. 2017; Azis et al. 2017). In various wound healing models, topical application (0.2%–0.4%), injection (1 mg) or ingestion (40 μg/disc) of asiaticoside has been shown to increase hydroxyproline content, improve tensile strength, increase collagen synthesis and remodelling of the collagen matrix, promote epithelialization, stimulate glycosaminoglycan synthesis and elevate antioxidant levels

(Somboonwong et al. 2012), stimulate the vascular endothelial growth factor production (Kimura et al. 2008) and promote fibroblast proliferation and extracellular matrix synthesis (Lu et al. 2004). Similarly, several other herbal extracts caused accelerated wound healing in rabbits (Fernandes et al. 2010) and carp (Vinoth et al. 2018).

24.4 Conclusion

The results of the dietary supplementation of CCE of *C. asiatica* at 10 mg/kg feed suggested that *C. asiatica* have the potency to improve the levels of non-specific immune parameters (ROB, phagocytic and myeloperoxidase activities and in vitro nitric oxide production) and specific immune parameter (lymphocyte proliferation) of *L. rohita*, as well as offer protection against *F. columnare* infection. The results also suggested that the CCE of *C. asiatica* can improve the liver and kidney functions of *L. rohita* when challenged with *F. columnare*, act as a stress reliever, prime the immune cells to offer an immunomodulating effect and heal the wounds faster. The use of CCE of *C. asiatica* in the diet at 10 mg/kg feed would, therefore, help to combat the columnaris disease or related flavobacteriosis disease in *L. rohita* and other closely related species. As the Indian fish farmers are innovative and environment-conscious, its use can be promoted for easy adoption in lieu of the negative effects of antibiotics and chemicals in aquaculture. The effect of *C. asiatica* as an immunomodulator of carps and other fish species needs to be demonstrated in the field or pond conditions. Yet, much more work is requisite on the aspects of commercialization of *C. asiatica* admixed feed. Further studies are also required on the mode of action of *C. asiatica* as an immunomodulator in carps and upregulation of genes. The WHO documented *C. asiatica* as one of the most important medicinal plants to be conserved and cultivated. The commercial cultivation of *C. asiatica* needs promotion from the agriculture sector for the large-scale production as well as the pharmaceutical sector on the commercial production of the final product either in the form of crude extract or the active component for application in aquaculture.

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Animal welfare statement All applicable guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA 2018), Government of India, New Delhi, were followed.

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Abbreviations

3D	Three dimensional
A-KG	α-ketoglutarate
AChE	Acetylcholinesterase
ACP	Alternative complement pathway
ACTH	Adrenocorticotropic hormone
AHLs	Acylated homoserine lactones
AHPND	Acute hepatopancreatic necrosis disease
AI	Abrasion immersion
AI-2	Autoinducer-2
ALF	Anti-lipopolysaccharide factor
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMP/AMPs	Antimicrobial peptides
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
APC	Antigen presenting cells
APD	Antimicrobial peptide database
ARGs	Antibiotic resistance genes
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AXOS	Arabinoxylo-oligosaccharides
BB	Brown bullhead
BCAHV	Blue catfish alloherpesvirus
BF2	Bluegill fry
BFT	*Biofloc technology*
BG	b-1, 3-glucans
BG	Bacterial ghost
BL	Bacterial lysate
bp	Base pair
BP2	Bactericidal peptide 2
BPI	Bactericidal permeability increasing

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BW	Bodyweight
C1qDC	C1q domain-containing
CA	Cytophaga agar
CAI-1	Cholerae *quorum-sensing* autoinducer-1
CAT	Catalase
CB	Cytophaga broth
CCE	Crude chloroform extract
CCO	Channel catfish ovary
CCP	Classical complement pathway
CCR-6	Chemokine receptor 6
CD8α	Cluster of differentiation 8α
CE	Capillary electrophoresis
CFU	Colony forming unit
CMD	Covert mortality disease
CMNV	Covert mortality nodavirus
CNTs	Carbon nanotubes
COVID-19	Coronavirus disease-19
COX-2	Cyclooxygenase-2
CPE	Cytopathic effect
CRDs	Carbohydrate recognition domains
CRE	Creatinine
CRH	Corticotrophin-releasing hormone
CRISPER	Clustered regularly interspaced short palindromic repeats
CRP	C-reactive protein
CTLs	C-type lectins
CTP	Cytidine 5′-triphosphate
DC	Dendritic cells
DGGE	Denaturing gradient gel electrophoresis
DI	Direct immersion
DNA	Deoxyribonucleic acid
DNV	Densovirus
DO	Dissolved oxygen
DOPA	Dihydroxyphenylalanine
DPBS	Dulbecco's phosphate buffered saline
dpc	Day post-challenge
dpf	Day post-feeding
DPmax	Degree of polymerization
dsRNA	Double-stranded RNA
ECP	Extracellular product
eDNA	Environmental DNA
EDTA	Ethylene diamine tetraacetic acid
EGC	Eosinophilic granular cells
EHP	*Enterocytozoon hepatopenaei*
EMS	Early mortality syndrome
EPC	Epithelioma papulosum cyprini

EPO	Eosinophil peroxidase
EPS	Exopolysaccharides
ESC	Enteric septicemia of catfish
FCR	Food conversion ratio
FFSc	Faculty of Fishery Sciences
FM	Fish meal
FOS	Fructooligosaccharides
FRP	Fibreglass reinforced plastic
FT-IR	Fourier transform infrared spectroscopy
GABA	Gamma-aminobutyric acid
GALT	Gut-associated lymphoid tissue
GAV	Gill-associated virus
GC	Gas chromatography
GCRV	Grass carp reovirus
Gdf9	Growth differentiation factor9
GDH	Glutamate dehydrogenase
gfp	Green fluorescence protein
GHS	Globally Harmonized System
GIT	Gastro-intestinal tract
GNBPs	Gram-negative binding proteins
GOS	Galactooligosaccharides
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSI	Gonadosomatic indices
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
hDNA	Human DNA
HI	Hypersonic infiltration
HMB	Beta-hydroxy beta-methylbutyrate
HPA	Hypothalamic-pituitary-adrenal
HPI	Hypothalamic-pituitary-interrenal
HPLC	High-pressure liquid chromatography
HPM	Hepatopancreatic microsporidiosis
HSF	Heat shock factor
HSPs	Heat shock proteins
HSR	Heat shock response
HTS	High-throughput sequencing
ICA-3	Ion channel activator protein 1-4
IFN	Interferon
Ig	Immunoglobulins
IGF	Insulin-like growth factor
IgM	Immunoglobulin M
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IHNV	Infectious haematopoietic necrosis virus
IL-1β	Interleukin 1β

IL-8	Interleukin 8
IM/i.m	Intramuscular
IMC	Indian major carp
IMNV	Infectious myonecrosis virus
IMO	Isomalto oligosaccharides
iNOS	Inducible NOS
IPM	Integrated pest (parasite) management
ISAV	Infectious salmon anaemia virus
ISKNV	Infectious spleen and kidney necrosis virus
ITS	Internal transcribed spacer
JAK/STAT	Janus kinase/signal transducer and activation of transcription
KHV	Koi herpesvirus
KMnO4	Potassium permanganate
LAB	Lactic acid bacteria
LBUSV	Largemouth bass ulcerative syndrome virus
LC	Liquid chromatography
LCDV	Lymphocystis disease virus
LD_{50}	Lethal dose 50
LGBP	Lipopolysaccharide and β-1,3-glucan-binding proteins
LGP2	Laboratory of genetics and physiology 2
LLE	Liquid-liquid extraction
LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
LSNV	Laem-Singh virus
MABs	Monoclonal antibodies
MAF	Macrophage activating factor
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MAMP	Microbial associated molecular pattern
MAPK	Mitogen-activated protein kinase
MAS	Motile *Aeromonas septicemia*
MBL	Mannose-binding lectin
MCP	Major capsid protein
MDA	Malondialdehyde
MDA5	Melanoma differentiation-associated gene 5
MDP	Muramyldipeptide
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
miRNAs	microRNAs
MjCRS	*M. japonicus* crustin
MOS	Mannanoligosaccharides
MPO	Myeloperoxidase
mRNA	Messenger RNA
MS	Mass spectrometry
MSRV	*Micropterus salmoides* rhabdovirus
MWCNTs	Multi-walled carbon nanotubes

$Na_2S_2O_3$	Sodium thiosulphate
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NCC	Non-specific cytotoxic cell
NETs	Neutrophil extracellular traps
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
NH_3	Ammonia
NLRs	Nod-like receptors
NMR	Nuclear magnetic resonance
NNV	Nervous necrosis virus
NOS	Nitric oxide synthase
NPs/Nps	Nanoparticles
Nramp	Natural resistance-associated macrophage protein
NSPAAD	National Surveillance Programme for Aquatic Animal Diseases
NTR	Neurotransmitters proteins
OMPs	Outer membrane proteins
OTUs	Operational taxonomic units
PAMPs	Pathogen-associated molecular pattern
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD	Pancreatic disease
PDB	Protein data bank
PER	Protein efficiency ratio
PG	Peptidoglycan
PGNs	Peptidoglycans
PGRPs	Peptidoglycan recognition proteins
PHA	Polyhydroxyalkanoates
PHB	Poly-beta-hydroxybutyrate
PHBV	Poly(β-hydroxybutyrate-β-hydroxyvalerate)
piRNAs	PIWI-interacting RNAs
PLGA	Poly D, L-lactide-co-glycolic acid
PLS	Partial least squares
PPAE	Prophenoloxidase activating enzyme
proPO	Prophenoloxidase
PRR	Pattern recognition receptors
PTGS	Post-transcriptional gene silencing
PVDF	Polyvinylidene fluoride membrane
QAC	Quaternary ammonium compounds
RAS	Recirculating aquaculture systems
RBA	Respiratory burst activity
RFLP	Restriction fragment length polymorphism
RIG-1	Retinoic acid-inducible gene I
RISC	RNA-induced silencing complex
RMS	Running mortality syndrome

RNA	Ribonucleic acid
RNAi	RNA interference
ROB	Respiratory oxidative burst
ROS	Reactive oxygen species
RPS	Relative percent survival
RSIV	Red sea bream iridovirus
SBD	Substrate binding domain
SBM	Soya bean meal
SCFA	Short-chain fatty acids
scFOS	Short-chain fructooligosaccharides
Sch B	Schisandrin B
SDF	Structure data file
SDGs	Sustainable Development Goals
Se	Selenium
SEP	Secretory/excretory proteins
SGR	Specific growth rate
SGR(L)	Specific growth rate (length)
SGR(W)	Specific growth rate (weight)
SI	Simulation index
SiRNAs	Small interfering RNAs
SMRV	Scophthalmus maximus rhabdovirus
SOD	Superoxide dismutase
SPE	Solid-phase extraction
SPF	Specific pathogen-free
SRS	Rickettsia septicemia
STAT	Signal transducer and activator of transcription
SVC	Spring viraemia of carp
SVCV	Spring viraemia of carp virus
SWCNTs	Single-walled carbon nanotubes
TAN	Total ammonia nitrogen
TC19	Thrombocidin-19
TCA	Tricarboxylic acid
TCID50	Median tissue culture infectivity dose
TEPs	Thioester-containing proteins
Tf	Transferrin
TGF-β	Transforming growth factor-β
TGIV	Iridovirus of Taiwan
TiLV	Tilapia lake virus
TLR	Toll-like receptor
TMA-N	Trimethylamine nitrogen
TNF	Tumour necrosis factor
TOS	Trans-galactooligosaccharides
TP	Total protein
TPP	Tripolyphosphate
TRAFs	Tumour necrosis factor receptor-associated factors

TRAP	Tumour necrosis factor receptor-associated protein
Treg	Regulatory T cells
TSA	Tryptic soy agar
TSB	Thai silver barb
TSV	Taura syndrome virus
USDA	United States Department of Agriculture
UTP	Uridine-5′-triphosphate
VHS	Viral haemorrhagic septicaemia
VHSV	Viral haemorrhagic septicaemia virus
WBC	White blood cells
WFS	White faeces syndrome
WHO	World Health Organization
WSD	White spot disease
WSSV	White spot syndrome virus
XOS	Xylooligosaccharides
YHD	Yellow head disease
Zn	Zinc
ZnO	Zinc oxide