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S. M. Sharifuzzaman *Editors*

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To Dawn Amy Austin and Tasnuba Sharmin.

Preface

Until half-way through the twentieth century, the control of diseases of fish and shellfish centerd on the use of therapeutic agents, principally antibiotics and inhibitory chemicals/disinfectants. Thereafter, prophylaxis involving vaccines entered the sphere of disease control although only comparatively few commercial products were developed. This was a serious constraint on what should have been a primary prophylactic tool. Subsequently, research has diversified into an increasingly wide range of disease control measures, including nonspecific immunostimulants, probiotics, prebiotics, and phytobiotics. The literature concerning probiotics in aquaculture has expanded considerably over the last 20 years, and it is timely to examine their role in depth. To date, a wide range of Gram-positive and Gram-negative bacteria and some eukaryotes, i.e., yeasts, unicellular algae and bacteriophages, have been reported to be beneficial to aquatic hosts. Often, publications described benefits associated with improved growth performance and protection against many bacterial and some parasitic diseases. The mode of action was initially thought to be competitive exclusion, whereby the microbial culture colonized the digestive tract of the host leading to in situ production of antimicrobial compounds, which inhibited pathogenic microorganisms, thereby reducing the risk of infection. In addition, studies have led to proposals for other reasons for the success of probiotics in disease control and include the provision of essential nutrients and immunomodulation. However, there is controversy about the nature of probiotics—are they food supplements or veterinary medicines? The designation has implications for licensing arrangements in numerous countries.

The current text has been developed to provide a detailed discussion of probiotics in fish and shellfish aquaculture. The text will highlight strengths and weaknesses in knowledge and discuss gaps that need to be addressed. We are grateful for the willing participation of the authors, who worked under a tight deadline.

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Abbreviations

3R	Replacement, reduction and refinement
ACP	Alternative complement pathway
AHL	N-acylhomoserine lactone
AHPND	Acute hepatopancreatic necrosis disease
AI-1	Autoinducer-1
AI-2	Autoinducer-2
AI-3	Autoinducer-3
AIP	Autoinducer peptide
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMR	Antimicrobial resistance
ASP	Aspartate aminotransferase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AXOS	Arabinoxylan oligosaccharide
BALO	*Bdellovibrio* and like organism
BFT	Biofloc technology
BMP	Best management practice
C AI-1	Cholerae autoinducer-1
CAS	Chrome Azurol S
CAT	Catalase
CE-TOFMS	Capillary electrophoresis mass spectrometry with time-of flight
CFU	Colony forming unit
C-N	Carbon–nitrogen
COD	Chemical oxygen demand
COS	Chitooligosaccharide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
EC	European Commission
EFSA	European Food Safety Authority

ELISA	Enzyme-linked immunosorbent assay
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus-PCR
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FAS	Fatty acid synthase
FCA	Freund's complete adjuvant
FCR	Feed conversion ratio
FIA	Freund's incomplete adjuvant
FISH	Fluorescence in situ hybridization
FOS	Fructooligosaccharide
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GH	Growth hormone
GIT	Gastrointestinal tract
GLP	Good laboratory practice
GMM	Genetically modified microorganism
GMP	Good manufacturing practice
GOS	Galactooligosaccharide
GPx	Glutathione peroxidase
GRAS	Generally recognized as safe
GSI	Gonado somatic index
Hb	Hemoglobin
HCT	Hemocrit
HHL	N-hexanoyl-L-homoserine lactone
HSP	Heat shock protein
HUFA	Highly unsaturated fatty acid
IGF-1	Insulin-like growth factor 1
IgM	Immunoglobulin M = macroglobulin
IHHNV	Infectious hypodermal and hematopoietic necrosis virus
IHNV	Infectious hematopoietic necrosis virus
IL	Interleukin
INF	Interferon
IPNV	Infectious pancreatic necrosis virus
JCM	Japan Collection of Microorganisms
KEGG	Kyoto Encyclopedia of Genes and Genomes
KHV	Koi herpesvirus
LAB	Lactic acid bacteria
Lb	*Lactobacillus*
LDH	Lactate dehydrogenase
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
MAS	Motile *Aeromonas* septicaemia
MBL	Metallo-β-lactamase
MCCB	Microbial culture collection bacteria
MCHC	Mean corpuscular hemoglobin concentration

MDP	Muramyl dipeptide
MIC	Minimum inhibitory concentration
MOS	Mannan oligosaccharide
MSP	Multi-strain probiotic
MTCC	The Microbial Type Culture Collection and Gene Bank, India
NAD	Nicotinamide adenine dinucleotide
NCBI	National Center of Biotechnology Information
NCIMB	National Collection of Industrial and Marine Bacteria
NTB	Nitroblue tetrazolium
ORF	Open reading frame
OTU	Operational taxonomic unit
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PER	Protein efficiency ratio
PFGE	Pulsed field gel electrophoresis
ppm	Part per million
pro-PO	Prophenoloxidase
PRR	Pattern recognition receptor
QAC	Quaternary ammonium compound
qPCR	Quantitative polymerase chain reaction
QPS	Qualified presumption of safety
QQ	Quorum quencher
QS	Quorum sensing
QSI	Quorum sensing inhibitor
R2A	Reasoner's 2A agar
RBC	Red blood cell
RMML	*Ruegeria Mobilis* Marine Lactonase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPS	Relative percent survival
rRNA	Ribosomal RNA
RT-PCR	Real-time reverse transcriptase polymerase chain reaction
SCFA	Short-chain fatty acid
SEM	Scanning electron microscopy
SFCA	Short-chain fatty acid
SGR	Specific growth rate
SOD	Superoxide dismutase
SPF	Specific pathogen free
SVC	Spring viremia of carp
T3SS	Type III secretion system
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TGGE	Temperature gradient gel electrophoresis
THB	Total heterotrophic bacteria
TLR	Toll-like receptor

TNF	Tumor necrosis factor
TSA	Tryptone soya agar
TSV	Taura syndrome virus
UV	Ultraviolet
VHSV	Viral hemorrhagic septicaemia virus
WBC	White blood cell
WCP	Whole cell protein
WGS	Whole genome sequencing
WHO	World Health Organization
WSD	White spot disease
WSV	White spot virus
XOS	Xylooligosaccharide

Introduction

S.M. Sharifuzzaman and B. Austin

Probiotic – from the Latin/Greek meaning "for life"

Abstract A wide range of bacteria, yeasts, micro-algae and bacteriophages has been examined as probiotics, in either cellular or acellular form, for use in aquaculture with the benefits including improved growth and health, immunomodulation and disease protection.

Keywords History · Competitive exclusion · Growth improvement · Immunomodulation · Definition

It is difficult to be sure of a precise starting point for interest in probiotics as there is anecdotal evidence that they have been used albeit unknowingly for millennia. Certainly, the ancient Roman naturalist, Pliny the Elder, appears to have used fermented milk as a remedy for gastro-intestinal problems. The reasons for success were unknown at that time. However, the modern starting point for the development of probiotics occurred with the astute observations by the Nobel Prize winning scientist, Elie Metchnikoff of the Institut Pasteur in Paris, France. While working in Bulgaria in 1907, he was curious why some impoverished Bulgarians, who lived in a harsh climate, were particularly long-lived. Focusing on individuals, who were over 100 year of age, he examined reasons for longevity and health. In particular, villagers from the Caucasus Mountains were observed to consume fermented milk, i.e., yoghurt, every day. The research led to the subsequent recovery and recognition of *Lactobacillus bulgaricus* from the yoghurt by a young Bulgarian physician, Stamen Grigorov. Metchnikoff considered that this lactic acid-producing organism could counteract the detrimental [= putrefactive] effects of metabolism in the digestive tract that contributed to ill health and premature aging (Gasbarini et al. 2016).

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This organism is regarded as the first probiotic and may have been responsible for the good health and longevity of the villagers (see Ozen and Dinleyici 2015). The reasoning was that the live microorganisms when administered in appropriate amounts conferred health benefits on the host (see Gasbarini et al. 2016).

So, what exactly is a probiotic? The term "probiotic" was used originally to describe compounds that are essential for healthy development (Kollath 1953). A refinement considered probiotics as compounds secreted by microorganisms that were stimulatory to other organisms (Lilly and Stillwell 1965). Subsequently, the term was used for tissue extracts that were stimulatory to the growth of microorganisms, and then a few years later, Parker (1974) described probiotics as organisms and substances that contribute to intestinal microbial balance. Thus, a link was made between probiotics and intestinal microflora. A further refinement stated that probiotics were live microbial food supplements that beneficially affect the host animal by improving its intestinal microbial balance (Fuller 1989). It should be noted that the important components of the revised definition were that probiotics were viable microorganisms that beneficially affected the microflora in the intestine (Fuller 1992). More recently, the World Health Organization published a definition in 2001 as: "live microorganisms, which when administered in adequate amounts confer a health benefit on the host."

Probiotics have entered everyday use for humans with yoghurt and other fermented milk products emphasizing the presence of beneficial bacteria, notably the lactic acid-producing bacteria, e.g., putative *Lactobacillus* spp. Also, *Bifidobacterium* and the yeast *Saccharomyces cerevisiae* have been included in the list of probionts. There is evidence that probiotics confer health benefits to humans for the control of multiple diseases/complaints including diarrhea, enterocolitis, constipation and irritable bowel syndrome (Hungin et al. 2018; Sanders et al. 2018).

Probiotics have found widespread use in terrestrial agriculture with benefits including growth promotion, improved flesh quality and the reduction in the number of zoonotic and enteric pathogens in the digestive tract (Hung et al. 2012; Hossain et al. 2017). Thus, probiotics were credited with moderating and maintaining a healthy microbial flora in the digestive tract. The scenario was that the local in situ production of antimicrobial compounds inhibited potentially harmful enteric pathogens (Shim et al. 2012; Menconi et al. 2014; Upadhaya et al. 2016). In brief, the mode of action could be described as competitive exclusion whereby the harmful organisms would be effectively excluded from the digestive tract by inhibition resulting from the probiotics. An example concerned the use of *Lactobacillus acidophilus* together with *Propionibacterium freudenreichii*, which when dosed at 10^9 CFU/animal/day reduced the shedding of the enteropathogen *Escherichia coli* O157:H7 in cattle feces (Wisener et al. 2015).

The use of probiotics in aquaculture is a relatively new concept, which was initiated in mid-1980s. Kozasa (1986) was the first to use the spores of *Bacillus toyoi*, a bacterium of soil origin, for enhancing the growth of yellowtail (*Seriola quinqueradiata*) and controlling the mortality of Japanese eel (*Anguilla japonica*) from edwardsiellosis caused by *Edwardsiella* sp. (see Gatesoupe 1999). Subsequently, a few studies were focused on the screening of antibiotic-producing strains for possible

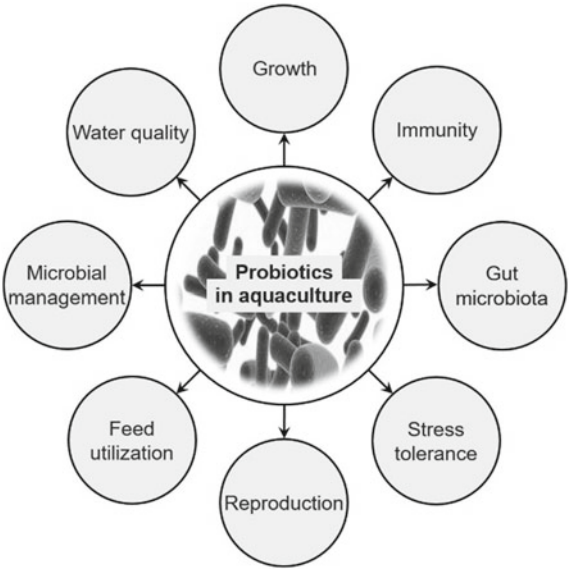

Fig. 1.1 Observed benefits of probiotics in aquaculture

use against epizootics in aquaculture. Dopazo et al. (1988) reported the antagonistic nature of seaweed-associated bacteria against fish pathogens *Aeromonas* spp., *Edwardsiella tarda*, *Pasteurella piscicida* (= *Photobacterium damselae* subsp. *piscicida*), *Vibrio* spp. and *Yersinia ruckeri*. Likewise, Kamei et al. (1988) described anti-infectious hematopoietic necrosis virus (IHNV) activity of some freshwater bacteria isolated from water and sediment of salmonid hatcheries. The interest in probiotics has further developed in the 1990s (e.g., Austin and Billaud 1990; Austin et al. 1992; Gatesoupe 1994; Rengpipat et al. 1998) and triggered much attention when alternative methods of disease control to replace antibiotics have been sought.

Prophylactic approaches in aquaculture have included the development and use of vaccines, non-specific immunostimulants, medicinal plant products and probiotics. The last mentioned offers a range of benefits and has been successfully evaluated for various farmed species including fish, crustaceans (shrimp, crab, lobster) and mollusks, either for larviculture, rearing juveniles or for broodstock maturation and reproduction. Evidence suggests that probiotics in aquaculture may be used to control growth, immunity, gut microbiota, reproduction and physiological stress of the cultured species, microbial diseases (pathogen inhibition, disruption of quorum sensing), water quality of the culture system and improve feed utilization/efficiency (Rollo et al. 2006; Nimrat et al. 2012; Carnevali et al. 2017; Sharifuzzaman and Austin 2017) (Fig. 1.1). As will be documented in ensuing chapters, the probiotics considered for use in aquaculture encompass a very wide range of Gram-positive and Gram-negative bacteria, including representatives from genera considered as fish pathogens, together with yeasts, micro-algae and bacteriophages. This range is much wider than the list of probiotics used for humans and agriculture.

It is speculative how often the viability of probiotics is examined as some works have pointed to the value of inactivated cells and subcellular components. Administration methods commonly included the oral uptake and application via water. Other than individual use, probiotics can be combined either with prebiotics, immunostimulants, functional ingredients or with a vaccine as adjuvant. Additionally, probiotics with quorum quenching ability offer a promising new concept for controlling emerging diseases in aquaculture. Quorum quenching probiotics not only suppress virulence factors in pathogens by interrupting the cell-to-cell communication systems, but also benefit the host in the same way as other useful microorganisms do. Some probiotics have the ability to control toxic nitrogenous compounds and pathogenic microorganisms in waters of aquaculture systems. In view of that, their use in biofloc systems is gaining importance in recent years. The modes of action of probiotics are complex, but extend beyond the principle of competitive exclusion and into the realms of immunomodulation. All these aspects will be considered in the following chapters.

Conclusion

Probiotics have been associated with better growth and some tangible health benefits including a reduced disease incidence in fish and shellfish farming. These data are certainly encouraging, and large-scale use of probiotics could deter negative consequences associated with the use of antimicrobial compounds in aquaculture. Therefore, eco-friendly prophylactic approaches like probiotics may well be useful not only to maintain the healthy microbial composition in host and/or culture systems, but also to produce safe and better quality aquatic foods.

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Methods Used for Selecting and Evaluating Probiotics

T. L. Korkea-aho and A. von Wright

Abstract There are many potential probiotic species as well as numerous target functions and technological applications in aquaculture, where probiotics may be used. Often, this fact defines the methodology used for evaluating candidate probiotics. Consequently, the research methods used for selecting and evaluating probiotics in aquaculture are very variable. There is an international recommendation published by the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) for the evaluation of probiotics for human consumption. Suggestions for a single guideline for evaluating probiotics in aquaculture are, however, more complicated, as differences in environments and multiplicity of host species need to be taken into consideration. Here, we list and compare the in vitro and in vivo methods used for selecting and evaluating probiotics in aquaculture. Furthermore, we point out some key issues that should be taken into account in probiotic research in aquaculture and make some suggestions for future work.

Keywords Aquaculture research · Probiotic selection · Probiotic evaluation · Probiotic characterization · Potential probiotic

1 Acquisition of Potential Probiotics

The acquisition of potential probiotics is made usually after isolating and screening microorganisms from various sources. The origin or host of the isolates is considered as important background information of a probiotic. If the potential probiotic occurs as a normal commensal of the host microbiota, it is more likely to be safe and capable of surviving in and is accepted by the host reaching the niches where it may proliferate

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and function. Beside these, many other microorganisms, not commonly found among the fish microbiota or marine or freshwater environments, are considered and used in aquaculture (Nikoskelainen et al. 2001a, b; Tarkhani et al. 2020).

The usual sites for screening of probiotic strains are the gastrointestinal tract (GIT) of healthy aquatic animals (Newaj-Fyzul et al. 2007), the mucus of the host GIT or skin (Boutin et al. 2012), eggs of the host (Korkea-aho et al. 2011) or culture water (Kewcharoen and Srisapoome 2019). For example, Boutin et al. (2012) selected the skin microbiota of stressed and unstressed brook charr (*Salvelinus fontinalis*) as a potential source for probiotics with effectiveness against *Flavobacterium*. These organisms include serious skin/external pathogens of a wide range of marine and freshwater fish. The assumption was that bacteria that comprise part of the skin microbiota could presumably be effective at the sites affected by the pathogens. Thus, members of the microbial community adapted to stressful environmental conditions would more likely attach to, establish themselves and function as probiotics on fish skin during infection processes.

Often, a large number of bacterial isolates are collected and purified, diluted in multipurpose culture medium, such as tryptone soya agar (TSA) or Reasoner's 2A agar (R2A), cross-streaked onto appropriate plates and characterized individually by phenotypic, biochemical and increasingly by molecular methods. The probiotic effects of microorganisms are, as a rule, genus and strain specific, so the taxonomy and background information of a potential probiotic culture need to be thoroughly investigated. This way valuable hints of possible health effects, pathogenicity and methodology of surveillance of the culture are obtained. The most common molecular identification method for unknown microorganisms is the sequencing of conserved regions within the genome, such as 16S ribosomal DNA in bacteria and ITS regions for yeasts and fungi (Reller et al. 2007). The identification is done by comparing the sequences with data stored in GenBank and in addition with phylogenetic analysis. Also, it is recommended to deposit the novel sequences in an official GenBank, such as the National Center of Biotechnology Information (NCBI) (Wanka et al. 2018). Often multiple tests are required to confirm the identity of a microorganism and to give more information of the characteristics of the potential probiotics (Chi et al. 2014; Kavitha et al. 2018). Other identification methods, such as metagenomics for potential probiotic organisms, are at the moment less often used in aquaculture research, but could provide vast amount of information, besides the identification, of the probiotic studied (Yi et al. 2018). Indeed, whole genome sequencing (WGS) is currently required by the European Food Safety Authority (EFSA) for bacteria and yeasts intended for authorization as animal feed additives (EFSA 2018).

2 Evaluation of Probiotic Properties and Functionality

2.1 Antipathogenic Activity

A potential probiotic may have antagonistic effects against pathogens by:

- competitive exclusion
- by producing bioactive compounds
- growing/proliferating faster, i.e., outcompeting the pathogen
- by acquiring nutrients that are essential for the growth and survival of the pathogen
- by attaching to and establishing itself on host surfaces more efficiently than pathogens.

The standard method to assess potential probiotics in aquaculture is to screen for the presence of antagonist effects against the target pathogens in vitro (Hai 2015). Several methods, which are based on the co-culture of pathogenic and probiotic microorganisms in the same medium and on the subsequent observation of inhibition and competitive growth of microorganisms, exist. For example, cross-streaking, overlays, spot-on-lawns, agar/well diffusion and broth co-culture methods are used for this purpose (Robertson et al. 2000; Spanggaard et al. 2001; Boutin et al. 2012). These are quite straightforward methods, saving time and resources, and because of this, they are often used as a primary selection step for putative probiotics. The disadvantages of these assays are artificial growth conditions that do not necessarily reflect the real growth situations in aquaculture environments. Moreover, some microorganisms do not grow or grow slowly in artificial growth media. Therefore, many potential probiotics may not be detected using these simple methods. Furthermore, nutrient requirements are often different for pathogenic and probiotic microorganisms, and a single nutrient medium can greatly affect the growth of either organism and might not give the correct information of inhibitory ability.

Many microorganisms produce antimicrobial components, such as enzymes, bacteriocins and/or small molecular weight compounds, that inhibit the growth or inactivate pathogenic bacterial cells. These extracellular products may be evaluated by several inhibition assays, such as agar diffusion or turbidometric assays with cell-free supernatants. A cell-free supernatant of a probiotic is acquired by centrifugation and filter sterilization of the culture medium after the growth of a microorganisms in optimal conditions (Schrader and Harries 2006; Korkea-aho et al. 2011; Muñoz-Atienza et al. 2013). When using these methods, it needs to be borne in mind that bioactive compounds could be produced by microorganisms at a specific time point or phase of growth, often when there is a limited availability of certain nutrients. Thus, bioactivity could be due to a mixture of several compounds in the supernatant. Consequently, methods need to be approached systematically to take account of all these variables (Vine et al. 2004a). When bioactive compounds with antipathogenic activity are found, they may be extracted, and their functional properties studied in more detail (Lategan et al. 2006).

Antipathogenic activity may result from events other than the presence of directly inhibitory compounds. For example, production of siderophores, e.g., iron chelators, gives bacteria an enhanced ability to acquire nutritional iron in iron-depleted environments, thus giving siderophore producers a competitive edge. This aspect may be studied by competitive growth of probiotic and pathogenic bacteria in iron-sufficient and iron-depleted growth conditions (Vijayan et al. 2006; Korkea-aho et al. 2011). Siderophore production of bacteria may be detected using chromeazurol S (CAS) medium (Schwyn and Neilands 1987).

Competitive exclusion of pathogens by probiotics involves many processes, although most commonly only the production of bioactive compounds is taken into account. Some studies have, however, been conducted to find out how fish mucus influences the competitive growth of probiotic and pathogenic bacteria (Vine et al. 2004a) and how radiolabeled pathogens and probiotics competitively attach themselves on fish mucus (Nikoskelainen et al. 2001b; Vine et al. 2004b). Adherence abilities of probiotic and pathogenic bacteria have been tested on primary cultures of intestinal cells from different intestinal segments (Lazado et al. 2011). Furthermore, less damaged epithelial cells were detected in histological samples of fish foregut after having been exposed to both pathogenic bacteria and probiotics in vitro. Here, comparison was made to histological samples after exposure only to pathogenic bacteria (Ringø et al. 2007).

Antipathogenic activity needs to be always tested in vivo, as in vitro results do not necessarily predict activity in the whole animal (Spanggaard et al. 2001; Cerezuela et al. 2012). This is usually tested by probiotic supplementation and the subsequent observation of the host for protection/survival after challenge with q virulent pathogen (Newaj-Fyzul et al. 2007; Korkea-aho et al. 2011; Zokaeifar et al. 2012; Ohtani et al. 2020). For efficient and reliable disease challenge experiments, specific target species, at the appropriate developmental stage (consideration needs to be given to the weight and age of the experimental animals), is used. The pathogenic strain must be specific for the target aquatic animal, and its effective challenge administration and dose need to be determined. For example, it is often a standard procedure to calculate the lethal dose of the pathogen for the target animal (Newaj-Fyzul et al. 2007; Korkea-aho et al. 2011). Indeed, even if the lethal dose is known from previous research already, it is prudent to verify the data for each successive group of experiments especially if different stocks/sizes/ages of the host animal are used. Simply put, one dose is not necessarily appropriate for all experiments. During in vivo challenges, the performance of the model needs to be compared with appropriate controls, where the adverse effects of the pathogen in terms of mortalities and/or overt disease signs may be recorded. Furthermore, the pathogen used in challenges needs to be re-isolated and its identity confirmed to ensure that the culprit has really been confirmed. Unfortunately, it is not unheard of for stocks to harbor pathogens that emerge and cause disease during experimentation. Experimenters need to ensure that the environment and husbandry of aquatic animals are identical in control and treatment groups. The correct dose of the probiotic present in treatments needs to be confirmed, and there should not be cross-contamination between treatments. Before starting a disease challenge experiment, the health status of aquatic animals used in

the experiment must be confirmed (Newaj-Fyzul et al. 2007; Korkea-aho et al. 2011; Zokaeifar et al. 2012; Ohtani et al. 2020). Beside challenge experiments, probiotics have been examined in naturally infected hosts (Boutin et al. 2012). It is essential to ensure that in vivo experiments are well planned, including consideration of ethical aspects. The possibilities of reducing the number of animals used in experimentation need to be considered; i.e., serious effort must be made to support the "3Rs"—replacement, reduction and refinement (Russell and Burch 1959). Furthermore, national legislation concerning animal experimentation needs to be considered, where appropriate permission obtained before starting the work.

3 Colonization and Stress Tolerance

For the potential probiotic, it is important to study the functional properties which improve its ability to withstand, attach and proliferate at the site of action. Most of the probiotics used in aquaculture are orally administered and enter the host via the GIT (Hai 2015). When administered this way, the ability to tolerate acidic conditions in the GIT environment is crucial for the survival of the probiotic. This aspect is sometimes addressed in the in vitro studies, by adding crude bile or synthetic gastric juices to the medium where viability and growth parameters of potential probiotics may be assessed (Nikoskelainen et al. 2001b; Kavitha et al. 2018). More often, this has been studied by in vivo tests, where recovery and number of viable probiotic bacteria are assessed by sampling and culturing fish GIT at different time points after feeding with the dietary supplements (Nikoskelainen et al. 2003; Merriefield et al. 2011; Korkea-aho et al. 2012). Also, these in vivo tests provide information about the adherence and colonization of the probiotic to the host mucosae and epithelia. This adherence to host surfaces is considered as one of the key functional properties of probiotics intended for terrestrial animals (Fuller 1989). Colonization and adhesion of probiotics to the GIT of aquatic animals have been demonstrated both in in vitro (Lazado et al. 2011) and in in vivo studies (Sugimura et al. 2011; Zokaeifar et al. 2012). The colonization patterns of probiotics in the intestine have been further examined using histological samples from the GIT (Merriefield et al. 2011). The adhesion and colonization properties of the probiotic may be affected by the method of administration. For example, the colonization properties on fish surfaces and in the GIT may be less effective when added via water rather than via feed (Korkea-aho et al. 2011; Kewcharoen and Srisapoome 2019). However, the colonization and adherence are often only transient. Despite this, even transient probiotic cells exert effects on the host when administered at a specified concentration and for an appropriate duration, e.g., with the correct dose (Nikoskelainen et al. 2003; Merriefield et al. 2011; Korkea-aho et al. 2012; Sharifuzzaman et al. 2014).

4 Immunostimulatory Properties

Many probiotics have been shown to function for the benefit of the host by stimulating the immune system and improving the host resistance to infectious diseases (for reviews, see Nayak 2010; Akhter et al. 2015). Innate immunity is the fundamental defense mechanism against pathogens in aquatic animals and is often assessed from blood samples obtained from the animal after exposure to a probiotic. Leukocytes are important components of cell-mediated immunity, and their elevated numbers are indicators of immunostimulation as a result of applying a probiotic. This proliferation of blood leukocytes, compared to other blood cells of the host, may be evaluated directly from blood samples by counting the number of erythrocytes and leukocytes microscopically, or from the percent hematocrit and leukocrit. Phagocytic activity of neutrophils and macrophages is activated during immunostimulation, and this may be studied by isolating macrophages from the head kidney and observing phagocytosis by microscopically counting engulfed stained yeast cells. Furthermore, the respiratory burst activity at phagocytosis may be measured from reactive oxygen species (ROS) of innate immune cells using a chemiluminescence method or by measuring nitroblue tetrazolium (NTB) released in the respiratory burst of these cells (Sharifuzzaman and Austin 2009; Cerezuela et al. 2012).

During immunostimulation, many important components of humoral immunity, such as bacteriolytic enzymes, interferons and complement components, may be assessed from serum prepared from the host's blood. Bacteriolytic effects may be measured by adding serum from a probiotic-fed host to the growth medium, which has been inoculated with pathogenic bacteria, and assessing the growth turbidometrically (Sharifuzzaman and Austin 2009). One of the important bacteriolytic enzymes is lysozyme to which many bacteria are sensitive; its activity may be assessed by using the especially lysozyme sensitive *Micrococcus lysodeikticus* (= a synonym of *M. luteus*) cells as an indicator (Korkea-aho et al. 2012). Lysozyme may be found as a first defense component in mucus and measured from (mucus) samples with this method (Newaj-Fyzul et al. 2007). In fish serum, there are antiproteases, which act as inhibitors for proteolytic enzymes secreted by pathogenic bacteria. These antiproteases may be measured as total antiproteases, α1-antiprotease and α-2 macroglobulin, when trypsin is added to fish serum, and antitrypsin activity measured (Newaj-Fyzul et al. 2007). In fish, complement activity may be initiated by several different pathways that may be measured from serum levels of the host (Nikoskelainen et al. 2003; Sharifuzzaman and Austin 2009; Sun et al. 2010).

Some antibodies are secreted as components of the first defense and exert an important role in innate immunity (e.g., primary antibody, immunoglobulin M—IgM), but in general immunoglobulins form the basis of acquired immune defense, are secreted and act specifically against certain pathogens. Total immunoglobulin level (Ig) and primary antibody M (IgM) in host serum can be measured by the enzyme-linked immunosorbent assay (ELISA) (Nikoskelainen et al. 2003; Cerezuela et al. 2012). Some activated leukocytes and other cells secrete signaling molecules, referred to as inflammatory cytokines, such as interleukins (ILs), tumor necrosis

factors (TNFs) and interferons (IFNs). Activation of these immune-related genes, for example, TNFα, IL1β, IL 4, IL6, IL8 and IFNγ, can be measured from gene expression by real-time reverse transcriptase polymerase chain reaction (RT-PCR) analyses (Kim and Austin 2006; Pérez-Sánchez et al. 2011; Cerezuela et al. 2012; Chi et al. 2014). Beside these, many other genes affecting fish homeostasis, and including stress and growth-related genes, are studied by real-time RT-PCR providing important information of up- and down-regulation activities that the probiotic exerts on these genes (Dawood et al. 2020). However, occasionally, when cellular immunostimulation has been detected, the expression of immunity-related genes has not been observed (Cerezuela et al. 2012). When studying the expression of genes after the exposure to probiotics, it is necessary to take into account that there are differences in time when the mRNA expression of genes is able to be detected (Chi et al. 2014; Yi et al. 2018), and in cells and organs which should be sampled (Kim and Austin, 2006). Usually, cells of immune-related organs, such as the head kidney cells of fish, express immune-related genes. Often, the sites where probiotics are in direct contact with the host cells, such as the intestinal epithelial cells, are crucial for research on the regulation of immune-related genes (Standen et al. 2013). The immune regulation of probiotic in smaller aquatic animals and larvae is often assessed by immune-related gene expression analysis by using the whole animals rather than component tissues (Zokaeifar et al. 2012).

Many studies have shown that elevated immune parameters indicate that the in vivo host has resistance against disease (Newaj-Fyzul et al. 2007; Korkea-aho et al. 2012; Zokaeifar et al. 2012; Chi et al. 2014). However, detectable disease resistance is not always acquired even when immune parameters of the host are elevated (Cerezuela et al. 2012).

5 Food Digestibility and Other Beneficial Health Effects

One of the functional properties of probiotics in the GIT is the improvement of feed digestibility and utilization. Enzyme activities, such as alginate lyases, amylases, lipases and proteases, improve the digestibility of food and may be studied in vitro by cultivating the potential probiotic on media containing potential feed components, such as starch, skimmed milk, peptone–gelatin and carboxylmethylcellulose, and quantifying the activities of the target enzymes spectrophotometrically (Kavitha et al. 2018). However, the enzyme production of a microorganism depends greatly on the environmental conditions, such as pH and temperature. Therefore, digestive enzyme activity should be confirmed by in vivo experiments. For example, this could be achieved by comparing activities of digestive enzymes from GIT samples of probiotic-fed hosts with those of controls, which have not been administered with probiotics (Zokaeifar et al. 2012; Tarkhani et al. 2020). Subsequently, the improved feeding and feed conversion can be visualized in in vivo experiments by better growth performance of the probiotic-fed host, as indicated by various parameters, such as improved survival, feed conversion ratio (FCR) and specific growth rate (SGR)

(Yanbo and Zirong 2006; Zokaeifar et al. 2012; Standen et al. 2013; Yanbo and Zirong 2006; Tarkhani et al. 2020).

The expanding aquaculture sector has posed limitations on fish meal availability, and this has led to some replacement with plant protein. However, anti-nutrients of plants, such as saponins, are of major concern to fish health and well-being (Krogdahl et al. 2010). Some studies have investigated the saponin degradation ability of potential probiotics by growth experiments in medium where the only energy sources are saponins. Thus, Wanka et al. (2018) screened 42 autochthonous bacterial isolates from the GIT of flatfish and determined that 7 cultures were able to degrade saponin in vitro.

Probiotics may be administered in water and used for improving the environment as well as the health status of the host. Changes in water quality are often monitored by standard water chemistry of cultured water with and without the addition of probiotics (Queiroz and Boyd 1998; Zhou et al. 2009; Thurlow et al. 2019). Nitrifying bacteria are well recognized and used in closed aquaculture systems for their beneficial ability of oxidizing toxic ammonia to non-toxic nitrate. Also, *Bacillus* sp. reduced ammonia when added in cultured water (Cha et al. 2013; Zokaeifar et al. 2014; Kewcharoen and Srisapoome 2019). However, not all studies have recorded a reduction in ammonia levels (Zhou et al. 2009). *Bacillus* spp. have been demonstrated to convert organic matter to carbon dioxide in water, but this ability has not been confirmed in in vivo experiments (Queiroz and Boyd 1998; Verschuere et al. 2000). Interestingly, total phosphorus, total nitrogen and nitrate in rearing water were significantly lower in ponds where fish were fed with probiotic *Bacillus velezensis* in comparison with water parameters in ponds where fish were not fed probiotic-supplemented feed (Thurlow et al. 2019). Benefits in survival and feed digestibility (Zhou et al. 2009), growth, immunity and disease resistance (Zokaeifar et al. 2014) have been demonstrated when probiotics were used as water additives in shrimp aquacultures. The concept of using probiotics as water additives is an attractive line of research, although the results so far have been variable, especially when water quality parameters are studied.

6 Safety Assessment

There is a lack of research on the possible adverse effects of probiotics used in aquaculture. For humans, some side effects have been described when probiotics have been administered, and include deleterious metabolic activities, horizontal gene transfer, infections and excessive immune stimulation (Marteau 2001). Research of the intrinsic properties of potential probiotic is valuable when assessing their safety. With some probiotics, an extensive body of in vivo research and experience concerning their use in aquaculture without evidence of any adverse effects has been accumulated. This provides important information on interactions between the probiotic and the host when considering safety aspects. EFSA has carried out the qualified presumption of safety (QPS) assessment for several microorganisms intended for deliberate use in the food chain and has included potential probiotics (EFSA

2013). QPS-qualified microorganisms are considered safe for the target species, consumer and the environment provided that they do not harbor transmissible resistance genes for relevant antibiotics. This type of information needs to be gathered so that meaningful safety determinations can be made.

If non-QPS microorganisms are used as feed additives, EFSA has published guidance on the performance of tolerance tests on different categories of animals, including aquatic species (EFSA 2017). According to the EFSA guidance (2017), probiotics should be assessed either (i) by a tolerance test, in which an overdose of the additive is fed to the animal for a relevant period of time, (ii) by a literature survey or (iii) by extrapolation from toxicological studies, if available. Overdosing of feed to fish is not always practical, and water quality could be compromised during overfeeding in small scale in vivo tests. In previous studies the potential probiotic have been injected intraperitonially or intramuscularly, and disease signs and/or mortalities observed. These approaches are often conducted to assess possible pathogenicity, infectivity and toxicity of the probiotic to the host in aquaculture (Irianto and Austin 2002b; Chi et al. 2014). The accumulation of the probiotic in internal organs of the host could be examined for potential damage, including inflammation. Also, microbiological examination of relevant tissues to determine the possible presence and longevity of viable (probiotic) cells is conducted (Irianto and Austin 2002b; Korkea-aho et al. 2011).

Possible virulence factors of putative probiotics may be examined using in vitro methods, such as screening for the production of hemolysis on blood agar and the production of proteinases in specific protein containing agar (Muñoz-Atienza et al. 2013; Chi et al. 2014). Furthermore, the bacterial ability to deconjugate bile salts and degrade mucin could be detrimental for the host, and the ability could be evaluated by streaking the potential probiotic bacteria on agar plates containing bile salts or mucus (Muñoz-Atienza et al. 2013). Genes encoding putative virulence factors may be detected using gene-specific primers in the PCR. However, care needs to be taken when interpreting the relevance of existing virulence genes in bacteria because not all genes are necessarily functional. Thus, genetic data gives information of potential rather than of actual virulence (Muñoz-Atienza et al. 2013). Indeed, some bacterial strains that have been considered to be pathogenic in certain situations have been determined to be effective probiotics in aquacultural use. This may be because of the absence of the expression of actual virulence in certain environments/conditions (Irianto and Austin 2002b).

Acquired antibiotic resistance of fish pathogens is not only threat for fish health, but could facilitate the potential transfer of resistance genes that poses a risk to the aquatic environment and to human health. Antibiotic susceptibility of bacteria may be examined by overlaying the potential probiotic with selected antibiotic-impregnated disks, and after incubation discrete zones of inhibition may be observed and measured (Kavitha et al. 2018). Another method for studying antibiotic susceptibility is the microdilution test, which is also required by EFSA. This test assesses the minimum inhibitory concentration (MIC), which is measured by preparing twofold serial dilutions of the antibiotic in either broth or agar, and examining the presence of growth or no growth of the seeded bacterial culture after incubation (Muñoz-Atienza et al.

2013). Genes conferring antibiotic resistance for bacteria may be tested with specific primers in the PCR; their presence in bacteria is often considered as a mark of horizontal transfer of these genes. For example using PCR methods, Munoz-Atienza et al. (2013) determined from antibiotic resistant lactic acid bacteria (LAB), which were isolated from aquatic animals, the presence of genes which could confer resistance to for example tetracycline (*tet*K, *tet*L, *tet*M), most types of aminoglycosides (*aac*(*6'*)-*Ie*-*aph*(*2"*)-*Ia*), erythromycin (*erm*(A), *erm*(B), *erm* (C), *mef*(A/E)) and lincosamides (*lnu*(A), *lnu*(B)).

7 Evaluation of Commercial Probiotics

Beside safety, there are several properties which need to be evaluated before probiotics are commercialized and made available for the use in aquaculture. Indeed, the effects of different preparation methods, shelf life and storage requirements of the potential probiotic are important to determine because these factors strongly influence the functionality of the probiotics and their industrial potential. Also, a cost–benefit analysis needs to be investigated. Commercial probiotics available in aquaculture are often mixed with feed or added directly into water. With research trials, probiotic feed preparation is carried out in laboratory conditions where commercial fish feed is coated with the potential probiotic. This will have been grown in broth media before washing with sterile saline, and the final suspensions adjusted to the desired number of cells. Counting is carried out by means of a hemocytometer slide, by optical density and even by colony count determination. Then, the potential probiotic suspension is added, for example by spraying and mixed with the feed (Newaj-Fyzul et al. 2007). The probiotic suspension may be mixed with oil before addition to the food. There may even be an extrusion/shaping state to form pellets (Cerezuela et al. 2012; Standen et al. 2013). The product can be air dried before storage. The desired outcome is that a known number of probiotic cells are added to a defined amount of feed. Thus, it is possible to equate the number of probiotic cells fed to the aquatic species.

The number of viable probiotic cells in feed may well decrease during storage; this scenario tends to be faster at higher temperatures and in cases of oxidative stress. Thus, it is necessary to determine viable cell counts in the feed throughout the life of the product. This procedure will typically involve colony counting on appropriate media with verification that any resultant growth is actually the probiotic and not a contaminant. If the number of viable cells declines and/or a contaminant is suspected, then preparation of a new probiotic batch may be needed to continue with the feeding trials (Pérez-Sánchez et al. 2011; Chi et al. 2014). In the selection of preparation and storage methods, it is necessary to consider that for some probiotic bacteria antimicrobial substances may be excreted during certain periods in the bacterial growth cycle. It is not unheard of that inhibitory compounds are often diminished over time and/or by subculturing (Nikoskelainen et al. 2001b). Commercial probiotics are often lyophilized, i.e., freeze-dried, so the storage and shelf life are longer. However, it seems that this cannot be applied to all bacterial strains and depends on the actual

probiotic as to whether or not it maintains its viability and effective properties after lyophilization (Panigrahi et al. 2005; Merrifield et al. 2011). Some probiotic bacteria have been shown to maintain their viability in terms of a stable number of colony counts for prolonged periods in feed. For example, Wanka et al. (2018) reported survival for 54 days when kept vacuum-packed at 4 °C. Microencapsulation, where microbes in high densities are encapsulated in a matrix, such as chitosan or cellulose, to protect them from adverse environmental changes, is also used in probiotic preparations (Cruz et al. 2012).

Many *Bacillus* isolates form endospores, which confer protection against adverse environmental conditions, such as high-temperature fluctuation and desiccation, which are conditions associated with feed processing and storage. This may well explain why *Bacillus* was one of the first commercially available feed supplements in aquaculture (Queiroz and Boyd 1998; Cruz et al. 2012). Commercial *Bacillus* probiotic preparations are usually stored as endospores, which are added directly to feed. However, in research experiments, *Bacillus* are inevitably cultured, and cell numbers are adjusted, before adding to the feed, so that both vegetative cells and endospores will be present when doses are considered (Zhou et al. 2009; Cha et al. 2013).

Monitoring is needed to control and evaluate viability, productivity and stability of commercial probiotics (Verschuere et al. 2000). As feed and water are favorable growth media for many microorganisms, such as bacteria, yeasts and molds, good manufacturing practices need to be followed when preparing large quantities of commercial probiotics to prevent the risk of contamination. Furthermore, the quality of commercial probiotics needs to be assured, for example, by monitoring the identity of the probiotic culture(s) in feed by culturing using appropriate selective agar and genetically by PCR or pulsed field gel electrophoresis (PFGE) methods. It is necessary to verify the identity of probiotics in samples obtained from the host (Balcázar et al. 2007; Korkea-aho et al. 2011) or water, as appropriate (Thurlow et al. 2019) after administration and to confirm the absence of contaminants. Certainly, experiments have been carried out to evaluate the functionality and safety of commercially available probiotics in aquaculture (Nikoskelainen et al. 2001b; Merrifield et al. 2011; Tarkhani et al. 2020). However, for the future, long-term studies are necessary to monitor probiotics in large-scale culture systems in order to confirm the quality and purity of the products used in aquaculture.

8 Conclusions and Suggestions for Further Work

The large variety of microorganisms have been evaluated as probiotics for use in aquaculture and include Gram-positive and Gram-negative bacteria, yeasts, microalgae and bacteriophages. However, there are some key issues which need to be investigated before any microorganism should be considered for use:

(i) It is necessary to have an accurate identification and background information of the probiotic culture, including its source and method of isolation and storage. This is important where concerns have been expressed about the possible association of prions with some media components, e.g., bovine heart.
(ii) Antipathogenic activity and/or other relevant functional properties.
(iii) Safety of the potential probiotic for host and the environment.
(iv) Commercializing properties, such as survival and stability in industrial processes (Fig. 2.1). It is essential to ensure that any beneficial properties are not lost during the scale-up process.

The probiotic cultures must have documented beneficial effects on the host when used in appropriate concentrations for a specified duration. Certainly, there is large amount of research on probiotics destined for use in aquaculture and the health benefits that these organisms confer on the host (for reviews, see Verschuere et al. 2000; Irianto and Austin 2002a; Vine et al. 2006; Kesarcodi-Watson et al. 2008; Nayak 2010; Akhter et al. 2015; Hai 2015). However, it would appear that safety aspects and commercialization requirements of the potential probiotics have received less attention by researchers. These requirements need to be routinely included in

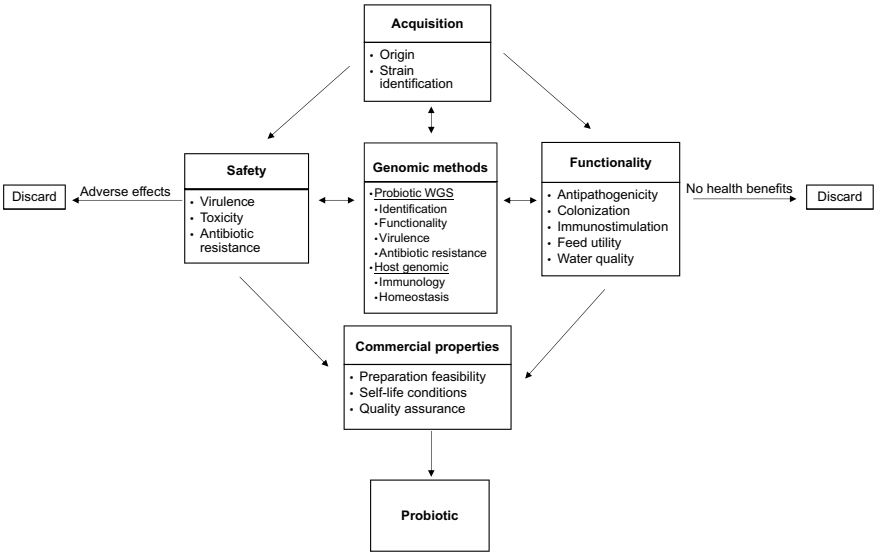

Fig. 2.1 Schematic diagram of key issues for evaluation by in vivo and in vitro methods of putative probiotics considered for use in aquaculture. After the acquisition and background studies of potential probiotics, functionality relevant to the use and safety of the potential probiotic needs to be investigated. If the potential probiotic has proven health benefits on the host and does not have any adverse effects on the host or environment, its commercial potential should be determined. The box in the center of the diagram illustrates the genomic methods utilized in identification, safety and functional genotyping research of potential probiotics (acknowledgements to Verschuere et al. 2000; Araya et al. 2002)

development studies of future probiotics destined for use in aquaculture in order to obtain both regulatory acceptance and commercial sustainability (Fig. 2.1).

Clearly, potential probiotics are selected initially on the basis of antipathogenic properties, for example, by screening to determine inhibition against certain pathogens in vitro with confirmation by in vivo experiments. Characterization of the probionts by phenotypic means has dominated early work and continues to find widespread use although the outputs in terms of accurate identifications may be questioned. The more modern approach of genotypic examination has led to greater confidence in identification with the information proving to be useful for functionality and safety during validation (Fig. 2.1). Recent developments in next-generation DNA sequencing technology have made WGS more accessible and enabled the study of the whole genome of bacteria to locate predictive beneficial probiotic genes in different bacterial strains. For example, WGS analyses of *B. subtilis* metabolic-related genes, such as antibiotic production genes, have been described (Stein 2005). Polyketide synthetase and nonribosomal peptide synthetase gene clusters, which synthesize novel bacteriocins, have been identified from potential probiotic *B. velezensis* genome (Yi et al. 2018). Furthermore, WGS of bacterial strains could be used for better identification and classification of the probiotic strains by comparing multiple loci in the genome because 16S rRNA sequences alone may not always provide enough variability to discriminate between species (Larsen et al. 2012).

The importance of probiotic development and research in aquaculture is often justified by their potential to replace antibiotics. It is clear that antibiotic usage in aquaculture conveys the risk for emerging resistant pathogens as well as the risk for the spreading of antibiotic resistance into the aquatic environment and to bacteria of relevance to humans by horizontal gene transfer. Genetic determinants of antibiotic resistance may be passed from bacteria to others, and also probiotic bacteria may possess resistance markers (Muñoz-Atienza et al. 2013). For these reasons, more research is needed to study potential probiotics for their resistance to antibiotics of human and veterinary significance. Genes related to antibiotic resistance need to be investigated with view to ascertain the risks associated with horizontal transfer of the antibiotic resistance. Certainly, PCR methods and/or WGS are ideal and have the advantage of being relatively fast and manageable (Muñoz-Atienza et al. 2013; Senan et al. 2015). In addition to the genes associated with antibiotic resistance, other safety aspects of potential probiotics may be assessed from the genome and include the genes related to virulence and production of harmful metabolites (Senan et al. 2015). It is noteworthy that for commercial probiotics, a WGS biosafety assessment is required also by EFSA (2018).

Genome analysis may be used in prediction of host-related conditions. Already, many studies utilize information about the activities of genes related to immune functions, stress responses, antioxidative activities and growth in aquatic animals in connection with probiotic administration (Dawood et al. 2020). Furthermore, "omics" (e.g., genomics, transcriptomics, proteomics and metabolomics) are utilized in probiotic studies. There has been a special interest in seeking probiotics with certain clearly defined specifications, such as selecting candidates with effectiveness

against certain diseases by using an "omics" approach to target the beneficial effects according to the genetic and metabolic profile of the host (Rebollar et al. 2016).

Similarly, the evolving technologies and expanding use of multidata give possibilities to future research directions using "omics" for specific selection of probiotics to enable optimizing nutrition and aquacultural production. However, genomic data generates information mainly on predicted features, so studies need to include more conventional approaches including use of in vitro and in vivo experiments to validate the selection for host and its environment.

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Application Methods of Probiotics and Options

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Abstract The success of using probiotics is conditioned not only by the species of microorganism, but also by the dose administered and the method of administration. In aquaculture, adding probiotics to water makes them easier to administer, but the dilution effect may reduce the number of microorganisms that animals ingest. Probiotics may also be used to improve the chemical and microbiological characteristics of water. When administered in food, the microorganisms may be inactivated as they pass through the digestive tract. To avoid their inactivation, probiotics may be encapsulated in several materials. The duration of treatment with probiotics as well as whether they are administered in one dose or in several pulses can also determine their effectiveness. Likewise, some strategies involve the use of several probiotics at the same time, having a synergistic effect on the host. Finally, the effectiveness of probiotics should be evaluated if they are administered to feed as inactivated cells or if subcellular components of them are used.

Keywords Dosage · Encapsulation · Inactivated cells · Microbial consortia · Paraprobiotics · Postbiotics · Probiotic administration · Subcellular components

1 Introduction

Once a probiotic has been chosen, the way in which it will be administered must be evaluated. The method used to apply the probiotics will influence their effectiveness, in addition to having legal and environmental connotations. Factors to be studied include the route and form of administration (live, dead, cell subunits), dosage, time and frequency of administration. The possibility of a formulation consisting of a pure strain or a combination of probiotics that can have a synergistic effect should also be considered.

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The viability of probiotics is influenced by the route of administration. For example the inclusion of live probiotics in feed may cause their inactivation. Conversely, the fact that a microbial strain is a probiotic for some species does not imply that it cannot be potentially pathogenic for other animals (Fu et al. 2020). Several probiotic strains have caused outbreaks of disease in the aquaculture industry, including the lactic acid bacteria *Weissella* sp. (Figueiredo et al. 2012); different strains of *Vibrio alginolyticus* have been proposed as probiotics despite this species having been described as pathogenic (Gomez-Gil et al. 2002; Arijo et al. 2008; Medina et al. 2020). In addition, a few probiotic species, such as *Rhodopseudomonas* sp. (Mitchell et al. 2017) and *Bacillus cereus* (Zhu et al. 2016), are associated with clinical infections in immunosuppressed patients. These limitations are reflected in the fact that very few probiotics are accepted as food additives, for example pursuant to European Regulation EC, No. 1831/2003 and European Commission Regulation (EU) 68/2013). To solve this problem, the use of inactivated probiotics or their subcellular components has been proposed. This chapter reviews and summarises information on the route, dosage, pattern of administration and formulation of probiotics in aquaculture.

2 Delivery Methods

The administration of probiotics will influence their viability. It is different if probiotics have to act on the skin or gills rather than if they have to enter the digestive tract of the fish. In the latter case, one of the problems of using probiotics is their inactivation when passing through the stomach and gut. So, it is important to determine which form is best suited for administering these microorganisms.

The main routes of administration of probiotics are by bath, direct inclusion in the fish feed, encapsulation in live animals (such as *Artemia*) and encapsulation in biopolymers. Other experimental routes, such as injection, have also been used.

2.1 Administration of Probiotics in Water

Inlet water is often pretreated via ozonation and UV sterilisation prior to use in aquaculture systems. However, the treatment may further disrupt complex microbial interactions and benefit opportunistic pathogen proliferation, mainly in larvae culture (Gonçalves and Gagnon 2011). Adding microorganisms with a probiotic effect to water may solve this problem. Probiotics are being used as water additives to improve water quality by affecting the microbiota and to stimulate the immune system of fish (Wang et al. 2020). In general, probiotics are suspended in farm water and spread over the water surface.

The administration of probiotics through water is the most suitable method for shellfish (Ringø 2020), including prawns and shrimps (Ali et al. 2018) and fish larvae (Tarnecki et al. 2019) and may lead to improvement in water quality parameters.

The skin and gills are involved in the first stages of infection, serving as a barrier to the host. An advantage of administering probiotics in water is that they may come into direct contact with the gills and skin more easily, leading to colonisation of these surfaces. This is especially the case if the probiotic bacteria have high affinity to mucosa (Lazado and Caipang 2014). In filter feeders, the probiotic suspended in water is also assimilated orally. In addition, this form of administration does not stress the fish, since they do not have to be manipulated.

The main disadvantage of administering probiotics in water is the large amount of product needed and is dependent on the volume of water in the facilities. Furthermore, in open aquaculture systems, this is not possible, as probiotics are quickly lost by dilution. On the other hand, the efficacy of administering probiotics in water seems lower than that obtained when administering them in feed. For example Kewcharoen and Srisapoome (2019) observed that adding *Bacillus* spp. to water did not improve shrimp growth and resistance to *Vibrio parahaemolyticus*. However, feed supplemented with the selected strain of *Bacillus* spp. and given to shrimp continuously for 5 weeks efficiently improved growth, as indicated by significant final weight gain, average daily growth, specific growth rates and feed conversion ratios.

2.2 Administration of Probiotics in Feed

This is the most common way of administering probiotics (Hai 2015; Gao 2018; Tachibana et al. 2020). Probiotics may be administered by mixing with mash feed that is either directly administered to animals or further prepared as pelleted/extruded feed during the manufacturing process (Neves et al. 2014; Opiyo et al. 2019; Tan et al. 2019). However, the high temperature used during pelleting and drying may thermally deactivate bacteria (Simon 2005). For example *Saccharomyces cerevisiae* supplemented in mash shrimp feed showed a reduction of approximately 3 log CFU/g after pelleting at 82 °C (Aguirre-Guzmán et al. 2002). Furthermore, pelleting shrimp feed supplemented with *Lactobacillus lactis* and gelatin as a feed binder at 21 °C followed by fluidized bed drying at an inlet temperature of 80 °C for 10 min caused approximately 2 log CFU/g viability loss (Wirunpan et al. 2016). Probiotics may also be administered by spraying onto the pellet surface once the feed has been manufactured (Sultana et al. 2020). This method allows the incorporation of sensitive compounds onto the feed. In this case, the probiotics must be suspended in saline solution or phosphate buffer or coated by a substrate, such as fish oil, before being added to the feed (Merrifield et al. 2011; Ghiasi et al. 2018).

One advantage of administering probiotics in feed is the ease of administration and dosage. The inclusion of probiotics in feed allows them to enter directly through the digestive tract along with the food. A disadvantage of this method is the impossibility of controlling the dose of probiotic that each fish consumes. On the other

hand, probiotics can be inactivated during the feed production process (temperature, changes in pressure during extrusion, osmotic shock and dehydration). To increase their viability during storage in feed, probiotics may be lyophilised before administration (Varela et al. 2010). Although freeze-drying remains the preferred technique for preserving probiotic bacteria, another technique for preservation is spray-drying. This consists of spraying the liquid feed in fine droplets (10–150 μm) that are directed into a flow of dry air heated to 150–250 °C. The increase in the air–liquid interface area subsequent to spraying quickly dries the biomass in seconds. When compared with freeze-drying, spray-drying represents a lower specific energy cost and higher productivity (Huang et al. 2017).

Once ingested by the fish, the probiotics are exposed to the action of digestive enzymes and the acidic pH of the stomach, which may decrease their survival when they reach the intestine. Some strategies, such as encapsulation, preserve microorganisms until they reach the gut. On the other hand, their use as a live additive for feed is very limited by the regulations of different countries. According to European Regulation (EC) No. 1831/2003, the live microorganisms that may be used as feed additives reflect several strains belonging to the following genera:

- *Bacillus* (*B. subtilis*, *B. cereus* var. toyoi).
- *Bifidobacterium* (*B. animalis* ssp. *animalis*).
- *Clostridium* (*C. butyricum*).
- *Enterococcus* (*E. faecium*).
- *Lactobacillus* (*L. acidophilus*, *L. brevis*, *L. buchneri*. *L. casei*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *L. farciminis*, *L. salivarius* ssp. *salivarius*, *L. lactis*).
- *Pediococcus* (*P. acidilactici*, *P. pentosaceus*).
- *Propionibacterium* (*P. acidipropionici*).
- *Saccharomyces* (*S. cerevisiae*).

Other microorganisms are used in the silage process. By this method, feed made with green foliage crops or fish waste is preserved by acidification produced by fermentative microorganisms. According to European Regulation (EC) No. 1831/2003, strains of the following microorganisms are authorised for use in silage processing:

- *Enterococcus faecium*
- *Lactobacillus* genus: *L. brevis, L. buchneri, L.* plantarum, *L. hilgardii*, *L. fermentum, L. casei, L. kefiri, L. diolivorans, L. paracasei, L. rhamnosus*
- *Lactococcus lactis*
- *Pediococcus* genus: *P. acidilactici, P. parvulus, P. pentosaceus*
- *Propionibacterium acidipropionici*

Other groups of microorganisms are used as additives for the reduction of contamination of feed by mycotoxins (*Coriobacteriaceae* family) or reduction of anti-nutritional factors in soybean (*Bacillus subtilis*).

Another way of using probiotics in food is by incorporating them as non-live additives, including previously inactivated probiotics in feed or using their subcellular components.

Table 1 lists the microorganisms and their derivatives that may be used as additives in feed according to European Commission Regulation (EU) 68/2013.

Table 1 Microorganisms and their derivatives allowed as additives in feed according to European Commission Regulation (EU) 68/2013

Name	Description
Products obtained from the biomass of specific microorganisms grown on certain substrates	May contain up to 0.3 % antifoaming agents. May contain up to 1.5 % filtration/clarifying agents. May contain up to 2.9 % propionic acid
Protein from *Methylophilus methylotrophus*	Protein product of fermentation obtained by culture of *M. methylotrophus* (NCIMB strain 10.515) (1) on methanol
Protein from *Methylococcus capsulatus* (bath), *Alcaligenes acidovorans*, *Bacillus brevis* and *Bacillus firmus*	Protein product of fermentation with *M. capsulatus* (Bath) (NCIMB strain *11,132*), *A. acidovorans* (NCIMB strain *12,387*), *Bacillus brevis* (NCIMB *13,288*) and *Bacillus firmus* (NCIMB strain 13,280) (1) on natural gas (approx. 91 % methane, 5 % ethane, 2 % propane, 0.5 % isobutane, 0.5 % n-butane), ammonia and mineral salts; the crude protein is at least 65 %
Bacterial protein from *Escherichia coli*	Protein product, by-product from the production of amino acids by culture of *E. coli* K12 (1) on substrates of vegetable or chemical origin, ammonia or mineral salts; it may be hydrolysed
Bacterial protein from *Corynebacterium glutamicum*	Protein product, by-product from the production of amino acids by culture of *C. glutamicum* (1) on substrates of vegetable or chemical origin, ammonia or mineral salts; it may be hydrolysed
Yeasts and parts thereof [brewers' yeast] [yeast product]	All yeasts and parts thereof obtained from *Saccharomyces cerevisiae*, *S. carlsbergensis*, *Kluyveromyces lactis*, *K. fragilis*, Torulaspora delbrueckii, *Candida utilis*/*Pichia jadinii*, *S. uvarum*, *S. ludwigii* or *Brettanomyces* ssp. (1) (2) on substrates mostly of vegetable origin such as molasses, sugar syrup, alcohol, distillery residues, cereals and products containing starch, fruit juice, whey, lactic acid, sugar, hydrolysed vegetable fibres and fermentation nutrients such as ammonia or mineral salts

(continued)

Table 1 (continued)

Name	Description
Mycelium silage from the production of penicillin	Mycelium (nitrogenous compounds), wet by-product from the production of penicillin by *Penicillium chrysogenum* (ATCC48271) (1) on different sources of carbohydrates and their hydrolysates, heat treated and ensiled by means of *Lactobacillus brevis*, *plantarum*, *sake*, *collinoides* and *Streptococcus lactis* to inactive the penicillin; nitrogen expressed as crude protein is at least 7 %
Yeasts from biodiesel process	All yeasts and parts thereof obtained from *Yarrowia lipolytica* (1) (2) grown on vegetable oils and degumming and glycerol fractions formed during biofuel production
By-products from the production of L-glutamic acid	By-products from the production of L-glutamic acid by fermentation with *Corynebacterium melassecola* (1) on substrate composed of sucrose, molasses, starch products and their hydrolysates, ammonium salts and other nitrogenous compounds
By-products from the production of L-lysine-monohydrochloride with *Brevibacterium lactofermentum*	By-products from the production of L-Lysine monohydrochloride by fermentation with *B. lactofermentum* (1) on substrate composed of sucrose, molasses, starch products and their hydrolysates, ammonium salts and other nitrogenous compounds
By-products from the production of amino acids with *Corynebacterium glutamicum*	By-products from the production of amino acids by fermentation with *C. glutamicum* (1) on substrate of vegetable or chemical origin, ammonia or mineral salts
By-products from the production of amino acids with *Escherichia coli* K12	By-products from the production of amino acids by fermentation with *E. coli* K12 (1) on substrate of vegetable or chemical origin, ammonia or mineral salts
By-product of enzyme production with *Aspergillus niger*	By-product of fermentation of *A. niger* (1) on wheat and malt for enzyme production

(1) The cells of the microorganisms have been inactivated or killed.
(2) The usage name of yeast strains may vary from the established scientific taxonomy; therefore, synonyms of the yeast strains listed could also be used.

2.3 Encapsulation of Probiotics

In addition to antimicrobial compounds, probiotic microorganisms may also be encapsulated. The low survival of probiotic bacteria during processing and storage of feeds remains a problem. Probiotics may be exposed to high temperatures, low pH, high osmotic pressure and freezing conditions that exert deleterious effects on

the beneficial microorganisms. The encapsulation of probiotics allows the organisms to remain viable when administered in feed. A biocompatible matrix should be employed to encapsulate and immobilise viable cells, protecting them from a hostile environment. Also, it facilitates their transit through the stomach without exposing them to acid or gastric juices (Shori 2017). Once in the intestine, the probiotics are released from the capsule, leaving them free for colonisation. Moreover, encapsulation protects against the host's immune response (Orive et al. 2013). On the other hand, the administration of encapsulated probiotics can be a nutritional strategy for improving the growth performance and immune status of fish (Amir et al. 2019).

Among the main techniques used to encapsulate probiotics are extrusion, emulsion, spray-drying, spray chilling and fluidized bed (Rodrigues et al. 2020). Many materials have been used to encapsulate probiotics for fish, i.e. calcium alginate, chitosan, cellulose, wax, whey protein and gum. One of the most commonly used materials for encapsulating probiotics is alginate. This material, extracted from algae, in addition to being economical, is easy to apply. Amir et al. (2019) evaluated chitosan-coated alginate microcapsules of *Geotrichum candidum* compared with free (un-encapsulated) probiotics by conducting an 11-week feeding trial in a semi-intensive earthen pond culture system. Fish (*Labeo rohita*) fed with *G. candidum*-supplemented diets had an improved growth rate, heightened intestinal enzyme activities, better haemato-immunological indices and a reduction in total cholesterol and triglycerides compared with those fed with a basal diet. Furthermore, diets supplemented with encapsulated *G. candidum* showed the most significant positive effect in comparison with un-encapsulated probiotics. *G. candidum* in encapsulated form was shown to have a marked effect on the growth, health status and immunity of fish suggesting its application as a feed additive in practical/commercial semi-intensive earthen pond culture systems. Alginate has been also used to encapsulate freeze-dried *S. cerevisiae*. The viability of encapsulated yeasts was significantly higher in simulated gastric and bile conditions and remained high after storage at room temperature for 14 days (Pinpimai et al. 2015). Moreover, calcium alginate macrocapsules have been tested to preserve and to administer probiotics. The results obtained by Rosas-Ledesma et al. (2012) indicate that the probiotic *Shewanella putrefaciens* Pdp11 could be encapsulated successfully in calcium alginate beads and could be stored at 4 °C for at least 1 month, with survival rates being above 90 %. On the contrary, storage of the capsules at 22 °C resulted in 40 % viability loss within 30 days. The survival of encapsulated probiotics through the fish gastrointestinal tract was also demonstrated. Cordero et al. (2015) fed gilthead seabream (*Sparus aurata* L.) specimens with calcium alginate beads containing commercial diet enriched with *S. putrefaciens* Pdp11 (at a concentration of 10^8 CFU/g). Fish were fed for 4 weeks. Results demonstrated that administration of alginate encapsulated Pdp11 had immunostimulant properties on humoral parameters (IgM level and serum peroxidase activity). Although no immunostimulant effects were detected on leucocyte activities, significant increases were detected in the level of mRNA of head-kidney leucocytes. The administration of strain Pdp11 encapsulated in alginate beads produced important changes in the intestinal microbiota, increasing the lactic acid bacteria, such *as Lactococcus* and *Lactobacillus* strains.

2.4 Probiotics Encapsulated in Living Organisms

Inclusion of probiotic cells in organisms, such as *Artemia,* is an easy way of administering probiotics to fry and fingerlings. Thus, marine *Streptomyces* were used to colonise *Artemia nauplii* prior to challenge with *Vibrio harveyi* and *V. proteolyticus.* A significant reduction in mortality ($p < 0.001$) was found after the addition of 1 % wet cell mass of *Streptomyces* strains to nauplii and adult *Artemia* when they were challenged with the pathogens (Das et al. 2010). In this way, the administration of probiotics to *Artemia* prevents them from accumulating pathogens, avoiding the infection of the fish fed with *Artemia*. Gatesoupe (2002) administered the probiotics Bactocell (*Pediococcus acidilactici*) and Levucell (*Saccharomyces cerevisiae*) in *Artemia* cysts and nauplii used as pollack (*Pollachius pollachius*) feed. The treatment increased the mean weight of pollack. Moreover, growth was even better with the combination of Levucell and Bactocell. Overall, a high bacterial load was found in the nauplii enriched with Levucell. Also, *Artemia* was used to encapsulate the probiotic *Shewanella putrefaciens* Pdp11 to feed *Solea senegalensis* larvae and fry (Lobo et al. 2014). Pdp11 supplied significantly modulated larval and fry gut microbiota. The probiotic produced a higher fish growth rate, a higher digestive proteolytic activity level and a fish body composition modulation along *S. senegalensis* rearing. In addition, less size variability was obtained from metamorphosis until the end of weaning. According to Jurado et al. (2018), the dietary administration of the bioencapsulated probiotic promoted transcriptional changes of genes involved in growth and immunity in *S. senegalensis* larvae.

Encapsulation in *Artemia* is also used to administer probiotics that inhibit the pathogen's virulence expression by quorum *s*ensing mechanisms. Thus, Nhan et al. (2010) investigated the effect of N-acyl homoserine lactone-degrading bacterial enrichment cultures on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. The application of the probiotics was performed via enriched *Artemia nauplii* used for larval feeding. The results of the study demonstrated that treatment had a similar positive effect on larval survival and larval quality, whereas they did not affect larval growth or the duration of the larval rearing process.

Rotifers have also been used as vectors for probiotics. Tarnecki et al. (2019) studied the effects of a mixed *Bacillus* (*B. licheniformis* and *B. amyloliquefaciens*) probiotic on rearing of larval common snook (*Centropomus undecimalis*). Experimental treatments included probiotics added to the water and live feed (rotifers). Data from trials indicated up to 2.5 times higher survival with probiotic addition, as well as 20 % higher survival 7 days following transportation. Microbiota analysis indicated the importance of system stabilisation prior to larval stocking to improve rearing success and probiotic performance. However, the probiotics did not promote faster growth or improve water quality parameters or innate immune enzyme activities.

2.5 Administration of Probiotics by Injection

The injection of probiotics has only been used to determine safety or to activate the immune system (Fu et al. 2020; Medina et al. 2020). Therefore, injection techniques are only limited to experimental systems; there is not any interest for industrial usage. In some cases, the use of probiotics has been proposed as a vaccination strategy, in which the pathogen is replaced by antigenically similar but harmless strains (Arijo et al. 2008; Medina et al. 2020).

3 Dosage, Frequency and Duration of Administration

The effectiveness of probiotics is affected not only by the route of administration, but also by the amount of probiotics administered and the length of exposure to the probiotics.

3.1 Probiotics Dosage

The definition of probiotics includes that they should be administered in adequate amounts to confer beneficial results to the host (FAO/WHO 2002), but this annotation does not incorporate specific parameters, such as dose, frequency or duration of administration. Using the correct dose allows probiotics to colonise and adhere to the digestive tract, with a consequent beneficial effect for the host (Merrifield et al. 2011). Therefore, since they are living microorganisms with self-replication capacity, a few cells could be enough to induce community growth (Tan et al. 2016). Studies in which probiotics are supplied to fish should determine tolerability and efficacy at different concentrations because excessive concentrations of these cells may cause perturbation in gastrointestinal microbiota or suppress beneficial probiotic activities (Ramos et al. 2015). Thus, an overdose or a dose that is too low causes economic losses and wasted energy and nutrients (Wang et al. 2019a; b). Adorian et al. (2019) fed specimens of Asian sea bass (*Lates calcarifer*) with different doses of the probiotics *Bacillus licheniformis* and *Bacillus subtilis* for 8 weeks, obtaining significantly lower values in growth parameters, liver activity and digestive enzymes of the animal at the lowest inoculated dose (10^3 CFU/g of feed); the 10^6 CFU/g dose of feed achieved the most promising results. Liu et al. (2018) tested the efficacy of the probiotic *Bacillus subtilis* at different doses in *Oplegnathus fasciatus* for 56 days, considering that the dose of 10^8 CFU/kg had the best probiotic efficacy and observed that at higher doses (10^{10} CFU/kg), there was repression of fish growth.

The weight of the fish treated should be taken into account when calculating the most beneficial dose of probiotics. Concentrations of 10^6 to 10^8 CFU/g of feed have been suggested in a large number of published articles. However, it is essential to

define the doses of probiotics for a specific fish and environment (Cordero et al. 2015; Lee et al. 2017; Wang et al. 2019a). Gobi et al. (2018) indicated that the supplementation of the probiotic *Bacillus licheniformis* Dahb1 for 4 *weeks* in tilapia (*Oreochromis mossambicus*) at a concentration of 10^7 CFU/g in diet improved survival against the pathogen *A. hydrophila* and also increased growth and immune parameters of the mucus. However, despite combining different doses of the probiotics *B. subtilis*, *L. fermentum*, *L. pentosus* and *Saccharomyces cerevisiae*, a higher dose (10^9 CFU/kg) of feed was needed to obtain improved growth and disease resistance in Asian sea bass (Lin et al. 2017). In this sense, the probiotic concentration supplied is directly related to a beneficial effect in the host. Nevertheless, a connection between dose and frequency or period of administration of the probiotic is necessary in order to obtain accurate results.

3.2 Frequency of Administration

Frequency of administration is an important factor related to proper maintenance and effectiveness of probiotics (Austin and Newaj-Fyzul 2017). Discontinuous administration could cause the probiotic to disappear from the host's system particularly at the beginning of the trial, where a daily addition is preferred to boost a better primary colonisation (Sharifuzzaman and Austin 2017). Furthermore, the continuous or repeated addition of probiotics to the host is recommended during the trial in order to help maintain immune system stimulation against possible infestations (Guo et al. 2009). In this sense, recent studies of feeding strategy compare different responses to growth and immune response when probiotics are administered continuously or by pulses (Tachibana et al. 2020). Nevertheless, it could depend on the species studied, the probiotic delivery method, the fish density and average body weight and the major outcomes of the study (Jahangiri and Esteban 2018).

3.3 Duration of Administration

Equally, the duration of administration of probiotic bacteria is considered a very significant factor, directly related to the above. According to research, the time interval for application of the potential probiotic can be as short as hours, as in the case of *Pseudomonas* sp. [isolate GP21], a probiotic isolated and tested on Atlantic cod (*Gadus morhua*) (Ruangsri et al. 2014), but it can also be as long as 8 months (Aly et al. 2008a, b), as occurs with *Bacillus pumilus* in *Tilapia nilotica* (*Oreochromis niloticus*). This factor depends on the parameters of study, such as the case of the probiotic *Shewanella putrefaciens* Pdp11, whose feeding time can vary during metamorphosis (10–21 days) until the end of weaning (23–73 days) of Senegalese sole specimens (Jurado et al. 2018). Purwandari and Chen (2013) studied the effects of the probiotic *Bacillus subtilis* on intestinal microbial diversity and immunity of the

orange-spotted grouper *Epinephelus coioides*. The innate cellular response and respiratory burst activity of the supplemental groups were significantly higher compared with the control at 10 and 20 days after feeding and even more significant at 30 days. The frequency of probiotic administration can vary when treating bacterial diseases in fish, depending on the species of probiotics studied and the potential pathogens. For example the probiotic *Lactobacillus* spp. exerts an antagonistic effect against the pathogen *Vibrio* spp. in shrimp *Penaeus monodon* (Ahmmed et al. 2018), and infected individuals showed a gradual decrease in *Vibrio* spp. after 4 and 12 h of probiotic treatment with a viable count drastically reduced in the gills and intestinal tracts of shrimp (Kabiraj et al. 2020). The addition of the probiotic *L. plantarum* to the diet of common carp (*Cyprinus carpio*) has been studied at different concentrations and durations. Here, the best effects were observed with higher doses and long-term administration of the probiotic (Soltani et al. 2017). These results and many others support the idea that the effects of probiotics are time and dose dependent (Vidal et al. 2016; Mohammadian et al. 2019; Klakegg et al. 2020) and vary depending on the species studied.

Although a short administration may produce short-term benefits, it can also cause a null or worse colonisation compared with a prolonged administration. However, the latter may induce immune suppression of continuous responses of nonspecific immune systems. In any case, the best parameters are those that allow probiotics to colonise the digestive tract and exert their beneficial effects on the host in a safe and effective way, promoting safety and good farming.

4 Use of Single Strains or Combinations of Two or More Cultures

A monostrain probiotic is defined as enclosing one strain of a certain species, and consequently multistrain probiotics enclose more than one strain of the same species or at least of the same genus. Arbitrarily, the term multispecies probiotics is used for preparations incorporating strains that belong to one or preferentially several genera. The approach to using a combination of probiotics should be systemic, based on a mixture of versatile strains capable of acting and interacting under a variety of conditions and able to maintain themselves in a dynamic way. In addition, as has been argued above, in aquaculture, the microbial habitat undergoes continuous alterations, allowing constant changes in the structural composition and functions of the microbial community (Verschuere et al. 2000). Therefore, through the combined application of multiple favourable probiotic candidates, it may be possible to produce greater benefits in aquaculture than by applying a single probiont (Nwogu et al. 2011; Ibrahem 2018).

Most research has focused on the use of single cultures, and it is largely speculative whether two or even multiple combinations of probiotics are beneficial (Newaj-Fyzul et al. 2015). Even, the evaluation of the combined administration of probiotics seemed

to be inadequate in comparison with the use of single cultures (Mohapatra et al. 2012). Irianto and Austin (2002) included a multistrain treatment alongside testing four strains of probiotic bacteria individually against *Aeromonas salmonicida.* They found that although each treatment improved the survival of rainbow trout against the pathogen, there was no advantage of multistrain administration. However, there is an increasing tendency to work with multistrain probiotics, in particular products with a high number of different strains. However, there is an increasing tendency to work with multistrain probiotics, in particular, products with a high number of different strains. There are some thoughts behind this: more strains imply more chances of success; it can mean a broader spectrum of efficacy, and there are often additive and even synergistic effects. The application of probiotics as multistrain or multispecies dietary supplements has often been shown to create improved benefits, such as an accumulation of multiple probiotic effects on the host (Kong et al. 2020).

4.1 Effect on Colonisation

An advantage of using a mixture of probiotics is that colonisation is promoted. A well-known effect of probiotics is that they can strengthen resistance to colonisation by pathogens. However, the probiotic has also to overcome the resistance to colonisation exerted by the microbiota already present. On the other hand, the anatomical and physiological characteristics of the surface of the host to be colonised (e.g. pH, enzymes, antibodies and surface receptors) determine the amount of probiotics supplied that can survive. Therefore, probiotic preparations containing several species and/or strains may have a better chance of survival. A multispecies probiotic preparation may create a niche that improves colonisation of damaged strains (Timmerman et al. 2004). It has been demonstrated that the gut microbiota of the group fed with a multispecies combination of probiotics had a higher number of operational taxonomic units (OTUs), whereas those fed a single-strain probiotic preparation had high Shannon index (H′) or diversity index (Ramos et al. 2013). In any case, the administration either by single species or by multispecies probiotic combinations had differing results.

4.2 Effect on Metabolism

Batista et al. (2015) evaluated the effect of using a monoculture probiotic (*Pediococcus acidilactis*) versus a multispecies supplementation (*Bacillus* sp.*, Pediococcus* sp.*, Enterococcus* sp. *and Lactobacillus* sp.) in Senegalese sole. The results show an improvement in the size of the animal and intestinal microvilli, as well as an increase in the thickness of the muscle layer, in the treatment with the probiotic combination compared with the group fed with a single probiotic strain. Similarly, the study on tilapia (*Oreochromis niloticus*) by Sutthi (2018) showed a synergistic effect in

the combination of *Bacillus* spp. (*B. subtilis, B. megaterium and B. licheniformis*) (10^6 CFU/g) and the yeast *Saccharomyces cerevisiae* (5.6×10^8 CFU/g) when added to the rearing tanks, significantly improving the weight and size of the animal. In addition, Niu et al. (2019) reported increased predicted gene functions related to lipid and carbohydrate metabolism in the intestinal microbiota of fish fed with low fishmeal diet supplemented with a multispecies probiotic formulation compared with those fed a diet devoid of probiotics. Also, combined applications result in increased benefits, such as in the case of the joint administration of *B. methylotrophicus* and *B. licheniformis,* which gives improved growth, immunity and disease resistance in rohu, *Labeo rohita* (Hamilton) (Mukherjee et al. 2019).

4.3 Effect on Immunity

Related to immunity, the combination of probiotics has been considered to have a greater effect in inhibiting pathogens than if treated with a single strain (Wang et al. 2019a; b). Results of combined *Bacillus* MCCB101 and *Micrococcus* MCCB104 administered in tiger shrimp (*Penaeus monodon*) supported maximum up-regulation of antimicrobial peptide gene expression against white spot virus (WSV) infection (Anthony et al. 2011). Besides improvements in growth performance, white shrimp fed a diet containing a mixture of probiotics (*Lactobacillus pentosus* BD6*, Lac. fermentum* LW2*, Bacillus subtilis* E20 and *Saccharomyces cerevisiae* P13) at 10^8 CFU/kg diet had better disease resistance against *V. alginolyticus* compared with the single probiotic in this study (*S. cerevisiae* P13). Similar results of improved disease resistance were also recorded in Asian sea bass fed with the same probiotic blend (Lin et al. 2017). Supplementation of the diet with a mixture of probiotic strains including *Bacillus* spp., *Lactobacillus* spp. and *Saccharomyces cerevisiae* resulted in increased lysozyme, myeloperoxidase and glutathione peroxidase as well as up-regulation of immune-related genes, including IL-1β, IL-6 and TNF-α in *Paralichthys olivaceus* specimens (Niu et al. 2019). In contrast, Díaz-Rosales et al. (2006) found no additional benefit from the combined use of two heat-inactivated probiotic strains of *Vibrio* sp. in the immune response of gilthead sea bream. Similarly, the administration of the probiotics *Shewanella putrefaciens* Pdp11 and *Shewanella baltica* Pdp13 in *Solea senegalensis* specimens did not show special or additional benefits from the combined use of the probiotic strains as reported by Díaz-Rosales et al. 2009.

4.4 Effect on Antimicrobial Activity

Although probiotics are appreciated for their antimicrobial activity, this characteristic may also be a potential fragility for probiotic mixtures. Secreted antimicrobial compounds, such as lactic acid, hydrogen peroxide and bacteriocins, not only

inhibit potential pathogens but also closely related species (Kailasapathy and Chin 2000). Despite evidence that probiotic species will inhibit each other when incubated together in vitro, in many cases, a probiotic mixture was more effective at inhibiting pathogens than its component species when tested at approximately equal concentrations of biomass.Please confirm if the section headings identified are correct.The section 16 belong to the section 12: Use of Single Strains or Combinations of Two or More Cultures.

4.5 Use of Multistrains and Multispecies

Some examples of the combined use of probiotics in different groups of aquaculture organisms include:

• Molluscs

Riquelme et al. (2001) observed that the inoculation of mixtures of the strains *Vibrio* sp. C33, *Pseudomonas* sp. 11 and *Bacillus* sp. B2 made the development of larval stages possible without antibiotics in *Argopecten purpuratus* larvae. They showed evidence that the antibiotics reduce the levels of bacteria in the water, but did not impede their proliferation into larvae, whereas the incorporation of selected bacteria allowed a modification of the microbiota associated with larvae.

Macey and Coyne (2005) tested a mixture of yeasts (isolates SS1, AY1) and bacteria (isolate SY9), which was added to dry feed at 10^7 CFU/g and fed to abalone (*Haliotis midae*) for 14 *days*. This led to 62 % survival after challenge with *V. anguillarum* compared with 25 % survival of the controls. In Catarina scallop (*Argopecten ventricosus*), evidence indicated that the mechanisms of action of the probiotic strains (10^6 CFU/mL) appear to be stage-specific and strain-specific and generated different responses by the host, including improved survival and growth and greater resistance to pathogens, likely from better nutrient assimilation and/or strengthening of the immune system. Compared with the controls, optimal results were achieved with the antibiotic treatment for survival of veliger larvae; *Lactobacillus graminis* for growth of veliger larvae; MIX-LB (1:1:1:1:1 mixture of *Lactobacillus graminis, L. plantarum, Bacillus cereus, B. flexus and B. firmus*) for settlement of pediveliger larvae; *L. plantarum* for growth and survival of early juveniles; and MIX-B (1:1:1 mixture of *Bacillus cereus, B. flexus and B. firmus*) for challenge against *V. alginolyticus*. A comparison of the strengths and weaknesses of all these strains places *L. graminis* and *L. plantarum* among the best for most developmental stages. In contrast, the *Bacillus* strains performed poorly when used as single treatments and with immature developing larvae (Abasolo-Pacheco et al. 2017).

• Crustaceans

Gómez and Shen (2008) studied the influence of *Bacillus* probiotics on the digestive enzyme activity and growth of *Litopenaeus vannamei*. The shrimps were treated with five concentrations of probiotic mix (*Bacillus subtilis, B. natto and B. licheniformis*) added to the feed and cultured for 45 *days*. The growth measured as the

weight gain at the end of culturing was significantly higher ($P < 0.05$) in probiotic-treated shrimps than in the control. Activities of protease and amylase, two digestive enzymes, were significantly higher ($P < 0.05$) in probiotic-treated shrimp than in the control. Combinations of several probiotics in Pacific white shrimp (*Litopenaeus vannamei*) diets, such as *E. faecium* and *L. pentosus,* or the combination of *L. pentosus*, *L. fermentum*, *B. subtilis* and *S. cerevisiae* significantly improved disease resistance against *V. parahaemolyticus* (Sha et al. 2016; Wang et al. 2019a, b), whereas the combination of *E. faecalis* and *E. faecium* showed significantly increased disease resistance of *L. vannamei* against *A. hydrophila* and *V. vulnificus* (Cui et al. 2010). These shrimp fed with *Shewanella haliotis* D4, *Bacillus cereus* D7 and *Aeromonas bivalvium* D15 at a ratio of 2:1:1, dosed at 10^7 cell/g, showed better growth performance and disease resistance compared with those fed with single probiotics (Hao et al. 2014). Moreover, Miandare et al. (2016) demonstrated that oral administration of multistrain probiotics (*L. acidophilus, L. casei, E. faecium* and *B. bifidum*) had beneficial effects on growth performance, feed utilisation, digestive enzyme activity, immune-related genes and survival of post-larvae *L. vannamei*. The oral administration of 0.5 and 1.0 *g*/kg multistrain probiotic enhanced the performance of *L. vannamei*, which may be due to the improvement of digestive enzyme activity and in turn improved intestinal microbiota.

• Fish

Aly et al. (2008a; b) noted significantly higher protection in tilapia against several pathogens (*A. hydrophila*, *P. fluorescens* and *S. iniae*) when fed with mixtures of *B. subtilis* and *L. acidophilus* for one month compared with groups that received either *B. subtilis* or *L. acidophilus* alone. Subsequently, a mixture (1:1 10^7 CFU/g) of *Lactococcus lactis* (BFE920) and *Lactobacillus plantarum* (FGL0001) served as an immune-stimulating feed additive protected Japanese flounder (*Paralichthys olivaceus*) against a challenge with *Streptococcus iniae* (Beck et al. 2015). Moreover, Dawood et al. (2016a; b) fed red sea bream (*Pagrus major*) fingerlings with a basal diet (control) supplemented with *L. rhamnosus* and *L. lactis* (D1813) for 56 days. Oral administration of both probiotics had positive effects on the growth, feed utilisation, health condition and immune system of the red sea bream. The multi-species formulation was more effective than any of the single-bacteria experimental diets. Furthermore, Maji et al. (2017) studied the probiotic effect of a consortium of putative lactic acid bacteria on *Labeo rohita.* They fed fish with a lactic acid bacteria-supplemented diet for a period of 30 days. At the end of the experiment, the probiotic fed group showed a significant improvement in weight gain percentage, specific growth rate and feed conversion ratio along with increased immune response. Challenge with *Aeromonas hydrophila* on day 30 of probiotic feeding showed a significant increase in the survival percentage of treated fish (93.3 %) over the control group (33.3 %). Finally, Mukherjee et al. (2019) evaluated the effectiveness of the *Bacillus* spp. (*B. methylotrophicus*, *B. amyloliquefaciens* and *B. licheniformis*) as prophylactic agent, either alone or in combination, in the diets for *L. rohita* fingerlings. The study appraised growth, blood-biochemical parameters, immunity and disease resistance in rohu, *Labeo rohita* fed with probiotic supplemented diets.

5 The Value of Using Inactivated, Viable Cells or Subcellular Components of Probiotics

Despite the proven health benefits of probiotics, some constraints are associated with their use, including the risk of microbial translocation, infection or enhanced inflammatory responses, which may be seen in some probiotics in hosts with imbalanced or compromised immune systems (Taverniti and Guglielmetti 2011). In addition, regulation and policy statements on the use of microorganisms make the acceptance of non-lactic acid bacterial species, which are Generally Recognised as Safe (GRAS) difficult and expensive. Therefore, interest is shifting towards alternatives including the use of inactivated or subcellular components of probiotics with advantages over living probiotic cells. Thus, better safety parameters, longer shelf life and ease in production and industrial scale-up have been pointed out (Nataraj et al. 2020; Tomar et al. 2015). In this sense, new terms such as paraprobiotics and postbiotics have emerged, which imply that microbial viability is not an essential requirement for health benefits, providing a potential opportunity in the field of functional foods.

5.1 Non-viable Probiotic Cells: Paraprobiotics

Taverniti and Guglielmetti (2011) used the term paraprobiotics (also called ghost cells) to define "non-viable microbial cells (intact or broken) or crude cell extracts (i.e. with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer". Methods for obtaining paraprobiotics include heat inactivation, ionisation and ultraviolet radiation, sonication, high pressure or formalin treatment (de Almada et al. 2016). The variety of methods used involves the acquisition of different types of paraprobiotics with effects that need to be determined (Choudhury and Kamilya 2019). In this sense, it is important to keep in mind that the potential health benefits of paraprobiotics depend on whether the mode of action of the probiotics relies on the viability of the cells. Since there is a number of potential mechanisms, different outcomes can be expected for each case. Thus, the diversity of probiotic strains means that the response to inactivation treatments may be diverse, with differences being observed even in the same bacterial groups (Ou et al. 2011). When analysing the effects of probiotic viability on the immune response, diverse results have been reported. Kamilya et al. (2015) observed that inactivated preparations of *Bacillus amyloliquefaciens* FPTB16 and *B. subtilis* FPTB13 exerted *in vitro* immunostimulatory effects in catla (*Catla catla*) head-kidney leukocytes. Similarly, Singh et al. (2017a; b) observed significant enhancement in immune parameters, such as serum lysozyme and myeloperoxidase as well as increased *il-1b*, *tnfa*, c3 and iNOS gene transcription in fish fed dietary *B. amyloliquefaciens* FPTB16. In this same way, increased superoxide anion generation, complement and phagocytic activity were reported in gilthead sea bream (*Sparus aurata*) fed dietary *Lactobacillus* and *Bacillus* paraprobiotic combinations (Salinas

et al. 2008). Moreover, other inactivated microorganisms including *Saccharomyces* sp. (Hoseinifar et al. 2011), *Enterococcus faecalis* (Rodriguez-Estrada et al. 2013) and formalin killed bacterial combinations (Taoka et al. 2006) have demonstrated the ability to modulate the immune system in different fish species. This immunomodulation may be based on microbial components, such as peptidoglycans, lipopolysaccharides, capsular polysaccharides, which are all stable under inactivation treatments and are immunostimulant agents for fish (Nayak 2010).

On the other hand, no significant increments in respiratory burst activity were observed in gilthead sea bream (Díaz-Rosales et al. 2006) and rainbow trout (*Oncorhynchus mykiss*) (Panigrahi et al. 2005) fed with diets supplemented with Gram-negative and Gram-positive heat-inactivated probiotics, respectively. Collectively, research carried out points to the fact that although the immune parameters modulated may be different, probiotic viability is not essential for immunomodulation.

Though stimulation of the immune system can be achieved with paraprobiotics, other modes of action described for probiotics cannot be expected in inactivated cells. This is the case of bacteriocin-mediated antagonism and other antimicrobial substances capable of inhibiting pathogens. Similarly, colonisation of fish mucosal surfaces is limited when probiotics are not alive. However, blocking of adhesion sites by ultraviolet radiation- and heat-inactivated cells has been reported (Ouwehand et al. 2000; Singh et al. (2017a, b). In this case, only a continuous supply of paraprobiotics to the fish could be effective in the interference of pathogen adhesion with host cells. Data by authors using inactivated probiotic cells reported that they may confer protection against infections in comparison with control fish receiving diets devoid of probiotic cells. In this way, susceptibility to *Streptococcus iniae* and *Lactococcus garvieae* infection was less reduced in rainbow trout and Chinese drum after treatment with inactivated probiotics compared with live cells (Brunt and Austin 2005). However, Pan et al. (2008) observed increased resistance to *Vibrio anguillarum* and *Aeromonas hydrophila* infection in Chinese drum (*Miichthys miiuy*) fed with inactivated *Clostridium butyricum* CB2 cells. Similarly, decreased mortality after *A. salmonicida* infection was reported in rainbow trout fed with inactivated *Enterococcus faecalis* compared with fish receiving diets devoid of probiotic cells (Rodriguez-Estrada et al. 2013).

Probiotics may represent a source of nutritional factors for the host. Feeding trials carried out with inactivated probiotics show that weight gain, higher efficiency ratio and specific growth rate can be obtained in species such as *Pagrus major* when using inactivated *Pediococcus pentosaceus* cells (Dawood et al. 2016a, b) and rainbow trout fed diets containing inactivated *Enterococcus faecalis* (Rodriguez-Estrada et al. 2013). The benefits derived may be related to increased tolerance to low salinity stress and nutrient input derived from dead probiotic cells, as the higher growth parameters were obtained for fish fed with diets containing higher paraprobiotic inclusion levels (Dawood et al. 2019). On the other hand, heat-killed probiotic combinations did not contribute in growth and feed conversion rate benefits for rohu (*Labeo rohita*) (Mohapatra et al. 2012). Thus, though it seems that paraprobiotics may improve growth parameters, the mechanisms involved need to be studied further.

5.2 The Use of Probiotic Subcellular Components: Postbiotics

The use of subcellular components of probiotics has also emerged as an alternative to administering live bacteria, and the study of their ability to confer health benefits to the host in aquaculture is on the rise. The term postbiotics is used for soluble factors resulting from the metabolic activity of a probiotic or any released molecule capable of conferring beneficial effects to the host in a direct or indirect way (Aguilar-Toalá et al. 2018). Postbiotics include a wide range of compounds, such as short chain fatty acids (SCFAs), enzymes, peptides, teichoic acids, cell surface proteins, endo- and exo-polysaccharides and organic acids (Tsilingiri et al. 2012; Ang et al. 2020).

Though less considered in aquaculture for now, human and veterinary uses of postbiotics have shown interesting properties, such as clear chemical structures, safety dose parameters and longer shelf life (Tomar et al. 2015). In addition, they have favourable absorption, metabolism, distribution and excretion abilities, which could indicate a high capacity to signal different organs and tissues in the host, inducing several biological responses. The different microbial-derived products included in the postbiotic composition can mimic the health effects of probiotics. Although the importance of postbiotics has been relatively overlooked, scientific evidence of their beneficial health effects is progressively increasing (Compare et al. 2017; Haileselassie et al. 2016; Wegh et al. 2019), even though their precise composition and underlying mechanisms are still under investigation, especially in the case of aquaculture, the information on the application of postbiotics is limited and mainly focused on Gram-positive microorganisms (Lieke et al. 2019; Ang et al. 2020).

Postbiotics include a great diversity of substances in their composition, and therefore, a wide range of effects can be expected. Firstly, many microbial-derived products represent sources of bioactive compounds for the host or the host microbiota, which could convert them into bioactive molecules (Banerjee and Ray 2017; Ray et al. 2012; Teame et al. 2020). On the other hand, it has been reported that dietary supplementation with exogenous enzymes such as carbohydrase, protease, phytase and xylanase increases performance and nutrient digestibility of diets with plant by-products in fish species such as Nile tilapia (*Oreochromis niloticus*) (Lemos and Tacon 2017; Maas et al. 2020). Probiotics with extracellular enzymatic activities capable of contributing to nutrient hydrolysis are currently being researched (Serra et al. 2019). These findings open the possibility of using alternative sources of fishmeal in aquafeeds, including postbiotics with enzymatic activities for the sake of aquaculture sustainability.

Postbiotics have also been considered viable alternatives to the use of antibiotics. The presence of bacteriocins and antimicrobial peptides has been documented in the extracellular products of several probiotic strains (Kuebutornye et al. 2020; Rather et al. 2017). Thus, nisin produced by *Lactobacillus lactis* and other bacteriocin-like products from lactic acid bacteria have been reported as potential biocontrol agents in aquaculture, with immunostimulant activity being detected in some cases (Lin et al. 2013; Sequeiros et al. 2015). Furthermore, Abbas et al. (2010)

pointed out the potential of using the cellular components of probiotics for protection of fish against bacterial diseases. These authors tested protection against *Yersinia ruckeri* infection of rainbow trout intraperitoneally injected with cell wall and outer membrane proteins, lipopolysaccharides (LPS) and whole cell proteins (WCP) of two probiotic strains (*Aeromonas sobria* GC2 and *Bacillus subtilis* JB-1), obtaining significantly higher survival percentages compared with the control group. Similarly, Sharifuzzaman et al. (2011) assessed the efficacy of the cellular components of probiotics *Kocuria* SM1 and *Rhodococcus* SM2, observing protection against vibriosis in rainbow trout. The results reported demonstrate that components of probiotics are beneficial to the host for protection against disease. The authors suggested that the mode of action may include ability to enhance innate immune response through the recognition of preserved microbial structures that constitute pattern recognition receptors (PRR) shared by pathogens and probiotic cells (Abbass et al. 2010). However, other mechanisms, such as the presence of common antigens in probiotic and pathogen cells, capable of inducing cross-reactive antibodies cannot be ruled out (Arijo et al. 2008). Recently, there have been reports of postbiotics modulating fish intestinal microbiota. The study showed increased bacterial diversity and richness within the rainbow trout intestinal ecosystem jointly with increased resistance to *Lactococcus garvieae* infection (Pérez-Sánchez et al. 2020). Thus, dietary inclusion of postbiotics may contribute to interference with pathogen colonisation through the modulation of fish microbiota.

6 Conclusions and Suggestions for Further Work

Multiple factors affect the efficacy of the administration of a probiotic, not only the species used. The addition of probiotics to water facilitates their administration and does not cause stress for the fish but requires large amounts of probiotics. The advantage of administering probiotics in feed is that it makes it easier for microorganisms to colonise the gut. However, the storage conditions of the feed and the passage of food through the stomach can inactivate the cells. To avoid this problem, techniques such as the conservation of the microorganisms by drying and encapsulation have been used. With regard to the dose and time of administration, the literature is extensive, with a very wide range of doses, which does not allow us to conclude which are the most adequate doses. The administration criterion seems to depend more on the probiotic strain used and the species to which it is to be administered. In the aquaculture industry, the trend to supply the animal with a combination of multiple probiotics is increasing, since many bacteria have symbiotic relationships with each other. Multistrain probiotic efficacy studies need to be performed, comparing single-strain and multistrain probiotics with each other and with placebo. The advantages of the probiotic mixture may be expected to increase when using low fishmeal diets as different microbial species can help the digestion of the vegetable protein sources included in aquafeeds. However, further research is needed to clarify

the optimal microbial composition as wells as the mechanisms involved in the interactions between microbial and host cells. Due to potential biosecurity problems, the use of live microorganisms as a therapeutic strategy is restricted to very few species. For this reason, it is important to consider the administration of inactivated probiotics (paraprobiotics) or the administration of subcellular components of these strains (postbiotics). This opens the possibility of using the wide range of probiotics that has been experimentally studied in the aquaculture industry. This is possibly the focus of future research on the commercial use of probiotics.

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Use of Probiotics in Finfish

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Abstract Aquaculture provides an excellent resource of animal protein of high nutritional value, and thus impacts favourably on food security. At present, one of the concurrent problems concerns the occurrence of diseases that results in significant economic losses globally of almost $9 billion USD per year. Thus, reducing the impact of disease on aquaculture production is an important necessity. To date, multiple strategies have been devised and implemented for the purposes of disease control of which the use of probiotics is gaining importance in many aquaculture-producing counties. It is apparent that apart from their role in moderating disease, probiotics may affect the host's digestive system leading to beneficial actions, including stimulation of the immune system and increasing resistance to stress. This chapter will address the use of probiotics on finfish, namely those associated with cold, temperate, and warm water environments.

Keywords Finfish · Immunomodulation · Improved growth · Feed supplement · Disease resistance

1 Introduction

The extensive use of antibiotics and other chemicals in non-medical use, including aquaculture, has led to the development and spread of resistant microbial strains and the interference by inhibition/killing of beneficial digestive tract microbiota (WHO 2006; Xia et al. 2020). Yet, there is an increasing demand for safe aquatic foods, which necessitate appropriate aquaculture development procedures for which the use of probiotics for fin fish and other aquatic species is important and timely (Dawood and Koshio 2016). Many microorganisms have been assessed for use as probiotics

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in aquaculture to enhance growth, improve digestion, and stimulate the immune response and resistance in aquatic species (Amir et al. 2019). Fish may be classified according to water temperature in which they occur and include warm, temperate, and cold water species. Warm water fish species include those that live at >27 °C, temperate water species exist at 15.5–26 °C; and cold water fish inhabit environments of <21 °C (Geraldi et al. 2019). Consequently, there are ongoing research projects to select suitable probiotics for these different culture systems. Probiotic efficiency depends on a variety of factors, including size and age of the aquatic species (Xiaolong et al. 2020). Probionts produce their beneficial action through a variety of measures, namely

- improving adhesion to intestinal cells
- enhancing the function of the epithelial barrier
- releasing antimicrobial substances
- inhabiting/colonizing locations that could otherwise allow attachment by pathogen
- influencing/stimulating immune functions (de Almada et al. 2015).

Overall, the dominant probiotics are applied in fish farming centre on the lactic acid bacteria (LAB), which are used extensively in human foods, including fermented milk products (Liu et al. 2017), *Bacillus* spp. (Giatsis et al. 2016) and the yeast, *Saccharomyces* (Xia et al. 2020a). The full range of probiotics used in finfish aquaculture is summarized in Table 1.

2 Use of Probiotics in Cultured Fish Species

2.1 Cold Water Fish Species

Probiotics are used in numerous cold water fish species; their use represents a safe approach for subsequent human consumption. However, the efficiency reflects the prevailing environmental conditions, including water temperature, that may be not ideal for all mesophilic probiotics that would prefer warmer conditions. Therefore, the temperature requirements of putative probiotics need to be considered against the water temperature needed to culture fish. The probiotics considered for use in cold water fish have included both Gram-positive (e.g. LAB, *Kocuria* and *Arthrobacte*r spp.) and Gram-negative bacteria (e.g. *Aeromonas*, *Pseudomonas* and *Vibrio* spp.) and yeasts (*Saccharomyces cerevisiae* and *Debaryomyces hansenii*; Nargesi et al. 2020). Much of the research work has focused on probiotics of value for single fish species. This approach will be used here to assess the literature dealing with the dominant farmed finfish species.

Table 1 Summary of prospective probiotic in finfishes

Fish species	Potential probiotic	Affected parameters	References
Commercially cultured finfish			
Cold water species			
Rainbow trout (*Oncorhynchus mykiss*)	*Lactococcus garvieae*	Controlling most important cold water bacterial disease	Vendrell et al. (2008)
	Flavobacterium psychrophilum		Burbank et al. (2012)
	Aeromonas salmonicida		Balcázar et al. (2009)
	Vibrio anguillarum		Harper et al. (2011)
	Yersinia ruckeri		Brunt et al. (2008)
	Bio-Aqua® *(Pediococcus acidilactici, Bacillus subtilis, Enterococcus faecium, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei,* and *Bifidobacterium bifidum)* and yeast *(Saccharomyces cerevisiae*	• Protein efficiency ratio • Feed conversion ratio • Cholesterol • Reproductive performance	Nargesi et al. (2020)
	lactic acid bacteria (LAB)	• Health status • Immune status • Production parameters	Popelka et al. (2020)
	Rhodococcus SM2 and *Kocuria* SM1	*Vibrio anguillarum* infection	Sharifuzzaman et al. (2011)
	Enterococcus faecium, Bacillus subtilis and *Bacillus licheniformis*	• Feed conversion ratio • Intestinal microbiota • Health status • Immune response	Merrifield et al. (2010)
	Aeromonas sobria GC2 and *Bacillus subtilis* JB-1	*Yersinia ruckeri* infection	Abbass et al. (2010)
Atlantic salmon (*Salmo salar* L.)	*Aliivibrio*	Growth and survivability	Klakegg et al. (2020)
	LAB, RII and RIII (lactic acid bacteria)	Intestinal microbial profile	Gupta et al. (2019)

(continued)

Table 1 (continued)

Fish species	Potential probiotic	Affected parameters	References
	Pediococcus acidilactici MA18/5 M		Jaramillo-Torres et al. (2019)
	Carnobacterium sp.	*Aeromonas hydrophila, Flavobacterium psychrophilum, A. salmonicida, Photobacterium damselae* and *Vibrio ordalii* infection	Robertson et al. (2000)
Turbot (*Scophthalmus maximus*)	LAB	• *Weissella cibaria, T. maritimum and V. splendidus* infection • Immune response genes	Muñoz-Atienza et al. (2014)
Olive flounder (*Paralichthys olivaceus*)	*Bacillus* sp. SJ-10 and *Lactobacillus plantarum*	• Plasma insulin • GH, and IGF-I • Lysosome activity • Intestinal health	Back et al. (2020)
	Lactobacillus multi strain	• Lipid retention • Growth and nutrient utilization • Myeloperoxidase and glutathione peroxidase • Lysozyme activities	Niu et al. (2019)
	Lactococcus lactis WFLU12	• Growth • Health • Feed utilization	Nguyen et al. (2018)
Temperate water species			
Flathead grey mullet (*Mugil cephalus)*	*Saccharomyces cerevisiae*	• Growth parameters • Protein efficiency • Survival	Akbary (2020)
	LAB	Growth and feed utilization	Abdulla (2014)
	Biogen®	General health and immune status	Elam (2004)
Channel catfish *Ictalurus punctatus*	*Bacillus velezensis AP193*	Ponds water quality	Thurlow et al. (2019)
	Bacillus subtilis	Broad inhibitory activity	Luo et al. (2014)
	Bacillus AP79, AP143, AP193 and AB01	• Growth • Immune response • Mortality	Ran (2013)

(continued)

Table 1 (continued)

Fish species	Potential probiotic	Affected parameters	References
	Pediococcus and *Enterococcus*	*Ictalurus punctatus* infection	Shelby et al. (2007)
Warm water finfish			
Tilapia	*Bacillus cereus NY5* and *Bacillus subtilis*	• Growth • Disease resistance • Gut immune status	Xia et al. (2020a)
	B. subtilis and B. licheniformis	• Haematology • Immune response • Antioxidants	Abarike et al. (2020)
	Saccharomyces cerevisiae	• Feed utilization • Growth performance • Resistance to *aeromonas hydrophila* infection	Dawood et al. (2020a)
	Lactobacillus rhamnosus JCM1136 and *L. lactis subsp. lactis* JCM5805	• Expression of immune-related genes • Growth and feed utilization • Gut health	Xia et al. (2020b)
	B. velezensis TPS3N, B. subtilis TPS4 and B. amyloliquefaciens TPS17	• Growth performance • Antioxidant response • Immune and biochemical parameters • Digestive enzymes • Growth- and immune-related genes • *S. agalactiae* infection	Kuebutornye et al. (2020)
	Aspergillus oryzae (ASP)	Oxidative status	Dawood et al. (2020b)
	L. plantarum	*A. hydrophila* infection	Ren et al. (2013)
Labeo rohita	*Pseudomonas aeruginosa* VSG-2	• Immune response • *A. hydrophila* infection	Ss et al. (2012)

(continued)

Table 1 (continued)

Fish species	Potential probiotic	Affected parameters	References
	Geotrichum candidum QAUGC01	• Growth rate • Intestinal enzyme activities • Hematological indices • Immunological parameters • Heat shock protein hsp 70 gene • Serum AST and ALT activities • Total cholesterol and triglyceride	Amir et al. (2019)
Gilthead seabream (*Sparus aurata* L.)	*Vibrio* (Pdp11 and 51M6)	Immunological parameters	Díaz-Rosales et al. (2006)
	Shewanella putrefaciens Pdp11 and *Bacillus* sp	Antioxidant response	Esteban et al. (2014)
	Bacillus subtilis	Proinflammatory genes	Cerezuela et al. (2013)
Sea bass (*Dicentrarchus labrax)*	*Vibrio lentus*	• Antioxidant response • Immunological parameters	Schaeck et al. (2017)
	Lactobacillus delbrueckii	• Cortisol level • Igf-i expression • Body weight	Carnevali et al. (2006)
	Pediococcus acidilactici	• Growth • Immune genes IL1β and COX-2 • Disease resistance	Torrecillas et al. (2018)
Asian seabass (*Lates calcarifer*)	*Bacillus thuringiensis QQ1 or Bacillus cereus QQ2*	• Growth performance • Digestive enzymes activity • Immunological parameters • Cholesterol	Ghanei-Motlagh et al. (2021)
	P. acidilactici	Immunological parameters	Ashouri et al. (2018)
Common carps (*Cyprinus carpio)*	*Lactobacillus fermentum*	• Growth • Antioxidant response • Immunological parameters	Ahmadifar et al. (2019)
	LAB	• Survivability under acidic (pH 2.5) • Immunological parameters	Feng et al. (2019)

(continued)

Table 1 (continued)

Fish species	Potential probiotic	Affected parameters	References
	Lactobacillus plantarum	• Immunological parameters • Growth • Immunological parameters • Resistance to motile *Aeromonas* septicemia	Kazuń et al. (2018) Soltani et al. (2017)
	Bacillus coagulans	• Growth performance • Survival • Immune response	Xu et al. (2014)
	Enterococcus faecium MC13	• *Aeromonas hydrophila* • Immune response	Gopalakannan and Arul (2011)
Ornamental species			
Angelfish (*Pterophyllum scalare,* Schultze, 1823)	LAB	Intestinal health and survival	Sousa et al. (2020)
	Enterococcus faecium	• Growth performance • Survival • Immune response	Dias et al. (2019)
	Pediococcus acidilactici	• Stress resistance (cold temperature and salinity) • Growth performance • Immune response	Azimirad et al. (2016)
	Bacillus (B1, B2 and B3)	• *Aeromonas hydrophila* • Survival	Monroy-Dosta et al. (2010)
Molly *Poecilia Sphenops*	*Lactobacillus delbrueckii* LABT1	Growth and survival	Selvaraj and Bogar (2019)
	Saccharomyces cerevisiae	• Growth • Reproductive performance • *Aeromonas hydrophila*	Aminlooi et al. (2019)
	Vibact	• Fecundity • Fry survival • Gonado somatic index	Chitra and Krishnaveni (2013)
Other finfish categories			
Zebrafish (*Danio rerio*)	*Lactobacillus rhamnosus* IMC 501®	• Hepatic stress tolerance • Growth	Gioacchini et al. (2014)
	L. rhamnosus	Reproduction	Gioacchini et al. (2010)

2.2 *Rainbow Trout (*Oncorhynchus mykiss *Walbaum)*

Rainbow trout production has grown steadily particularly in Chile and Norway, followed by France, Iran, Germany, Denmark, Italy, USA, Spain, Finland and the UK (Lauzon et al. 2014). Nevertheless, trout production is facing great challenges among which is the issue of disease. This is compounded with limitations on the use of chemotherapeutic strategies and the development and spread of antibiotic-resistant microorganisms (Romero et al. 2012). For trout, probiotics have been regarded as an environmentally friendly strategy to control diseases. A wide range of probiotics has been evaluated for controlling the most important bacterial diseases, including *Lactococcus garvieae* (lactococcosis; Vendrell et al. 2008), *Flavobacterium psychrophilum* (cold water disease, rainbow trout fry syndrome; Burbank et al. 2012), *Aeromonas salmonicida* (furunculosis; Balcázar et al. 2009), *Vibrio (Listonella) anguillarum* (vibriosis; Harper et al. 2011) and *Yersinia ruckeri* (enteric redmouth; Brunt et al. 2008).

Various nutrients, namely proteins, fatty acids, lipids, carotenoids and vitamins, have been found to affect reproductive performance of various fish species and suggest a relation between the intestinal microbiota and reproductive performance (Mehdinejad et al. 2019). It was demonstrated that supplementation of feed with Bio-Aqua® (this probiotic comprises a mixture of eight bacterial cultures, i.e. *Pediococcus acidilactici*, *Bacillus subtilis, Enterococcus faecium*, *Lactobacillus rhamnosus, Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Bifidobacterium bifidum*) and yeast (*Saccharomyces cerevisiae*)) at a concentration of 2×10^9 CFU/g led to enhanced protein efficiency ratio, decreased feed conversion ratio, lowered cholesterol and enhanced reproductive performance. This reflected increased egg diameter, improved hatching rates and fertilization; and enhanced survival of eyed eggs. In addition, there were improvements to the eyed egg stages, to hatching and absorption of yolk sac in female rainbow trout brood-stock (Nargesi et al. 2020).

LAB has been evaluated as probiotics in rainbow trout leading to improvement in health, immunomodulation and enhanced production parameters including feed conversion and weight gain (Popelka et al. 2020).

Rhodococcus SM2 and *Kocuria* SM1 protected rainbow trout against *Vibrio anguillarum* infection through initiation of immune factors attributed to cell wall proteins and whole cell proteins, which led to increased respiratory burst, peroxidase and bacterial killing activities and increased leucocyte count (Sharifuzzaman et al. 2011).

Originally, it was considered that probiotics acted by producing inhibitory compounds in the digestive tract led to the prevention of diseases. Specifically, it was considered that there was colonization of the digestive tract by the probiotics, and in situ production of the inhibitory compounds; this was referred to as *Competitive Exclusion*. Also, probiotics were considered to scavenge for essential nutrients and compete for adhesion sites with potential pathogens. Studies with probiotics, i.e. *Enterococcus faecium, Bacillus subtilis* and *Bacillus licheniformis*, alone or in

combination when administered for 10-weeks enhanced feed conversion ratio and improved the intestinal microbiota and thus health status of rainbow trout. Research pointed to an increase in serum lysozyme activity and elevated leukocyte levels in rainbow trout (Merrifield et al. 2010).

Aeromonas sobria GC2 and *Bacillus subtilis* JB-1 probiotics protected rainbow trout against challenge with *Yersinia ruckeri* with the mode of action reflecting the whole cell proteins, outer membrane proteins, cell wall proteins/and or lipopolysaccharides (LPS) in the probiotics (Abbass et al. 2010).

2.3 *Atlantic Salmon (*Salmo salar *L.)*

Various dietary probiotics have been evaluated to see if they may modulate the composition and improve the status of the intestinal microbiota in Atlantic salmon. For example, bathing with *Aliivibrio* strains resulted in increased growth and survival and enhanced the feed conversion ratio (FCR) in Atlantic salmon post-smolts (Klakegg et al. 2020).

LABs were evaluated to determine their effect on the status of the lower intestinal microbial flora of Atlantic salmon by use of rRNA gene amplicon sequencing. The results showed that LAB supplementation shifted the intestinal microbial profile and increased the intestinal bacterial diversity (Gupta et al. 2019). Another study evaluated the dietary supplementation of *Pediococcus acidilactici* MA18/5 M (at 1.19×10^6 CFU/g) on the salmon gut bacterial communities. Here, results showed a profound effect on intestinal microbiota with significantly increased survival after pancreatic necrosis virus infection (Jaramillo-Torres et al. 2019).

Carnobacterium sp. isolated from Atlantic salmon intestine was antagonistic to *Aeromonas hydrophila, Flavobacterium psychrophilum, A. salmonicida, Photobacterium damselae*, *Vibrio ordalii* and *V. anguillarum* and was effective in reducing disease caused by *A. salmonicida, Y. ruckeri and V. ordalii but not V. anguillarum* (Robertson et al. 2000).

2.4 *Turbot (*Scophthalmus maximus *L.)*

Turbot is considered a valuable flatfish. However, production has been affected adversely because of bacterial diseases, notably vibriosis. The use of LAB achieved promising results as an effective alternative to chemotherapy with antimicrobial activity against a minimum of four strains of *V. splendidus.* LAB inhibited the attachment of turbot pathogens to the mucus. In particular, *Weissella cibaria* and *Leuconostoc mesenteroides* led to the best resistance against infections by *T. maritimum* and *V. splendidus*, reflecting the ability to reduce the linkage of turbot pathogens to mucus. Also, LAB stimulated the expression of non-specific immune response genes in mucosal tissues of turbot juveniles (Muñoz-Atienza et al. 2014).

2.5 *Olive Flounder (*Paralichthys olivaceus *Temminck)*

Olive flounder is a marine carnivorous finfish (Niu et al. 2019). *Bacillus* sp. SJ-10 and *Lactobacillus plantarum* were heat-killed and used as supplement in olive flounder diet leading to better plasma insulin, growth hormone (GH), insulin-like growth factor 1 (IGF-I), lysosome activity and intestinal health (Back et al. 2020). Juvenile olive flounders were supplemented with 10^8–10^9 CFU/kg of a *Lactobacillus* multi-strain probiotic (MSP) and showed lower lipid retention, better growth and nutrient utilization and higher myeloperoxidase, glutathione peroxidase and lysozyme activities. In addition, there was evidence of enhanced expression of immune-related genes involving IL-1β, IL-6 and TNF-α indicating that the product could be used an immunostimulant for olive flounder (Niu et al. 2019). Similarly, *Lactococcus lactis* WFLU12 was examined with regard to the intestinal and serum metabolome of olive flounder using capillary electrophoresis mass spectrometry with time of flight (CE-TOFMS). Results for the group receiving WFU12 showed that 53 out of 200 metabolites of the intestinal luminal metabolome and 5 from 171 metabolites of the serum metabolome were in higher concentrations compared to the controls. The outcome was better growth, health and feed utilization (Nguyen et al. 2018).

3 Temperate Water Finfish Species

3.1 *Caspian White Fish (*Rutilus frisii Kutum *Kamenskii)*

Caspian white fish is an important fresh water species found in the Caspian Sea. The PrimaLac® probiotic was evaluated in fingerlings and revealed enhanced growth, immune responses and digestion. Thus, the product was regarded as beneficial when used as a dietary supplement for early stages of Caspian white fish (Mirghaed et al. 2018).

3.2 *Flathead Grey Mullet (*Mugil Cephalus *L.)*

Saccharomyces cerevisiae was used as a dietary supplement and evaluated for its effect on growth performance, biochemical activities and digestion. The outcome was that a dose of 5×10^6 yeast cells/g gave significantly improved growth parameters, protein efficiency and survival (Akbary 2020). *Lactobacillus* sp., which was isolated from the gut of grey mullet demonstrated good tolerance to various salt concentrations. This observation deserves more attention in order to assess use of the LAB as a probiotic in mariculture (Abdulla 2014). Use of another *Lactobacillus* sp. as a feed supplement revealed significantly higher growth and feed utilization leading to a reduction in the cost of fish feeds (El-Tawil et al. 2012). This theme of

improved growth was repeated with use of Biogen® when fed at 1 and 2 kg/tonne with data revealing decreased serum glucose and cholesterol levels and improved serum albumin and globulin concentrations. The indication was that the product enhanced general health and the immune status of fish (Elam 2004).

3.3 *Channel Catfish (*Ictalurus punctatus *Rafinesque)*

Bacillus velezensis AP193 was examined with regard to its effect on growth, water quality and gut health. In particular, the data revealed significant (40.4% or 32.6%) growth compared to the controls and improved water quality in the fish ponds as manifested by significant decrease in total phosphorus (19%), total nitrogen (43%) and nitrate (75%) (Thurlow et al. 2019). As a result of 6S rRNA gene sequencing and phenotyping, an organism which was isolated from channel catfish gut and identified as *Bacillus subtilis* was determined to be an efficient probiotic. Thus, the culture demonstrated broad inhibitory activity, a marked ability to produce amylase and protease as extracellular products, the ability to withstand stomach conditions, and was safe to use in channel catfish (Luo et al. 2014). Also, *Bacillus* AP79, AP143, AP193 and AB01, which were isolated from channel catfish intestine, showed promising results for controlling disease, increasing immune parameters and improving growth. Thus, there was a 12% decrease in mortality compared with controls when administered as feed supplements (Ran 2013). Some success resulted with administration of *Pediococcus* and *Enterococcus* either alone or in combination when added to commercial catfish diets. However, in contrast to other putative probiotics, use of these organisms did not lead to significant improvements in growth or enhancement of immune parameters (Shelby et al. 2007).

4 Warm Water Finfish Species

4.1 *Nile Tilapia (*Oreochromis niloticus *L.)*

Tilapia is the most common cultivated fish in freshwater aquaculture. However, there have been heavy losses resulting from disease, including those caused by *Streptococcus* spp. (Amal and Saad 2011). A study investigated the effects of dietary incorporation of *Bacillus cereus* NY5 and *Bacillus subtilis* on growth performance, intestinal condition and resistance to *Streptococcus agalactiae* in Nile tilapia. Here, the results showed that *B. cereus* NY5 and the mixture of *B. subtilis* and *B. cereus* NY5 at 1×10^8 CFU/g feed improved growth (weight gain and FCR) and disease resistance accompanied by overall improvement of the immune status of the gut, intestinal histology and microbial community in the intestinal tract (Xia et al. 2020a). Moreover, use of *Bacillus* spp. (*B. subtilis* and *B. licheniformis* spores) led to effects on

the physiological response of Nile tilapia whereby there were increased hematocrit values; red and white blood cells counts; decreased glucose, cortisol, alanine aminotransferase, pyruvate kinase, lactate dehydrogenase and aspartate aminotransferase activities, increased superoxide dismutase, myeloperoxidase and catalase activities; and upregulation of the expression of HSP-70 and hypoxia-inducible factor-1-alpha (Abarike et al. 2020). In another study, the effects of dietary *Saccharomyces cerevisiae* were investigated on growth performance, the immune response and disease resistance of juvenile Nile tilapia. The outcome was that use of the yeast enhanced feed utilization, growth performance and resistance to *Aeromonas hydrophila* infection (Dawood et al. 2020a). The individual and synergetic effects of *Lactobacillus rhamnosus* JCM1136 and *L. lactis* subsp. *lactis* JCM5805 were evaluated on the growth, intestinal health, intestinal morphology, immune response and disease resistance of juvenile Nile tilapia. Data revealed improvements in growth, feed utilization and gut health. In addition, there was increased expression of immune-related genes, namely TNF-α, IFN-γ, hsp70 and IL-1β (Xia et al. 2018). These beneficial actions result from modifications to the gut microbial structure and the control of tilapia immunity and growth (Xia et al. 2020b). Yet another publication has highlighted the value of *B. velezensis* TPS3N, *B. subtilis* TPS4 and *B. amyloliquefaciens* TPS17 in enhancing growth performance (final weight, specific growth rate [SGR], weight gain and FCR], antioxidant response (superoxide dismutase and catalase), improved immune and biochemical parameters (nitric oxide, immunoglobulin M, lysozyme) and digestive enzyme activity (lipase and trypsin). Moreover, there was upregulation of growth-related genes GHR-1 and IGF-1 and immune-related genes (TNF-α, TLR-2, IgM and C-LYZ). The overall outcome was the demonstration of significant protection against challenge with *S. agalactiae* (Kuebutornye et al. 2020). Ren et al. (2013) used *L. plantarum* to protect tilapia against challenge with *A. hydrophila;* promising results were obtained which were attributed to competition for adhesion sites along with improved immunity. Finally, it should be mentioned that the yeast, *Aspergillus oryzae* (dosed at 1×10^6 cells/g) improved oxidative status and gene expression of HSP in Nile tilapia under hypoxia conditions (Dawood et al. 2020b).

*4.2 Major Carp/rohu (*Labeo rohita *Hamilton)*

Rohu is one of the three major carp species, representing ~70% of Indian aquaculture production. The fish faces serious challenges with bacterial infections, mainly the septicaemic diseases caused by *Aeromonas hydrophila.* The pathogen is capable of gaining entry through abrasions leading to the development of haemorrhages, ulcers, exophthalmia and abdominal distension (Giri et al. 2012). Thus, much emphasis has been placed on researching control measures as alternatives to the use of chemicals.

Dietary supplementation with *Pseudomonas aeruginosa* VSG-2 for 60 *days* led to enhancement of immune systems and protection against challenge with *A. hydrophila.* Here, three experimental diets included *P. aeruginosa* VSG-2 at 10^5, 10^7 and 10^9 ^{CFU}/g with the results—notably for the 10^7 dose—revealing markedly

enhanced serum lysozyme, phagocytosis, respiratory burst activity and superoxide dismutase (SOD) and increased serum IgM levels and alternative complement pathway (ACP) activities. The results were accompanied by increased survival rates against challenge with *A. hydrophila* (Giri et al. 2012).

Encapsulation of probiotics, i.e. (*Geotrichum candidum* QAUGC01, was used to improve the growth performance and immune response of fingerlings reared in earthen ponds in a semi-intensive culture system. Here, the results revealed significant ($P < 0.05$) improvement of growth rate, intestinal enzyme activities (protease, amylase and cellulase), haematological indices (red blood cells [RBC], haemoglobin [Hb], haemocrit [HCT], white blood cells [WBC] and mean corpuscular haemoglobin concentration [MCHC]), immunological parameters (respiratory burst, phagocytic and lysozyme activities and IgM levels), upregulation of heat shock protein HSP 70 gene and decrease in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and total cholesterol and triglyceride levels (Amir et al. 2019).

*4.3 Gilthead Seabream (*Sparus aurata *L.)*

The value of two probiotics belonging to the family *Vibrionaceae,* i.e. Pdp11 and 51M6, was assessed with respect to their effect on the immune response. The results revealed a significant improvement of serum peroxidase, complement, phagocytic and cytotoxic activities (Díaz-Rosales et al. 2006). Furthermore, using *Shewanella putrefaciens* Pdp11, which was isolated from seabream skin, and *Bacillus* sp. as feed supplements resulted in upregulation of antioxidant genes and protection against intracellular reactive oxygen species (ROS) (Esteban et al. 2014). Dietary administration of Pdp11 led to significantly decreased serum IgM levels and peroxidase activity, but the upregulation of INFγ and IL-1β genes and growth stimulation (Guzmán-Villanueva et al. 2014). It is noteworthy that use of *Bacillus subtilis* led to the significant upregulation of proinflammatory genes, interleukin-8 (IL-8), caspase-1 (CASP-1) and cyclooxygenase-2 (COX-2) genes (Cerezuela et al. 2013).

*4.4 European Sea Bass (*Dicentrarchus labrax *L.)*

European sea bass is a marine fish that is recognized for its high value, but faces great challenges from disease. Dietary administration of *Lactobacillus delbrueckii* led to decreased cortisol level, improved IGF-I expression and enhanced body weight (Carnevali et al. 2006). Administration of *Vibrio lentus* revealed upregulation in gene expression related to cell proliferation, cell adhesion, ROS, iron transport and immune functions (Schaeck et al. 2017). A similar theme occurred with feeding *Pediococcus acidilactici,* which led to improved growth, regulation of immune genes IL1β and COX-2 and disease resistance (Torrecillas et al. 2018).

4.5 *Asian Seabass (*Lates calcarifer *Bloch)*

Asian seabass is an economically significant euryhaline fish in the western Pacific region and highly suffers losses to production from infections particularly with *Vibrio* spp., *V. harveyi* and *V. alginolyticus* (Mohamad et al. 2019). Success with disease control occurred using probiotics associated with quorum quenching (QQ), and included *Bacillus thuringiensis* QQ1 and *Bacillus cereus* QQ2. Feeding with these cultures led to significantly improved growth performance, enhance digestive enzyme activity (i.e. amylase, lipase, trypsin and alkaline phosphatase), Hct and respiratory burst activity and reduced serum total cholesterol (Ghanei-Motlagh et al. 2021). In addition, Ashouri et al. (2018) reported that *P. acidilactici* was an effective feed additive and immunostimulant in juveniles fish.

4.6 *Common Carp (*Cyprinus carpio *L.*)

The viral disease spring viraemia of carp (SVC) is a highly contagious and lethal disease, especially of common carp resulting in severe economic losses. The disease is endemic in Europe, America and in areas of Asia. Moreover, chitosan-alginate encapsulated probiotic *Lactobacillus plantarum* expressing the viral G protein administered orally induced antigen-specific immune responses and provided protection against SVC (Jia et al. 2020). Use of *Lactobacillus fermentum* as an oral probiotic led to improved weight gain, SGR and haematological parameters, increased antioxidant enzymes and lysozyme and serum respiratory burst activities and better survival against challenge with *A. hydrophila* (Ahmadifar et al. 2019). Similarly, *Lactobacillus plantarum* led to increased levels of γ-globulins and total protein, enhanced B-lymphocytes and phagocytic activity, especially the potential killing activity of head kidney cells, and there was greater resistance to challenge with *A. hydrophila* (Kazuń et al. 2018). Again, research with a probiotic culture of *Lactobacillus plantarum* generated data revealing increased growth, Hct, Hb, respiratory burst activity, complement, lysozyme and serum bacteriocidal activities, ALT, AST, alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) levels and better resistance to motile *Aeromonas* septicaemia caused by *A. hydrophila* (Soltani et al. 2017). As a further example, feeding with a LAB culture resulted in enhanced growth and immunomodulatory properties, improved survivability under acidic conditions (pH 2.5) and increased expression of cytokines and resistance to challenge with *A. hydrophila* (Feng et al. 2019).

Bacillus coagulans was examined for a role as a dietary probiotic on growth performance, survival immune response, with results revealing that recipient carp had significantly higher ($P < 0.05$) daily weight gain, final weight, rate of weight gain, lysozyme, myeloperoxidase and respiratory burst activities compared with the controls (Xu et al. 2014).

Probiotic *Enterococcus faecium* MC13, which was isolated from grey mullet (*Mugil cephalus*) intestine, led to commendable protection against challenge with *A. hydrophila* (Gopalakannan and Arul 2011).

5 Use of Probiotics in Ornamental Fish Species

Ornamental fish production has been steadily increasing to meet the worldwide market demands. With this increasing production, the problem of infectious diseases has been exacerbated, and effective control measures are actively sought. The possibility of using probiotics orally has been addressed, and there is every possibility that the approach is useful for improving growth and immunity of ornamental fish (Prang 2008).

*5.1 Angelfish (*Pterophyllum scalare*, Schultze)*

Angelfish is a very important ornamental fish because of its attractive shape and colouration. The species is in high demand in many countries and has been the subject of interest for the development of probiotics (Prang 2008). *Bacillus* B1, B2 and B, which were isolated from the digestive tract of angelfish and applied orally, were completely effective at protecting against challenge by *A. hydrophila.* Compared to controls which did not receive probiotics, all the experimental group survived challenge with almost no clinical signs of disease (Monroy-Dosta et al. 2010). Other research has sought to enrich *Artemia* nauplii, which are used to fed angelfish, with probiotics. Using this approach, *Artemia* enriched with *Pediococcus acidilactici* led to improved growth and immunity, i.e. lysozyme and protease activities, and total immunoglobulin, and resistance to stress, namely cold temperatures and salinity (Azimirad et al. 2016). In parallel, a commercial LAB probiotic improved reproductive performance, intestinal health and survival (Sousa et al. 2020). Also, five strains of *Enterococcus faecium* improved growth performance and survival from challenge with *A. hydrophila* (Dias et al. 2019).

5.2 Molly (Poecilia sphenops Valenciennes*)*

(Black) mollies are popular ornamental fish that are noted for their attractive colours and ease of maintenance within home aquaria. The fish are comparatively easy to breed (Dernekbasi et al. 2010). Vibact, which is a commercial probiotic, led to significantly enhanced fecundity, fry survival and gonado somatic index (GSI) (Chitra and Krishnaveni 2013). Also, *Lactobacillus delbrueckii* LABT1 probiotic supplement has been evaluated for mollies with data pointing to improved growth and

excellent (100%) survival in recirculating aquaculture systems (Selvaraj and Bogar 2019). Moreover, *Artemia* supplemented with 2-β-mercapto-ethanol-treated yeast, *Saccharomyces cerevisiae,* has been examined for growth and reproductive performance, lysozyme activity and resistance of challenge with *A. hydrophila* with results demonstrating improved reproductive indices, immune responses and survival after challenge (Aminlooi et al. 2019).

6 Miscellaneous Finfish Categories

*6.1 Zebrafish (*Danio rerio *Hamilton)*

Zebrafish have become widely adopted as the standard fish model that may be used for many scientific purposes including studying host-microbe-immune interactions. The attraction is that there are many and diverse research tools available for the species; its physical transparency allows in vivo imaging of specific cell populations (López Nadal et al. 2020). Therefore, it is not surprising that zebrafish have been used for the study of probiotics. *Lactobacillus rhamnosus* has been investigated with respect to the study of zebrafish fecundity with data revealing modulation of the gene expression of neuropeptide hormones and enhanced fecundity (higher number of ovulated eggs, increase in oocyte maturation). This was accompanied by increased transcription of genes coding for signals, which induce maturation, i.e. *lhcgr, cbr1l* and *paqr8* genes. Thus, there are indication that the approach could be used as a new technology to improve reproduction in fish (Gioacchini et al. 2010). In terms of a more conventional understanding of probiotics, use of *Lactobacillus rhamnosus* IMC 501® led to enhanced innate immune responses and improved hepatic stress tolerance (Gioacchini et al. 2014).

7 Conclusion

A diverse range of probiotics has been evaluated for use with finfish with data pointing to benefits with growth, immunomodulation and resistance to infectious diseases. However, there is only little information on the effect of probiotic cultures on different finfish species in culture conditions. Researchers have tended to study putative probiotics in single fish species; therefore, it is difficult to know if there would be equal effectiveness in other aquatic animal species, between freshwater and marine, and from tropical to cold water environments. Nevertheless, probiotics have emerged as effective oral agents of disease control.

8 Suggestions for Further Work

- More work is needed to clarify the nature of effective doses. Specifically, what is the optimum number of cells of each probiotic to be used, and how long should they be administered?
- How long does the benefit remain after the cessation of administration of the probiotics?
- What is the effective life of the probiotic? Is there any deterioration in the activity of laboratory or commercial preparations?
- What is the ideal way of applying probiotics to feed? Do saline suspensions of cells suffice or is it necessary to include binders, such as alginates or oils?
- What is the shelf life of the supplemented diets? Should probiotics be applied to feed directly before use on the farm? Do probiotics survive during the feed manufacturing process?

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Use of Probiotics in Shellfish

S. M. Sharifuzzaman, Chaminda N. Walpita, and Md. Tawheed Hasan

Abstract A diverse range of microorganisms including Gram-positive and Gram-negative bacteria, which have been often recovered from aquatic animals and their environment, have been evaluated as probiotics in shellfish culture. Research has examined the use of single and multiple bacterial cultures alone, and in combination with prebiotics, plants products or other functional ingredients. Probiotics have been applied continuously or a short pulses, and found to promote water quality and reduce bacterial load, notably of *Vibrio* spp. Often recipients fare better in terms of improved survival, increased tolerance/resistance to stress and disease, higher growth, and faster rate of metamorphosis. Some probiotics have been commercialized and are available to the shellfish industry particularly in the Far East.

Keywords Shellfish · Water quality · Growth · Survival · Disease resistance · Metamorphosis · Commercial products

1 Introduction

Since the end of World War 2, disease control in aquaculture focused initially on the use of antimicrobial compounds/antibiotics until concerns about the development and spread of resistance and tissue residues prompted large-scale reduction in usage. With finfish aquaculture, vaccines achieved widespread attention until the current diversification to probiotics, prebiotics, nonspecific immunostimulants, and medical

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plant products. True, there has been some research on vaccines for use in shrimp aquaculture with commercial products, for example, Vibriomax which was developed for pathogenic vibrios (Amatul-Samahah et al. 2020). However, probiotics have garnered widespread attention. Certainly, Ecuador has a long history of using probiotics, including *Vibrio alginolyticus* with effectiveness against white spot syndrome virus (WSSV) (Rodríguez et al. 2007, 2011) and *Bolitas nigricans* (Vandenberghe et al. 1999). Moreover, there is anecdotal evidence suggesting a concomitant >90% reduction in the need for antibiotics. Here, the approach progressed from artisanal, whereby probiotics were isolated and cultured on individual shrimp farms (Rodríguez et al. 2007) to the availability of commercial products, e.g., Sanolife® MIC and PrimaLac® (Decamp et al. 2008; Miandare et al. 2016). Clearly, from these beginnings, probiotics have developed into potent disease control agents for aquatic invertebrate culture in multiple countries, especially in Asia and South America.

In shellfish aquaculture, different types of microorganisms have been administered as probiotics, consisting mainly of bacteria belonging to the genera *Bacillus* and *Lactobacillus* (*Lb*). Other bacteria and some strains of yeast also have probiotic properties (Table 1) (Sajeevan et al. 2006; van Hai and Fotedar 2010; Ringø 2020). These microorganisms are generally obtained from their natural environments, including culture water (such as farm pond, rearing tank), hepatopancreas, or intestine of different aquatic species and sediment. For example, members of the lactic acid bacteria (LAB; *Lactococcus*, *Lactobacillus,* and *Pediococcus*) *Bacillus* and *Vibrio* are commonly isolated from the intestinal tract of penaeid shrimp, freshwater prawns, crabs, and bivalves (Balcázar et al. 2007; Talpur et al. 2013; Maeda et al. 2014; Wu et al. 2014; Adel et al. 2017a; Gao et al. 2018; Wee et al. 2018; Zuo et al. 2019), whereas *Arthrobacter*, *Pseudoalteromonas*, *Pseudomonas,* and *Streptomyces* are dominant in marine water and sediment (Vijayan et al. 2006; Das et al. 2010; Aftabuddin et al. 2013; Xue et al. 2016; Sorieul et al. 2018). In addition, strains sourced from guts of fish (*Lb. plantarum*; Valipour et al. 2019) and chicken (*Lb. acidophilus*, *Lb. bulgaricus*, *Lb. casei*, *Lb. casei* subsp. *tolerans,* and *Lb. jensenii*; Phianphak et al. 1999), culture collections (such as *C. butyricum*; Li et al. 2019a), fermented soybeans (such as *B. subtilis*; Liu et al. 2010) and homemade curd (*Lb. acidophilus*; Sivakumar et al. 2012) were beneficial to shrimp. Among the probiotics belong to yeasts, *Debaryomyces hansenii*, *Rhodosporidium paludigenum,* and *Rhodotorula* sp. were isolated from the coastal waters and shrimp ponds (Yang et al. 2010; Nimrat et al. 2011). In general, intestinal non-pathogenic commensal bacteria are considered as the most suitable candidates for probiotics.

2 Probiotic Microorganisms

Probiotic can be composed of single-strain (such as *Lactococcus lactis*; Maeda et al. 2014), multi-strain/species mixture (such as combination of *Bacillus licheniformis*, *B. megaterium*, *B. polymyxa*, *B. subtilis*, *B. thuringiensis*, *D.hansenii,* and *Rhodotorula*; Nimrat et al. 2011) or can be combined with prebiotics, plants products, or other

Table 1 Probiotics used in shellfish aquaculture

Bacteria			Other microorganism
Lactic acid bacteria	*Bacillus* species	Others	
Enterococcus faecalis	*Bacillus* sp.	*Aeromonas media*	Yeast: *Candida parapsilosis*, *Can. sake*, *Debaryomyces hansenii*, *Rhodosporidium*, *Rh. paludigenum*, *Rhodotorula*, *Saccharomyces cerevisiae*
Ent. faecium	*B. aquimaris*	*Alteromonas* sp.	
Lactobacillus sp.	*B. aryabhattai*	*Alt. macleodii*	
Lb. acidophilus	*B. cereus*	*Arthrobacter* sp.	
Lb. brevis	*B. circulans*	*Art. enclensis*	
Lb. bulgaricus	*B. coagulans*	*Bifidobacterium* sp.	
Lb. casei	*B. flexus*	*Bifi. bifidum*	
Lb. casei subsp. *tolerans*	*B. fusiformis*	*Bifi. thermophilum*	
Lb. fermentum	*B. licheniformis*	*Clostridium butyricum*	
Lb. jensenii	*B. megaterium*	*Microbacterium aquimaris*	
Lb. pentosus	*B. mesentericus*	*Neptunomonas* 0536	
Lb. plantarum	*B. polymyxa*	*Paenibacillus* sp.	
Lb. rhamnosus	*B. pumilus*	*Phaeobacter* sp.	

(continued)

Table 1 (continued)

Bacteria			Other microorganism
Lactic acid bacteria	*Bacillus* species	Others	
Lb. salivarius	*B. pumilus*	*P. gallaeciensis*	
Lb. sporogenes	*B. stratosphericus*	*P. daeponensis*	
Lactococcus garvieae	*B. subtilis*	*P. inhibens*	
Lac. lactis	*B. thuringiensis*	*Pseudoalteromonas* sp.	
Pediococcus acidilactici	*B. vireti*	*Pseudomonas* sp.	
Streptococcus cremoris		*Ps. aeruginosa*	
Str. phocae		*Ps. aestumarina*	
		Ps. synxantha	
		Roseobacter	
		R. gallaeciensis	
		Streptomyces sp.	
		Stm. rubrolavendulae	
		Vibrio sp.	
		V. alginolyticus	

functional ingredients [such as *Bacillus* and isomalto-oligosaccharides (Li et al. 2009); mixture of *Pediococcus parvulus*, *Candida parapsilosis* and antiviral plants *Echinacea purpurea*, *Uncaria tomentosa* (Peraza-Gómez et al. 2014)]. Commercial products are available in liquid or powder forms. The liquid preparation contains live cultures and is readily useable. In contrast, probiotics powder requires to be germinated before application and generally contains cells at high density. There are also microencapsulated probiotics and other forms.

As a prophylactic agent, probiotics require to administer before disease outbreaks or developing any undesirable bio-physicochemical condition in the culture system. There are several ways to introduce probiotics, for example, (i) applying directly into the rearing water, (ii) supplementation with artificial feed, and (iii) through bioencapsulation, i.e., enrichment of live feeds brine shrimp nauplii or rotifers with probiotics (Farzanfar 2006; Sharifuzzaman and Austin 2017; Ringø 2020). The infeed route, where probiotics are added to the inert diet, is a more practical method than others for growing shellfish, whereas bioencapsulated probiotics are preferable for larviculture. Overall, dietary supplementation (oral administration) is advantageous in conveying probiotics into culture organisms at any stage of rearing (Verschuere et al. 2000; Hai 2015).

The level of exposure, i.e., dose, frequency, and duration of administration are important variables influencing the efficacy of probiotics. To realize the expected benefits, it is necessary to administer probiotics in adequate amounts, ranging 10^5–10^9 colony-forming units (CFU)/g feed or 10^5–10^6 CFU/mL as water additives. Daily application is recommended as probiotic cultures which do not colonize the gut or rearing system spontaneously (Guo et al. 2009; Skjermo et al. 2015). There is also evidence of pulse-administration, i.e., using probiotics at regular intervals, for example, alternating between 2 weeks probiotics and 1 week control diets in Pacific white shrimp, *Litopenaeus vannamei* (Kesselring et al. 2019). The duration of application varies (ranging from 7 to 100 days; Rengpipat et al. 2003; Liu et al. 2010; Maeda et al. 2014; Talpur et al. 2013; Adel et al. 2017a, b; Wee et al. 2018) depending on their intended use. A mixture of multi-strain probiotics is better over a single-strain preparation, with the former being more active in wide-ranging environmental conditions and hosts (Ringø 2020).

3 Application in Larviculture

Most shellfish hatcheries use probiotics with the overall aim of reducing opportunistic pathogens, i.e., the predominantly vibrio population in culture water, improving/maintaining water quality, and decreasing the incidence of diseases, leading to higher survival of larvae. For example, use of a commercial probiotic product, Sanolife®MIC, i.e., a mixture of *Bacillus* strains which are able to inhibit pathogenic vibrios, grows in hatchery rearing conditions and degrades waste products, was beneficial to black tiger shrimp (*Penaeus monodon*) and whiteleg shrimp (*L. vannamei*) in hatcheries in Asia and Latin-America (Decamp et al. 2008). After

germination, the product (1–5 × 10^4 CFU/mL) was immediately applied to the tanks daily and found to improve water quality and reduce *Vibrio* load in the water column.

When *Arthrobacter* sp. CW9 (at 10^5, 10^6, 10^7 CFU/mL) applied to *L. vannamei* breeding tank, the probiotic increased survival rate, growth rate and immune status (phenoloxidase activity, phagocytic activity and clearance efficiency) of larvae (Xia et al. 2014). In the same way, *B. subtilis* E20, added to the rearing water at 10^9 CFU/L for 14 days, found to improve larval development and survival rate during breeding of *L. vannamei* (Liu et al. 2010). Moreover, larvae had enhanced immune-related gene expressions (prophenoloxidase I, prophenoloxidase II, lysozyme) and tolerance to environmental stressors, such as low temperature, exposure of freshwater and 60 ppt salt water, and nitrite-N at 300 mg/L.

In a study with newly hatched larvae of giant river prawn (*Macrobrachium rosenbergii*), Keysami et al. (2007) investigated the effect of *B. subtilis* as a dietary supplement. The authors reported higher survival, growth, and faster rate of metamorphosis when larvae were fed enriched *Artemia salina* nauplii with *B. subtilis* for 40 days. Talpur et al. (2013) introduced *Lb. rhamnosus* into water daily at doses of 1.0 × 10^6, 5.0 × 10^6, and 1.0 × 10^7 CFU/mL for the larviculture of swimming crab (*Portunus pelagicus*). All the three treatments significantly increased survival rate of swimming crab larvae, with the highest survival conferred by the dose 5.0 × 10^6 CFU/mL. Moreover, probiotics positively affected activities of protease and amylase enzymes, pH, nitrogen content, and bacterial load in rearing water. In larviculture, probiotics may promote larval development (metamorphosis) and growth, and provide essential nutrients. The bioencapsulation of probiotics into live feed (e.g., brine shrimp = *Artemia*) is considered as an ideal route for introducing probiotics to the larvae, whereas delivery through rearing water is also recommended and practiced widely.

4 Use in Larval and Juvenile Rearing

Many studies have demonstrated positive effects of probiotics in raising postlarvae and juvenile shellfish, primarily crustaceans and bivalve molluscs. The benefits included better larval growth, higher survival, increased tolerance/resistance to stress and disease, modulation of digestive enzymes and immunity, reduced pathogen load, and improvement of water quality.

4.1 Black Tiger Shrimp

Supplementation of *Bacillus* S11 as fresh cells, fresh cells in normal saline solution and a lyophilized form in the diets of black tiger shrimp, *P. monodon* for 100 days led to improved growth and survival following experimental challenge with *V. harveyi* (Rengpipat et al. 1998). Similar results were reported for dietary *Streptococcus phocae* P180, which resulted in significantly improved growth and

protection against *V. harveyi* although the probiotic did not protect *P. monodon* postlarvae when challenged with *V. parahaemolyticus* (Swain et al. 2009). In another study with tiger shrimp, Vaseeharan and Ramasamy (2003) revealed enhanced resistance against *V. harveyi* following bath treatment of postlarvae with cell-free extracts of *B. subtilis* BT23 at 10^6–10^8 CFU/mL.

A probiotic bacterium, *B. cereus*, which was isolated from the gut of wild *P. monodon*, was able to enhance the immune status (phenoloxidase, lysozyme, respiratory burst, bacteriocidal activity) in tiger shrimp (Navin Chandran et al. 2014). Moreover, the immunomodulatory effect of *B. cereus* led to increased survival against *V. harveyi*. In contrast, *Pseudomonas* PM 11 and *V. fluvialis* PM 17 added into culture water (at 10^3 CFU/mL) did not positively affect the immune system of tiger shrimp (Alavandi et al. 2004).

Tiger shrimp postlarvae (PL30) fed with mixed strains of *Bacillus* (*B. licheniformis*, *B. polymyxa*, *B. megaterium*, *B. subtilis,* and *B. thuringiensis*), either as live-sprayed *s* or freeze-dried preparations, led to higher specific growth rate, feed efficiency, and *Bacillus* counts in the hepatopancreas and intestines (vs. lower in culture water) with significantly lower number of *Vibrio* in the system compared to those in a control group (Boonthai et al. 2011). When supplemented infeed for 30 days, *Lb. acidophilus* 04 (Sivakumar et al. 2012) and *Lb.* AMET1506 (Karthik et al. 2014) improved growth parameters (body weight, weight gain, specific growth rate) and feed utilization in *P. monodon*. Probiotic *Lb. acidophilus* 04 increased *Lactobacillus* count in culture water, and lessened *Vibrio* numbers in the gastrointestinal tract. Conversely, AMET1506 improved both *Vibrio* and *Lactobacillus* numbers, and reduced *Escherichia coli* and total heterotrophic bacteria. The use of probiotics and their effects on postlarvae and juvenile tiger shrimp is summarized in Table 2.

4.2 Whiteleg Shrimp

A combined feeding of photosynthetic bacteria and *Bacillus* sp. for 28 *days* led to increased weight gain and digestive enzyme activities (amylase, protease, lipase, and cellulase) in whiteleg shrimp, *L. vannamei* (Wang 2007). Likewise, *B. fusiformis* at 10^5 CFU/mL, added daily or every other day, increased survival of whiteleg shrimp larvae (zoea 1) when reared until postlarva 1 (PL1) (Guo et al. 2009).

A 1:1 mixture of *B. subtilis* and *B. licheniformis* was capable of improving growth, feed utilization, survival, hemato-biochemical parameters (glucose and cortisol) and immunological parameters (serum lysozyme, total hemocyte count) in *L. vannamei* postlarvae (Madani et al. 2018). Moreover, mixed *B. subtilis* L10 and *B. subtilis* G1, either as dietary or water supplements, promoted growth, survival, digestive enzyme activities, immune-related gene expressions and protection against *V. harveyi* (Zokaeifar et al. 2012, 2014). Furthermore, improved water quality parameters and feed conversion ratio were observed only when those probiotics were added into water. In a 21-day feeding trial, dietary *B. licheniformis*, *B. flexus,* and *B. licheniformis* + *B. flexus* at a dose of 2×10^9 CFU/g elevated growth, immune parameters,

Table 2 Effects of probiotics on *Penaeus monodon* larvae and juveniles

Probiotics	Dose/duration	Effects	Reference
[2]*Bacillus* S11	Dietary; 10^{10} CFU/g; 100 days	↑ Growth, *Bacillus* S11, protection against *V. harveyi*	Rengpipat et al. (1998)
[2]*Bacillus* sp. JL47	Dietary; 4 g wet bacterial weight/kg; 30 days	↑ Growth, survival, protection against *V. campbellii* → Ammonia stress	Laranja et al. (2014)
[1]*B. cereus*	Dietary; 0.4/100 g; 90 days	↑ Survival, growth, SGR, immunological parameters → Water quality parameters	NavinChandran et al. (2014)
[1]*B. subtilis* BT23	Water; 10^6–10^8 CFU/mL; 6 days	↑ Protection against *V. harveyi*	Vaseeharan and Ramasamy (2003)
[3]*C. butyricum*	Dietary; 10^9 CFU/g (1–2%); 56 days	↑ Growth, intestine digestive enzyme, resistance against nitrite stress	Duan et al. (2019)
[1]*Ent. faecium* MC13	Dietary; 10^7 CFU/mL; duration N/A	↑ Protection against *V. harveyi*, *V. parahaemolyticus*	Swain et al. (2009)
[2]*Paenibacillus* sp.	$10^{4,5}$ CFU/mL; 1 day	↑ Protection against *V. harveyi*	Ravi et al. (2007)
[1]*Str. phocae* PI80	Dietary; 10^7 CFU/mL; duration N/A	↑ Growth, protection against *V. harveyi*	Swain et al. (2009)
[2]*Stm. fradiae*	Dietary and water; 10^9 cells/g, 10^9 cells/mL; 60 days	↑ Growth performance, FCR ↓ THB, *Vibrio* counts	Aftabuddin et al. (2013)

(continued)

Table 2 (continued)

Probiotics	Dose/duration	Effects	Reference
[1]*B. subtilis*, *Enterococcus* sp.	Dietary; 10^{10} CF/mL (3 mL/kg); 84 days	↑ Weight, survival, trypsin activity, protection against *V. harveyi*	Nimrat et al. (2012)
[1]*Pseudomonas* sp., *V. fluvialis*	Water; 10^3 CFU/mL; 45 days	↓ Hemocyte counts, phenol oxidase, antibacterial activity	Alavandi et al. (2004)
[1,2]*B. licheniformis*, *B. megaterium*, *B. polymyxa*, *B. subtilis*, *B. thuringiensis*	Dietary; 9.98 log CFU/mL, 9.29 log CFU/g; 120 days	↑ SGR, feed efficiency, *Bacillus* numbers → Culturable bacteria counts ↓ *Vibrio* counts	Boonthai et al. (2011)

Symbol: (↑) positive effect; (↓) negative effect; (→) no effect.
Source: 1 = from host; 2 = from environment; 3 = commercial product.
B. (*Bacillus*); *C.* (*Clostridium*); *Ent.* (*Enterococcus*); *Str.* (*Streptococcus*); *Stm.* (*Streptomyces*); *V.* (*Vibrio*); FCR (feed conversion ratio); SGR (specific growth rate); THB (total heterotrophic bacteria); N/A (not available)

survival, digestive enzyme activities, water quality parameters, tolerance to stress and protection against *V. harveyi* (Cai et al. 2019). Also, Wang et al. (2019) evaluated comparative effects between single and mixtures of probiotics (*Lb. pentosus* BD6, *Lb. fermentum* LW2, *B. subtilis* E20, and *S. cerevisiae* P13) and concluded that a mixture at 10^8 CFU/kg improved growth, feed utilization, immune parameters, and resistance against *V. alginolyticus* challenge compared to single strains and the control.

Juvenile whiteleg shrimp naturally infected with WSSV (white spot syndrome virus) and IHHNV (infectious hypodermal and haematopoietic necrosis virus) was fed with multi-strain probiotics (*B. licheniformis* MAt32, *B. subtilis* MAt43, and *B. subtilis* subsp. *subtilis* GAtB1) at doses of 10^6, 2×10^6, 4×10^6, and 6×10^6 CFU/g (Sánchez-Ortiz et al. 2016). The probiotic mixture reduced the prevalence of WSSV and IHHNV as well as enhancing specific growth rate and upregulated immune-related gene expressions (prophenoloxidase, LvToll1 gene, superoxide dismutase) in a dose-dependent manner, without any effect on weight gain and survival.

In other studies, *C. butyricum* CBG01 in its different forms, i.e., fermentation supernatant, live cells, cell-free extract, spray-dried spores, mixture of live cells and supernatant, modulated growth and feed utilization, immune parameters, gut structure (VH and intestinal wall thickness), and controlled vibriosis (Li et al. 2019a, b). Supernatant of the probiotic had no effect, whereas a mixture of live cells and supernatant showed the opposite effect. Moreover, dietary administration of *C. butyricum* (10^9 CFU/g) for 56 days not only led to a positive effect on specific growth rate, feed conversion ratio, digestive enzyme activities (amylase, protease, lipase) and immune-related gene expressions (Toll, HSP70) in *L. vannamei* but also enhanced epithelium height, scFA of intestine, and tolerance to ammonia stress at different concentrations (Duan et al. 2017). The use of other probiotics, such as commercial multi-strains product PrimaLac® (*Lab. acidophilus*, *Lab. casei*, *E. faecium,* and *B. bifidium*), *Pediococcus pentosaceus,* and their effects on postlarvae and juvenile whiteleg shrimp is summarized in Table 3.

4.3 Giant River Prawn

Venkat et al. (2004) studied the benefits of *Lb. acidophillus* and *Lb. sporogenes* supplementation in the diet (either infeed or through encapsulation into *Artemia*) of *M. rosenbergii* postlarvae. After 60 days feeding, the authors found that probiotics sustained a better growth in prawn, and that *Lb. sporogenes* encapsulated into live feed promoted significantly higher weight gain, feed efficiency, and protein gain. In a similar feeding period with probiotics used separately, *Lb. sporogenes*, *B. subtilis,* and yeast *S. cerevisiae* ($10–15 \times 10^7$ CFU at 3–4% of feed), Seenivasan et al. (2016), demonstrated improved survival, weight gain, specific growth rate, protein efficiency rate and digestive enzyme activities (such as protease and lipase) in prawn postlarvae (PL30). Notably, diet incorporated with 4% *S. cerevisiae* led to greater benefits including a lower feed conversion rate. Moreover, Kumar et al. (2013) observed

Table 3 Effects of probiotics on *Litopenaeus vannamei* postlarvae and juveniles

Probiotics	Dose/duration	Effects	Reference
[2]*Bacillus*	Dietary; $10^{7,9}$ CFU/g; 30 days	↑ Weight gain, survival, digestive enzymes, gut structure, immune status, protection against WSSV	Chai et al. (2016)
[1]*B. aquimaris*	Dietary; >3 × 10^6 CFU/g; 4 weeks	↑ Weight gain, phenoloxidase activity, astaxanthin level	Ngo et al. (2016)
[3]*B. aryabhattai*	Dietary; 10^8 CFU/g; 6 weeks	↑ Innate immunity, antioxidant activity, protection against *V. harveyi* ↓ *Vibrio* counts	Tepaamorndech et al. (2019)
[2]*B. subtilis*	Dietary; $10^{6,7,8}$ CFU/kg; 98 days	↑ Immune status, protection against *V. alginolyticus*	Tseng et al. (2009)
[1]*B. subtilis*	Dietary; 5 × 10^4CFU/g; 40 days	↑ Growth, immune function, antioxidant capacity	Shen et al. (2010)
[4]*C. butyricum*	Dietary; 10^9 CFU/g (0.25, 0.5, 1%); 56 days	↑ Growth, FCR, intestinal digestive and immune functions, intestinal structure, resistance to ammonia stress	Duan et al. (2017)
[1]*Lb. pentosus*	Dietary; 10^7 CFU/g; 28 days	↑ Growth, feed utilization, digestive enzyme activity, and protection against *V. campbellii*, *V. rotiferianus*, *V. vulnificus*	Zheng and Wang (2016)
[4]*Lb. plantarum*	Dietary; 10^9 CFU/mL; cell-free extract; 45 days	↑ Growth, survival rate, tolerance to acute low salinity	Zheng et al. (2017)
[1]*Lac. lactis* subsp. *lactis*	Dietary; 10^8 CFU/g; 8 weeks	↑ Growth, body protein, digestive enzyme activity, *Lactobacillus* and *Bacillus* counts, protection against *V. anguillarum* ↓ *Vibrio* counts	Adel et al. (2017a)
[5]*Pb. polymyxa*	Dietary; 10^8 CFU/g; 8 weeks	↑ Growth, FCR, immune and antioxidant activity, intestinal health, protection against *V. parahaemolyticus*	Amoah et al. (2019)
[1]*Pc. pentosaceus*	Dietary; 10^8 CFU/g; 8 weeks	↑ Growth, digestive enzyme activity, immunity, protection against *V. anguillarum*	Adel et al. (2017b)
[1]*Pseudoalteromonas*	Dietary; 10^7 CFU/kg; 21 days	↑ Protection against *V. parahaemolyticus* ↓ *Vibrio* counts in hindgut	Wang et al. (2018)

(continued)

Table 3 (continued)

Probiotics	Dose/duration	Effects	Reference
[1] *Ps. aestumarina*	Dietary; 10^5 CFU/g; 28 days	↑ Survival, FCR, protection against *V. parahaemolyticus*	Balcázar et al. (2007)
[1] *R. gallaeciensis*	Dietary; 10^5 CFU/g; 28 days	↑ Survival, FCR, protection against *V. parahaemolyticus*	Balcázar et al. (2007)
[3] *Rh. paludigenum*	Dietary; 10^8CFU/g; 42 days	↑ Weight gain, SGR, survival rate, antioxidant competence	Yang et al. (2010)
[1] *Sh. algae*	Dietary; 10^6 CFU/g; 60 days	↑ Final weight, protection against *V. parahaemolyticus*	Interaminense et al. (2019)
[3] *Streptomyces* N7, RL8	Dietary; 10^8CFU/g; 30 days	↑ Growth, hemocyte count, protection against *V. parahaemolyticus* ↓ *Vibrio* in hepatopancreas	Bernal et al. (2017)
[1] *V. alginolyticus*	Dietary; 10^5 CFU/g; 28 days	↑ FCR, protection against *V. parahaemolyticus*	Balcázar et al. (2007)
[1] *A. bivalvium*, *B. cereus*, *Sh. haliotis*; their mixture	Dietary; 10^7 CFU/g; 28 days	↑ Growth, innate immunity, protection against *V. harveyi*	Hao et al. (2014)
[3] *B. flexus*, *B. licheniformis*; *B. flexus* + *B. licheniformis*	Dietary; 2×10^9 CFU/g; 21 days	↑ Growth, innate immune and digestive enzyme activities, stress tolerance, water quality, protection against *V. harveyi*	Cai et al. (2019)
[2] *B. subtilis* L10 + *B. subtilis* G1	Water; $10^{5,8}$ CFU/mL; 8 weeks	↑ Water quality, SGR, FCR, digestive enzyme activity, immune response, protection against *V. harveyi*	Zokaeifar et al. (2014)
[4] *Lb. pentosus* + *Lb. fermentum* + *B. subtilis* + *S. cerevisiae*	Dietary; 10^8 CFU/kg; 56 days	↑ Growth, immune response, protection against *V. alginolyticus* → Carcass composition	Wang et al. (2019)
[4] PrimaLac® (*Lb. acidophilus*, *Lb. casei*, *Ent. faecium*, *Bifi. bifidium*)	Dietary; 0.5, 1.0 g/kg; 60 days	↑ Growth, FCR, body protein, digestive enzyme activity, immune gene expression ↓ Body lipid, body moisture	Miandare et al. (2016)

Symbol: (↑) positive effect; (↓) negative effect; (→) no effect
Source: 1 = from host; 2 = from other organism/product; 3 = from environment; 4 = commercial product; 5 = culture collection
A. (*Aeromonas*); *B.* (*Bacillus*); *Bifi.* (*Bifidobacterium*); *C.* (*Clostridium*); *Ent.* (*Enterococcus*); *Lb.* (*Lactobacillus*); *Lac.* (*Lactococcus*); *Pb.* (*Paenibacillus*); *Pc.* (*Pediococcus*); *Ps.* (*Pseudomonas*); *R.* (*Roseobacter*); *Rh.* (*Rhodosporidium*); *S.* (*Saccharomyces*); *Sh.* (*Shewanella*); *V.* (*Vibrio*); FCR (feed conversion ratio); SGR (specific growth rate); N/A (not available)

improvements in growth, immune response, number of total bacterial and *Bacillus* in the intestinal tract, and reduction of pathogens *Aeromonas* and *Pseudomonas*, including disease resistance against *V. alginolyticus* when juveniles of *M. rosenbergii* were fed *B. licheniformis* (1×10^9 CFU/g).

Likewise, host-associated *Bacillus* NL110 and *Vibrio* NE17 (either through feed, water, or both ways) not only had a positive influence on rearing water quality parameters (such as nitrate and ammonia) but also improved growth, survival, and immune parameters, such as total hemocyte count, phenoloxidase activity, and respiratory burst in prawn juveniles (Rahiman et al. 2010).

However, the inclusion of heat-killed *Lb. plantarum* (10^8 CFU/g) in diets of prawn juveniles for a period of 90 days was insignificant for enhancing growth and feed utilization parameters but sufficient enough to significantly improve immunity (viz., total hemocyte count, phenol oxidase activity, respiratory burst activity, hemolymph, and bacterial clearance efficiency) and disease resistance of *M. rosenbergii* against *Aeromonas hydrophila* (Dash et al. 2015). In addition, there are investigations on the use of other probiotics concerning growth, pathogen exclusion, immunomodulation, disease protection (*Vibrio*, *Aeromonas*), microbial modulation, and enzymatic activity in prawns (Table 4).

4.4 Bivalve Molluscs

Probiotics as means of vibriosis control were studied both through the diet and rearing water. Dietary supplementation of *Agarivorans albus* F1-UMA, *Vibrio* F15-UMA and *Vibrio* C21-UMA for seven months in red abalone, *Haliotis rufescens* (Silva-Aciares et al. 2013), and *S. colwelliana* WA64 and S. *olleyana* WA64 for 28 days in Japanese abalone; *H. discus* (Jiang et al. 2013) conferred an elevated protection against pathogens *V. parahaemolyticus* and *V. harveyi*. All of these probiotics increased total hemocyte count in abalone, whereas the former group increased phagocytic activity and wheat germ agglutinin cells, and the latter group elevated serum lysozyme and respiratory burst activity. Feeding probiotics (*A. albus* F1-UMA, *Vibrio* F15-UMA, and *Vibrio* sp. C21-UMA) for seven months not only improved growth but also increased transcription levels of caspase 8 and peptidyl-lys metalloendopeptidase genes.

Similarly, single *Lactobacillus* NS61 improved growth, and a mixture of *P. aeruginosa* YC58 and *Burkholderia cepacia* Y021 improved survival and immune parameters in cortez oyster (*Crassostrea corteziensis*) after 30 days feeding at 5×10^4 CFU/mL (Campa-Córdova et al. 2009). When eastern oyster (*C. virginica*) larvae and juveniles were treated with either *Phaeobacter* S4 or *B. pumilus* RI06-95 (Karim et al. 2013; Sohn et al. 2016a) separately, and in combination (*Phaeobacter* S4 + *B. pumilus* RI06-95) (Sohn et al. 2016b) at $10^{2,4,6}$ CFU/mL via water, both probiotics at 10^4 CFU/mL increased larval and juvenile survival after challenge with *V. tubiashii* and *Roseovarius crassostreae* (Karim et al. 2013) and *V. coralliilyticus* RE22 (Sohn et al. 2016a, b), respectively. Moreover, probiotics *Phaeobacter* S4 and

Table 4 Effects of probiotics on *Macrobrachium rosenbergii* postlarvae and juvenile

Probiotics	Dose/duration	Effects	Reference
[1]*B. cereus*	Dietary; 10^4 CFU/g; 28 days	↑ Growth, SOD activity, propionic acid level, hepatopancreatic condition → Protection against *A. hydrophila*	Wee et al. (2018)
[3]*B. coagulans*	Dietary; 10^9 CFU/g; 60 days	↑ Growth, feed utilization, lysozyme andrespiratory burst activities, digestive enzymes, protection against *V. harveyi*	Gupta et al. (2016)
[2]*B. pumilus*	Dietary; $10^{8,9}$ CFU/g; 60 days	↑ Growth, SGR, immunity, digestive enzyme activities	Zhao et al. (2019)
[1]*B. vireti*	Dietary; 10^8 CFU/mL; 14 days	↑ Antioxidant defense enzymes, protection against *Ps. aeruginosa*	Hindu et al. (2018)
[2]*C. butyricum*	Dietary; 2×10^7 CFU/g (500 mg/kg), for 5 days after 2 days of basal diet; 8 weeks	↑ Weight gain, FCR, antioxidant capacity, nonspecific immune response	Wangari et al. (2021)
[3]*Lb. cremoris*	Dietary; 8.5, 11.4×10^{11} CFU/100 g; 60 days	↑ Weight gain, SGR, FCR,	Suralikar and Sahu (2001)
[1]*Lac. lactis* LC-I, LC-II	Dietary; 10^8 CFU/g; 50 days	↑ Weight gain, SGR, FCR, immune response	Kader et al. (2021)
[1]*Pc. acidilactici* + [2]*S. cerevisiae* + [2]β-glucan	Dietary; 10^8 CFU/g; 60 days	↑ Growth, feed coefficiency, protease activity, immune gene expressions, protection against *A. hydrophila*	Miao et al. (2020)

Symbol: (↑) positive effect; (↓) negative effect; (→) no effect
Source: 1 = from host; 2 = commercial product; 3 = culture collection
A. (*Aeromonas*); *B.* (*Bacillus*); *C.* (*Clostridium*); *Lb.* (*Lactobacillus*); *Lac.* (*Lactococcus*); *Pc.* (*Pediococcus*); *Ps.* (*Pseudomonas*); *S.* (*Saccharomyces*); *V.* (*Vibrio*); FCR (feed conversion ratio); SGR (specific growth rate); SOD (superoxide dismutase)

B. pumilus RI06-95 were used with hard clam (*Mercenaria mercenaria*), bay scallop (*Argopecten irradians*), blue mussel (*Mytilus edulis*), and razor clam (*Ensis directus*) at 10^4 CFU/mL (Sohn et al. 2016b). After 24 h treatment with *Phaeobacter* S4 and *Phaeobacter* S4 + *B. pumilus* RI06-95, there was an increase resistance to infectious disease, i.e., *V. coralliilyticus*, in bay scallop. The daily addition of *B. pumilus* RI06-95 (10^4 CFU/mL) into rearing water significantly reduced *Vibrio* spp. in a hard clam hatchery but not in bay scallop. After challenge with the same pathogen, bay scallop treated with *Phaeobacter* S4 and *Phaeobacter* S4 + *B. pumilus* RI06-95 increased protection, but not for hard clam, suggesting a species-specific action of the probiotics.

4.5 Crab

There are only a few studies related to probiotics that evaluated immunity and disease resistance in crabs. Mud crab (*Scylla paramamosain*) fed with LAB (*E. faecalis* Y17 and *P. pentosaceus* G11; Yang et al. 2019) and *Bacillus* (*B. subtilis* DCU, *B. pumilus* BP, and *B. cereus* HL7; Wu et al. 2014) had elevated immune-related gene expressions (i.e., catalase, prophenoloxidase and superoxide dismutase) and demonstrated protection against vibriosis after immersion challenge with *V. parahaemolyticus*. In a separate study, *S. paramamosian* fed *B. subtilis* E20-containing diet ($10^{9–10}$ CFU/kg) had significantly increased phenoloxidase activity, phagocytic activity, and disease resistance compared with those reared with the control and mixed probiotics diet, *B. subtilis* E20 and *Lb. plantarum* 7–40 (Yeh et al. 2014).

4.6 Lobster

Feeding with bacteriocin producing probiotics, a single strain (*B. pumilus* B3.10.2B) and a three strain (*B. pumilus* B3.10.2B, *B. cereus* D9, *Lb. plantarum* T13), for 60 days resulted in increased growth, reduced feed conversion rate and increased survival against *V. owensii* in juvenile ornate spiny lobster, *Panulirus ornatus* (Nguyen et al. 2014). In addition, *Bacillus* spp. at 100 mg/L was administrated via *Artemia* nauplii in European lobster (*Homarus gammarus*) for 12 and 30 days (Daniels et al. 2010, 2013). After 7 days of a 12-day trial period, larvae had better growth and survival. Moreover, 30 days of supplementation led to increased *Bacillus* content and microvillous height density in the intestine leading to improved feed utilization. Conversely, use of *Bacillus* spp. (3.5×10^7 CFU/L) had no effect on weight gain and carapace length after 18 days supplementation as a water additive (Middlemiss et al. 2015). Dietary supplementation of that probiotic improved salinity stress in *H. gammarus* but not as a water additive. Instead of lowering opportunistic *Vibrio* concentration, *Bacillus* spp. favored an increase in opportunistic pathogens in the culture environment.

5 Use in Grow-Out Culture

Scientific data are scant on the use of probiotics in grow-out culture. Therefore, the benefit of using probiotics in grow-out ponds or open systems is poorly understood. Yet, a plethora of commercial products has been used for farming shrimp and prawn particularly in developing countries. Manufacturers claim about product quality, and the apparent success of those products may have motivated farmers to use the commercial probiotic products regularly, even in the absence of supporting scientific evidence. Members of the genera *Bacillus*, *Nitrobacter*, *Nitrosomonas*, *Pseudomonas*, *Rhodobacter*, *Rhodococcus*, *Saccharomyces,* and *Streptococcus* are commonly found in commercial products, and the intended actions of these probiotic microorganisms are wide ranging in shrimp farming (Table 5).

A study by Matias et al. (2002) examined the impact of different commercial microbial products on the water quality of *P. monodon* grow-out ponds. When a mixed *Bacillus* sp. and *Saccharomyces* sp. were applied to the pond water, there was relatively better water quality (lower concentrations of total nitrogen and ammonia) in the early culture phase and higher shrimp biomass compared to a mixture of *Bacillus* sp., *Nitrosomonas* sp., and *Nitrobacter* sp., and the control. In a similar context, Devaraja et al. (2002) noted high number of *Bacillus* spp. in pond water, and presumptive sulfur oxidizing bacteria in sediment, and better feed conversion ratio and shrimp production using a mixture of probiotics containing *Bacillus* sp. and *Saccharomyces* sp. Moreover, probiotic *Str. phocae* PI80, after enrichment during fermentation with molasses or glucose as extra carbon source plus yeast extract as nitrogen source, led to improve growth performance and feed utilization of *P. monodon* when supplemented through

Table 5 Probiotic microorganisms in commercial products used for shrimp farming in Sri Lanka and their intended benefits

Species/strains in commercial formulations	Intended actions
Acinetobactor calcoaceticus	Outcompete pathogens, mostly *Vibrio*
Aerobacter sp.	Increase appetite
Bacillus megaterium	Increase feed consumption, growth
B. licheniformis	Increase digestibility, feed conversion
B. subtilis	Degrade organic pollutants
B. mesentericus	Degrade bottom sludge
Clostridium butyricum	Reduce H_2S, toxic gasses
Nitrobacter spp.	Optimize water quality
Nitrosomonas spp.	Optimize soil parameters
Ochrobactrum anthropic	Enhance natural nitrification
Paenibacillus polymyxa	Oxidize ammonia
Pediococcus spp.	Reduce viscosity in water
Phodopseudomonas palustris	Inhibit pathogens in shrimp gut
Pseudomonas stutzeri	Reduce white gut syndrome
Rhodobacter spp.	Reduce white feces syndrome
Rhodococcus spp.	Maintain healthy hepatopancreas
Saccharomyces cerevisiae	Facilitate tissue repair, gut healing
Streptococcus faecalis	Probable immunostimulation

feed (6.5×10^{13} CFU/mL) and pond water (5 L/pond; 8000 m^2) over 120 days of culture period (Pattukumar et al. 2014). The probiotic not only enhanced immune response (total haemocytes count, phenoloxidase activity, intracellular superoxide anion and phagocytic activity) of shrimp but also reduced presumptive *Vibrio* and luminous bacterial counts in ponds. Furthermore, a commercial probiotic formulation containing *Bacillus*, *Pseudomonas*, *Nitrobacter,* and *Aerobacter* spp. used at 5-day intervals could effectively suppress total *Vibrio* counts in 1 ha shrimp ponds (Wijerathne and Walpita 2013). These data suggest that under pond conditions, some microbial products have the potential to enhance/improve the pond environment and shrimp yields.

Using a commercial probiotic mixture (*Bacillus* sp., *Nitrobacter* sp., *Nitrosomonas* sp., and *Saccharomyces cerevisiae*; 3, 5, 10 mg/L at different time intervals), Wang et al. (2005) revealed higher survival rate, feed conversion rate, and final production in treated *P. vannamei* ponds compared to the controls after 109 days. Also, probiotics increased population density of beneficial bacteria, i.e., *Bacillus* sp. and reduced concentrations of nitrogen, phosphorus, and the number of presumptive vibrios, thereby improved the quality of water. In blue shrimp *Litopenaeus stylirostris* (= *Penaeus stylirostris*), reared in floating cages (14 m^2 each) in earthen ponds, *dietary* probiotic *P. acidilactici* treatment for over 10 weeks was able to improve shrimp production due to increased survival rate and the final biomass (Castex et al. 2008). Moreover, shrimp fed with *P. acidilactici* resulted in lower feed conversion ratio, higher hepatosomatic index, and digestive gland weight enhanced α-amylase and trypsin activities, and lower incidence of 'Summer syndrome' caused by *Vibrio nigripulchritudo*. In the shrimp gut, total bacterial counts were lower, whereas the number of probiotic cells (10^4–10^5 CFU/g of gut) was higher for 4 h after feeding before decreasing until the next meal.

The usefulness of probiotics Zymetin (a mixture of *Streptococcus faecalis*, *C. butyricum*, *Bacillus mesentericus*, protease, lipase, and beer yeast) and Super PS (a mixture of *Rhodobacter* sp. and *Rhodococcus* sp.) was evaluated for growing *M. rosenbergii* (1.04 g) in ponds over a period of 8 months (Ghosh et al. 2016). Combined application of Zymetin and Super PS through feed and water, respectively, led to maximum growth and survival of prawns. The total production (35% higher) of prawns and feed conversion ratio was also improved. However, the result was less encouraging when Zymetin and Super PS were used separately. During pond culture of juvenile *M. rosenbergii* (2.56 g), Sumon et al. (2018) supplemented *Clostridium butyricum* (2×10^9 CFU/g) in diets and revealed beneficial effects on growth, digestive protease and amylase activities, and immune response (total haemocyte count) of prawn.

The application of probiotics in shrimp farming is recognized as a best management practice (BMP) for controlling white spot disease (WSD) and pathogenic *Vibrio*. Importantly, the rearing of specific pathogen-free (SPF) shrimp postlarvae leads to focusing more on critical BMPs during grow out. The near zero water exchange at least during the first 2–3 months may reduce the risk of diseases. Hence, the frequent need to use probiotics for pond bottom and water quality management has received due attention. Use of probiotics supplemented with a carbon source has led

to profound reductions of sludge and organic waste materials accumulating in pond bottoms while improving water quality parameters and maintaining healthy algal blooms during the culture cycle. The addition of sugar/molasses into the pond, preferably 2–3 days after probiotic application, may increase the time interval between two probiotic applications as the activity could confer better survival of the added probiotics under high levels of carbon sources. In general, by maintaining a healthy C:N ratio (close to 30–40), the interval of repeat application of probiotics in ponds may be increased.

6 Use in Broodstock Maturation and Reproduction

In recent years, the benefits of probiotics in fish reproduction related to gonadal development, sperm and embryo quality, oocyte maturation, ovulation, embryonic development, fecundity, hatching rate, and fry survival have been highlighted (Ghosh et al. 2007; Carnevali et al. 2013, 2017; Gioacchini et al. 2013; Nargesi et al. 2020). This suggests that probiotics may influence the induction of reproduction and spawning activities, but such information has not been so well studied in shellfish. The only available data are about the giant river prawn, *M. rosenbergii*. Thus, using commercial probiotics Sanolife® MIC (composed of *B. licheniformis*, *B. pumilus* and *B. subtilis*; dosed at ~5×10^4 CFU/mL), Barua et al. (2017) reported higher numbers of eggs and hatchlings and improved hatching rate in berried prawn. The length of time required to complete the embryonic development (13–14 vs. 14–15 days in control) was also reduced after administration of probiotics. This outcome may be explained by the nutritional effects of probiotics, which may be a source of proteins, fatty acids, biotin, and vitamin B12 (Irianto and Austin 2002; Balcázar et al. 2006; Vine et al. 2006; Merrifield and Ringø 2014). The association between nutrition and reproduction is well known due to the fact that all reproductive events are synchronized with the availability of nutrients to produce viable progeny (Scaramuzzi et al. 2006; Carnevali et al. 2013). Essentially, a balanced nutrition, modulation of gut microbiota, and reduced physiological stress after probiotic treatment may have caused a better stimulation for broodstock to mature and produce healthy eggs and larvae.

7 Probiotics in Biofloc Shrimp Culture

Biofloc technology is a promising method to promote efficient nutrient recycling and has become an economically viable and relatively inexpensive way for sustainable aquaculture. It is primarily a waste treatment system, whereby the toxic wastes and nutrients loaded into the culture water are converted into microbial proteins, which in turn are used as feed, and leads to improved feed utilization and growth of farmed species, better waste recycling, and lower water consumption due to minimum water exchange. In biofloc systems, heterotrophic bacterial growth is encouraged through

elevated carbon levels by addition of extra carbon through direct supplementation or by feeds. Thus, a rise in the carbon and nitrogen ratio (C:N) to 12–15 may stimulate the growth of heterotrophic bacteria (Crab et al. 2007, 2012; Hargreaves 2013; Xu et al. 2016; Liu et al. 2019). The bacterial composition has been regarded to mostly belong to the genera *Actinobacteria*, *Bacillus* (*B. cereus*, *B. licheniformis*, *B. subtilis*, *B. thuringiensis*), *Bacteroidetes*, *Cyanobacteria*, *Marinobacter goseongensis*, *Photobacterium*, *Planctomycetes*, *Proteus mirabilis*, *Rhodotorulla*, *Verrumibrobia,* and *Vibrio* (Ferreira et al. 2015; Kathia et al. 2017; Liu et al. 2019). Some of these organisms were recognized to be potential probiotics (see Table 1).

Bacterial isolates, namely *B. cereus*, *B. licheniformis,* and *B. thuringiensis,* from the biofloc of *L. vannamei* culture system were capable of reducing *Vibrio* counts in pond water. Moreover, *B. licheniformis* showed in vitro inhibition of pathogenic *V. alginolyticus*. These isolates were effective in improving immunological parameters of shrimp and important for growth, maintaining health, and culture conditions (Ferreira et al. 2015). Furthermore, supplementation of probiotics into the culture water of biofloc system alone or in combination with feed has been studied. Thus, probiotics in diets of *L. vannamei* under biofloc systems improved the homeostasis of shrimp and prevented outbreaks of vibrio disease (Aguilera-Rivera et al. 2014). Similarly, use of multi-strains of *Bacillus*, *Enterococcus*, *Thiobacillus,* and *Paracoccus* applied directly into the culture water and dietary *Bacillus*, *Enterococcus,* and *Lactobacillus* led to a higher growth and survival of *L. vannamei*. Also, the treatment resulted in better feed conversion although it was not clear whether the addition of probiotics into the water or to the feed or both contributed to the positive outcome (Krummenauer et al. 2014). However, addition of named probiotics or commercial formulations into the biofloc system for *L. vannamei* culture gave results with a different perspective. Commercial probiotics *B. licheniformis* and *B. subtilis* used for *L. vannamei* did not have any effect on bacteria, phytoplankton, water quality, or shrimp growth, suggesting that the existing microflora in the biofloc system are adequate for effective bioremediation and biocontrol (Ferreira et al. 2017). Likewise, application of commercial probiotics neither improves water quality nor growth of *L. vannamei*, and thus, the bioflocs in a zero-water exchange farm may be sufficient to maintain water quality and growth of shrimp (Arias-Moscoso et al. 2018).

Overall, biofloc technology serves as a robust microbial system in high-density aquaculture, promoting zero-water exchange facility (<1% water exchange in a well-managed system; Hargreaves 2013), better nutrient utilization, host immunostimulation, biosecurity and biocontrol of pathogenic microorganisms, and growth of cultured species (Crab et al. 2007, 2012; Hargreaves 2013; Ekasari et al. 2014; Kathia et al. 2017). The addition of probiotics into such systems as feed supplement has shown promising results although introduction into the culture water is less effective.

8 Conclusions and Suggestion for Further Work

Considering the unique role and as an alternative strategy to chemotherapy with antibiotics, the use of probiotics has a promising future with the intended development of the shellfish industry at the hands of SPF broodstock, larviculture and grow-out farming. There are potential probiotics, which control diseases in aquaculture, and development of such effective strains is highly desirable due to the fact that with a primitive immune system, shrimp is very susceptible to pathogens. Due to limited data from actual field trials, extensive evaluation of probiotics under different farming conditions and changing environments as well as in biofloc culture system is necessary to build concrete evidence on the efficacy and viability of probiotics. In addition, product quality of commercial preparations remains questionable as manufacturers often (but not always) exaggerate claims that merits further investigations. Nevertheless, being multifunctional microorganisms, probiotics can ensure both shellfish health and environmental management holistically.

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Probiotics for Controlling Infectious Diseases

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Abstract One of the activities of probiotics is their ability to control the onset of infectious diseases. The most common mechanism is the production of substances that inhibit microbial growth, including bacteriocins and organic acids. These substances are synthesised as a mechanism of competition for nutrients and adhesion sites. Although the range of bacteriocin-producing bacteria is broad, few putative probiotics are used in commercial aquaculture. This chapter reviews the latest research on pathogen-antagonistic microorganisms. After bacteriocidal activity, one of the most outstanding properties of probiotics is their ability to activate the immune response. The use of probiotics as a pathogen biocontrol mechanism is also compared with other strategies, such as the use of medicinal plants, immunostimulants and vaccines. Despite the existence of a great diversity of microorganisms with probiotic potential, a deeper understanding of their safety in animals, including humans, and the environment is required, so that they can be used on an industrial scale in the future.

Keywords Antagonistic effect · Immunostimulants · Infectious diseases · Medicinal plants · Pathogens · Vaccines

1 Introduction

Disease outbreaks in aquaculture are traditionally treated with antibiotics and chemotherapeutics. To decrease the use of these drugs, alternative strategies have been developed for improving fish health in aquaculture systems whilst reducing the potential spread of antimicrobial resistance (Gudmundsdóttir and Björnsdóttir 2007; Nayak 2010; Dawood et al. 2019). One of the most common activities of probiotics is the ability to control infectious diseases. The most common mechanism is the production of substances like bacteriocins, which inhibit microbial growth. Bacteriocins are a heterogeneous group of antimicrobial peptides with the ability to kill

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closely related microorganisms (narrow spectrum) or a wide range of microorganisms (broad spectrum) (Gálvez et al. 2014). Bacteriocins are synthesised by many bacteria as a mechanism of competition for nutrients and adhesion sites. They act at low concentrations, and may be biodegraded and digested by animals, which is not harmful to health. Probiotics may also produce and release organic acids and hydrogen peroxides to defend the host against the invasion of pathogens (Gaspar et al. 2018). Furthermore, probiotics control pathogen virulence by inhibiting their communication systems (by quorum sensing). Interference with the quorum sensing signal, called quorum quenching, might offer a new alternative for preventing and/or treating bacterial infections via inhibition of virulence factor expression and biofilm formation (Kim et al. 2018).

To use probiotics as a control mechanism for infectious disease, the benefits and drawbacks of their use must be compared with those of other disease control systems, such as immunostimulants, medicinal plants or vaccines. On the other hand, in the process of selecting a probiotic, it is necessary to evaluate which pathogens it can affect, since the antimicrobial range of action depends on the antimicrobial substances it releases.

2 Probiotics Effective Against Aquaculture Diseases

There is a wide range of pathogenic microorganisms whose growth has been affected by potentially probiotic bacteria, either in in vitro experiments or in animal tests.

Most probiotics put forward as biological control agents in aquaculture belong to the lactic acid bacteria (*Lactobacillus*), and to the *Vibrio* and *Bacillus* genera (Hoseinifar et al. 2018). Table 1 summarises some recent research on probiotics and their effect against some aquaculture pathogens. Unlike probiotics used in terrestrial animals, a large number of Gram-negative bacteria have been proposed for use in aquaculture. The number of species with probiotic potential is very high and includes strains of species that are even described as pathogenic (Arijo et al. 2008; Allameh et al. 2017; Medina et al. 2020; Wang et al. 2020). Several probiotic species have caused disease outbreaks in the aquaculture industry, including *Vibrio* sp. and *Weissella* sp. (Figueiredo et al. 2012). This implies a limitation of the use of these strains, since a probiotic strain useful for one fish species could be pathogenic for another animal especially if virulence genes are acquired. For example, *Vagococcus lutrae* has been used as a probiotic for seabream and seabass, but it has been observed to cause skin lesions in warm-blooded animals (Fu et al. 2020). On the other hand, there is also the possibility of plasmid transfer between pathogens and potential probiotics, which could give the probiotic virulence factors (van Reenen and Dicks 2011). This can be dangerous in the case of transmission of antibiotic resistance genes between probiotics and pathogens (Patel et al. 2012), which is why, in fact, legal provisions limit the use of probiotics to very few species. For example, the European Regulation (EC) No 178/2002 laying down the general principles and requirements of food law,

Table 1 Range of probiotics effective against infective fish diseases

Probiotic	Animal tested	Mode of administration/effective dose in feed	Length of treatment	Pathogen (antibacterial effect against)	Reference
Administration of a single probiotic strain					
Bacillus aerius	*Pangasius bocourti*	Oral/10^7 CFU/g	60 days	*Aeromonas hydrophila*	Meidong et al. (2018)
Bacillus aerophilus	*Labeo rohita*	Oral/10^8 CFU/g	6 weeks	*A. hydrophila*	Ramesh et al. (2017)
Bacillus amyloliquefaciens	*L. rohita*	Oral/10^9 CFU/g	70 days	*A. hydrophila*	Nandi et al. (2018)
	Paralichthys olivaceus	Oral/1.4×10^6 CFU/g	30 days	*Streptococcus iniae*	Kim et al. (2017)
	Danio rerio	Oral/2×10^6 CFU /g	1 month	*A. hydrophila, Streptococcus agalactiae*	Lin et al. (2019)
Bacillus cereus	*Clarias gariepinus*	Oral/10^7 CFU/g	30 days	*Aeromonas sobria*	Reda et al. (2018)
	Colossoma macropomum	Oral/10^6 CFU/g	120 days	*A. hydrophila*	Dias et al. (2018)
Bacillus licheniformis	*Oreochromis mossambicus*	Oral/10^7 CFU/g	4 weeks	*A. hydrophila*	Gobi et al. (2018)
Bacillus pumilus	*Oreochromis niloticus*	Oral/10^8 CFU/g	4 months	*S. agalactiae*	Srisapoome and Areechon (2017)
Bacillus spp.	*O. niloticus*	Oral/3×10^8 CFU /g	4 weeks	*S. agalactiae*	Sookchaiyaporn et al. (2020)
Bacillus subtilis	*Acipenser dabryanus*	Oral/2×10^8 CFU /g	8 weeks	*A. hydrophila*	Di et al. (2019)
	L. rohita	Oral/10^8 CFU/g	4 weeks	*A. hydrophila*	Ramesh and Souiss, (2018)
	Anguilla japonica	Oral/10^8 CFU/g	8 weeks	*Vibrio anguillarum*	Lee et al. (2017)
	O. niloticus	Oral/3.8×10^7 CFU /g	6 weeks	*S. agalactiae*	Zhu et al. (2019)

(continued)

Table 1 (continued)

Probiotic	Animal tested	Mode of administration/effective dose in feed	Length of treatment	Pathogen (antibacterial effect against)	Reference
	P. olivaceus	Oral/5 × $10^{7\ CFU}$ /g^{-}	12 weeks	*S. iniae*	Lee et al. (2020)
	Oplegnathus fasciatus	Oral/10^{10} CFU/kg	56 days	*Vibrio alginolyticus*	Liu et al. (2018)
	In vitro/*Litopenaeus vannamei*	Oral/10^{6} CFU/g	45 days	*V. alginolyticus, Vibrio parahaemolyticus*	Interaminense et al. (2018)
Bacillus velezensis	Hybrid grouper (*Epinephelus lanceolatus*♂ × *E. fuscoguttatus*♀)	Oral/10^{4} CFU/g	4 weeks	*Vibrio harveyi*	Li et al. (2019)
	O. niloticus	Oral/10^{9} CFU/g	9 weeks	*S. agalactiae*	Zhang et al. (2019)
	Scophthalmus maximus L	Oral/10^{8} CFU/g	42 days	*V. anguillarum*	Chen et al. (2016)
Chromobacterium aquaticum	*D. rerio*	Oral/10^{6} CFU/g	8 weeks	*A. hydrophila, S. agalactiae* and others	Yi et al. (2019)
Enterococcus casseliflavus	*Oncorhynchus mykiss*	Oral/10^{9} CFU/g	8 weeks	*S. iniae*	Safari et al. (2016)
Enterococcus faecalis	*Puntius gonionotus*	Oral/10^{7} CFU/g	15 days	*A. hydrophila*	Allameh et al. (2017)
Enterococcus faecium	*Cyprinus carpio*	Oral/10^{8} CFU/g	60 days	*Pseudomonas aeruginosa*	Arun and Singh (2019)
	Sander lucioperca	Oral/10^{10} CFU/g	6 weeks	*A. hydrophila*	Faeed et al. (2016)
Exiguobacterium acetylicum	*Carassius auratus*	Oral/10^{9} CFU/g	4 weeks	*A. hydrophila*	Jinendiran et al. (2019)
Geotrichum candidum	*C. auratus*	Oral/10^{6} CFU/g	60 days	*A. hydrophila*	Noor-Ul et al. (2020)
Lactobacillus casei	*Tor grypus*	Oral/5 × 10^{6} CFU/g	75 days	*A. hydrophila*	Mohammadian et al. (2020)

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Table 1 (continued)

Probiotic	Animal tested	Mode of administration/effective dose in feed	Length of treatment	Pathogen (antibacterial effect against)	Reference
Lactobacillus fermentum	*C. carpio*	Oral/2 × 10^8 CFU/g	60 days	*A. hydrophila*	Krishnaveni et al. (2020)
Lactobacillus plantarum	*C. carpio*	Oral/10^8 CFU/g	6 weeks	*Aeromonas veronii*	Zhang et al. (2020)
	O. niloticus	Oral/1.02 × 10^6 CFU/g	56 days	*E. faecalis*	Foysal et al. (2020)
	Acipenser baerii	Oral/10^8 CFU/g	8 weeks	*S. iniae*	Pourgholam et al. (2017)
	C. carpio	Oral/1.2 × 10^6 CFU/g	80 days	*A. hydrophila*	Soltani et al. (2017)
	C. carpio	Oral/10^8 CFU/g	14 days	*A. hydrophila*	Kazuń et al. (2018)
	O. niloticus	Oral/10^7 CFU/g	58 days	*S. agalactiae*	Yamashita et al. (2017)
Lactococcus lactis	*O. niloticus*	Oral/10^8 CFU/g	6 weeks	*S. agalactiae*	Xia et al. (2018)
	P. olivaceus	Oral/10^9 CFU/g	8 weeks	*Streptococcus parauberis*	Nguyen et al. (2017)
	C. carpio	Oral/5 × 10^8 CFU/g	8 weeks	*A. hydrophila*	Feng et al. (2019)
	Cromileptes altivelis	Oral/10^8 CFU/g	4 weeks	*V. harveyi*	Sun et al. (2018)
Paenibacillus ehimensis	*O. niloticus*	Oral/10^6 CFU/g	2 months	*A. hydrophila*, *S. iniae*	Chen et al. (2019)
Paenibacillus polymyxa	*C. carpio*	Water/10^3 CFU/mL	8 weeks	*A. hydrophila*	Gupta et al. (2016)
Pseudomonas putida	*O. niloticus*	Oral/10^8 CFU/g	60 days	*A. hydrophila*	Abomughaid (2020)
Rummeliibacillus stabekisii	*O. niloticus*	Oral/10^6 CFU/g	8 weeks	*A. hydrophila*, *S. iniae*	Tan et al. (2019)
Shewanella algae	In vitro/*L. vannamei*	Oral/10^6 CFU/g	45 days	*V. alginolyticus*, *V. parahaemolyticus*	Interaminense et al. (2018)

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Table 1 (continued)

Probiotic	Animal tested	Mode of administration/effective dose in feed	Length of treatment	Pathogen (antibacterial effect against)	Reference
Streptomyces amritsarensis	*Ctenopharyngodon idella*	Oral/10^9 CFU/g	28 days	*A. veronii*	Li et al. (2020)
Vibrio lentus	In vitro/*Dicentrarchus labrax*	Water/10^6 CFU/mL	10 days	*V. harveyi*	Schaeck et al. (2016)
Combinations of several probiotic strains					
B. cereus + *B. subtilis* (1:1)	*O. niloticus*	Oral/10^8 CFU/g	6 weeks	*S. agalactiae*	Xia et al. (2020)
	Piaractus mesopotamicus	Oral/10^8 CFU/g	60 days	*A. hydrophila*	Farias et al. (2016)
Bacillus spp. + *L. casei* (4:1)	*C. idella*	Oral/1.68 g $kg^{/}$	60 days	*A. hydrophila*	Chen et al. (2020)
B. subtilis + *B. licheniformis* (1:1)	*O. niloticus*	Oral/10 g/kg	4 weeks	*S. agalactiae*	Abarike et al. (2018a)
B. subtilis + *B. licheniformis* + *B. pumilus* (1:1:1)	*L. vannamei*	Oral/10^{10} CFU/g	33 days	*V. parahaemolyticus*	Lee et al. (2019)
B. subtilis + *L. pentosus* + *S. cerevisiae* + *L.fermentum* (1:1:1:1)	*Lates calcarifer*	Oral/10^9 CFU/kg	56 days	*A. hydrophila*	Lin et al. (2017)
B. velezensis + *Rhodotorula mucilaginosa* (1:10)	*Salmo salar* L	Oral/5 × 10^6 CFU /g	62 days	*Aeromonas salmonicida*	Wang et al. (2019a)
B. velezensis + *B. subtilis* + *B. amyloliquefaciens* (1:1:1)	*O. niloticus*	Oral/10^8 CFU/mL	4 weeks	*A. hydrophila*	Kuebutornye et al. (2020)

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Table 1 (continued)

Probiotic	Animal tested	Mode of administration/effective dose in feed	Length of treatment	Pathogen (antibacterial effect against)	Reference
Lactobacillus delbrukei subsp. bulgaricus + *Lactobacillus acidophilus*	*O. mykiss*	Oral/5 × 10^7 CFU /g	60 days	*Lactococcus garvieae*	Mohammadian et al. (2019)
Lactobacillus pentosus + *L. fermentum* + *B. subtilis* + *Saccharomyces cerevisiae* (1:1:1:1)	*L. vannamei*	Oral/10^8 CFU/kg	56 days	*V. alginolyticus*	Wang et al. (2019b)
L. plantarum SM16, *L. plantarum* SM33, *L. fermentum, Lactobacillus brevis, and Pediococcus pentosaceus* (1:1:1:1:1)	*L. rohita*	Oral/10^8 CFU/g	30 days	*A. hydrophila*	Maji et al. (2017)

establishing the European Food Safety Authority (EFSA) and laying down procedures in matters of food safety (art. 14 and 15), and the European Regulation (EU) 68/2013 about feed additives. In the absence of a list of authorised microorganisms, the Qualified Presumption of Safety (QPS) list of the EFSA is taken as a reference for their safe use in food, a list that is periodically reviewed (Herman et al. 2019). The list includes as safe microorganisms Gram-positive bacteria, i.e. *Bacillus*, *Bifidobacterium*, *Carnobacterium*, *Lactobacillus*, *Leuconostoc* and *Streptococcus*. However, there are no Gram-negative bacteria listed as safe to use as a living organism. This legal limitation implies that future research will have to focus on observing the potential adverse effects of probiotics proposed for use in aquaculture, otherwise it will not be possible to use all these probiotics in the aquaculture industry.

3 Probiotics Compared with Other Disease Control Measures

3.1 Probiotics verses Non-specific Immunostimulants

The concept of immunostimulation first appeared in 1970 as part of the vaccination process, and was later followed by the concept of probiotics (Portalès and Clot 2006). Indeed, it is difficult to separate the concept of immunostimulation from vaccination, as immunostimulants have been administered in combination with vaccines as adjuvants for boosting the immune response (Anderson 1992). However, they have been used independently since the 1980s (Olivier et al. 1985; Siwicki 1987). The use of immunostimulants for the prevention of diseases in fish culture has been extended since the beginning of the 1990s when these products were considered a new promising treatment against diseases (Kitao et al. 1987; Siwicki 1989; Anderson 1992). Anderson (1992) defined 'immunostimulant' as a chemical substance, drug, stressor or action that elevates the non-specific defence mechanism or the specific immune response. This is because an innate immune response is initiated upon recognition of pathogen-associated molecular patterns (PAMPs) (Wangkahart et al. 2019), molecules that mimic some cellular or extracellular pathogenic bacterial components. Immunostimulant agents were first used with whole bacteria, such as *Cryptosporidium parvum*, and later used with high molecular weight substances (LPS or peptidoglycans) (Werner 1986). Therefore, the link with the effect of probiotic bacteria is very close.

The first immunostimulant product developed was Ribomunyl® in 1980, and its composition was based on proteoglycans from *Klebsiella pneumoniae* and purified ribosomes from pathogens (Dussourd d'Hinterland et al. 1980). One decade later, immunostimulants began to be used in the aquaculture industry, and are now based on biological and/or synthetic compounds (Siwicki et al. 1994). Synthetic substances include compounds, such as Levamisole (Olivier et al. 1985) or FK-565 (Kitao and Yoshida 1986). Meanwhile, Mehana et al. 2015 classified the biological substances in

bacterial derivatives, polysaccharides, animal and plant extracts, nutritional factors, such as vitamins and hormones, cytokines and others. All of them may be effective in preventing diseases when administered alone, without the need to be coupled with a vaccine (Hungin et al. 2018), or use of antibiotics and chemotherapeutics. Also, they are widely applied to improve fish welfare and production (Mehana et al. 2015).

Immunostimulants exert a non-specific response, including macrophage and phagocytic activity, killing activity, reactive oxygen species (ROS), chemiluminescent response, and humoral response, which includes increases in serum complement, lysozyme and immune substances associated with non-specific and specific immune responses (Gannam and Schrock 1999). Meanwhile, probiotics exert their mode of action in many aspects of fish physiology (Tapia-Paniagua et al. 2012; Soltani et al. 2019), including the immune system, microbiota, nutrition, growth, maturation or reproductive aspects (Irianto and Austin 2002; Gatesoupe 2008; Zorriehzahra et al. 2016; Chauhan and Singh 2019).

The benefits of immunostimulants assayed in vivo include increased survival when affected by viral, bacterial and parasitic diseases, growth enhancement, increased antibody production following vaccination and increased lysozyme levels (Barman and Nen 2013; Wang et al. 2017; Dawood et al. 2018). Also, these products may be obtained from a natural source in large amounts, such as glucans from yeast or chitosan from arthropods, which are low-cost ingredients. However, the use of immunostimulants has some disadvantages: (i) some of the molecules have a high cost and limited efficiency; (ii) the memory component developed by these substances and the duration of the immune response is very short or unknown; (iii) they are not effective against all diseases; (iv) overdoses of some products can induce immunosuppression or toxicity (Bullock et al. 2000). Sometimes the mode of action and effects are not clearly defined, or the effects of long-term oral administration remain unclear. Other authors claim that the benefits described are numerous, but theoretical. For example, in larvae culture, there is controversy between authors that defend that the early use of immunostimulants in fish larvae can induce immune tolerance (Bricknell and Dalmo 2005). However, large quantities of live probiotic cells may interfere with the associated eco-systems (Sharifuzzaman et al. 2011), or the risk of lateral gene transfer of antibiotic resistance genes (Gueimonde et al. 2013; Sharma et al. 2016; Tan et al. 2016). This is why new strategies are being set up, such as the use of microbial cellular components with immunostimulant effects on fish (Kum and Sekki 2011; Giri et al. 2015, 2018).

Some bacterial derivatives are considered to be immunostimulants (Giri et al. 2015). Examples include, but are not limited to, muramyl dipeptide (N-acetyl-muramyl-L-alanyl-D-isoglutamine, MDP), derived from *Mycobacterium* lipopolysaccharide (LPS; Kodama et al. 1993) that is a cell wall component of Gram-negative bacteria (Neumann 1995; Nya and Austin 2010); Freund's complete adjuvant (FCA) that contains killed *Mycobacterium butyricum* (Sakai 1999); *V. anguillarum* whole cell inactivated vaccine [= bacterin] (Norqvist et al. 1989), *Clostridium butyricum* and *Achromobacter stenohalis* cells and other components, such as flagellin (Wangkahart et al. 2019) or cell wall proteins of *Kocuria* SM1 and *Rhodococcus* SM2 (Sharifuzzaman et al. 2011); bacterial DNA (Giri et al. 2015) and unmethylated CpG dinucleotides (Jørgensen et al. 2001).

The efficacy of immunostimulants and probiotics depends on the effective dose, exposure time and, in some cases, the feeding regime of each type of fish. For example, in Atlantic salmon, injection with a high dose of glucans (100 mg/kg) led to absence of protection for 1 week, but maximum benefits occurred after 3–4 weeks, whilst the injection of a low dose (2–10 mg/kg) gives protection for only one week (Kum and Sekki 2011). There are three main ways to deliver immunostimulants: (i) injection, (ii) immersion and oral uptake and (iii) bioencapsulation. The advantages and limitations are similar to those of probiotics. Injection is not usual when administering probiotics, but immunostimulants provide potent immunisation and can be administered in large fish. It is, however, a complicated task, which is costly and is highly stressful for the animals. Immersion and oral uptake are the simplest methods, making it possible to treat many fish of any size at the same time. However, the substances can lose activity due to their dilution in water, and it is difficult to measure the amount of feed ingested by the fish. The potency is not as high as with the injection route, and large amounts of immunostimulants are needed to achieve good protection. Currently, bioencapsulation is a good alternative, since it protects against the digestive system and environmental conditions. Table 2 shows the effects of probiotics compared with immunostimulants.

Table 2 Effects of probiotics compared with immunostimulant substances on cultured fish

	Probiotics	Immunostimulant
Prophylactic effect	Duration variable	Short duration, require more treatments
Efficacy	Variable	Good
Spectrum of activity benefits	Wide	Wide
Improved immune response	Yes	Yes
Stimulation of growth	Yes	No described
Water quality	Yes	–
Improved digestion	Yes	No described
Improve intestinal barrier	Yes	No described
Control microbiota	Yes	No directly
Toxicity	No described	No described
Accumulation of toxic residues	No	No
Environmental impact	No	Interfere with the associated eco-systems horizontal gene transference
Administration (main routes)	Feed or oral directly to culture ponds or immersion bioencapsulation	Feed or oral directly to culture ponds or immersion bioencapsulation injection

3.2 Probiotics verses Medicinal Plant Products

Medicinal plants comprise herbs, seaweeds, herbal extracted compounds, spices, commercial plant-derived products and traditional Chinese herbs (Van Hai 2015). There is growing interest in the use of medicinal herbs in aquaculture because of their promising effects, and they look like a promising alternative method for controlling fish diseases (Van Hai 2015; Abarike et al. 2018b). Plants have been reported to produce various effects, such as growth promotion, appetite stimulation, immunostimulation, and to have antipathogenic properties in aquaculture (Citarasu 2010; Reverter et al. 2014; Bulfon et al. 2015; Awad and Awaad 2017). The mode of action of these plants and their derivatives is attributed to the presence of many bioactive compounds, such as alkaloids, steroids, phenolics, tannins, terpenoids, saponins, glycosides and flavonoids (Harikrishnan et al. 2011a; Mendam et al. 2015).

Plants may be administered as a whole or in parts (leaf, root, bark, fruit), and can either be used fresh or as herbal extract preparations with different solvents (water, methanol, ethanol, chloroform) (Kim et al. 2011; Pan et al. 2013; Fridman et al. 2014; Hu et al. 2014; Zhang et al. 2014; Thanigaivel et al. 2015; Zhou et al. 2016). Their effects are variable amongst fish species, and depend mainly on different factors, such as route of administration, dosage and time (Zakęś et al. 2008; Harikrishnan et al. 2011a; Bulfon et al. 2015). Like other immunostimulants, medicinal plants and their extracts may be administered via injection (Harikrishnan et al. 2011a), bathing/immersion (Çek et al. 2007) or oral administration (Wang et al. 2015), which is the most practical and commonly used in aquaculture (Pourmoghim et al. 2015; Bilen et al. 2016; Öz et al. 2018). The review performed by Bulfon et al. (2015) presented a great variety of different dosages including up to 25% of the diet, although the most common doses ranged from 0.01 and 0.5%. However, there is not any positive correlation between dosage and its effect on the immune response (Jian and Wu 2004). Similarly, the length of feeding time is fundamentally important. To date, studies with medicinal plants and/or their bioactive compounds have involved different feeding durations, ranging from 1 to 16 weeks (Awad and Awaad 2017), but the basis for choosing these periods is often unclear.

One of the main problems of using medicinal plants as a chemotherapeutic is that the biological activity and chemical compositions of plants and extracts vary according to their characteristics (location, age, climate, cultivars, temperature and growth regulators) and sampling methods (plant part, drying, distillation and storage) (Wang et al. 2014). The antimicrobial activity of a plant against bacteria is determined by its mechanism of action, which is determined by the chemical composition (Chouhan et al. 2017; Cui et al. 2019). Thus, differing antimicrobial activities of plants with different chemical profiles are expected. In this sense, in vitro studies evaluating the cytotoxicity and the antibacterial effects of herbs have examined several bacterial fish pathogens (Vaseeharan et al. 2013; Alizadeh Behbahani and Imani Fooladi 2018; Da Cunha et al. 2018; Assane et al. 2020), highlighting their potential use for controlling bacterial disease in cultured fish.

A key aspect for proposing a natural substance as an antimicrobial agent is whether it has active compounds that may be toxic for the host. There have been reports that some plants and their major components are toxic for different animals (Malekmohammad et al. 2019), including fish (Spanghero et al. 2019; Tavares-Dias 2018).

The administration of medicinal plants for disease control in aquaculture may be achieved singly or in combination with other plants. Some studies show that medicinal plants (such as *Allium sativum*, *Azadirachta indica, Curcuma longa, Ocimum basilicum*, *Ocimum sanctum*, *Cinnamomum zeylanicum*, *Juglans regia*, *Mentha piperita, Radix astragalus* and *Radix angelicae*) enhance growth, immune responses and survival against a wide range of pathogen infections in farmed fish, such as *O. mykiss*, *L. calcarifer*, *C. carpio* and *Pseudosciaena crocea* (Jian and Wu 2003; Harikrishnan et al. 2009; Nya and Austin, 2009a, 2009b; Mohamad and Abasali 2010; Talpur and Ikhwanuddin 2012; Talpur et al. 2013; Awad and Awaad 2017; Stratev et al. 2018; Hayatgheib et al. 2020; Kuebutornye and Abarike 2020).

Medicinal plants may be incorporated with a probiotic. Thus, fenugreek seed (*Trigonella foenum graecum*) in combination with probiotic strains *B. licheniformis*, *L. plantarum* and *B. subtilis* enhanced growth performance, skin mucosal immunity response, humoral immune response and the expression of immune-associated genes of gilthead seabream (*Sparus aurata*) after three weeks of a feeding regime (Bahi et al. 2017; Guardiola et al. 2017). A diet enriched with *Scutellaria baicalensis*, and/or *Lactobacillus sakei* BK19 in rock bream, *O. fasciatus,* demonstrated that the maximum protection against *Edwardsiella tarda* was recorded in the mixed (plant + probiotic) diet group (Harikrishnan et al. 2011b). The synergistic effect of *M. piperita* and the probiotic *Bacillus coagulans* improved the growth performance, nutritional physiology and resistance of Indian carp (*Catla catla*) when challenged against *A. hydrophila* (Bhatnagar and Saluja 2019). The effect of herbal-probiotic mixtures of *Astragalus membranaceus*, *Angelica sinensis*, *Crataegus hupehensis* and probiotics *B. subtilis* and *B. lincheniformis* improved growth and enhanced immune responses and survival of Nile tilapia (*O. niloticus*) when challenged against *S. agalactiae* (Abarike et al. 2018b). Moreso, in *O. niloticus*, a mixture of Chinese medicinal herbs and probiotics (*Bacillus*, *Lactobacillus* and *Yeast*) enhanced growth performance, innate immune response and antibacterial activity against *E. tarda* (Hwang et al. 2019).

There are some advantages and disadvantages when using probiotics instead of medicinal plants. On the one hand, probiotics may colonise the gut and adhere to the epithelial surface, and consequently interfere with the adhesion of pathogens (Zorriehzahra et al. 2016). Furthermore, they can consume the nutrients that are essential for the growth of a number of pathogens (Brown 2011). However, safety regulations and marketing authorizations are very restrictive regarding the use of live microorganisms. Conversely, medicinal plants are easily accessible and economical, and there is no need for significant investment in their biotechnological development, which is also an encouraging factor for large scale usage in aquaculture. Moreover, although plant products have a natural origin, and most of these medicinal plants do not represent a hazard for human health, animal health, or the environment (Stratev

et al. 2018), some constituents are unstable (e.g. they are photo- and/or thermo-labile) (Burt 2004). Finally, little is known regarding the interaction of the plants with the host microbiota.

In contrast to plant extracts and the other protein-based antimicrobial preservatives, bacteriocins, produced by some probiotic bacteria tolerate high thermal stress and are active over a wide pH range, remaining effective at fairly low concentrations (Wang et al. 2019c).

3.3 Probiotics verses Vaccines

Modern vaccines can be classified as killed, attenuated, DNA, synthetic peptide, recombinant vector, genetically modified and subunit vaccines, but although whole vaccines showed a better advantage than other types (Assefa and Abunna 2018), all showed disadvantages, especially with regard to the route of administration. Although it is a very efficient for achieving protection against pathogens, the intraperitoneal inoculation of vaccines combined with adjuvants (Harikrishnan et al. 2011c) may be the cause of stress, feed intake reduction (Lillehaug 2014), lesions such as inflammation, deformities and granulomas (Berg et al. 2006), and growth alterations (SØrum and Damsgård 2004; Berg et al. 2007). In addition, staff with experience in the application of this type of vaccines is required. On the other hand, the oral vaccination route is favoured because of its ease of administration, but not all fish can eat/take the same amount of antigen so it may not provide a uniform protection. It may also become more expensive if it is necessary to protect the antigen by encapsulation (Vallejos-Vidal et al. 2014).

Probiotics may be used to reduce disease outbreaks in aquaculture. Some probiotics are characterised by their antagonistic activity against pathogens or the stimulation of the fish immune response, including the production of specific antibodies. Immune cross-reactions amongst phylogenetically-related bacteria are widely documented, and they play an important role in protection against pathogens (Medina et al. 2020). Some vaccines use non-pathogenic microorganisms that contain antigens similar to those of pathogenic strains (Brunt and Austin 2005; Brunt et al. 2007; Arijo et al. 2008; Abbass et al. 2010). If a probiotic shares antigens with a certain pathogen, it could produce antibodies with a cross-reaction to that pathogen. Therefore, a probiotic with these characteristics could be used in a similar way to a live vaccine.

The ability of probiotic bacteria administered through diet to modulate the innate and adaptive immune system of farmed fish has been reported (Brunt and Austin 2005; Nayak 2010; Hemaiswarya et al. 2013; Foey and Picchietti 2014), even when some probiotic microorganisms were supplied as heat-killed cells (Biswas et al. 2013). There is information that a probiotic strain of *E. faecium* increased the transcription of genes encoding complement system, lysozyme activity, protease activity and proinflammatory cytokines in specimens of *P. olivaceus* infected with *L. garvieae* (Kim et al. 2013). On the other hand, significant increases in T lymphocytes

(Romano et al. 2007; Picchietti et al. 2009), granulocytes (Sharma et al. 2013), and immunoglobulins (Sharifuzzaman and Austin 2010; Neissi et al. 2013; Xing et al. 2013) have been reported in farmed fish receiving probiotics, and include *D. labrax, Rachycentron canadum* and *O. mykiss*. However, different studies have reported the ability of the subcellular components obtained from probiotics to exert an immunostimulant effect on the specific and non-specific immune responses of farmed fish (Arijo et al. 2008; Chi et al. 2014; Giri et al. 2015, 2018). All these studies strongly suggested that probiotics may be used as adjuvants in aquaculture. In this sense, the reduction of the side effects of vaccines administered with adjuvants is a challenging goal for fish vaccination (Dadar et al. 2017), and the use of probiotics as potential adjuvants is a very interesting possibility, especially because they can be easily administered through the diet as spores (Soltani et al. 2019), freeze-dried (Tapia-Paniagua et al. 2015) and using some type of encapsulation (Martínez Cruz et al. 2012; Rosas-Ledesma et al. 2012). Another interesting aspect in comparison with vaccines is that the use of the probiotic is not limited by the size of fish, because they have been supplied in all growth stages even during larviculture (Lobo et al. 2014).

However, new terms, such as postbiotic, have emerged that imply that bacterial viability is not an essential requirement for health benefits. Postbiotics are soluble factors resulting from the metabolic activity of a probiotic or any released molecule capable of conferring beneficial effects to the host in a direct or indirect way (Tsilingiri et al. 2012), and include a wide range of compounds (Aguilar-Toalá et al. 2018; Ang et al. 2020). In human and veterinary uses, postbiotics have shown beneficial health effects (Nakamura et al. 2016; Compare et al. 2017) indicating a high capacity to modulate different organs and tissues in the host, inducing several biological responses such as an immune response (Kearny et al. 2015), and suggesting that they could mimic the health effects of probiotics.

Therefore, the use of postbiotics may represent a valid and safer alternative to avoid risks linked to live probiotic bacteria for treating many diseases, and the scientific evidence of their beneficial health effects is increasing (Haileselassie et al. 2016; Nakamura et al. 2016; Compare et al. 2017; Zółkiewicz et al. 2020). However, especially in the case of aquaculture, the information on the application of postbiotics is limited (Lieke et al. 2020; Ang et al. 2020), and mainly focused on Gram-positive microorganisms. Studies on the relationship between the immune system and postbiotics can be very relevant, because they could imply a more efficient application of probiotics.

4 Conclusion and Suggestions for Further Work

In conclusion, there is a wide range of probiotics that has been studied for the control of infectious diseases. Probiotics have shown the ability to act against pathogens at the same level as other treatments, such as immunostimulants, medicinal plants and vaccines. However, most probiotics are not legally recognised for use in aquaculture. This represents a limitation for the commercial use of the strains studied. More

research is needed to demonstrate that the wide range of probiotics used experimentally are safe for farmed fish, other animals (including humans) and the environment in general.

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Paraprobiotics in Aquaculture

Shengkang Li and Ngoc Tuan Tran

Abstract Probiotics are live microorganisms and friendly to the environment, conferring health benefits on hosts. However, in some cases, the live form of probiotics may lead to the establishment of potential risks to wild aquatic organisms by releasing viable bacteria into ambient environments. Non-viable probiotics are termed as "paraprobiotics," and exhibit the benefits in similar ways to their viable counterparts. This suggests that there is benefit for the use of paraprobiotics in aquaculture. Remarkably, evidence has demonstrated the positive effect of paraprobiotics on growth performance, digestibility, feed utilisation, gut commensal microbiota and physiological changes of aquatic animals. This chapter focuses on the application of paraprobiotics in aquaculture, with aspects related to the definition, inactivation methods, method of application, effective dosage, duration of administration, common parameters used to assess the health benefits, mechanisms of action, receptor mediated immunostimulation and the efficacy of paraprobiotics in aquaculture.

Keywords Paraprobiotics · Heat-killed probiotics · Growth · Feed utilisation · Immune response · Disease resistance · Stress resistance · Gut microbiota

1 Introduction

Aquatic animals are a rich source of protein, minerals and essential fatty acids, which provide 16% of the animal protein consumed by humans and are the main source of protein for ~950 million people worldwide (Pradeepkiran 2019). Noteworthy, the share of world fish production used for human protein consumption rapidly increased from 6.7% in the 1960s to 8.7% (more than 146 million tonnes) in 2014 (FAO 2016) . In response to the global demand, the aquaculture industry has developed to a greater extent both in terms of technology and practical measures (Zorriehzahra et al. 2016). However, the intensification in aquaculture has given rise to an increase in aquatic animal and environmental stress, and infectious diseases are the most important

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factors resulting in heavy economic losses (Bondad-Reantaso et al. 2005; Carbone and Faggio 2016; Dawood et al. 2019a; Hai 2015; Tuan et al. 2013). To reduce the economic losses, chemicals, including antibiotics, disinfectants, parasiticides, probiotics, and other feed additives, have been used commonly for preventing and treating disease outbreaks, as well as improving growth performance and/or water quality (Rico et al. 2013). Nevertheless, the unscientific use of veterinary drugs (including antibiotics) may lead to disadvantages for the cultured host, human health, and the surrounding environment through the overgrowth and spread of drug-resistant bacteria and environmental residues of toxic substances (Hasan et al. 2019a; Pérez-Sánchez et al. 2018; Rico et al. 2013). The development of alternative methods friendly to the environment and able to improve the growth and health status in aquatic animals has become crucially important (Dawood et al. 2019a). Of these methods, the application of probiotics is particularly suitable for sustainable aquaculture (Azevedo et al. 2015; Hai 2015; Putra and Utomo 2015; Shewita et al. 2011).

Probiotics are known as live microorganisms capable of conferring health benefits on the hosts (FAO/WHO, 2006) . In aquaculture, the literature has reviewed and discussed the benefits of probiotics, including improving growth, providing nutritional and enzymatic contributions to the digestion of the host, enhancing immune responses and increasing disease resistance (Dawood et al. 2019a; Hasan et al. 2019b; Li 2018; Newaj-Fyzul et al. 2014; Ringø et al. 2020; Ringø et al. 2018; Soltani et al. 2019; Tran et al. 2020b; 2022; Wu et al. 2014; Yang et al. 2019). However, in some cases, using live probiotic bacteria may result in a potential risk to wild aquatic organisms by the release of large numbers of bacteria into the ambient environment during the process (Díaz-Rosales et al. 2006). Therefore, a new direction has opened up, namely the use of non-viable bacteria as a promising alternative approach (Tran et al. 2022). The evidence supported that non-viable probiotics may cause similar effects in hosts compared to their viable counterparts (Choudhury and Kamilya 2019; Nayak 2010). Non-viable probiotics are termed "paraprobiotics" (Choudhury and Kamilya 2019; Taverniti and Guglielmetti 2011), which have proven to have positive effects on the health of aquatic animals, such as improving growth, feed efficiency, immune responses, disease and stress resistance and survival rate, as well as modulating the gut microbiota (Dawood et al. 2015a; Dawood et al. 2019b; Duc et al. 2020; Hasan et al. 2019a; Nguyen et al. 2019; Pan et al. 2008; Tung et al. 2009; Tung et al. 2010; Wu et al. 2020; Yang et al. 2016).

In this chapter, aspects involving the definition, inactivation methods, administration methods, parameters used for evaluating the health benefits, modes of action, receptor mediated immunostimulation and the efficacy of paraprobiotics in aquatic animals will be discussed.

2 Definition of Paraprobiotics

Probiotics contain both viable and non-viable microbial cells that have the ability to provide beneficial effects on the recipient host (Lahtinen 2012; Nayak 2010). The

non-viable (dead) probiotic cells are able to be fractionated and produce a range of biological responses to mammalian and avian species (Adams 2010; de Almada et al. 2016). The term "paraprobiotics" has been coined to interpret the concept of non-viable beneficial microbes, and are defined as "non-viable microbial cells (intact or broken) or crude cell extracts (i.e. with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer" (Taverniti and Guglielmetti 2011). The prefix "para" has its origins in Greek meaning "alongside of" or "atypical," possibly simultaneously referring to the similarity and difference from the conventional probiotic definition (Taverniti and Guglielmetti 2011). Paraprobiotics have also been regarded as "inactivated probiotics" or "ghost probiotics" (Choudhury and Kamilya 2019; de Almada et al. 2016). In some cases, paraprobiotics may be regarded as "postbiotics" that is defined as "non-viable bacterial products or metabolic by-products from probiotic microorganisms that have biological activity in the host" (de Almada et al. 2016; Patel and Denning 2013). In fact, paraprobiotics are derived from microorganisms, which had their viability compromised followed by exposure to factors that change the cell structures and physiological functions (Barros et al. 2020; de Almada et al. 2016). Also, cell structural components (mainly cell wall components) of the probiotics are referred to as paraprobiotics (Teame et al. 2020). However, Taverniti and Guglielmetti (2011) have excluded the "purified molecules of microbial origin or pure microbial cell products" from the concept of paraprobiotics because of their use in conventional pharmaceutical methodologies.

In aquaculture, the concept of paraprobiotics is a relatively new one (Choudhury and Kamilya 2019). It is argued that the products may be safe in use rather than their probiotic counterparts insofar as there is reduced risk of microbial translocation, infection or inflammation, and the acquisition of virulence genes is eliminated between the pathogens and probiotic bacteria (Aguilar-Toalá et al. 2018; Newaj-Fyzul et al. 2014). Paraprobiotics have been applied widely in aquaculture and shown to be beneficial to the health of aquatic animals.

3 Paraprobiotic Preparation

Inactivation of probiotic bacteria to produce paraprobiotics is principally carried out by methods, such as heat, gamma radiation, ultraviolet (UV) radiation, high hydrostatic pressure, ultrasonication, lyophilisation or chemical/acid deactivation (Aguilar-Toalá et al. 2018; Barros et al. 2020; de Almada et al. 2016; Tran et al. 2022). Of these methods, heat, UV-light, formalin and sonication are applied frequently to preparations destined for use in aquaculture, with heat treatment being the most commonly used method (Choudhury and Kamilya 2019). Regarding heat inactivation, different bacteria are inactivated by heating at temperatures ranging from 60 to 121°C, and the duration of heating may also vary from 10 min to 2 h (Table 1). It is

Table 1 Summary on the application of paraprobiotics in aquatic animals

Heat-killed probiotic	Originated from	Inactivation method	Host species	Dosage	Application method and duration	Studied parameters	Reference
Eight species of lactic acid bacteria	Fish, seafood and fish products	Heat (80 °C, 30 min)	Turbot (*S. maximus*) (700 g)	10^8 CFU/mL	In vitro (head-kidney leucocytes)	Immune parameters	Munoz-Atienza et al. (2015)
L. plantarum 06CC2	Mongolian dairy products	Heat (boiling water, 1 h)	Japanese pufferfish (*T. rubripes*) (205 ± 8 g)	20 mg/mL	In vitro (head-kidney cells)	Immune-related genes expression	Biswas et al. (2013b)
L. plantarum (LP20)	Commercial products	Heat (70 °C, 10 min)	Red sea bream (*P. major*) (11 ± 0.03 g)	0, 1, 10, 100, 1000 and 2000 mg/kg diet	Diet; 56 days	Growth parameters, immune parameters and stress resistance	Dawood et al. (2015a)
L. plantarum (HK-LP)	Commercial products	Heat	Red sea bream (*P. major*) (11 ± 0.03 g)	0.025, 0.05 and 0.1% of dry diet	Diet; 56 days	Growth parameters, digestibility and immune parameters,	Dawood et al. (2015c)
L. plantarum L-137	Commercial products	Heat	Nile tilapia (*O. niloticus*) (13.7 ± 0.4 g)	Reduced from 0, 10, 20 and 50 ppm (for 50 days) to 0, 2, 4 and 4 ppm (for 70 days), respectively	Diet; 120 days	Growth parameters, immune parameters and stress resistance	Nguyen et al. (2019)
L. plantarum L-137	Commercial products	Heat (70 °C, 10 min)	Genetically improved farmed tilapia (GIFT, *O. niloticus*) (16 ± 0.03 g)	0, 50, 100 or 1000 mg/kg diet	Diet; 12 weeks	Growth, digestive enzyme activity, intestinal morphology and oxidative status	Dawood et al. (2019b)
L. plantarum L-137	Commercial products	Heat	Striped catfish (*P. hypophthalmus*) (0.06 g)	0, 10, 20 and 50 ppm	Diet; 8 weeks	Growth parameters, immune parameters and disease resistance	Duc et al. (2020)
L. plantarum L-137	Commercial products	Heat	White shrimp (*L. vannamei*) (post-larvae and juvenile stages)	Reduced from 0, 0.5 and 1.0 g/kg feed (Phase 1, PL_1 to PL_{15}) to 0, 0.1 and 0.25 $_g$/kg feed (Phase 2, PL_{16} to PL_{45}), respectively	Diet; 45 days	Water parameters, growth parameters, immune parameters and disease resistance	Duc et al. (2017)

(continued)

Table 1 (continued)

Heat-killed probiotic	Originated from	Inactivation method	Host species	Dosage	Application method and duration	Studied parameters	Reference
L. plantarum	Commercial products	Heat (65 °C, 30 min) or sonication (2 k Hz, 40 min)	White shrimp (7.96 ± 0.59 g)	10^{10} CFU/kg	Diet; 45 days	Growth parameters and stress resistance	Zheng et al. (2017)
L. plantarum	Commercial products	Heat (65 °C, 30 min) or sonication (2 k Hz, 40 min)	White shrimp (7.96 ± 0.59 g)	10^{10} CFU/kg	Diet; 15 days	Growth parameters, digestive enzyme activity, gut morphology and gut microbiota	Zheng et al. (2018) and Zheng et al. (2020)
L. plantarum (HK-LP)	Commercial products	Heat (70 °C, 10 min)	Kuruma shrimp (*M. japonicas*) (4.86 ± 1.5 g)	1, 10, 100 and 1000 mg/kg diet	Diet; 60 days	Growth parameters, immunological parameters, stress resistance and bacteria count in both rearing water and faeces	Tung et al. (2009)
L. plantarum (HK-LP)	Commercial products	Heat (70 °C, 10 min)	Kuruma shrimp (*M. japonicas*) (larval and post-larval stages)	0, 0.001, 0.01, 0.1 and 1 g/kg	Diet; Larval stage: 8 days and post-larva: 30 days	Larvae: growth performance, survival rate, stress resistance Post-larvae: growth performance, digestive enzyme activity, survival rate and stress resistance	Tung et al. (2010)
L. plantarum (MTCC no. 1407)	Culture collection	Heat (water bath at 60 °C, 30 min)	Giant freshwater prawn (*M. rosenbergii*) (0.54 ± 0.03 g)	10^{7}, 10^{8} and 10^{9} CFU/g diet	Diet; 90 days	Growth parameters, immune parameters and disease resistance	Dash et al. (2015)
L. plantarum L-137	Commercial products	Heat (70 °C, 10 min)	Sea cucumber (*A. japonicas*) (1.35 ± 0.04 g)	0, 0.005, 0.025, 0.05 and 0.25 g/kg diet	Diet; 60 days	Growth parameters, digestibility and immune parameters	Yang et al. (2016)
L. rhamnosus JCM 1136	Culture collection	Heat (water bath at 75 °C, 1 h with continuous stirring)	Rainbow trout (*O. mykiss*) (126 g)	10^{11} CFU/g	Diet; 30 days	Immune response, expression of cytokine genes and immune gene	Panigrahi et al. (2005) and Panigrahi et al. (2011)
L. paracasei spp. *paracasei* (06TCa22)	Mongolian dairy products	Heat (boiled for 1 h)	Japanese pufferfish (*T. rubripes*) (50.4 ± 2.2 g)	0.5, 1 and 2 mg/g	Oral administration; 3 days	Immune response, expression of cytokine genes and disease resistance	Biswas et al. (2013a)

(continued)

Table 1 (continued)

Heat-killed probiotic	Originated from	Inactivation method	Host species	Dosage	Application method and duration	Studied parameters	Reference
L. paracasei spp. *paracasei* (06TCa22)	Mongolian dairy products	Heat (boiled for 1 h)	Japanese pufferfish (*T. rubripes*) (205 ± 8 g)	20 mg/mL	In vitro (head-kidney cells)	Expression of pro-inflammatory, cell-mediated immune regulators, antiviral and regulatory cytokine genes	Biswas et al. (2013b)
L. delbrüeckii subsp. *lactis* (CECT 287)	Culture collection	Heat (60 °C, 1 h)	Gilthead seabream (*S. aurata*) (65 g)	5×10^5, 5×10^6 and 5×10^7 CFU/mL	In vitro (head-kidney leucocytes)	Cellular innate immune responses	Salinas et al. (2006)
Bacillus sp. SJ-10	Fermented fish, "Jeotgal"	Heat (121 °C, 15 min)	Olive flounder (*P. olivaceus*) (~9.64 g)	1×10^8 CFU/g diet	Diet; 8 weeks	Growth parameters, immune parameters, microvilli length and disease resistance	Hasan et al. (2019a)
B. pumilus SE5	Gut of *E. coioides*	Heat (water bath at 95 °C, 1 h)	Grouper (*E. coioides*) (14.57 ± 0.05 g)	1.0×10^8 cells/g	Diet; 60 days	Microbial community and expression of mucosal immune genes	Yang et al. (2014)
B. pumilus SE5	Gut of *E. coioides*	Heat (water bath at 95 °C, 1 h)	Grouper (*E. coioides*) (14.6 ± 0.2 g)	1.0×10^8 cells/g	Diet; 60 days	Growth parameters and immune parameters	Yan et al. (2016)
B. amyloliquefaciens FPTB16	Fermented fish product "Shidal"	Heat (60 °C, 2 h), UV-light (2.5 h) or formalin (1.0 v/v, 24 h, 4 °C)	Catla (*C. catla*) (35 g)	10^7, 10^8 and 10^9 cells/mL	In vitro (head-kidney leucocytes)	Cellular immune responses	Kamilya et al. (2015)
B. amyloliquefaciens FPTB16	Fermented fish product, "Shidal"	Heat (60 °C, 2 h)	Catla (*C. catla*) (25.98 ± 2.57 g)	10^7, 10^8 and 10^9 cells/g diet	Diet; 4 weeks	Immune response and expression of immune relevant genes	Singh et al. (2017)
B. subtilis CECT 35	Culture collection	Heat (60 °C, 1 h)	Gilthead seabream (*S. aurata*) (65 g)	5×10^5, 5×10^6 and 5×10^7 CFU/mL	In vitro (head-kidney leucocytes)	Cellular innate immune responses	Salinas et al. (2006)
B. subtilis FPTB13	Fermented fish product, "Shidal"	Heat (60 °C, 2 h)	Catla (*C. catla*) (35 g)	10^7, 10^8 and 10^9 cells/mL	In vitro (head-kidney leucocytes)	Cellular immune responses	Kamilya et al. (2015)
B. subtilis VSG1	Gut of *L. rohita*	Heat (water bath at 80 °C, 30 min)	*L. rohita* (43 ± 1.07 g)	1 mg	Intraperitoneal injection; 21 days	Immune parameters and disease resistance	Giri et al. (2015)

(continued)

Table 1 (continued)

Heat-killed probiotic	Originated from	Inactivation method	Host species	Dosage	Application method and duration	Studied parameters	Reference
B. subtilis AB1	Gut of rainbow trout	Formalin (2.0 v/v, 48 h) or sonication	Rainbow trout (*O. mykiss*) (30 g)	10^7 cell/ g	Diet; 14 days	Disease resistance	Newaj-Fyzul et al. (2007)
E. faecalis	Commercial products	Heat	Rainbow trout (*O. mykiss*) (36.3 ± 0.42 g)	0, 2.5 and 5 g/kg	Diet; 12 weeks	Growth parameters, immune parameters and disease resistance	Rodriguez-Estrada et al. (2013)
E. gallinarum L-1	Gut of gilthead sea bream	Heat (60 °C, 2 h) or UV-light (2.5 h)	Gilthead sea bream (*S. aurata*), European sea bass (*D. labrax*), meagre (*A. regius*) and red porgy (*P. pagrus*) (200 g)	10^6, 10^7 and 10^8 CFAU/mL	In vitro (head-kidney leucocytes)	Immune parameters	Román et al. (2015)
E. faecalis KH2	Commercial products	Heat	Ginbuna crucian carp (*C. auratus langsdorfii*) (15–20 g)	500 mg	Intraperitoneal injection; 7 days	Cell-mediated immunity, in vitro and i*n vivo*	Matsuura et al. (2017)
L. lactis CECT 539	Culture collection	Heat (boiling water, 2 h)	Turbot (*S. maximus*) (40–60 g)	10^6 cells/mL (in vitro study) 10^5 cells/mL (in vivo study)	In vitro (head-kidney macrophages) and in vivo study (diet; 7 days)	Immune parameters	Villamil et al. (2002)
L. lactis D1813	Commercial products	Heat and being lyophilised	Nile tilapia (*O. niloticus*) (Laboratory experiment: 11.07 ± 0.07 g; Field experiment: 13.7 ± 0.2 g)	Laboratory experiment: 0.25, 0.50, 1.00 and 2.00 g/kg Field experiment: L0.25, L0.5, L1.0 and L2.0	Diet; Laboratory experiment: 8 weeks, Field experiment: 22 weeks	Growth parameters, feed utilisation, immune parameters and disease resistance	Suprayudi et al. (2017)
P. aeruginosa VSG2	Gut of Indian major carp (*L. rohita*)	Heat (water bath at 80 °C, 30 min)	Indian major carp (*L. rohita*) (43 ± 1.07 g)	1 mg	Intraperitoneal injection; 21 days	Immune parameters and disease resistance	Giri et al. (2015)

(continued)

Table 1 (continued)

Heat-killed probiotic	Originated from	Inactivation method	Host species	Dosage	Application method and duration	Studied parameters	Reference
P. aeruginosa VSG2	Gut of Indian major carp (*L. rohita*)	Heat (water bath at 80 °C, 1 h)	Indian major carp (*L. rohita*) (190 g)	20 mg/mL	In vitro (head-kidney macrophages)	Immune parameters	Giri et al. (2016)
P. aeruginosa VSG2	Gut of Indian major carp (*L. rohita*)	Heat (water bath at 80 °C, 10 min)	*C. carpio* (6.3 ± 0.28 g)	0, 10, 20, 30, and 40 mg/kg	Diet; 8 weeks	Immune parameters and disease resistance	Giri et al. (2020)
C. butyricum CB2	Gut of chickens	Heat (150 °C, 15 min)	Chinese drum (*M. miiuy*) (200–260 g)	10^8 cells/g	Diet; 30 days	Immune parameters and disease resistance	Pan et al. (2008)
Vibrio-like bacteria of the *Shewanella* genus (Pdp11 and 51M6)	Skin of gilthead seabream	Heat (60 °C, 1 h)	Gilthead seabream (*S. aurata*) (65 g)	5×10^5, 5×10^6 and 5×10^7 cfu/mL	In vitro (head-kidney leucocytes)	Immune parameters	Salinas et al. (2006)
Vibrio-like bacteria of the *Shewanella* genus (Pdp11 and 51M6)	Skin of gilthead seabream	Heat (60 °C, 1 h)	Gilthead seabream (*S. aurata*) (65 g)	$10^{8\ CFU}$/g Pdp11, 10^8 cfu/g 51M6 or 0.5×10^8 CFU/g Pdp11 plus 0.5×10^8 CFU/g 51M6	Diet; 4 weeks	Immune parameters	Díaz-Rosales et al. (2006)
Pseudomonas sp. GP21 *Psychrobacter* sp. GP12	Gut of Atlantic cod	Heat (60 °C, 1 h)	Atlantic cod (*G. morhua*) (300–400 g)	10^7 CFU	In vitro (intestinal epithelial cells)	Immune parameters	Lazado and Caipang (2014)
Pseudomonas sp. GP21 *Psychrobacter* sp. GP12	Gut of Atlantic cod	Heat (60 °C, 1 h)	Atlantic cod (*G. morhua*) (800–1000 g)	10^8 CFU	In vitro (head-kidney leucocytes)	Immune response and antioxidant defence	Lazado et al. (2010)
Psychrobacter sp. SE6	Gut of juvenile grouper	Heat (water bath at 95 °C, 1 h)	Grouper (*E coioides*) (14.57 ± 0.05 g)	1.0×10^8 cells/g	Diet; 60 days	Intestinal microbiota and expression of in TLR signalling pathways	Sun et al. (2014)
V. fluvialis	Gut of gilthead sea bream	Heat (60 °C, 2 h), UV-light (2.5 h)	Gilthead sea bream (*S. aurata*) and European sea bass (*D. labrax*) (200 g)	10^9 CFU/mL	In vitro (head-kidney leucocytes)	Immune response and antioxidant defence	Román et al. (2012)

(continued)

Table 1 (continued)

Heat-killed probiotic	Originated from	Inactivation method	Host species	Dosage	Application method and duration	Studied parameters	Reference
V. fluvialis	Gut of gilthead sea bream	Heat (60 °C, 2 h), UV-light (2.5 h)	European sea bass (*D. labrax*) (100 g)	10^9 CFU/mL	In vitro (head-kidney leucocytes)	Expression of immune-related genes	Román et al. (2013)
S. cerevisiae var *ellipsoideus*	Commercial products	No data	Beluga sturgeon (*Huso huso*) (11.44 ± 0.56 g)	1%, 2%	Diet; 6 weeks	Growth parameters, physiological responses and gut microbiota	Hoseinifar et al. (2011)
A mixture of *L. farciminis* CNCM MA27/6R and *L. rhamnosus* CNCM MA27/6B	Rumen goat	Heat	Sea bass (*D. labrax*) (250 mg)	50 g/kg diet	Diet; 103 days	Survival, growth, conformation, digestive metabolism (enzymatic, ultrastructural and microbial aspects)	Frouël et al. (2008)
A mixture of *B. subtilis*, *L. lactis* and *S. cerevisiae*	Culture collection	Heat	Rohu (*L. rohita*) (4.54 g)	10^{11} CFU/kg diet	Diet; 60 days	Growth parameters, nutrient digestibility and retention, digestive enzyme activity and gut microbiota	Mohapatra et al. (2012)
A mixture of *R. minuta* and *Ct. somerae*	Commercial products	Heat	Hybrid sturgeons (*A.aerii* × *A. hrenckii*) (7–12 cm)	5 g/kg diet	Diet; 3 weeks	Growth parameters, feeding efficiency and microbiota	Wu et al. (2020)
A mixture of live *P. acidilactici* and bacterial paraprobiotics or live *P. acidilactici* and yeast paraprobiotics or live *Bacillus* and yeast paraprobiotics	No data	No data	Rainbow trout (*O. mykiss*) (2.06 ± 0.07 g)	Bacterial paraprobiotic: 0.003%; Yeast Paraprobiotic: 0.15%	Diet; 63 days	Feed performance and disease resistance	Villumsen et al. (2020)
A mixture of *E. faecalis* and MOS	Commercial products	Heat	Rainbow trout (*O. mykiss*) (36.3 ± 0.42 g)	0, 2.5 + 2.5 g/kg and 5 + 5 g/kg	Diet; 12 weeks	Growth parameters, immune parameters and disease resistance	Rodriguez-Estrada et al. (2013)
A mixture of *L. plantarum* (HK-LP) and β-glucan (BG)	Commercial products	Heat	Red sea bream (*P. major*) (11 ± 0.03 g)	HK-LP (0.025, 0.05 or 0.1% of dry diet) combined with either 0% or 0.1% BG	Diet; 56 days	Growth parameters, immune parameters and stress resistance	Dawood et al. (2015c)

(continued)

Table 1 (continued)

Heat-killed probiotic	Originated from	Inactivation method	Host species	Dosage	Application method and duration	Studied parameters	Reference
A mixture of *L. plantarum* (LP) and vitamin C (VC)	Commercial products	Heat	Red sea bream (*P. major*) (11 ± 0.03 g)	0 or 1 g LP/kg combined with either 0.5 or 1 g VC/kg	Diet; 56 days	Growth parameters, immune parameters and stress resistance	Dawood et al. (2016)
A mixture of *L. plantarum* (HK-LP) and soybean meal (SBM)	Commercial products	Heat	Amberjack (*S. dumerili*) (25.05 ± 0.1 g)	0.0 or 0.1% HK-LP combined with either 0%, 15%, 30%, or 45% SBM	Diet; 56 days	Growth performance, digestibility and parameters, immune parameters	Dawood et al. (2015b)

noted that the efficacy of heat inactivation depends mainly on the species of microorganism, the culture media (type and pH of the media and water activity), growth and developmental stages and heating modes (Choudhury and Kamilya 2019).

UV radiation has been recognised to damage the DNA and protein of organisms, causing significant effects on growth and reproduction (Angélica Garrido-Pereira et al. 2013). UV rays at 200–400 nm can effectively inactivate vegetative bacterial cells and endospores (Choudhury and Kamilya 2019). In the case of the inactivation of probiotics used in aquaculture, UV-light treatment with a 2.5 h exposure has been applied with *Vagococcus fluvialis* (Román et al. 2013, 2012), *Bacillus amyloliquefaciens* FPTB16 (Kamilya et al. 2015) and *Bacillus subtilis* FPTB13 (Kamilya et al. 2015).

Formalin, which is commonly used as a disinfectant for eliminating infectious agents in aquaculture, inactivates microorganisms through the interaction with amino acids and nucleic acids (Leal et al. 2018). Inactivation of probiotic bacteria by formalin treatment has been reported for *B. subtilis* AB1 (using 2.0 v/v formalin for 48 h) (Newaj-Fyzul et al. 2007) and *B. amyloliquefaciens* FPTB16 (using 1.0 v/v formalin for 24 h at 4°C) (Kamilya et al. 2015).

Sonication refers to the use of sound waves at the higher limit of human hearing (>16 kHz) to disrupt intermolecular interactions by breaking the microbial cell wall, thinning cell membranes and causing DNA damage (Choudhury and Kamilya 2019). The effectiveness of sonication depends on the microbial species, especially on the cell wall structure (Sesal and Kekeç 2014). In aquaculture practice, studies showed that sonication method has been used successfully to inactivate the cells of *B. subtilis* AB1 (Newaj-Fyzul et al. 2007) and *L. plantarum* (Zheng et al. 2017).

The inactivation methods can cause the death of probiotic microorganisms, and each method may affect the viable cells in different ways in order to change the cell structures and biological activities (de Almada et al. 2016; Piqué et al. 2019). Thus, the methods and conditions used for the inactivation of probiotic bacteria are based on the intended use (Teame et al. 2020). It is necessary to consider the retention of cell structures and the natural benefits of probiotic microorganisms (Choudhury and Kamilya 2019; Teame et al. 2020). However, assessment of the purification, composition, and activities of paraprobiotics in the gut of hosts is difficult to ascertain because of the natural characteristics of non-viable microorganisms. Specifically, the isolation of the paraprobiotics based on traditional microbiological techniques cannot be done. Therefore, further research is needed to standardise appropriate analytical methods.

4 Method of Administration

In aquaculture, to gain optimal efficacy of paraprobiotics, issues relating to administration modes, dosage levels and duration of application should be carefully considered. Paraprobiotics may be administered to aquatic animals by either oral administration or injection. Paraprobiotics may be orally administrated indirectly *via* the

feeds which lead to improvements in the immune response and disease resistance of hosts (Dash et al. 2015; Dawood et al. 2015a, 2019b; Giri et al. 2020; Tung et al. 2009, 2010). Certainly, the oral administration of dead probiotic cells to fish or shrimp species has led to demonstrable health benefits (Pan et al. 2008; Patil et al. 2014). Furthermore, the intraperitoneal injection of heat-killed probiotics (such as *Pseudomonas aeruginosa* VSG2) as adjuvants for vaccines resulted in protection against infection in fish (Giri et al. 2015).

Paraprobiotics may be used alone or in combination and have been demonstrated to exert synergistic benefits on the health status of hosts. In aquatic animals, the use of single paraprobiotics is most common although a mixture of different paraprobiotics or paraprobiotics and other components (such as prebiotics and vitamins) possesses health-promoting effects and gives great benefits to hosts (Dawood et al. 2015c; Dawood et al. 2016; Frouël et al. 2008; Rodriguez-Estrada et al. 2013; Salinas et al. 2008). The success in mixed paraprobiotics may be related to the optimal combination of strain-specific properties (Pérez et al. 2010). Additionally, in the cases of in vitro studies, the heat-killed bacteria were directly incubated with the host tissues in culture media to assess their immunostimulant effects (Biswas et al. 2013b). Thus, the selection of administration modes is based on the specific purposes of the paraprobiotic application.

The dosage is an important factor that helps to achieve optimum beneficial effects of probiotics in the host. This is not only needed for the establishment and subsequent proliferation in the gut but also for conferring health benefits (Nayak 2010). In aquaculture, the doses of paraprobiotics differ with respect to probiotic species, host species, and type of parameters investigated. Dash et al. (2015) showed the effective dose of heat-killed *Lactobacillus plantarum* to be 10^8 CFU/g diet (when compared to applications of 10^7 or 10^9 CFU/g diet), which led to enhanced immune response and disease resistance of *Macrobrachium rosenbergii*. Moreover, Nguyen et al. (2019) reported that feeding Nile tilapia with diets supplemented with 20–50 ppm of heat-killed *L. plantarum* L-137 promoted the growth, immune responses and stress resistance of fish. The dosage of the probiotic needs to be carefully considered to avoid under- and over-dosing, the latter of which leads possibly to unexpected outcomes and unnecessary costs (Dash et al. 2015). Thus, the suitable dosages of paraprobiotics are determined from their capacity of improving the growth and protection of hosts.

The duration of application is also an important factor influencing the effectiveness of paraprobiotics in aquatic animals. This period varies with respect to host species, probiotic species and health parameters and has ranged from 3 to 103 days (Table 1). In an *in vitro* study, the head-kidney leucocytes of gilthead seabream (*Sparus aurata*) were incubated for 30 min with heat-killed probiotics to investigate the cellular innate immune parameters (Salinas et al. 2006). Thus, the duration of administration of paraprobiotics is most likely to depend on the specific goals of the supplementation.

5 Common Parameters Used for Assessing the Health Benefits of Paraprobiotics

Growth performance and feed utilisation are crucially important factors to assess the effectiveness of aquaculture practice. It has been noted that there are several parameters (such as final weight, body weight gain, specific growth rate, feed intake, protein efficiency ratio, feed conversion ratio, apparent digestibility coefficients and digestive enzyme activity), which are used for evaluating the roles of paraprobiotics in the growth of aquatic animals (Dash et al. 2015; Dawood et al. 2015a, 2015b, 2015c; Duc et al. 2020; Tung et al. 2009, 2010; Yang et al. 2016; Zheng et al. 2017). These parameters indicate the significance of the ingredients added to diets used for cultured animals to encourage weight gain, thereby affecting the final production levels.

The immune system plays an important role in protecting the host from invasion by pathogens. Interestingly, the use of paraprobiotics has the capacity of stimulating the immune system of the host; the immune responses have been evaluated through numerous parameters. These include complement activity, lysozyme, phagocytosis, respiratory burst and antioxidant capacity. These have been used for evaluating the effects of paraprobiotics on:

- innate immunity
- the level of antibodies (including immunoglobulin-Ig), which are used for assessing their effects on the adaptive immune responses
- the levels of cytokines used for investigating their influences on both innate and adaptive immune immunity of hosts (Biswas et al. 2013a, 2013b; Dash et al. 2015; Dawood et al. 2015c, 2016; Kamilya et al. 2015; Munoz-Atienza et al. 2015; Panigrahi et al. 2005; Panigrahi et al. 2011; Salinas et al. 2006; Singh et al. 2017; Tung et al. 2009).

In fish, phagocytosis plays an important role in the early activation of the inflammatory response and is mediated by neutrophils, monocytes, and macrophages. The activation of phagocytic cells may lead to the secretion of many biologically active molecules (i.e. enzyme inhibitors, cationic peptides and complement components) and to the production of reactive oxygen (ROS) and nitrogen species (Pérez et al. 2010). Moreover, lysozymes are universally distributed amongst living organisms and released by leucocytes, which are capable of protecting hosts against invasion and colonisation of both Gram-positive and Gram-negative bacteria in the presence of complement (Giri et al. 2015; Pimpimol et al. 2012; Ramesh and Souissi 2018; Saurabh and Sahoo 2008). Lysozyme and phagocytic activities are indicators of the body defence system of fish, highlighting the health status and responses of fish to stressful conditions (Nguyen et al. 2019). For the complement system, the activation of complement components results in a cascade of biochemical reactions and leads to antigen elimination (Holland and Lambris 2002; Pérez et al. 2010). Complement activity is used to evaluate the influence of paraprobiotics on the humoral immune response of hosts (Panigrahi et al. 2011).

Amongst the immune cell parameters, the number of red and white blood cells is important in both innate and adaptive (immune) responses (Opiyo et al. 2019; Standen et al. 2013). The application of haematological techniques has received increased attention with regard to assessing the beneficial effect of paraprobiotics in aquatic animals (Dawood et al. 2019b; Duc et al. 2020; Tung et al. 2009).

Additionally, the parameters of cortisol and glucose content, total bilirubin, blood urea nitrogen, glutamyl oxaloacetic transaminase and glutamic-pyruvate transaminase activities and triglycerides have been used to evaluate the stress levels of hosts and the capacity of the host defence system to tolerate the stressors (Dawood et al. 2015c; Nguyen et al. 2019; Singh et al. 2017).

6 Modes of Action

The paraprobiotics serving as beneficial dietary supplements have been demonstrated to be important in conferring health benefits in aquatic animals (Figs. 1 and 2). Moreover, paraprobiotics are directly or indirectly associated with the composition and/or activity of the gut microbiota (Hoseinifar et al. 2011; Wu et al. 2020; Yang et al. 2014; Zheng et al. 2020). It has been determined that paraprobiotics serve as growth factors that selectively stimulates the growth of beneficial microbes (such as lactic acid bacteria) by the provision of enzymes, RNA and free nucleotides, B-complex vitamins and/or amino acids (Hoseinifar et al. 2011). Alternatively, paraprobiotics may inhibit the growth of potential pathogens in the gut epithelium by activating the mucosal immunity of fish (Yang et al. 2014), which positively influence health.

Interestingly, paraprobiotics have beneficial effects on the improvement of feed utilisation and growth performance in aquatic animals (Dawood et al. 2015a, 2015b, 2015c, 2016, 2019b; Duc et al. 2017, 2020; Hasan et al. 2019a; Nguyen et al. 2019; Rodriguez-Estrada et al. 2013; Tung et al. 2010; Yan et al. 2016; Yang et al. 2016; Zheng et al. 2017). Thus, Dawood et al. (2015a) reported significantly higher growth, feed intake, feed efficiency ratio, protein retention and apparent digestibility coefficients in red sea bream (*Pagrus major*) after dietary supplementation with heat-killed *L. plantarum* (*L. plantarum* LP20, containing 20% *L. plantarum* HK L-137 and 80% dextrin on a dried-weight basis) for 56 days. The mechanism of paraprobiotics for improving the growth performance may be related to an enhanced secretion of intestinal enzymes. These enzymes play a role in promoting the digestive capacity of fish to hydrolyse feed ingredients (i.e. carbohydrate, protein and lipid), thereby improving growth performance and feed efficiency (Dawood et al. 2015b). In accordance with this observation, Yang et al. (2016) demonstrated that the activities of protease and amylase were increased in sea cucumber (*Apostichopus japonicas*) fed with heat-killed *L. plantarum* L-137-supplemented diets.

Another aspect of using paraprobiotics is the stimulation of changes in the morphology of the digestive tract. The mucosal thickness, villus length and muscle thickness increased significantly when genetically improved farmed Nile tilapia (*Oreochromis niloticus*) were fed with heat-killed *L. plantarum* (HK L-137) (at 50,

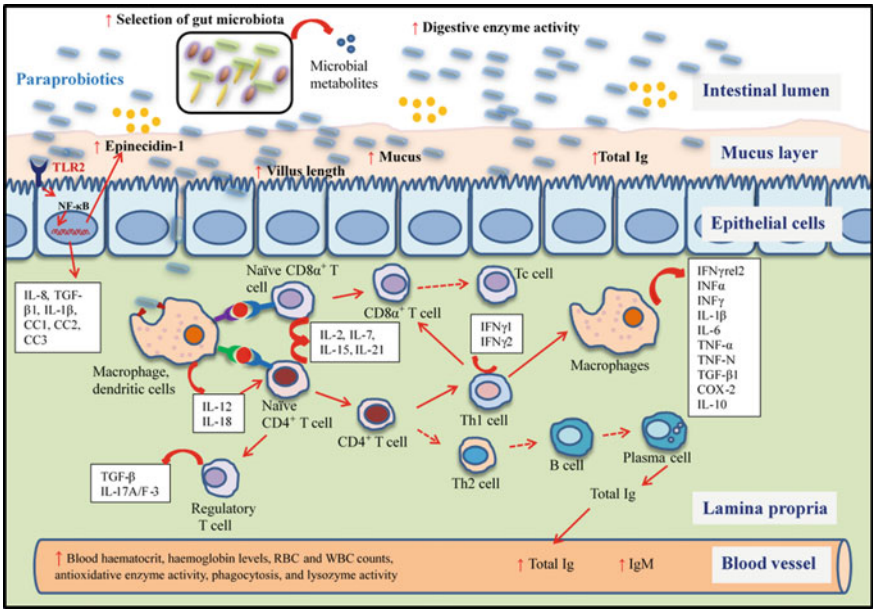

Fig. 1 Beneficial effects of paraprobiotics on fish health following dietary administration (Biswas et al. 2013a; Dawood et al. 2015b; Dawood et al. 2019b; Giri et al. 2016; Hoseinifar et al. 2011; Matsuura et al. 2017; Nguyen et al. 2019; Pan et al. 2008; Sun et al. 2014; Yan et al. 2016; Yang et al. 2014; Yang et al. 2016). The solid arrows indicate the evidence of the mechanisms; the dashed arrows indicate the hypothetical evidences. Abbreviations: IgM, immunoglobulin M; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor; IFN, interferon; NF-κB, nuclear factor kappa B; COX-2, cyclooxygenase-2; RBC, red blood cell; WBC, white blood cell; CC, CC chemokine 1

100 or 1000 mg/kg feed) compared to the controls (Dawood et al. 2019b). The increase in intestinal villus length gives rise to the absorptive surface area, leading to better nutrient utilisation and growth improvement (Dawood et al. 2019b; Khojasteh 2012). Furthermore, there was an increase in the number of endocytotic vesicles in sea bass (*Dicentrarchus labrax*) fed with a diet supplemented with heat-inactivated bacteria. This preparation comprised a mixture of *L. farciminis* CNCM MA27/6R and *L. rhamnosus* CNCM MA27/6B, and the outcome may be related to the transportation of antigens from the gut lumen through the enterocytes to intra-epithelial lymphoid cells or macrophages. This process would lead to a stimulation of the immune system of the fish (Frouël et al. 2008).

Certainly, immunostimulation is one of the most important roles of paraprobiotics in aquatic animals. Several studies have reported the effects of paraprobiotics on innate and cellular immunity. Specifically, an increase of blood haematocrit, haemoglobin levels and red blood cell and white blood cell counts, antioxidative enzyme (serum superoxide dismutase-SOD and catalase-CAT) activity, total serum protein and IgM levels, phagocytosis and lysozyme activity was recorded in Nile

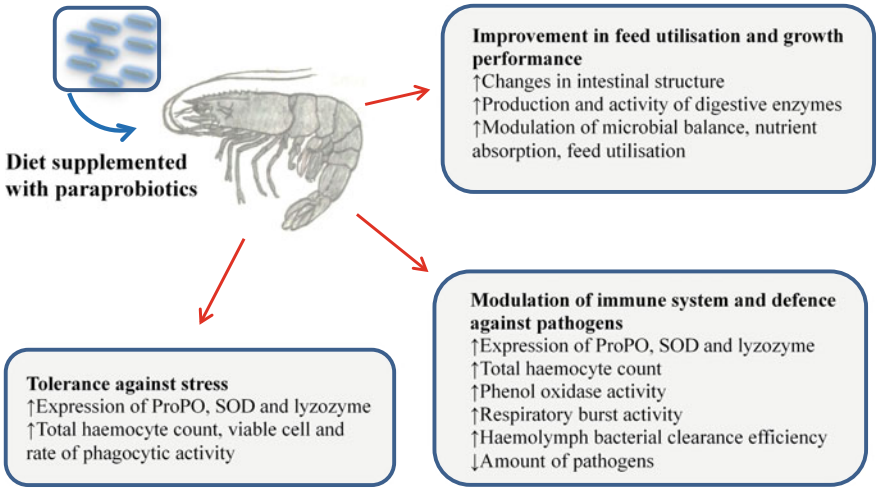

Fig. 2 Beneficial effects of paraprobiotics on shrimp health following dietary administration (Dash et al. 2015; Tung et al. 2009, 2010; Zheng et al. 2018, 2020). Abbreviations: ProPO, prophenoloxidase; SOD, superoxide dismutase

tilapia after feeding with diets supplemented with HK L-137 (Dawood et al. 2019b; Nguyen et al. 2019). With Japanese pufferfish (*Takifugu rubripes*) orally administrated with *Lactobacillus paracasei* (06TCa22), there was an increase in the expression of pro-inflammatory cell-mediated immunity inducing antiviral/intra-cellular pathogen killing, anti-inflammatory and peripheral T-cell expansion and the production of superoxide anion and phagocytic activity (Biswas et al. 2013a). At the gut level, both viable and heat-inactivated *Bacillus pumilus* SE5 probiotics were capable of inducing activation of intestinal mucosal immunity through the expression of antibacterial epinecidin-1 in grouper (*Epinephelus coioides*) (Yang et al. 2014). Moreover, heat-killed probiotics have been reported to induce cell-mediated immunity in Ginbuna crucian carp (*Carassius auratus langsdorfii*) (Matsuura et al. 2017). Intraperitoneal injection of heat-killed *Enterococcus faecalis* induced an increase in CD4-1$^+$ and CD8α^+ lymphocytes and macrophages in vivo. Moreover, the expression of Th1 cytokine genes (IL-12, IFNγ1, IFNγ2 and IFNγrel2) was enhanced in vitro and in vivo (Matsuura et al. 2017). These authors suggested that heat-killed *E. faecalis* induced cell-mediated immunity in fish. Furthermore, Pan et al. (2008) reported an increase in total Ig level in the serum and gut mucus of Chinese drum (*Miichthys miiuy*) orally administered with live or dead cells of *Clostridium butyricum* CB2. Dawood et al. (2019b) reported increased IgM levels in the serum of Nile tilapia fed with HK L-137. Thus, there was improvement in the immune functions in aquatic animals leading to the enhancement in protection against the onslaught of pathogens.

Although crustaceans do not possess adaptive immune systems, they have efficient innate immune systems associated with humoral responses, i.e. melanisation, coagulation and production of antimicrobial peptides, and cellular responses (namely

encapsulation, phagocytosis and autophagy) (Tran et al. 2020a). The mechanisms of action of the paraprobiotics are mainly related to the activation of the prophenoloxidase (proPO) system and respiratory burst activity. Serving as a source of pathogen-associated molecular patterns through the pattern recognition receptors (PRRs), paraprobiotics activate haemocytes to produce melanin *via* proPO and other bacteriocidal substances (such as hydrogen peroxide-H_2O_2 and superoxide anion-O_2^-) through respiratory burst which increased disease resistance (Dash et al. 2015; Zheng et al. 2017). Under stress stimulation, SOD exerted an important role in converting O_2^- into water and H_2O_2, which was then converted into oxygen and water involving the catalysis of antioxidant enzymes, CAT and glutathione peroxidase (Zheng et al. 2017). Furthermore, there was an improvement in total haemocyte count, phenol oxidase and respiratory burst activities and clearance efficiency found in the giant freshwater prawn (*M. rosenbergii*) fed heat-killed *L. plantarum* (MTCC no. 1407) (Dash et al. 2015).

So far, the available evidence demonstrated the benefits of paraprobiotic administration in aquaculture. However, the modes of action of paraprobiotics are not yet fully understood. Further, studies basing on "omics" studies, i.e. transcriptomic, metabolomics and proteomics, are needed to provide knowledge on the beneficial effects of praraprobiotics on the health of aquatic animals.

7 Toll/toll-Like Receptors as Pattern Recognition Receptors Recognise Paraprobiotics

Toll/toll-like receptors (TLRs) are type I transmembrane receptors with three types of domains (LRR domain, transmembrane helix and TIR domain), which are expressed by innate immune cells of the intestinal epithelium and the lamina propria either at the cell surface or in endosomes (de Medina et al. 2013). TLRs serve as one of the most important PRRs and play a vital role in the innate immune responses of both invertebrates and vertebrates (Lin et al. 2012; Tran et al. 2020a). In mammals, TLRs are responsible for recognising not only the microbial components but also damaged host cell components and activate the mucosal immune system (Abreu 2010; de Medina et al. 2013). TLRs associate with intestinal homeostasis by modulating the production of Ig, maintenance of gut integrity tight junctions, and expression of antimicrobial peptides (de Medina et al. 2013). The roles of fish TLRs in the mucosal immune response upon probiotic stimulation have recently received attention (Pérez et al. 2010). Interestingly, the gene expression of TLR2, but not TLR5, was significantly increased in grouper (*E. coioides*) fed either with heat-inactivated *B. pumilus* SE5 (Yan et al. 2016; Yang et al. 2014) or heat-inactivated *Psychrobacter* sp. SE6 (Sun et al. 2014). This indicates the association of the immunostimulatory effects of heat-killed probiotics in fish with the activation of the TLR2 signalling pathway (Fig. 1). Generally, the recognition of pathogen-associated molecular pattern molecules by TLRs leads to an assembly of cytosolic TIR domain-containing adaptor molecules.

These include MyD88, MyD88 adaptor-like protein (MAL/TIRAP), TRIF/TICAM1 and TRIF-related adaptor molecule (TRAM/TICAM2), which transduce signals from the membrane surface to the nucleus. This process is through the activation of downstream transcription factors in the cytosol and then activation of the induction of critical antimicrobial peptides (Tran et al. 2020a). However, in the case of heat-inactivated *B. pumilus* SE5 and heat-inactivated *Psychrobacter* sp. SE6, the immune system of grouper was stimulated through a MyD88-independent pathway (Sun et al. 2014; Yang et al. 2014). The activation of TLR2 may result in the production of cytokines (IL-8, IL-1β and TGF-β1) and antimicrobial peptide (Epinecidin-1) in grouper (Yan et al. 2016; Yang et al. 2014). Furthermore, Lazado and Caipang (2014) reported that heat-inactivated *Pseudomonas* sp. GP21 and GP12 up-regulated the expression of CC chemokine 1, CC chemokine 2 and CC chemokine 3 in the intestinal epithelial cells of Atlantic cod (*Gadus morhua*).

In most cases, the mechanisms that modulate the immunity of fish species through TLR signalling is not fully described. However, the evidence has demonstrated that inactivated probiotics are capable of inducing the production of cytokines, participating in the immune responses of aquatic animals (Biswas et al. 2013a, 2013b; Giri et al. 2015, 2016; Hasan et al. 2019a; Lazado et al. 2010; Matsuura et al. 2017; Panigrahi et al. 2011; Román et al. 2013; Singh et al. 2017; Yang et al. 2014).

8 Microorganisms Used as Paraprobiotics and Their Efficacy in Aquaculture

The microorganisms used as paraprobiotics in aquaculture have been reported to include both Gram-negative and Gram-positive bacteria and yeasts (Table 1). It is noted that the bacteria originated from several sources, such as the gut of aquatic animals (Giri et al. 2015, 2016; Lazado and Caipang 2014; Lazado et al. 2010; Newaj-Fyzul et al. 2007; Román et al. 2013, 2012; Sun et al. 2014; Yan et al. 2016; Yang et al. 2014), fish body (Díaz-Rosales et al. 2006; Salinas et al. 2006), seafood- and fish-derived products (Kamilya et al. 2015; Munoz-Atienza et al. 2015; Singh et al. 2017), culture collections (Dash et al. 2015; Panigrahi et al. 2005, 2011; Villamil et al. 2002) and commercial products (Dawood et al. 2015a, 2015b, 2015c, 2016, 2019b; Duc et al. 2017, 2020; Frouël et al. 2008; Mohapatra et al. 2012; Nguyen et al. 2019; Rodriguez-Estrada et al. 2013; Suprayudi et al. 2017; Tung et al. 2009, 2010; Yang et al. 2016; Zheng et al. 2017; Zheng et al. 2018, 2020) have been used as paraprobiotics. Furthermore, the bacteria isolated from the intestine of healthy chicken (Pan et al. 2008) and Mongolian dairy products (Biswas et al. 2013a, 2013b) have been used for the preparation of paraprobiotics used in aquaculture. Remarkably, albeit the sources of probiotic bacteria serving as paraprobiotics are different, they can all be applied effectively in aquatic animals.

8.1 Lactic Acid Bacteria

Eight heat-inactivated lactic acid bacteria (LAB), including *Enterococcus faecium* CV1, *E. faecium* LPP29, *Lactobacillus curvatus* subsp. *curvatus* BCS35, *Lactococcus lactis* subsp. *cremoris* SMF110, *Leuconostoc mesenteroides* subsp. *cremoris* SMM69, *Pediococcus pentosaceus* SMM73, *P. pentosaceus* TPP3, and *Weissella cibaria* P71, were incubated singly with the head-kidney leucocytes of turbot (*Scophthalmus maximus*). The outcome was that heat-inactivated LAB significantly increased leucocyte respiratory burst activity and did not have any cytotoxic effect on apoptosis of turbot phagocytes and lymphocytes (Munoz-Atienza et al. 2015).

8.2 **Lactobacillus plantarum**

L. plantarum is a rod-shaped, Gram-positive, catalase-negative, non-endospore-forming, fermentative, facultative anaerobic lactic acid bacterium. Use of viable cells has led to an improvement of growth, digestive enzyme activities, feed utilisation, immunity, disease resistance and survival, as well as inhibiting the adhesion and growth of pathogens in aquatic animals (Dash et al. 2015). Heat-killed *L. plantarum* 06CC2, which was isolated from the Mongolian dairy products, induced the expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and TNF-N), cell-mediated immune regulators (IL-12p40 and IL-18), antiviral (I-IFN-1) and other regulatory (IL-2, IL-7, IL-15, IL-21, IL-10 and TGF-β1) cytokines in the head-kidney cells of Japanese pufferfish (*T. rubripes*), *in vitro* (Biswas et al. 2013b). In red sea bream (*P. major*), heat-killed *L. plantarum* (HK-LP) enhanced growth parameters, feed utilisation, serum lysozyme activity, total serum protein, serum alternative complement pathway activity and mucus secretion, as well as the survival rate after low salinity stress (Dawood et al. 2015a; Dawood et al. 2015c). However, the dietary supplementation of HK-LP did not affect changes in protein body content, somatic parameters, total bilirubin, blood urea nitrogen, glutamyl oxaloacetic transaminase, glutamic-pyruvate transaminase, triglycerides and mucus bacteriocidal activity of the fish (Dawood et al. 2015c). Use of HK L-137 led to improved growth, immune responses and oxidative status, induced upregulation of growth-related gene (IGF-I) and the glucose regulation gene (G6PD) and the downregulation of fatty acid synthase (FAS), as well as having positive effects on preserving health and facilitating the enhancement of defence against pathogens and ammonium exposure in Nile tilapia (Dawood et al. 2019b; Nguyen et al. 2019). It was noted that the appropriate doses of 20–50 ppm HK L-137 in diets enhanced not only growth but also immune responses and stress resistance of Nile tilapia (*O. niloticus*) (Nguyen et al. 2019). In juvenile striped catfish (*Pangasianodon hypophthalmus*) (initial weight 0.06 g), dietary administration of HK L-137 significantly improved final weight, weight gain and specific growth rate, survival rate, protein efficiency ratio, feed conversion ratio,

immune response (*viz.* an increase in lysozyme activity and the number of red blood cells and white blood cells) and resistance to *Edwardsiella ictaluri* (Duc et al. 2020).

At the post-larvae and juvenile stages of white shrimp (*Litopenaeus vannamei*), dietary *L. plantarum* LP20 (fed at 0.5 or 0.1 g/kg of feed) enhanced growth and immune responses (Duc et al. 2017). Also, feeding white shrimp with diets supplemented with heat-killed *L. plantarum* for 45 days increased growth performance, survival rate and stress resistance to acute low salinity (Zheng et al. 2017). White shrimps fed with heat-killed *L. plantarum* for 15 days revealed improved final weight, weight gain, feed conversion ratio, digestive enzymes activity (including amylase, lipase and pepsin), the height of enterocytes (Zheng et al. 2018) and the abundance of the bacterial phylum Verrucomicrobia (Zheng et al. 2020).

In kuruma shrimp (*M. japonicas*), *L. plantarum* HK-LP Prep at 0.1 and 1 g/kg diets significantly improved survival of the larvae, whereas HK-LP Prep at 1 g/kg diet significantly enhanced growth and formalin stress resistance of the post-larvae (Tung et al. 2010). At the juvenile stage, *L. plantarum* HK-LP Prep (at 100 and 1000 mg/kg diet) improved growth and feed efficiency, as well as, increased survival rate, total haemocyte count, viable cell and rate of phagocytic activity following low salinity shock (Tung et al. 2009).

In giant freshwater prawn (*M. rosenbergii*), heat-killed *L. plantarum* (MTCC no. 1407) (at 10^7, 10^8 and 10^9 CFU/g diet) was administered for 90 days and revealed a significant increase in total haemocyte count, phenoloxidase activity, respiratory burst activity, clearance efficiency and resistance against *Aeromonas hydrophila* (Dash et al. 2015).

In sea cucumber (*A. japonicas*), there was a significant enhancement in body weight gain, specific growth rate and protease activity after feeding with either 0.05 or 0.25 g/kg diet of *L. plantarum* HK L-137 (Yang et al. 2016). The diet supplemented with 0.25 g/kg diet enhanced amylase, lysozyme, and phagocytic activities; whereas the diet containing 0.05 g HK L-137/kg of diet improved the SOD enzyme and alkaline phosphatase activity. The optimal dose, 0.05 g/kg diet of HK L-137, led to health benefits on growth, digestive enzyme activity and non-specific immune responses of sea cucumber (Yang et al. 2016).

8.3 Lactobacillus rhamnosus

L. rhamnosus is a facultative, lactic acid bacterium in the phylum Firmicutes, which is generally considered beneficial and has been used as a probiotic in humans (Boonma et al. 2014). In juvenile rainbow trout (*Oncorhynchus mykiss*) (average weight 126 g), heat-killed *L. rhamnosus* JCM 1136 induced cellular and humoral immune responses (*viz.* phagocytic activity, superoxide anion, complement activity, lysozyme activity, and plasma Ig level) during a 15-day feeding period. Interestingly, the bacterial cells disappeared from the gut of fish 15 days after ceasing the feeding regime (Panigrahi et al. 2005). Furthermore, heat-killed *L. rhamnosus* JCM 1136 induced the expression

of cytokine genes (TGF-β and IFN) and immune-related gene (Ig) in rainbow trout (Panigrahi et al. 2011).

8.4 Lactobacillus paracasei *subsp.* paracasei

L. paracasei subsp. *paracasei* (Lpp) 06TCa22 was isolated from traditional Mongolian dairy products and determined to possess probiotic properties, including tolerance to low pH and bile acid, gas production from glucose, adherence to Caco-2 cells and carbohydrate utilisation (Takeda et al. 2011). Biswas et al. (2013b) incubated Japanese pufferfish (*T. rubripes*) head-kidney cells with heat-killed Lpp and revealed significant expressions of pro-inflammatory (IL-1β, IL-6, IL-17A/F-3, TNF-α and TNF-N), cell-mediated immune regulators (IL-12p35, IL-12p40 and IL-18), antiviral (I-IFN-1 and IFN-γ) and other regulatory (IL-2, IL-7, IL-21, IL-10 and TGF-β1) cytokines. Also, the heat-killed Lpp enhanced the pro-inflammatory, cell-mediated immunity inducing, antiviral/intra-cellular pathogen killing, anti-inflammatory and peripheral T-cell expansion and survival controlling cytokine gene expression, superoxide anion production, phagocytic activity and disease resistance against *V. harveyi* in Japanese pufferfish (Biswas et al. 2013a).

8.5 Lactobacillus delbrüeckii *subsp.* lactis

Heat-killed *L. delbrueckii* ssp. *delbrueckii* was incubated with the head-kidney leucocytes of gilthead seabream (*S. aurata*) with the results revealing the positive effect of heat inactivation on the cellular innate immune responses of the fish. Thus, there was an increase in the leucocyte peroxidase content, phagocytosis, respiratory burst activity and cytotoxicity (Salinas et al. 2006). It was concluded that the bacterial concentration of 5×10^7 CFU/mL gave a strong stimulatory effect (Salinas et al. 2006).

8.6 Bacillus *sp.*

The members of the genus *Bacillus* (belonging to the Order Bacillales, Class Bacilli and Phylum Firmicutes) are Gram-positive, rod-shaped, catalase-positive, endospore-producing aerobic or facultative anaerobes (Ringø 2020). The live bacteria have been used widely as environmental and dietary probiotics in aquaculture systems (Hong et al. 2005; Moriarty 1998; Ringø 2020; Wu et al. 2014). Heat-killed *Bacillus* SJ-10, which was isolated from traditional Korean fermented fish ("Jeotgal") (at 1×10^8 CFU/g diet), showed positive effects on growth performance (i.e. enhanced weight gain and protein efficiency ratio) and humoral innate immune (i.e. increased

lysozyme and SOD activities), as well as induced the relative expressions of TNF-α, IL-1β and IL-6 in the liver and gill of olive flounder (*Paralichthys olivaceus*) (Hasan et al. 2019a). In addition, the results revealed an improved survival rate (18.52%) compared to the control group (0%) after challenge with *S. iniae* for 13 days (Hasan et al. 2019a).

8.7 Bacillus pumilus

B. pumilus is a Gram-positive, aerobic, rod-shaped endospore-forming bacterium (Hill et al. 2009). The heat-inactivated *B. pumilus* SE5 (at 1.0×10^8 cells/g) demonstrated the capability of inducing the expression of mucosal immune and antibacterial genes (including TLR2, IL-8, TGF-β1 and epinecidin-1) and reducing the diversity of pathogenic bacterial species, i.e. *Psychroserpens burtonensis* and *Pantoea agglomerans* (Yang et al. 2014). In another study, Yan et al. (2016) found that dietary administration of heat-inactivated *B. pumilus* SE5 to grouper (*E. coioides*) juveniles for 60 days significantly improved the final weight, weight gain, specific growth rate, feed conversion ratio and immune parameters (phagocytic activity, serum complement C_3 and IgM levels and SOD activity), as well as stimulated the expression of TLR2 and pro-inflammatory cytokines (IL-8 and IL-1β).

8.8 Bacillus amyloliquefaciens

B. amyloliquefaciens (strain FPTB16), which is a closely related species to *B. subtilis*, was isolated from an indigenous fermented fish product ("Shidal") and has been reported to be a potential probiotic in catla (*Catla catla*) to improve health and disease resistance (Das et al. 2013). Kamilya et al. (2015) determined the immunostimulatory effects of heat-, UV-light- and formalin-inactivated *B. amyloliquefaciens* FPTB16 in catla (*C. catla*), increasing the respiratory burst activity, nitric oxide production, leucocyte peroxidase content and proliferative response in head-kidney leucocytes. Later, Singh et al. (2017) showed that *B. amyloliquefaciens* FPTB16 (at 10^7, 10^8 and 10^9 cells/g of diet) increased oxygen radical production, serum lysozyme activity, total serum protein content, myeloperoxidase activity, alkaline phosphatase activity and expression of IL-1β, TNF-α, C3 and iNOS in liver or head-kidney, remained unchanged glucose content, glutamate pyruvate transaminase and glutamate oxaloacetate transaminase activities in serum and down-regulated the expression of IFN-γ.

8.9 Bacillus subtilis

B. subtilis has been identified in the intestine of several finfish species and reported to be beneficial in aquaculture (Soltani et al. 2019). Heat-killed *B. subtilis* CECT 35 stimulated the leucocyte peroxidase content, respiratory burst and cytotoxic activity in a dose-dependent manner and phagocytosis in the monocyte-macrophages and acidophilic granulocytes of gilthead seabream (*S. aurata*) (Salinas et al. 2006). In another study using head-kidney leucocytes of catla (*C. catla*), Kamilya et al. (2015) reported that heat-killed *B. subtilis* FPTB13 isolated from an indigenous fermented fish product ("Shidal") stimulated the superoxide anion production, nitric oxide production, myeloperoxidase content and proliferative response of the head-kidney leucocytes albeit not significantly. However, the effective stimulation was dose dependent with the highest values observed at a concentration of 10^7 cells/mL (Kamilya et al. 2015). Newaj-Fyzul et al. (2007) fed rainbow trout (*O. mykiss*) with *B. subtilis* AB1 (either as viable, formalised or sonicated cells or as cell-free supernatant) at 10^7 cells/g for 14 days and challenged the fish with *Aeromonas* sp. The data revealed protection of fish against challenge. In a later study using heat-killed whole cell products of *B. subtilis* VSG2 administered to *Labeo rohita*, Giri et al. (2015) revealed a significant enhancement in humoral immune response (*viz.* total serum protein, albumin, globulin levels, lysozyme activity, alternative complement pathway activities), cellular immune response (*viz.* respiratory burst activity and phagocytic activity), expression of immune-related genes (IL-1β, TNF-α, COX-2, NF-κB, iNOS and IL-10) and disease resistance against challenge with *A. hydrophila.* The authors concluded that the cellular components of *B. subtilis* VSG2 may be useful as adjuvants for vaccines in aquaculture (Giri et al. 2015).

8.10 Enterococcus faecalis

The Gram-positive *E. faecalis* is grouped with the lactic acid bacteria and is a commensal inhabiting the gastrointestinal tracts of many organisms (Van Tyne et al. 2013). In rainbow trout (*O. mykiss*), inactivated *E. faecalis* increased the growth performance (an increase in weight gain, specific growth rate, feed gain ratio and protein efficiency ratio), immunity system (*viz.* an increase in haematocrit value, phagocytic activity and mucus weight) and resistance against challenge with *Aeromonas salmonicida* after a 12-week feeding trial (Rodriguez-Estrada et al. 2013). In ginbuna crucian carp (*C. auratus langsdorfii*), heat-killed *E. faecalis* led to stimulation of the expression of Th1 cytokine genes and an increase in the number of CD4-1$^+$ lymphocytes, CD8α$^+$ lymphocytes and macrophages, as well as the expression of Th1 cytokines in the kidney leucocytes (Matsuura et al. 2017).

8.11 Enterococcus gallinarum

E. gallinarum L-1 was isolated from gilthead sea bream (*S. aurata*) intestine and has been characterised to be suitable for fish as a potential probiotic (Sorroza et al. 2013). In a study using head-kidney leucocytes of gilthead sea bream (*S. aurata*), European sea bass (*D. labrax*), meagre (*Argyrosomus regius*) and red porgy (*Pagrus pagrus*), Román et al. (2015) determined that heat- or UV-light-inactivated *E. gallinarum* L-1 (at 10^6, 10^7 and 10^8 CFU/mL) stimulated the cellular immune system in the leucocytes of sea bream, sea bass and red porgy but not meagre. The stimulation was dose dependent in most cases with the highest values found at a dose of 10^8 CFU/mL. There were not any significant differences amongst the different doses.

8.12 Lactococcus lactis

L. lactis is a Gram-positive spherical, homolactate, non-sporulating and facultative anaerobic organism found in the gut (Song et al. 2017). Heat-killed *L. lactis* stimulated significantly the chemiluminescent response of turbot (*S. maximus*) macrophages and nitric oxide production (Villamil et al. 2002). Moreover, in Nile tilapia (*O. niloticus*), a commercial probiotic (PowerLac^{TM} consisting of heat-killed and lyophilised *L. lactis* D1813) improved the final body weight and growth and feed conversion ratio (in an 8-week laboratory experiment of probiotic feeding at 0.25 or 0.5 g/kg) and promoted growth, protein and lipid retention, feed conversion ratio and mortality of fish after challenge with *A. hydrophila* (in a 22-week field experiment feeding with probiotic at 0.5 g/kg) (Suprayudi et al. 2017).

8.13 Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative rod-shaped organism that is an opportunistic pathogen causing diseases in non-immunocompetent individuals (de Bentzmann and Plésiat 2011). Heat-killed *P. aeruginosa* VSG2, which was isolated from the intestine of Indian major carp (*L. rohita*), improved the immune responses, induced the expression of immunity-related genes and increased the disease protection in *L. rohita* (Giri et al. 2015, 2016). Subsequently, Giri et al. (2020) administered heat-killed *P. aeruginosa* VSG2 to common carp (*Cyprinus carpio*) at 0, 10, 20, 30 and 40 mg/kg for 8 weeks and revealed an increase in lysozyme, protein level, alkaline phosphatase and alternative complement pathway, SOD, glutathione, glutathione peroxidase (GPx), myeloperoxidase levels, protease activity, the expression of SOD, GPx and CAT and disease resistance to challenge with *A. hydrophila.*

8.14 Clostridium butyricum

The Gram-positive anaerobic bacterium *Clostridium butyricum* may be found in the intestine of healthy humans and animals (Juan et al. 2017) and widely used as a probiotic in aquatic animals (Tran et al. 2020b). Chinese drum (*M. miiuy*) fed dead *C. butyricum* CB2, which was isolated from the gut of chickens, demonstrated a significant enhancement in head-kidney leucocyte phagocytic activity, serum lysozyme activity and total Ig level, gut mucus and survival rate after challenge with either *Vibrio anguillarum* or *A. hydrophila* (Pan et al. 2008).

8.15 Vibrio-*Like Bacteria of the* Shewanella *genus (Pdp11 and 51M6)*

In a study with head-kidney leucocytes of gilthead seabream (*S. aurata*), heat-killed bacteria, *Shewanella* Pdp11 and 51M6, which were isolated from the skin of gilthead sea bream, enabled an increase in peroxidase content, respiratory burst and cytotoxic activity and phagocytic activity (in the monocyte-macrophages and acidophilic granulocytes) (Salinas et al. 2006). Of these probiotic, use of *Shewanella* 51M6 led to better immunostimulatory effects than Pdp11; a dose of 5×10^7 CFU/mL was optimum (Salinas et al. 2006). Moreover, using heat-killed *Shewanella* Pdp11 and 51M6 applied singly or in combination, Díaz-Rosales et al. (2006) observed that the serum peroxidase content and natural haemolytic complement activity increased with time. The use of single or combined probiotics significantly enhanced phagocytic ability, and only, the use of single heat-killed *Shewanella* 51M6 increased the cytotoxic activity of gilthead seabream (Díaz-Rosales et al. 2006).

8.16 Pseudomonas *sp.*

The defence mechanisms of heat-killed *Pseudomonas* sp. (GP21) in the modulation of the immune system of Atlantic cod (*G. morhua*) were found after incubation with fish head-kidney leucocytes (Lazado et al. 2010) or intestinal epithelial cells (Lazado and Caipang 2014).

8.17 Psychrobacter *sp.*

The incubation of *Psychrobacter* GP12 with Atlantic cod (*G. morhua*) head-kidney leucocytes (Lazado et al. 2010) or intestinal epithelial cells (Lazado and Caipang 2014) led to an increase in the expression of immunity-related genes. In another

study, heat-inactivated *Psychrobacter* SE6 was fed at 1.0×10^8 cells/g to grouper (*E. coioides*) for 60 days, leading to an elevated expression of TLR2, epinecidin-1 and IgM and a decreased abundance of gut microbiota in the fish (Sun et al. 2014).

8.18 Vagococcus fluvialis

In a study with head-kidney leucocytes of gilthead sea bream (*S. aurata*) and European sea bass (*D. labrax*), heat- or UV-light-inactivated *V. fluvialis* (at 10^7 and 10^8 CFU/mL) increased respiratory burst activity in sea bream leucocytes, whereas the heat- or UV-inactivated bacteria (at 10^8 CFU/mL) increased the peroxidase content in sea bass leucocytes. Only, UV-inactivated bacteria increased the phagocytic activity in sea bass macrophages (Román et al. 2012). Also, Román et al. (2013) revealed that heat- or UV-inactivated *V. fluvialis* L-21 stimulated the expression of genes involving in the early inflammatory response (such as IL-1, TNF-α and COX-2) in head-kidney leucocytes of European sea bass (*D. labrax*).

8.19 *Brewer's Yeast* Saccharomyces cerevisiae *var.* ellipsoideus

Hoseinifar et al. (2011) carried out an investigation with juvenile Beluga sturgeon (*Huso huso*) (11.44 ± 0.56 g), which were fed with inactive brewer's yeast *S. cerevisiae* var. *ellipsoideus* (0, 1 or 2%) for 6 weeks. The outcome was a significant improvement in the final weight, weight gain, specific growth rate, feed conversion ratio and the level of lactic acid bacteria but not in survival rate, haematological parameters and serum biochemical parameters in fish fed with the yeast at 2%.

8.20 *Combined Dietary Paraprobiotics*

A commercial product HWF™, which contained a mixture of non-viable bacteria *Rhodotorula minuta* and *Cetobacterium somerae*, was fed to hybrid sturgeon (*Acipenser baerii* × *Acipenser schrenckii*) for 3 weeks. The results revealed a significant improvement in the rate of weight gain and feed conversion ratio, stimulation of the expression of genes related to growth, inflammation and non-specific immunity and antiviral-related genes and changes to the composition of gut microbiota (increasing relative abundance of the bacterial phylum Firmicutes and decreased levels of Proteobacteria, Actinobacteria and Chlamydiae) (Wu et al. 2020).

Frouël et al. (2008) conducted a 103-day feeding trial to determine the effects of a live and heat-inactivated bacterial mixture containing *Lactobacillus farciminis*

CNCM MA27/6R and *L. rhamnosus* CNCM MA27/6B on the growth and digestive metabolism of juvenile sea bass (*D. labrax*) (250 mg). Use of both live and heat-inactivated bacteria led to an increase in survival rate, trypsin and acid phosphatase activities and the number of endocytosis vesicles at the apical pole of enterocytes but not in the gut microvilli length and the number of heterotrophic microbiota in water as well as heterotrophic microbiota and lactic acid bacteria in gut contents.

In a study with rohu (*L. rohita*) fingerlings (6.0 ± 0.06 g), a mixture of heat-killed *B. subtilis*, *L. lactis* and *S. cerevisiae* (10^{11} CFU/kg of feed) was administered for 60 days. The outcome was a significant increment in the apparent digestibility coefficient of dry matter and lipid productive values and a significant decrease in total heterotrophic bacterial populations in the gut (Mohapatra et al. 2012).

Using gilthead seabream (*S. aurata*), Salinas et al. (2008) reported that the administration of heat-killed *Lactobacillus delbrüeckii* ssp. *lactis* and *B. subtilis* was more effective together than as separate preparations. This was demonstrated by the increase of natural complement, serum peroxidase, phagocytic activities, total serum IgM and numbers of gut IgM^+ cells and acidophilic granulocytes in the fish fed with the mixture of the two heat-killed probiotics.

Feeding rainbow trout (*O. mykiss*) (weight = 36.3 ± 0.42 g) with a combination of inactivated *E. faecalis* and mannan oligosaccharide, Rodriguez-Estrada et al. (2013) reported improved weight gain, specific growth rate, feed gain ratio and protein efficiency ratio, haematocrit value, phagocytic activity, mucus weight and resistance to *A. salmonicida.*

Dawood et al. (2015c) administrated a mixture of *L. plantarum* HK-LP and β-glucan (0.025, 0.05 and 0.1% of HK-LP combined with either 0 or 0.1% of β-glucan) to juvenile red sea bream (*P. major*) for 56 days. The data revealed that dietary *L. plantarum* HK-LP and β-glucan had a significant effect on improved growth, digestibility and immune responses. Also, a mixture of heat-killed *L. plantarum* (at 0 and 1 g/kg diet) combined with vitamin C (0.5 or 1 g/kg diet) fed to red sea bream (*P. major*) revealed a significant effect on increasing growth performance and resistance to low salinity stress by immunomodulation (Dawood et al. 2016). Moreover, *L. plantarum* HK-LP combined with soybean meal increased growth performance, digestibility, blood parameters (haematocrit, peroxidase, and bacteriocidal activities) and tolerance against low salinity stress in amberjack (*Seriola dumerili*) (Dawood et al. 2015b).

Villumsen et al. (2020) reported that lipid utilisation was increased in rainbow trout (*O. mykiss*) fed a mixture of live *Pediococcus acidilactici* and bacterial paraprobiotics. Conversely, there was a decrease in lipid utilisation after feeding with a mixture of live *P. acidilactici* and yeast paraprobiotics or a mixture of *Bacillus* spp. and yeast paraprobiotics. Unfortunately, the diets did not significantly improve survival of fish infected with *Y. ruckeri*.

9 Conclusions and Suggestions for Further Work

The studies showed that a diverse range of Gram-negative and Gram-positive bacteria and yeasts derived from various sources has been inactivated and used as paraprobiotics in aquaculture. Paraprobiotics may be used alone or in combination *via* oral administration or intraperitoneal injection. The health benefits include changes in intestinal structure, improvement in feed utilisation and growth performance, immunity, tolerance to stressors and infectious diseases and modulation of the gut microbiota in aquatic animals.

Overall, issues are similar with viable (Nimrat et al. 2012; Tuan et al. 2013) and non-viable probiotics, i.e. paraprobiotics. Concerns revolve around the direct application of paraprobiotics to the culture system in order to modulate the diversity and abundance of beneficial bacteria therein and/or existing in the cultured animals. Specifically, the overall effect of the addition of paraprobiotics on water quality in aquaculture sites and the surrounding aquatic environment both during and after administration needs to be researched. In short, consideration needs to be given to the development of environmental impact statements. It is noted that the use of vaccination in fish farming has generated excellent evidence of protection against infections (Sommerset et al. 2005). So, the development and use of paraprobiotics, which could be argued to serve as potential oral vaccines, merit further investigation. Certainly, many paraprobiotics have been developed, commercialised and used in aquaculture. However, the quality and selection of host-specific paraprobiotic products need to be considered and controlled carefully to ensure their safety and efficacy for use in aquaculture. Also, researchers need to explore the interactions between paraprobiotics and the gut microbiota and/or host physiological changes.

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Quorum Quenching Bacteria as Probiotics

I. Natrah, S. Muthukrishnan, and P. Bossier

Abstract Bacteria regulate their own gene expression by producing, releasing and sensing chemical signals from the environment through a mechanism known as quorum sensing (QS). This bacterial cell-to-cell communication is responsible for controlling various biological traits in bacteria including the regulation of certain phenotypes and the expression of virulence factors that are responsible for pathogen–host association. Quorum quenching (QQ) or quorum sensing inhibitors (QSI) have been suggested as non-antibiotic therapeutic control to combat bacterial infections in aquaculture. Quorum quenchers are non-bacteriostatic organisms/molecules that can restrain the virulence of pathogens through interference with QS, enabling the host to use its own defence. Bacteria with quorum quenching capabilities are found in diverse environments and secretes secondary metabolites that interfere with the QS system which could render the bacterial infections in the host.

Keywords Quorum sensing · Quorum quenching · Signal molecules · Microbes · Therapeutics

1 Introduction

The rapid increase of the world population has led to high demand for continuous food supply. The aquaculture sector is a key solution to ensure continuous global food and nutritional security. Aquaculture is not only the fastest-growing food sector

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for the production of high quality and bioavailable protein for human consumption but is also able to close the "fish-gap", i.e. the disparity between sea food supply and demand (Ellis et al. 2016). Thus, aquaculture is considered as an important complement to global capture fisheries. As a result, production of aquaculture has increased at an average annual rate of 8% in the past three decades, compared to other major animal food production sectors. The world fish production was estimated to be around 179 million tonnes in 2018, of which 82 million tonnes was derived from aquaculture (FAO 2020). However, increased intensification of aquaculture to meet the demands of human has led to several issues, such as high-stress conditions and an increased incidence of disease outbreak (Lara-Flores 2011; Cruz et al. 2012; Dawood and Koshio 2016; Ina-Salwany et al. 2018). Bacterial diseases are among the major causes of disease outbreak in aquaculture. For example, *Vibrio* species are well-known endemic bacteria causing vibriosis outbreaks leading to mass mortalities in aquaculture (Novriadi 2016; Baker-Austin et al. 2018). The bloom of *Vibrio* species is often associated with high-stress conditions (Dawood and Koshio 2016). During the last decade, antibiotic usage was a great boon to the farmers and have been used traditionally to control bacterial diseases in the culture system (Krishnan 2014). However, inappropriate usage of antibiotics leads to the emergence and spread of antibiotic resistance (Assefa and Abunna 2018) and the presence of antibiotic residues in the aquaculture products. Furthermore, uncontrollable usage of antibiotics in aquaculture could aid in the development of a reservoir of (antibiotic) transferable resistance genes between microbes from the aquatic environments via horizontal gene transfer that eventually reach human pathogens (Heuer et al. 2009). Considering these factors, there has been heightened research in developing natural alternative treatment to antibiotics for sustainable aquaculture for the past few decades.

The use of beneficial microorganisms with quorum quenching properties, which target the communication systems of the pathogens, is seen as a novel and potential strategy to control emerging diseases in aquaculture. Several studies reported on the potential application of quorum quenching bacteria as an alternative strategy to reduce the expression of virulence factors in aquatic pathogens by disrupting their communication systems (= quorum sensing) (Morohoshi et al. 2008; Tinh et al. 2008; Defoirdt et al. 2011; Torres et al. 2013; Zhao et al. 2019; Chen et al. 2019; Muthukrishnan et al. 2020). The use of quorum quenching bacteria as probiotics in aquaculture has gained importance in recent years because of the rapid development of multidrug resistance among pathogenic bacteria.

2 Quorum Sensing

Quorum sensing (QS) is a mechanism in which bacteria regulate their own behaviour and control the gene expression of various biological processes through the presence or absence of small signal molecules known as autoinducers (Defoirdt et al. 2004). To date, different hypotheses on the role of QS in bacteria have been reported.

Originally, QS was considered as "density-dependent-sensing" by which, through signal molecules, bacteria determine the population cell density in the environment and express certain biological traits accordingly (Miller and Bassler 2001). Others coined it as "diffusion-sensing", where the diffusion rate of an individual bacterial cell is monitored based on the signal-molecules-sensing in the surrounding medium. Therefore, the expression of extracellular products from the bacteria relies on the assessment of diffusion rate, which results in minimal losses of energy due to extracellular diffusion and mixing (Redfield 2002). At low diffusion rates, a QS response could be attained in small communities. These two concepts were combined and redefined into a more ecologically relevant description as "efficiency sensing" (Hense et al. 2007). Bacterial biofilm formation (Cvitkovitch et al. 2003; Merritt et al. 2003; Parsek and Fuqua 2004), bioluminescence (Miller and Bassler 2001; Von Bodman et al. 2008), virulence factors (Natrah et al. 2011), swarming (Shrout et al. 2006; Tremblay et al. 2007), sporulation, competence, motility, resistance to antibiotic and transfer of genetic material (Fuqua et al. 2001) are among the bacterial activities that are found to be regulated by QS. Several phenotypes and gene products associated with virulence in aquacultural pathogens have been shown to be QS-regulated (Table 1).

The QS system generally involves three main factors including signal production (AI synthase), signal molecules (AI) and signal detection (AI receptor) (Defoirdt 2018). The QS signals produced are dependent on the type of communications, viz. intraspecies-, interspecies- and interkingdom-communication. The QS pathways/signals are categorised into three main groups in bacteria based on their structure and specific functions (Borges and Simoes 2019). The autoinducer-1 (AI-1) also known as N-acylhomoserine lactone (AHL) is mediated by the lux-type quorum-sensing system and is an archetypal mechanism used by species-specific (intraspecies) communication by Gram negative bacteria (Fuqua et al. 2001, Liaqat et al. 2013). The AHLs are small chemical molecules consisting of a lactone ring and variable acyl side chain (from 4 to 18 carbons). The acyl chain may contain a substitution of oxo or hydroxyl at the third carbon position. The system, which commonly uses AI-1, is known as LuxI/LuxR-type QS system. The LuxI/R system consists of two components, LuxI (AHL synthase) and LuxR (AHL receptor) proteins (Papenfort and Bassler 2016; Vadakkan et al. 2018). The AHLs are recognised by the LuxR proteins that are localised in the cytoplasm and able to trigger the transcription of QS-regulated genes once the quorum is achieved. The first marine bacterium identified with the AHL-mediated LuxI/R-type QS system was the Gram-negative *Vibrio fischeri*. The luminescence production in this bacterium is controlled by the LuxI/R-type QS system (Li and Nair 2012).

The second class of QS signal is autoinducer peptides (AIPs) produced by small post-translationally modified peptides and are widely found in Gram-positive bacteria. The AIPs that are produced in the bacterial cell are short peptide chains, and the presence of membrane transport proteins enables AIPs to cross the membrane cells (Henke and Bassler 2004). AIPs are usually an integral component of a histidine-kinase signal transduction system. In certain cases, AIPs are secreted and imported back to the cells. The secreted AIPs are recognised by cytoplasmic transcription

Table 1 Quorum sensing involvement in aquaculture bacterial pathogens

Pathogen	Host	QS molecules	Phenotypes and virulence factor	Impact	References
Genus: *Aeromonas*					
Aeromonas hydrophila	*Carassius auratus gibelio*	HAI-1	Biofilm Motility Haemolysin Protease Lipase	Fish challenged with wild type of *A. hydrophila* demonstrated 100% mortality within four days and typical clinical sign of haemorrhagic septicaemia observed	Chu et al. (2011)
A.*hydrophila* *A. salmonicida*	Burbot (*Lota lota* L.)	C4-HSL	Unknown	The highest mortality was observed in burbot larvae challenged with wild-type of *A. hydrophila*	Natrah et al. (2012)
A. hydrophila	Water surface	C4-HSL C6-HSL AI-2	Protease	QS-modulated protease production	Jahid et al. (2015)
A. hydrophila	*Xiphophophorus helleri Hecke*	HAI-1 (*ahyR* gene)	Exoprotease Hemolysin Amylase Dnase		Bi et al. (2007)
Genus: *Vibrio*					
Vibrio anguillaram	Sea bass (*Dicentrarchus labrax*)	Indole	Biofilm		Li et al. (2014)
V. alginolyticus	Zebrafish (*Danio rerio*)	HAI-1 AI-2 CAI-1	Motility Biofilm Secretion system	Fish infected with 2.0 × 10^7 CFU/fish via intramuscular injection demonstrated 100% mortality within 24 h	Liu et al. (2020)

(continued)

Table 1 (continued)

Pathogen	Host	QS molecules	Phenotypes and virulence factor	Impact	References
	Marine	C4-HSL 3-OH-C4-HSL 3-oxo-C6-HSL 3-OH-C8-HSL C10-HSL 3-oxo-C10-HSL C12 3-OH-C12 3-oxo-C12 3-oxo-C14	Biofilm	Out of 11 different AHLs characterised in 47 V. *alginolyticus* strains, only 3-oxoC10-HSL plays a functional role in biofilm formation	Liu et al. (2017)
V. cholerae	Common carp (*Cyprinus carpio*)	CAI-1	Haemolysin Biofilm CtxA TcpA rtxC toxR zot acfA Nag-ST	Fish injected with 10^8 and 10^7 CFU/mL (48 h of post-injection) died within 12 h	Gao et al. (2017)
V. harveyi	Black Tiger shrimp (*Penaeus monodon*)	HBHL AI-2	Extracellular toxins	Increased mortality in infected prawn	Manefield et al.(2000)
	Brine shrimp (*Artemia franciscana*)	HAI-1[a] AI-2 CAI-1	Luminescence	Increased mortality in infected larvae	Defoirdt and Sorgeloos (2012)

(continued)

Table 1 (continued)

Pathogen	Host	QS molecules	Phenotypes and virulence factor	Impact	References
	A. *franciscana*	HAI-1	Flagellar motility	Flagellar motility significantly affects the virulence of *V. harveyi* in gnotobiotic brine shrimp model	
	In vitro	HAI-1 AI-2 CAI-1	Metalloprotease Extracellular toxins Chitinase* Phospholipase* Siderophore* Type III secretion system (T3SS)*		Natrah et al. (2011)
V. parahaemolyticus	*P. monodon*	AI-2	Biofilm	*V. parahaemolyticus* produces AI-2 and significantly correlates with biofilm production	Mizan et al. (2018)
Genus: *Edwardsiella*					
Edwardsiella tarda	Fish	AI-2	Regulates the growth phase and growth conditions	*Edw. tarda* of a LuxS/AI2-mediated QS pathway regulates the production of virulence-associated elements	Zhang et al. (2008)
E. tarda LTB-4	Turbot (*Scophthalmus maximus*)	-C4-HSL -C6-HSL -3-oxo-C6-HSL	Unknown	LTB-4 produces four types of signalling molecules which may play a role in virulence associated elements	Han et al. (2010)

(continued)

Table 1 (continued)

Pathogen	Host	QS molecules	Phenotypes and virulence factor	Impact	References
E. ictaluri Ei-151	Striped catfish (*Pangasianodon hypophthalmus*)	HAI-1 AI-2	Virulence factor	Infected catfish exhibited white spot disease with swollen and pale internal organs. It is the causative agent of enteric septicaemia of catfish (ESC)	Yang et al. (2012)
E. piscicida	Fish	AI-2/LuxS[a]	Biofilm Motility Growth	Increased pathogenicity through an increase in physiological activities	Sun et al. (2020)
Genus: *Pseudomonas*					
Pseudomonas aeruginosa	Nile tilapia (*Oreochromis niloticus*)	*lasI*, *lasR* *rhlR* *rhlAB* and *rhlI*	Biofilm Antimicrobial resistance (AR) Virulence genes	Biofilm, AR and virulence genes in *Pseudomonas* spp. are being regulated by QS	Osman et al. (2020)
Genus: *Streptococcus*					
Streptococcus agalactiae	*O. niloticus*	AI-2/LuxS	Biofilm Haemolytic activity Antiphagocytosis	LuxS is associated with both *S. agalactiae* virulence and immune evasion	Cao et al. (2020)

[a] Not a dominant signal
[*]Not controlled by QS
↓ Negatively regulated
HAI-1: homoserine lactone, C4-HSL: *N*-butyryl-L-homoserine lactone, C6-HSL: *N*-hexanoyl-L-homoserine lactone, AI-2: autoinducer-2, CAI-1: cholera autoinducer-1, 3-OH-C4-HSL: *N*-3-hdroxy-hexanoyl-L-homoserine lactone, 3-oxo-C6-HSL: *N*-(3-oxo-hexanoyl)-L-homoserine lactone, 3-OH-C8-HSL: *N*-3-hydroxyoctanoyl-L-homoserine lactone, C10-HSL: *N*-decanoyl-L-homoserine lactone, 3-oxo-C10-HSL: *N*-(3-oxodecanoyl)-homoserine-L-lactone, C12-HSL: *N*-dodecanoyl-L-homoserine lactone, 3-OH-C12: *N*-(3-oxo-dodecanoyl)-homoserine lactone, 3-oxo-C12-HSL: *N*-(3-oxododecanoyl)-homoserine lactone, 3-oxo-C14-HSL:*N*-(3-Oxotetradecanoyl)-L-homoserine lactone, ctxA: cholera toxin, tcpA: toxin coregulated pilus, OmpW: outer membrane protein, rtxC: repeat in toxin, hlyA: hemolysin, toxR: transcriptional regulatory proteins, ace: accessory cholera toxin, zot: zonula occludens toxin, acfA: accessory colonisation factors and Nag-ST: heat-stable enterotoxin

factors, which turn the secreted precursor-AIPs to mature AIPs by extracellular proteases. The mature AIPs enter the cells back and modify the activity of the corresponding transcription factor (Bhatt 2018).

The third signal molecule produced by bacteria is known as autoinducer-2, a furanosyl borate diester, 3A-methyl-5,6-dihydro-furo(2,3-$_D$)(1,3,2) diox-aborole-2,2,6,6A-tetraol (Chen et al. 2002), which enables interspecies communication (Pereira et al. 2013). The genes responsible for AI-2 activity, designated *luxS,* is widespread and has been found in several Gram-negative and Gram-positive bacterial species (Surette et al., 1999, Vendeville et al. 2005). The AI-2 is synthesised by the LuxS/AI-2 system and mediates both interspecies and intraspecies interactions among Gram-positive and Gram-negative bacteria (Wang et al. 2018). The LuxS protein is a homodimeric metalloenzyme consists of two identical tetrahedral metal-binding sites and is found in *Streptococcus* spp. and *E. coli,* among others (Wang et al. 2018; Borges and Simoes 2019).

Recently, biochemical pathways and the chemical structure of another type of QS signal molecule, known as Autoinducer-3 (AI-3) in pathogenic *E. coli,* were characterised. This signal molecule in *E. coli* upregulates the locus of enterocyte effacement (LEE), which encodes the type III secretion system (T3SS) (Kim et al. 2020). In addition to these QS signalling molecules, another signalling molecule known as Cholerae autoinducer-1 (CAI-1) a (S)-3-hydroxytridecan-4-one through the CqsA/CqsS system (Higgins et al. 2007; Ng et al. 2011) is widely found in *Vibrio* species, including *Vibrio cholerae*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio furnissii* and *Vibrio anguillarum* (Henke and Bassler 2004). The CqsA/CqsS homologues have been found also in other bacteria, such as *Legionella pneumophila* and *Burkholderia xenovorans* (Tiaden et al. 2007; Spirig et al. 2008). Other reported autoinducers include 4-quinolones, indole, pyrones, dialkylresorcinols, fatty acids and fatty acid methyl esters (For a review, refer to Defoirdt 2018).

3 Quorum Quenchers

Quorum quenchers (QQs) are non-bacteriostatic organisms or molecules that could be a new anti-infective strategy to control pathogenic bacteria without interfering with the growth of the bacteria. Quorum quenchers are also known as quorum sensing inhibitors (QSIs), which are capable of down-regulating the virulence of pathogens by interfering with QS signals and enabling the host to use its own defence mechanisms to control the pathogen. Quorum quenching bacteria disrupt the QS system by (1) prevention of QS signal biosynthesis, (2) signal degradation (chemical, metabolic and enzymatic), (3) receptor blocking/antagonists and (4) modification of signal and receptor interactions (Kalia and Purohit 2011; Paluch et al. 2020) (Fig. 1).

(i) Prevention of QS signal biosynthesis: the intermediates from fatty acid (S-adenosyl methionine and an acylated acyl carrier protein) biosynthetic pathway are involved in the biosynthesis of AHL. The synthesis of AHL

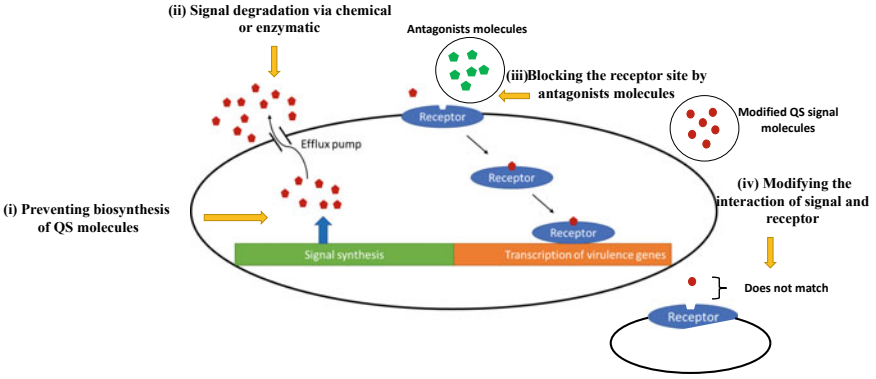


Fig. 1 Mechanisms of quorum sensing (QS) inhibition in bacteria

could be inhibited by blocking the fatty acid pathway (Geske et al. 2007; Lade et al. 2014).

(ii) Signal degradation: Three types of QS signal degradation have been reported, namely chemical and enzymatic (Delago et al. 2015). Chemicals, such as furanones, cinnamaldehyde, chlorolactones, thiopheones, pyrogallol and boronic, could degrade the QS signals by competing with the binding site (Defoirdt et al. 2004, 2007) or modifies the lactone ring which results in loss of activities (Yates et al. 2002). Certain QQ bacteria are capable of metabolising lactone-containing compounds via enzymatic process (Safari et al. 2014) through the production of enzymes such as acylases, lactonases, oxidase and reductase enzyme) that enable complete degradation or inactivation of AHL (Lade et al. 2014).

(iii) Receptor blocking/antagonists: antagonist molecules compete or bind to the receptor thus inhibits the binding of the actual signal molecules, which results in failure to express the virulence factor (Ni et al. 2009).

(iv) Modification of signal and receptor interactions inhibit the transcription by structural modification or competitive inhibition, which interrupt the interaction of receptor-signal molecules (Vadakkan et al. 2018).

4 Quorum Quenching Bacteria

A number of bacteria have been reported to produce quorum quenching enzymes responsible for QS signalling molecule degradation that interrupt the bacterial communication resulting in failure to express virulence genes (Chu et al. 2014). For example, AHL-degrading enrichment cultures isolated from the gut of European sea bass, *Dicentrarchus labrax* (EC5(D)) and Asian seabass, *Lates calcarifer* (EC5(L)) (Cam et al. 2009) improved the survival of *M. rosenbergii* larvae challenged with *V. harveyi* (Nhan et al. 2010). The AHL degrading enrichment cultures (ECs) could

possibly be applied in different fish or shrimp species because the ECs isolated from the fish gut are effective also in the prawn-rearing environment.

In aquaculture, the commonly used probiotic candidates are from the genus *Bacillus* (Hong et al. 2005; Decamp et al. 2008). Bacilli are well known as prolific producers of secondary metabolites with unique chemical structures highly potential for pharmacological properties (Teasdale et al. 2011). The efficacy of quorum quenching bacteria may be tested in host–pathogen settings. Previous studies demonstrated the application of quorum quenching bacteria increased survival of fish, shrimp and brine shrimp larvae when challenged with pathogenic *V. campbellii* (Niu et al. 2014; Pande et al. 2015). Defoirdt et al. (2011) identified five *Bacillus* strains from two AHL-degrading ECs that were previously isolated from white shrimp (Tinh et al. 2008) and European seabass (Cam et al. 2009). These *Bacillus* isolates were closely related to *Bacillus cereus*, *Bacillus subtilis*, *Bacillus anthracis* and *Bacillus thuringiensis* with AHL degradation rates between 0.7 and 0.9 mg/L/h in Luria–Bertani medium supplemented with 5 mg/L N-hexanoyl-L-homoserine lactone. Also, Pande et al. (2015) found that *Bacillus* sp. NFMI-C, which was isolated from the microalga *Chaetoceros muelleri,* is able to degrade the AHL (***N***-3-hydroxy butanoyl-L-homoserine lactone). Moreover, the bacterial strain protected *M. rosenbergii* prawn larvae from QS regulated luminescence *V. campbellii* in a challenge test (Pande et al., 2015). Meanwhile, *Bacillus* sp. QSI-1 blocked the AHL signal of *Aeromonas hydrophila* and demonstrated significant increase in survival rate (83.3%; 25/30 fish) in zebrafish fed with QSI-1 compared to the control group (13.3%; 4/30 fish) when challenged with *A. hydrophila.* In addition, the strain inhibited biofilm formation (77.3%), haemolytic activity (77.6%) and protease of *A. hydrophila* within 24 h (Chu et al. 2014). Similar QSI protective effects of *Bacillus* spp. were reported against: (1) *A. hydrophila* infecting catfish (*Clarias gariepinus*) (Novita et al. 2015); (2) *V. harveyi* infecting Asian sea bass (*Lates calcarifer*) (Ghanei-Motlagh et al. 2020) and (3) *A. hydrophila* infecting goldfish (*Carrassius auratus gibelio*) (Vadassery and Pillay 2020).

Other bacteria from different families and genera demonstrated QSI activity. For example, *Shewanella* sp. isolated from the gut of ayu (*Plecoglossus altivelis*) was capable of degrading AHL and inhibited biofilm formation of *V. anguillarum* (Morohoshi et al. 2008). Similarly, *Halobacillus salinus* C42, which was isolated from seagrass, was capable of suppressing the virulence genes of *V. harveyi.* The two phenethylamides from *H. salinus* C42, which were identified as 2,3-methyl-N-(2'-phenylethyl)-butyramide and N-(2'-phenylethyl)-isobutyramide, inhibited violacein production from *C. violaceum* CV026 and green fluorescent protein (GFP) of *E. coli* JB525. The QQ activity was claimed to be due to competition with signal molecule AHLs for receptor binding because both metabolites mimiced the AHL structure (Teasdale et al. 2009). Similarly, Chen et al. (2018) reported another two phenethylamides, namely 2-methyl-N-(2'-phenylethyl)-butyramide, 3-methyl-N-(2'-phenylethyl)-butyramide and one benzyl benzoate produced by *Oceanobacillus* sp. XC22919, which were isolated from the marine environment, and inhibited violacein from *C. violaceum* ATCC12472. These QSI molecules from *Oceanobacillus* sp. XC22919 were capable of inhibiting pyocyanin, elastase, proteolytic and biofilm

formation in *P. aeruginosa*. Another potential quorum quenching bacteria, which was identified as *Ruegeria mobilis* YJ3, produced a novel marine-derived AHL lactonase with strong AHLs degradative activity, and designated as *Ruegeria mobilis* marine lactonase (RMML) (Cai et al. 2018). Examples of QQ bacteria in aquaculture and its effect on the host are shown in Table 2.

5 Quorum-Quenching Enzymes from Bacteria

Bacteria have been widely reported to have the capability of quenching QS signals via enzymatic reactions. In fact, the expression of QQ enzymes by α-proteobacteria, β-proteobacteria, γ-proteobacteria and in some Gram-positive bacteria has been extensively discussed (e.g. Czajkowski and Jafra 2009). Lactonase (Lu et al. 2006; Huang et al. 2012; Mayer et al. 2015; Tang et al. 2015; Zhang et al. 2015) and acylase (Kem et al. 2015; Liu et al. 2017) are the two widely studied QQ enzymes. These enzymes are ubiquitous in prokaryotes and eukaryotes (Dong and Zhang 2005). Furthermore, Muras et al. (2018) reported high prevalence of QQ enzyme sequences retrieved from the metagenomic samples collected from the Mediterranean Sea. The lactonase sequences were found to be distributed uniformly at different depths of seawater compared to acylase sequences, which were found abundantly in the deep sea. Acylase inactivates AHLs by cleaving the amide bond between the acyl chain and the homoserine lactone moiety resulting in a fatty acid and homoserine lactone. Meanwhile, lactonase hydrolyses the lactone bond and forms acylated homoserine (Dong and Zhang 2005). Another QQ enzyme, oxidoreductase (e.g. P-450/NADPH-P450 reductase of *B. megaterium* CYP102A1) reduces carbonyl to hydroxyl that targets the acyl side chain by oxidative or reducing activities (substitutes the oxo-group at C3 with the hydroxyl group), which may be degraded by amidohydrolase to form homoserine lactone and hydroxydecanoic acid (Uroz et al. 2005, Bzdrenga et al. 2017) and therefore catalyses a QS signal structure modification instead of degradation (Chen et al. 2013). The enzymatic inactivation mechanisms are shown in Fig. 2. Additionally, Dong et al. (2020) further described the production of AHL lactonase enzyme from *Lactobacillus casei*.

AHL-degrading enzymes were widely examined for disrupting the QS in aquaculture pathogens (Chen et al. 2010; Cao et al. 2012; Vinoj et al. 2014; Liu et al. 2017; Dong et al. 2020). Infection of *A. hydrophila* was found to be reduced in the zebrafish fed with purified lactonase extracted from *Bacillus* sp. A191 (Cao et al. 2012). Similarly, oral administration of *Bacillus* isolates (TS1, TS2 and TA23) to catfish improved survival rate, feed conversion ratio (FCR), specific growth rate (SPR) and the non-specific immune system compared to the controls. In addition, all the *Bacillus* isolates were positive for QSI screening using *Chromobacterium violaceum* indicating the production of lactonase enzyme (Novita et al. 2015). A number of *Vibrio* spp. have caused massive losses to the shrimp industry over the last decade because of the disease outbreak known as acute hepatopancreatic necrosis disease (AHPND) (Muthukrishnan et al. 2019) with estimated total losses

Table 2 Bacterial quorum quenchers in aquaculture

QQ species	Source	Beneficial effect	References
Shewanella sp.	Ayu fish (*Plecoglossus altivelis*)	Inhibited biofilm formation of fish pathogen *Vibrio anguillarum*	Morohoshi et al. (2008)
EC5 cultures	Whiteleg shrimp (*P. vannamei*) shrimp gut	EC5 bacteria cultures able to degrade AHLs and enhance the survival of first-feeding turbot larvae (*Scophthalmus maximus L.*)	Tinh et al. (2008)
Halobacillus salinus C42	Seagrass	*H. salinus* C42 capable of inhibiting bioluminescence produced by *V. harveyi*	Teasdale et al. (2009)
Gut microbes	European sea bass (*Dicentrarchus labrax L*) and Asian sea bass (*Lates calcarifer*)	Increase the survival rate of the *Macrobrachium rosenbergii* larvae	Cam et al. (2009) Nhan et al. (2010)
Bacillus spp.	*P. vannamei*) and *D. labrax L*	All the isolates are able to degrade *N*-hexanoyl-L-homoserine lactone (HHL) down to below detection limit within 6–9 h	Defoirdt et al. (2011)
Cobetia sp. MM1IDA2H-1	Seawater samples	QS in fish pathogen *A. salmonicida* repressed by *Cobetia* sp. MM1IDA2H-1	Ibacache-Quiroga et al. (2013)
Bacillus sp. QSI1	Goldfish (*Carrassius auratus gibelio*)	Supernatant of *Bacillus* sp. QSI1 showed haemolytic activity, inhibited protease, and biofilm formation of fish pathogen *Aeromonas hydrophila*	Chu et al. (2014)
Bacillus sp. NFMI-C	*Chaetoceros muelleri*	*Bacillus* sp. NFMI-C was able to degrade *N*-hydroxybutanoyl-L-homoserine lactone, the AHL produced by *Vibrio campbellii*	Pande et al. (2015)
B.amyloliquefaciens *Lysinnibacillus sphaericus* *B. cereus*	Unknown	Catfish fed with the mixture of the three QQs demonstrated higher survival rate (93%) compared to the control (31%) in the *A. hydrophila* challenged experiment	Novita et al. (2015)
Ruegeria mobilis YJ3	Healthy shrimp	Capable of degrading both short- and long-chain AHLs. Moreover, *R. mobilis* YJ3 reduces production of QS regulated pyocyanin of *Pseudomonas aeruginosa* PAO1	Cai et al. (2018)

(continued)

Table 2 (continued)

QQ species	Source	Beneficial effect	References
Phaeobacter inhibens S4Sm	Inner surface of an oyster shell	*P. inhibens* S4Sm secretes secondary metabolites that hijack the QS ability of pathogenic *V. coralliilyticus* RE22Sm and protects oyster larvae from by suppressing the virulence gene expression	Zhao et al. (2019)
B. thuringiensis QQ1 and *B. cereus* QQ2	Barramundi fish	Both the strains showed high capacity to degrade AHL produced by *Vibrio harveyi* and *V. alginolyticus*. Increased digestive enzyme activity, growth performance and resistance against *V. harveyi* in Asian seabass	Ghanei-Motlagh et al. (2019, 2020)
B.thuringiensis	Soil samples from tilapia culture pond	Goldfish fed with 10^8 and 10^{10} CFU/g of *B. thuringiensis* QQ17 demonstrated 73–83% of survival when challenged with pathogenic *A. hydrophila*	Vadassery and Pillay (2020)
Lactobacillus casei MCJ∆1	Dead grass carp (*Ctenopharyngodon idellus*)	AHL lactonase AiiK produced *by L. casei* inhibited the QS activity, swimming motility, extracellular proteolytic activity, haemolytic activity and biofilm formation of *A. hydrophila* AH-1 and AH-4	Dong et al. (2020)

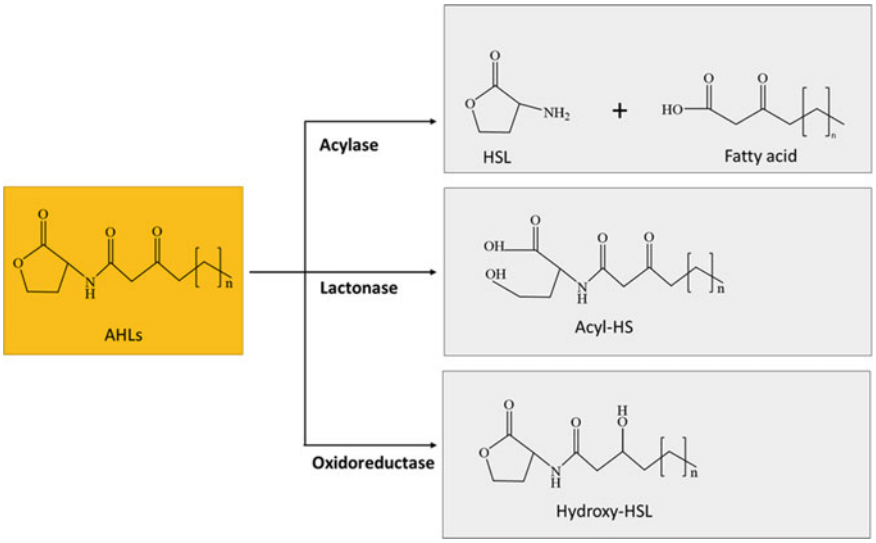


Fig. 2 Enzymatic inactivation of AHLs. The degradation mechanism of acylase, lactonase and oxidoreductase

of US$ 23.58 billion from 2009 to 2016 (Shinn et al. 2018). A study conducted by Vinoj et al. (2014) showed reduction of *Vibrio* colonisation in shrimp injected with AHL-lactonase extracted from *B. licheniformis* DAHB1. Likewise, AHL-lactonase produced by *Bacillus* sp. B546 demonstrated a protective effect towards carp (Chen et al. 2010). In another study, mortality of zebrafish due to *A. hydrophila* infection reduced significantly when treated with *Bacillus* sp. QSI-125. Therefore, application of AHL-degrading bacteria could be a sustainable strategy to control the expression of virulence factors by pathogenic bacteria.

In addition, specific genes encoding quorum quenching enzymes have been widely studied. Several *Bacillus* spp. isolated from soil, marine sediments (Teasdale et al. 2011) and marine sponges (Phelan et al. 2012) were screened positive for AHL lactonase encoding gene *aiiA*. Kem et al. (2015) reported that two genes (*mhtA* and *mhtB*) from *Marinobacter nanhaiiticus* and gene *bntA* from *Marinobacter* sp. were homologous to acylase gene *pvdQ* in *P. aeruginosa*. Rehman and Leiknes (2018) demonstrated degradation and modifications of AHLs by the seven bacteria isolated from Red Sea sediment. All the seven bacteria belonged to the phylum Proteobacteria. Interestingly, genome sequence of the three bacteria strains from the same study revealed the presence of AHL lactonase open reading frames (ORFs) from metallo-β-lactamase (MBL) superfamily and AHL acylase ORFs. AHL lactonase *Lc*Aiik extracted from *L. casei* proved to specifically degrade C_6-HSL, which was produced by *A. hydrophila*. In addition, *Lc*Aiik reduced the production of virulence factors in *A. hydrophila*.

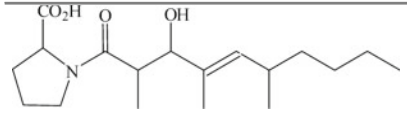
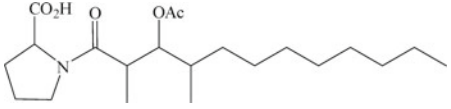
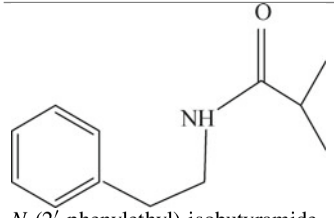
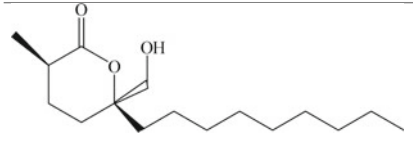
6 Small Quorum Sensing Inhibitory Molecules from Bacteria

A number of natural small molecules with different structures from various organisms with QS inhibitory activities have been documented. The structures of the small molecules produced by bacteria producing and its QSI mechanism are outlined in Table 3. The small QSI molecules could be categorised into five groups, including AHL analogues, cyclic or linear peptides, fatty acid or phenol derivatives, amides and others. Th QS inhibitions of the molecules could be divided into four ways:

(a) competition with the receptor proteins,
(b) binding to AHL synthase thus inhibiting the signal molecules synthesis,
(c) reduction of receptor stability
(d) blockage of signal molecules protein expression (Zhao et al. 2019).

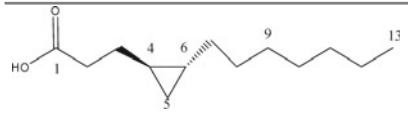
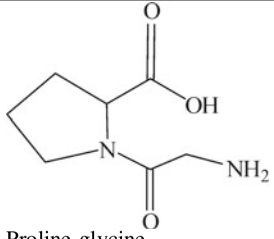
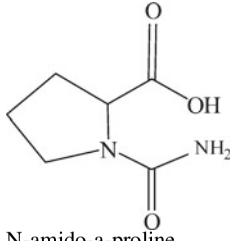
Certain probiotic bacteria produced AHLs (Zhao et al. 2019), which could act as antagonists and compete for receptors binding with the pathogenic bacteria in the vicinity (Bruns et al. 2018). For example, Ma et al. (2018) identified a compound known as DL-homocysteine thiolactone isolated from *Staphylococcus hominis* D11, which is an analogue of the AHLs. DL-homocysteine thiolactone inhibits QS activity by competing with AHLs for the same receptor-binding site. Similarly, Abed et al.

Table 3 Small molecules QS inhibitors

Small molecules	Type of bacteria	QSI inhibitory mechanism	Reference
 Tumonoic acid E  Tumonoic acid F	*Blennothrix cantharidosmum*	Inhibit bioluminescence	Clark et al. (2008)
 *N*-(2′-phenylethyl)-isobutyramide	*Halobacillus salinus* C42	Interfere with *Vibrio harveyi* bioluminescence	Teasdale et al. (2009)
 Malyngolide	*Lyngbya majuscula*	Inhibit violacein production and luminescence	Dobretsov et al. (2010)

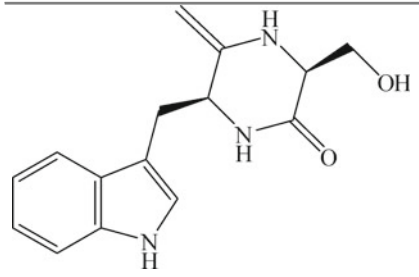
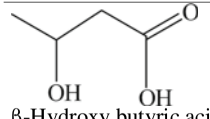
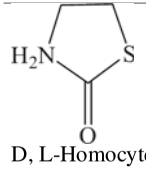
(continued)

Table 3 (continued)

Small molecules	Type of bacteria	QSI inhibitory mechanism	Reference
 Lyngbyoic acid	*Lyngbya majuscula*	Reduce pigment pyocyanin and the elastase LasB in *Pseudomonas aeruginosa*	Kwan et al. (2011)
 Proline-glycine  N-amido-a-proline	*Streptomyces* sp. NIO 10,068	Downregulation of QS-mediated virulence factors like swarming, biofilm formation, pyocyanin, rhamnolipid and LasA production in *P. aeruginosa* ATCC 27,853	Naik et al. (2013)

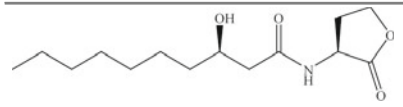
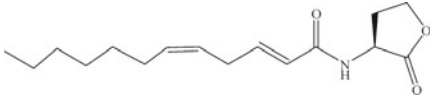
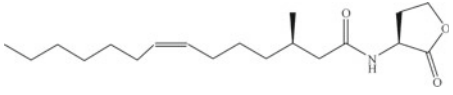
(continued)

Table 3 (continued)

Small molecules	Type of bacteria	QSI inhibitory mechanism	Reference
 Cylo (Trp-Ser)	*Rheinheimera aquimaris* QSI02	Inhibit biofilm formation, pyocyanin production and elastase reduction in *P. aeruginosa* PA01	Sun et al. (2016)
 β-Hydroxy butyric acid	*Brevibacterium casei* MSI04	Control biofilm, motility, bioluminescence, haemolysin production, colonisation capacity and virulence expression in *Vibrio* sp.	Seghal Kiran et al. (2016)
 D, L-Homocyteine thiolactone	*Staphylococcus hominis*	An analogue of the acyl-homoserine lactones (AHLs) which occupy the receptors	Ma et al. (2018)

(continued)

Table 3 (continued)

Small molecules	Type of bacteria	QSI inhibitory mechanism	Reference
 *N*-(3-Hydroxydecanoyl)-L-homoserine lactone  *N*-(Dodecanoyl-2,5-diene)-L-homoserine lactone  *O*-(3-Hydroxytetradecanoyl-7-ene)-L-homoserine lactone	*Phaeobacter inhibens*	Competitive inhibition of AHL-mediated QS	Zhao et al. (2019)

(2013) reported that four different diketopiperazines (DKPs), Cyclo(L-Pro-L-Phe), Cyclo(L-Pro-L-Leu), Cyclo(L-Pro-L-isoLeu) and Cyclo(L-Pro-D-Phe) produced by *Marinobacter* sp. SK-3 isolated from hypersaline cyanobacterial mat inhibited luminescence of *E. coli* pSB401 by competing with the same AHLs receptor-binding site. Also, the DKPs were previously reported as QS signal molecules of *Burkholderia* sp. The structural and functional similarities of these signal molecule analogues may well serve as both QQ and QS signal molecules, either for their producing bacteria or for other surrounding bacteria, indicating cross-species manipulation (Zhao et al. 2019). Another DKP, Cyclo(Trp–Ser), was discovered from marine sediment bacteria *Rheinheimera aquimaris* QSI02 and is reported to inhibit QS-regulated pyocyanin production, biofilm formation and elastase activity of *P. aeruginosa*, possibly interfering in the LasR receptor stability (Sun et al. 2016).

Two novel cyclodepsipeptides, Solonamide A and Solonamide B, produced by *Photobacterium halotolerans,* which were isolated from mussel, interfere in the expression of *agr* (virulence gene)*, hla* (haemolysin) and *rnaIII* (encoding RNAIII, an *agr* effector molecule) and downregulate the virulence gene expression in *Staphylococcus aureus* (Mansson et al. 2011; Nielsen et al. 2014). The interference with *agr* QS system is possibly due to competition with signal molecule AIP for binding to sensor histidine kinase AgrC (Mansson et al. 2011; Nielsen et al. 2014). Another four novel cyclodepsipeptides, Ngercheumicin F-I produced by the same *P. halotolerans* also reported to interfere with *agr* QS by inhibiting the *rnaIII* expression in methicillin-resistant *S. aureus* community as well as reducing virulence genes expression of *hla* and *rnaIII* of agr in *S. aureus* (Kjaerulff et al. 2013). Ngercheumicins and Solonamides were reported as AIPs analogues that have potential as QQ metabolites showing similar chemical structure with the AIPs of *S. aureus* (Mannson et al. 2011; Kjaerulff et al. 2013).

Three types of AHLs isolated from *Phaeobacter inhibens* S4Sm downregulate the transcription of virulence factor in pathogenic *V. coralliilyticus* cultures by disrupting the QS pathways. These compounds possess structural similarities with the QS signalling molecule produced by the pathogenic bacterium and have antagonistic activity against *V. coralliilyticus* (Zhao et al. 2019). Additionally, tumonoic acids, which were isolated from marine cyanobacterium *Blennothrix cantharidosmum* in Papua New Guinea, demonstrated modest QSI activity (inhibition of bioluminescence) (Clark et al. 2008). Similarly, malyngolide isolated from *Lyngbya majuscule* obtained from the Indian River Lagoon, Florida, USA (Dobretsov et al. 2010) and *N*-(2′-phenylethyl)-isobutyramide isolated from *Halobacillus salinus* C42 obtained from a seagrass sample collected from Point Judith Salt Pond, South Kingstown, RI (Teasdale et al. 2011) could effectively inhibit *V. harveyi* bioluminescence; a QS-controlled phenotype. Like AHLs, the *N*-(2′-phenylethyl)-isobutyramide isolated from *H. salinus* metabolites possesses a ring system with a side chain connected via an amide bond. Several studies indicated that the synthetic QS antagonists were synthesised based on AHL structural motifs by having phenyl rings appended to either the end of the acyl chain or replacing the lactone ring (Teasdale et al. 2009).

7 Conclusion and Future Perspectives

Disease outbreaks are a major drawback for the aquaculture industry leading to high mortalities and huge economic losses. Although antibiotics were used traditionally for disease control, the use of these agents has led to the emergence and spread of antimicrobial resistance (AMR) affecting animals and humans. Quorum quenching bacteria have opened up new lines of research and a search for novel molecules derived from bacteria to control the virulence factors of bacterial pathogens is gaining much interest in a recent years. A number of small molecules isolated from QQ bacteria proved to be effective against aquatic pathogens in the laboratory. Also, enzymes produced by QQ bacteria aid in degrading the QS signals, which eventually help in controlling disease outbreaks by downregulating the transcription of virulence factors produced by pathogens. However, most of the tests have been done in laboratory settings warranting the need of an experimental design of mimicking the real environment of mixed communities in realistic field tials. Furthermore, the route of delivery of the QQ bacteria into culture systems as disease-control agents needs to be further elucidated.

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Probiotics for Biofloc System and Water Quality

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Abstract Probiotics from various taxonomic divisions are often used directly in waters of aquaculture systems, in biofloc systems, in addition to their use as dietary additives. The direct effect of probiotics on water quality includes an important role in reducing the concentration of toxic nitrogenous compounds, such as ammonia, nitrite, and nitrate, reducing the level of organic matter and pH, reducing the level of pathogenic microorganisms, and modulating the microbial community of water and sediment. Also, probiotics exert an indirect function in improving the survival rate and growth performance of farmed animals. Moreover, the problems or disputes in using probiotics have been discussed, and suggestions are made for the direction of future studies in terms of improving water quality.

Keywords Probiotics · Biofloc system · Water quality · Nitrogen removal · Nitrite · Ammonia · Microbial community structure · Aquaculture

1 Introduction

In aquaculture systems, leftover/uneaten feed, animal faeces, urea, and the residues of dead animals or plants contribute a great deal of organic matter and nitrogen-related compounds. All nitrogen forms, including organic and inorganic forms, may accumulate in the culture unit and transform into toxic compounds. These may become a serious problem because of the detrimental effects on the cultured species (Barbieri, 2010; Romano and Zeng, 2013; Waslelesky, et al. 2017). Therefore, an important task is to ensure adequate water quality in the culture system by controlling the level of organic matter and toxic nitrogenous compounds, such as ammonia and nitrite.

Though the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) define probiotics as "live microorganisms which when administrated in adequate amounts confer a health benefit on the host". Probiotics for use in aquaculture have diverged from their counterparts used for terrestrial animals

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including humans because of the intricate relationship between aquatic animals and the ambient environment. Therefore, it is accepted that probiotics for use in aquaculture have subtle differences. Consequently, Verschuere et al. (2000) suggested a definition for probiotics destined for use with aquatic animals as "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". In short, the previously accepted definition of a probiotic as a feed supplement was extended to include application in water. Therefore, within this amended definition, probiotics could be considered to have functions involved with degrading organic matter or removing ammonia and nitrite from aquaculture systems, and thereby improving the water quality. This action is of fundamental importance to the success of aquacultural operations.

A biofloc technology (BFT) system has been described as an exceptionally eco-friendly technology because it relies on the activities of aquatic microorganisms that may be expected to be found naturally within the aquaculture system. These functions focus on:

(1) controlling water quality through the immobilization of nitrogen, resulting in incorporation in microbial proteins
(2) nitrogenous compounds incorporated into microbial protein consequently serve as a source of nutrition for cultured aquatic species
(3) suppressing the growth of pathogens through competition (Avnimelech 2009; Emerencianno et al. 2017).

Any BFT system is designed as a zero exchange or minimal exchange (water) system based on recycling and reuse of nutrients within the same system (Godwin et al. 2020). Consequently, BFT has emerged as an outstanding technology capable of solving some of the environmental and economic challenges faced by traditional aquaculture production systems. Many commercial probiotics have been used in BFT systems to set up or enhance the initial microbial communities in aquatic habitats. The outcomes have been significant.

The focus in this chapter will be to introduce the taxonomic types, methods, and effect of probiotics used for improving water quality in aquaculture and/or biofloc systems. Also, there will be discussion of the problems and/or disputes with using probiotics. This will be followed by suggestions for the future development of probiotics for use in aquaculture.

2 Probiotics Types Used in Biofloc Systems for Improving Water Quality

A diverse range of probiotics has been considered for used in biofloc systems and for improving water quality. Most of these probiotics have been either derived from the intestines of the host animals or isolated from the aquacultural environments. As

reviewed by Wang et al. (2019), the microorganisms used as probiotics or examined as potential candidates for improving water quality belong to various taxonomic divisions, including Bacteroidetes, Firmicutes, Proteobacteria, and yeasts.

3 Firmicutes

3.1 Bacillus

Bacillus species have been among the most widely used groups of probiotics in aquaculture since the 1990s (Gatesoupe 1999; Irianto and Austin 2002). Besides their use as dietary supplements, *Bacillus* strains have been frequently reported to improve water quality when used as water additives because of their nitrification and denitrification functions. For example *B. licheniformis* was reported to remove nitrogen and modulate the microbial community in grass carp (*Ctenopharyngodon idellus*) pond water (Liang et al. 2015). Moreover, when added to the culture water of Pacific white shrimp, *B. subtilis* FY99-01 improved the water quality by reducing the levels of pH, nitrite, and soluble reactive phosphorus and decreased the abundance of Vibrionaceae representatives (Wu et al. 2016). Some *Bacillus* strains exhibited similar beneficial effects to the aquatic animals when used as water additives as when used as dietary supplements. Thus, Zhou et al. (2009) reported that probiotic *B. coagulans* SC8168 used as a water additive could significantly increase survival rate of shrimp larvae and enhance the activity of some of the digestive enzymes.

3.2 Lactic Acid Bacteria (LAB)

Xie et al. (2017) reported that a *L. plantarum* isolate, which was recovered from the aquaculture environment, demonstrated high nitrite removal ability. This suggested that the organism was a promising candidate for water purification in aquaculture.

4 Proteobacteria

4.1 Bdellovibrio

Bdellovibrio comprises a group of parasitic Proteobacteria that prey on Gram-negative bacteria for growth, reproduction, and survival (Rotem et al. 2014). *Bdellovibrio* and *Bdellovibrio* and like organisms (BALOs) contribute to improving water quality (Zhang et al. 2009) and reduce the total bacteria numbers and especially *Vibrio* populations in the rearing water (Zhang et al. 2009; Li 2014; Wen et al.

2014). Because of its beneficial effects, *Bdellovibrio* has been approved as a probiotic/biocontrol agent for animal use by the Ministry of Agriculture and Rural Affair of China since 1994.

4.2 Ectothiorhodospira

Ectothiorhodospira is a genus of photosynthetic purple sulphur bacteria that comprises spiral cells with red coloration that deposit sulphur globules extracellularly (Trüper and Inhoff 1981). *Ectothiorhodospira shapashnikovii* WF, which was isolated from a marine shrimp pond, acted as both a bioremediation agent and nutrient source for white shrimp larvae (Wen 2014).

4.3 Paracoccus

Paracoccus marcusii DB11, which was isolated from sea cucumber culture ponds, demonstrated the ability to reduce the chemical oxygen demand (COD), ammonia, and titrate in sea cucumber feed leachates (Yan et al. 2011).

4.4 Pseudomonas

Pseudomonas stutzeri, which is distributed widely in the environment and occupies diverse ecological niches, has been proposed as a model organism for denitrification studies (Lalucat et al. 2006). For example *P. stutzeri* SC221-M was shown to improve water quality by decreasing nitrogen levels and microbial community structures in farmed carp and grass carp systems (Deng et al, 2014; Fu et al. 2017).

4.5 Rhodopseudomonas

Rhodopseudomonas comprises a genus of purple non-sulphur photosynthetic bacteria. Of relevance, *R. palustris* was added to water in grass carp farms leading to significantly reduced nitrogen levels and modulated microbial communities (Zhang et al. 2014).

5 Farmed Animals in Biofloc Systems or Waters Which Contain Probiotics

5.1 Shrimp

5.1.1 Pacific White Shrimp (*Litopenaeus Vannamei*)

Wu et al. (2016) added *B. subtillis* FY99-01 to the culture water of Pacific white shrimp leading to improvements of the water quality in terms of a reduction in the level of nitrite, pH, and soluble reactive phosphorus and decreased the abundance of Vibrionaceae representatives. Additionally, the addition of *B. coagulans* to water significantly increased the survival rate and some digestive enzyme activities of larvae shrimp (Zhou et al. 2009). Furthermore, the body length and growth rate of white-leg shrimp was significantly higher in treatments with sucrose and *Bacillus* compared with controls (Lukwambe et al. 2019; Zhang, et al. 2020).

5.2 Fish

5.2.1 Grass Carp (*Ctenopharyngodon Idellus*)

Liang et al. (2015) reported that using *B. licheniformis* in grass carp (*Ctenopharyngodon idellus*) pond water led to removal of nitrogen and modulation of the microbial community. Also, *Pseudomonas stutzeri* F11 has been used with some success in an experimental grass carp aquaculture system (Fu et al. 2017).

6 Methods to Use Probiotics in Biofloc Systems or the Water in Aquaculture Systems

A typical scenario is that a known volume of the bacterial suspension is added to the water in the biofloc system on a daily basis for a specified number of days. For example Fu et al. (2017) added *Pseudomonas stutzeri* F11 at 1×10^5 CFU/ml to an experimental grass carp aquaculture system at 3-day intervals, with the trial lasting for 9 days. In comparison, Liang et al. (2015) used the probiotic *Bacillus licheniformis* BSK-4, which has a nitrogen removal function and was fed to the fish at a dose of $1 \times 10^{8\ \mathrm{CFU}}/\mathrm{m}^3$/week for 18 days.

7 Effect of Probiotics on a Biofloc System in Terms of Water Quality

7.1 Reduction in the Level of Harmful Nitrogenous Compounds and Organic Matter

Wang et al. (2005) reported that commercial probiotics used in *Litopenaeus vannamei* ponds could decrease the concentrations of nitrogen and phosphorus. Thus, the administration of mixed *Bacillus* preparations has been found to be effective for some water quality parameters, namely controlling pH, ammonia, and nitrite levels during the rearing of white shrimp (Nimra et al. 2012). In parallel, use of *Bacillus subtilis* SC02 (Zhang et al. 2013) and photosynthetic bacteria (Zhang et al. 2014) led to a decrease in the nitrogen levels in grass carp culture water (Liang et al. 2015). In particular, the amount of ammonia–nitrogen decreased by 26.27% ($P < 0.05$) and 26.33% ($P < 0.05$) on the third and ninth day, respectively, whereas nitrite-nitrogen decreased by 59.54% ($P < 0.05$) and 39.04% ($P < 0.05$) on the sixth and ninth day, respectively, during the 9-day experiment (Fu et al. 2017). Similarly, use of *Bacillus licheniformis* BSK-4 in the grass carp culture water led to decreased nitrite, nitrate, and total nitrogen levels in water significantly over an extended period, whereas the ammonia level increased.

7.2 Reduction the Level of Potentially Pathogenic Microorganisms

Wu et al. (2016) reported that the abundance of Vibrionaceae representatives in Pacific white shrimp culture water was reduced after continually adding *B. subtilis* FY99-01 for 84 days.

8 Changes in the Microbial Community

Many researchers have found that the addition of some probiotics to aquaculture systems led to a change in the microbial communities in the water. Thus, Deng et al. (2014) reported that the addition of *Pseudomonas stutzeri* SC2210M to an experimental aquaculture system increased significantly the number of bacterial species. Similarly, Zhang et al. (2013) and Liang et al. (2015) determined that the addition of a B. *subtilis* preparation to a grass carp aquaculture system significantly increased the microbial diversity of the water. Also, Zhang et al. (2014) highlighted that the addition of photosynthetic bacteria to aquaculture water could increase the

number of Actinobacteria and decrease the number of nitrite reducers and anaerobic bacteria thus improving the water quality. Subsequently, Huerta-Rábago et al. (2019) added a commercial probiotic preparation to a biofloc system in a shrimp farm and determined that the number of phyla in one of the treated ponds was greater than the controls. However by the end of the study, the bacterial diversity in the trial ponds was very similar to that of the controls. Generally, these results are in agreement with the 9-day study involving an experimental grass carp culture system in which *Pseudomonas stutzeri* F11 exerted a significant impact on the microbial composition. Here, there were two different clusters at phylum and genus level for the treatment and control groups. Thus, the relative abundance of Bacteroidetes and Firmicutes increased in the treatment group, whereas Proteobacteria, Actinobacteria, and Verrucomicrobia decreased (Fu et al. 2017). A similar theme was reported by Lukwambe et al. (2019) after using a commercial preparation of *Bacillus.* Here, there was evidence for the succession, redistribution of beneficial microalgae, inhibition of the growth of harmful cyanobacteria, and sustained presence of microalgae community structure in shrimp aquaculture sites. However, in a 30-day experiment reported by Arias-Moscoso et al. (2018), the addition of commercial probiotics allowed the development of similar concentrations of heterotrophic bacteria, including *Vibrio*-like organisms, and ammonia oxidizers to the controls.

9 Improvements in the Growth Performance of Cultured Animals

The survival rate, body length, growth rate, and final weight of *Litopenaeus vannamei Boone* were significantly higher in treatments with sucrose and *Bacillus* compared with controls (Lukwambe et al. 2019; Zhang et al. 2020).

10 Problems with the Use of Probiotics in Biofloc Systems or Water in Aquaculture Sites

Much of the work about probiotics and bioflocs has involved comparatively brief experiments. Generally, there has been a dearth of studies using the approach in large-scale aquaculture sites for extended periods. It would appear that the effect of probiotics on water quality and microbial community structure is less significant in the long term rather than deduced from comparatively brief experiments, i.e. the commonly used 3–7 days for adding probiotics to water. The question to be resolved concerns the longevity of the added probiotics in the aquatic systems—do the probiotics survive and multiply in water and if so, for how long? Are the probiotics able to survive and compete with members of the natural aquatic microflora, or are

the bacterial cells, which have been cultured in in vitro systems, outcompeted by the resident organisms? Research is needed to address these points.

To date, many studies have been carried out to demonstrate the beneficial effect of microorganisms, including commercial products, to improve water quality in aquaculture sites. However, there is lack of official standards for commercial products and their use in aquaculture to improve pond water and sediment quality in China, which is the world's biggest producer of farmed aquatic species destined for human consumption (Wang 2019). Not surprisingly, the quality of commercial probiotic products varies considerably, and farmers have difficulty with making informed decisions about which product to choose. There is anecdotal evidence that decisions may be made according to the persuasiveness of company representatives or the conclusions reached by colleagues, friends, and/or neighbours.

The possible risk of transferring antibiotic resistance from probiotics to pathogenic bacteria should not be ignored. In this connection, some probiotic *Lactobacillus* isolates have been reported to transfer antibiotic-resistance genes in vitro and in rodent models (Egervärn et al., 2010; Cohen 2018). Some studies have indicated that a probiotic that is regarded as safe for human and terrestrial animal use may not be necessarily safe or suitable for use in aquaculture (Salma et al. 2011; He et al. 2017). Thus, any probiotic considered for aquaculture needs to be fully evaluated in the aquatic host (Goodwin et al. 2020) and the aquatic environment.

11 Conclusions and Suggestions for Further Work

Probiotics destined for use to control water quality need an appropriate set of criteria to determine their appropriateness for use in aquaculture. These criteria need not be the same as those appertaining to use as feed additives in terrestrial or aquatic animals. The proposed basic selection criteria for probiotics involved with water quality include:

(a) Safety. The candidate probiont should be safe for both aquatic animals and humans, without plasmids encoding virulence and/or antibiotic-resistance genes.
(b) Adaptability. The culture should be able to survive in the aquatic environment with the ability to adapt to the fluctuations in pH, salinity, temperature, and dissolved oxygen levels.
(c) Function. The cultures should have the ability to improve water and/or sediment quality by decreasing the amount of organic matter and affecting levels of nitrogen, as ammonia and nitrite.
(d) Convenience. The cultures should be readily applicable for large-scale production without the loss of important characteristics, storage, and administration on aquaculture sites (Wang et al., 2019).

Many articles have reported the significant effect of probiotics on improving water quality of aquaculture systems and their beneficial role in biofloc systems. However, there is a general lack of data on:

– the mechanisms of action of probiotics to reduce the presence and effect of pollutants in and around aquaculture sites
– effect on the structure of microbial communities
– the potential negative effects on aquatic ecosystems.

Further studies are warranted to determine the nature/mechanism of the beneficial effects and possible harmful effect of the probiotics. These data would be invaluable for decision making concerning the selection and administration of probiotics for use in aquaculture. Moreover, more work is needed to determine criteria to allow the maximum survival of probiotics in aquaculture environments and to understand fully possible competitive interactions with other members of the aquatic microflora. The fate of probiotics in and around aquaculture sites is largely unknown. Therefore, attention needs to be given to maximizing the benefit associated with using probiotics to improve water quality.

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Probiotics as Vaccine Adjuvants

Dibyendu Kamilya and Mukta Singh

Abstract Infectious diseases have become a major stumbling block for successful and profitable aquaculture. Vaccination is one of the most appropriate and effective methods currently available to the aquaculture industry for preventing infectious diseases. To be more effective, the vaccine antigen should not only be highly immunogenic, but it should be administered along with an effective adjuvant. The majority of the fish vaccines are administered through parenteral routes. Mucosal vaccination is more appropriate in fish as parenteral routes may not be the optimal route to deliver some vaccines. However, lack of effective adjuvants and knowledge of mucosal immune response are major limitations in developing effective mucosal vaccines in fish. The use of probiotics as adjuvants, either in combination with a vaccine or as a vaccine vector, is a promising new concept in mucosal vaccinology. This concept has gained significant momentum in human and animal vaccination programmes, but its application is still limited in the aquaculture sector. This chapter provides an overview of the published literature on the potency of probiotics as the vaccine adjuvant in fish vaccinology.

Keywords Adjuvants · Probiotics · Vaccine · Immunity · Fish

1 Introduction

Aquaculture is one of the fastest-growing animal production sectors catering for the demands for protein-rich diets of the growing global population. The growth of the aquaculture industry has accelerated rapidly worldwide over the past decades resulting in challenges to develop a productive, feasible, and sustainable aquaculture. According to the Food and Agricultural Organization (FAO 2020), the world aquaculture production reached 177.8 million metric tonnes in the year 2018–19 with an annual growth rate of 7.53%. Increasing intensification and commercialization of aquaculture practices have negatively affected the fish farming industry due

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to frequent disease outbreaks (Hai 2015). The outbreaks of numerous fish/shellfish diseases have marred the rapid increase in farm production due to high stock mortality (Kurath 2008). Thus, infectious diseases have become a major hindrance in the growth of the aquaculture industry, influencing both the socio-economic status of the farmers and the economic progress of a country.

In aquaculture, chemotherapeutic agents, namely antibiotics and chemicals, are the conventional cures for microbial infections. The frequent use of these chemotherapeutic agents results in harmful effects, such as the emergence of drug and antibiotic-resistant bacteria, the accumulation of antibiotic residues in the flesh, the destruction of beneficial gut microbes, and changes in the natural microbiota of the aquatic environment (Azevedo et al. 2015). Thus, the benefits of using chemotherapeutants in aquaculture have become uncertain, not only due to their adverse effects but also due to the decreasing consumer preference for drug-treated aquatic products. Therefore, various non-antibiotic-based and environmental-friendly approaches for disease treatment are increasingly being adopted for aquaculture health management.

The use of vaccines, probiotics, and immunostimulants are some of the promising alternative approaches for the control of infectious diseases in aquaculture (Newaj-Fyzul and Austin 2015). Vaccination is considered the most appropriate method currently available to the aquaculture sector for preventing infectious diseases (Tafalla et al. 2013). Nevertheless, there are still many diseases against which no effective vaccine is available, and a lot of reasons why some vaccines are effective and others fail. Making antigens more immunogenic, inducing the appropriate response to elicit protection, and determining the effectiveness of pilot vaccines are some of the issues faced by fish vaccinologists (Secombes 2008). Moreover, the majority of the commercially available fish vaccines are administered through different parenteral routes, which may not be the ideal route to deliver some vaccines (Adams 2019). Therefore, mucosal vaccines have gained prominence in the last decade in view of their ability to provide a longer duration of protective immunity in the vaccinated fish and ease of delivery (Munang'andu et al. 2015). However, one of the major challenges limiting the progress of the development of a protective mucosal vaccine for fish is the lack of effective adjuvants (Munang'andu et al. 2015; Adams 2019). Besides the fact that the vaccine antigen should be highly immunogenic, use of a potent adjuvant is another important factor for a successful vaccine (Soltani et al. 2019a). Adjuvants increase vaccine effectiveness by increasing antigen uptake and presentation by antigen presenting cells and by providing a co-stimulatory signal for lymphocyte activation (Barr et al. 2006). Different licenced and experimental adjuvants have been investigated in fish vaccinology, especially with injectable vaccines (Secombes 2008; Tafalla et al. 2013).

One of the most commonly purported benefits of probiotics is their ability to stimulate the immune system of the host (Nayak 2010). The beneficial attributes of probiotics in terms of growth and health promotion are well recognized (Balcazar et al. 2006; Kesarcodi-Watson et al. 2008; Doan et al. 2020). Recent evidence suggests that probiotics may be used either in combination with a vaccine or as a vaccine vector to improve the effectiveness of vaccination, especially in the higher vertebrate models (Licciardi and Tang 2011; Vitetta et al. 2017). The use of probiotic bacteria as novel

adjuvants in fish vaccination, especially in mucosal vaccination strategies, offers an exciting new approach, and researchers have started to work in this area.

2 Probiotics in Aquaculture

The unregulated use of antibiotics has exerted a very strong selection pressure on the resistance amongst bacteria, which have adapted to this situation, mainly by a horizontal flow of resistance genes (Cabello 2006). Therefore, several biological propositions, particularly microbial interventions, have gained significant momentum to fight diseases and avoid reliance on antibiotics (Panigrahi and Azad 2007). A promising and emerging alternative approach to prevent fish diseases is the use of probiotics, which helps fish to fight against pathogens by various mechanisms. From both the nutritional and immunological perspectives, probiotics may be utilized as the beneficial microbes that manipulate the intestinal microbiota through dietary supplementation.

A diverse group of Gram-positive and Gram-negative bacteria has been reported as probiotics in aquaculture. The recent findings suggest members from approximately 20 bacterial genera as potential probiotic candidates, and the majority of the species belong to *Bacillus* and lactic acid bacteria (LAB) groups (Knipe et al. 2020). The probiotics used in aquaculture play an important role in improving an organism's overall health. Besides promoting growth by contributing to digestion and nutrition, probiotics enhance the resistance of fish against infectious microorganisms by stimulating immunity (Balcázar et al. 2006; Nayak 2010). Additionally, probiotics have an extended application in aquaculture. Probiotics may improve the water quality when directly applied in the aquaculture pond water. Thus, probiotics are also described as microbial 'water additives' to substantiate this extended application (Moriarty 1998). Amongst the various benefits attributed to probiotics, the most widely believed benefits are regulation of the immune system, ability to promote systemic and local immunity under in vitro and in vivo conditions, and antipathogenic activity.

3 Probiotics and Fish Immunity

In recent fish health management research, much attention has been devoted to the immunostimulating effects of beneficial probiotic bacteria in piscine systems. Many immunological studies have been conducted in several fish species using different probiotics, and the ability of probiotics to induce fish immunity is noteworthy. Like mammals, the immune system of fish defends the host against a pathogenic challenge. Stimulation of the immune system by probiotic application, therefore, renders the fish more resistant to infectious microorganisms. Several probiotics, either individually

or in combination, enhance both systemic and mucosal immunity of fish to provide protection against pathogens (Nayak 2010).

Probiotic bacteria may interact directly with different immune cells, particularly phagocytes, to activate the innate immune responses. In fact, a plethora of studies indicate either probiotic induced enhancement in a number of immune cells or innate immune responses, such as respiratory burst, lysozyme, phagocytic, antiprotease, and myeloperoxidase activity (Irianto and Austin 2002; Salinas et al. 2008; Newaj-Fyzul and Austin 2015; Kamilya et al. 2015; Sangma and Kamilya 2015). Apart from the classical phagocytic functions, probiotics may effectively modulate different immune cells to produce several pro-inflammatory and other cytokines, such as IL-1, IL-6. IL-8, IL-10, IL-12, IFN-γ, TNF-α, and TGF-β (Kim et al. 2016; Panigrahi et al. 2007; Beck et al. 2015). Furthermore, probiotics promote changes in cell physiology, including neutrophil migration, improvement of neutrophil adherence, and plasma bacteriocidal activity (Soltani et al. 2019a). These probiotic effects on systemic immune components ultimately result in the modulation of several immune effector functions. Besides systemic immunity, fish possesses a well-defined mucosal immunity that is also crucial for defence against pathogenic invasion. Improving mucosal immunity provides an important avenue for preventing pathogen adhesion to the host tissue, which is an essential precursor to certain invasive diseases (Bogaert et al. 2004). Probiotics colonize the intestinal milieu and execute immunomodulatory activity, besides exerting other beneficial functions. The fish gut immune response is an outcome of cross talk between the gut mucosal epithelial cells, mucus, antimicrobial products, gut-resident commensal organisms, and mucosal/submucosal immune cells. It is now realized that the key mediator of gut mucosal immunity is gut-associated lymphoid tissues (GALT; Lazado and Caipang 2014). Probiotics stimulate the fish gut mucosal immunity by way of lymphocyte-mediated response and phagocytic and lysozyme activity (Nayak 2010; Lazado and Caipang 2014).

4 Adjuvants in Fish Vaccination

The types of vaccines that are used currently in the aquaculture industry include killed, live attenuated, DNA, subunit, or recombinant vaccines and are primarily administered parenterally (Adams 2019). However, problems with some of these vaccines include low immunogenicity, and a reduced capacity to trigger mucosal and cell-mediated immunity. Strategies to improve existing immunization regimens include alteration of the vaccine antigen, changing the number and timing of doses, alternative routes of administration, use of improved vaccine delivery systems, and/or adjuvants to increase immunogenicity. Vaccine adjuvants are used widely to increase the immunogenicity of vaccines. In order to increase individual immunity to that antigen, logical vaccine design has historically included the co-presentation of an adjuvant with a vaccine antigen (Schijns 2000; Mahon 2001; Bramwell and Perrie 2005). An adjuvant that modulates the humoral or cellular immune response to the vaccine antigen is an integral part of current parenteral and mucosal vaccines (Schijns

2003). It remains to be thoroughly elucidated about the exact nature of the specific mechanisms by which adjuvants enhance immune responses to co-presented vaccine antigens. However, it is considered that the adjuvant enhances the presentation of antigen and complex formation of antigen–antibody (depot effect) and also confers immunomodulatory effects (Isolauri et al. 2000; Kukkonen et al. 2008; O'Hagan and De Gregorio 2009). A range of effective adjuvants, such as oil emulsions, Freund's complete (FCA and incomplete (FIA) adjuvants, nano/microparticles, aluminium salts, cytokines, and immunostimulants, has been examined for use in fish vaccination (Secombes 2008; Tafalla et al. 2013; Adams 2019). Certainly, the majority of these adjuvants are used in injection vaccines but are limited for mucosal vaccination (Adams 2019).

Owing to the operational and practical difficulties of injectable vaccines, mucosal vaccination has emerged as a major area in fish vaccinology. Whereas injectable vaccines have continued to dominate in the vaccination of fish, research in mucosal vaccination has gained interest in the last decade due to their potential to lengthen the duration of protective immunity in vaccinated fish (Munang'andu et al. 2015). The major challenges hindering the progress in developing highly protective mucosal vaccines for fish are choice of antigen delivery system, optimization of antigen dose, route of delivery, oral tolerance, and choice of adjuvants (Munang'andu et al. 2015). The adjuvants that have been explored in mucosal vaccination include nano/microparticles, alginates, cytokines, β-glucan, and lipopolysaccharide (LPS) (Lavelle et al. 1997; Huttenhuis et al. 2006; Adomako et al. 2012; Galindo-Villegas et al. 2013; Kadowaki et al. 2013; Chen et al. 2014). However, developing an effective adjuvant that would allow an adequate antigen uptake in mucosal organs and enhance the immunogenicity of the mucosal vaccine is one of the key challenges.

5 Probiotic as Adjuvants in Fish Vaccination

The targeted administration of live probiotic cultures, either directly or as adjuvants, is an advancing area of potential therapeutics (Vitetta et al. 2015). The use of probiotics as adjuvants can support the intestinal commensal cohort to beneficially participate in the intestinal microbiome-intestinal epithelia-innate-cell-mediated immunity axes with vaccines aimed at preventing infectious diseases whilst conserving immunological tolerance (Vitetta et al. 2017). Several features of probiotics, such as their ability to induce immune cell types, promote immune responses, and acceptable safety profile makes them ideal and promising mucosal vaccine adjuvants. The ease of administration of probiotics is another advantage for the implementation of improved vaccines. Immunostimulating probiotics may be able to enhance immune responses to a vaccine antigen in the setting of reduced vaccination schedules, thereby conferring protection against infectious diseases where vaccine coverage is low. In animal models (other than fish), bifidobacteria and lactobacilli have been studied to a large extent to show potent adjuvant effects to vaccines. It has been suggested that a combination of both structural components and secreted factors belonging

to these probiotic groups are responsible for enhanced immunological responses to vaccination (Vitetta et al. 2017). In addition to the co-administration of probiotics (as adjuvant) with the vaccine, another way to use probionts as vaccine adjuvants is to use recombinant probiotics co-expressing antigen/adjuvant. Recombinant lactic acid bacteria (LAB) have been investigated as mucosal vaccine vectors in many animal studies to realize the adjuvant strategies necessary to induce a robust and long lasting protective immune response (Vilander and Dean 2019). Like other animal models, the probiotic adjuvant strategy in fish vaccinology has also been explored, although not extensively. The use of probiotic bacteria as adjuvants in fish vaccinology is detailed below:

Nile tilapia (*Orechromis niloticus*) fry were fed with two different probiotic products, such as Organic Green™ (1×10^{11} bacterial cells each from *Lactobacillus acidophilus, Bacillus subtilis, Saccharomyces cerevisiae,* and *Aspergillus oryzae*) and Vet-Yeast™ (1×10^{9} *S. cerevisiae* dried cells), for five months. Fish fed with the probiotics and vaccinated with *Aeromonas hydrophila* whole cell inactivated vaccine showed significantly higher survival after challenge, compared to the positive control group. However, the antibody titre did not show any significant difference between the groups (Aly et al. 2016).

Rainbow trout (*Oncorhynchus mykiss*), bath vaccinated with a streptococcosis/lactococcosis vaccine and fed with *L. plantarum* (10^{8} CFU/g feed) for two months, showed significantly higher agglutination antibody titre compared to the group that received only vaccine (Kane et al. 2016). However, haematological and innate immune parameters did not show any significant difference. Enhancement of specific antibody titre in the probiotic supplemented group indicated the adjuvant potency of the probiotic. Contrarily, rainbow trout fed with the probiotic *L. plantarum* (2×10^{7} CFU/g feed) and vaccinated against *Yersinia ruckeri* failed to show significant antibody titre compared to vaccinated fish that did not receive the probiotic (Soltani et al. 2019b).

A genetically engineered *L. plantarum* co-expressing glycoprotein (G) of spring viraemia of carp virus (SVCV) and ORF81 protein of Koi herpesvirus (KHV) was tested to induce protective immunity in carp (*Cyprinus carpio* and *C. carpio koi*) via oral vaccination. Compared to the control, the immunized carp showed significant levels of immunoglobulin M (IgM) and effective protection against virus challenge. The results demonstrated the ability of recombinant *L. plantarum* as an oral vaccine against SVCV and KHV infection in carp (Cui et al. 2015).

Olive flounder (*Paralichthys olivaceus*) was vaccinated by feeding a pellet feed onto which a membrane protein antigen of *Streptococcus iniae* (SiMA)-expressing *Lactococcus lactis* BFE920 was adsorbed (Kim et al. 2016). The vaccinated flounder showed significantly elevated antigen-specific antibodies, T-cell marker mRNAs, and T-cell effector functions. Also, relative per cent survivals (RPS) of 84% and 82% were observed in the vaccinated fish after intraperitoneal infection and bath immersion with *S. iniae*, respectively. The protective effect of the vaccine was confirmed even 3-months after vaccination in a field study, indicating immuno-potency of the feed vaccine.

Infectious pancreatic necrosis virus (IPNV) VP2/VP3 capsid proteins were expressed successfully and separately in *L. casei*. Rainbow trout was immunized with *Lactobacillus*-derived VP2/VP3, and subsequent challenge with IPNV showed a significant reduction in viral loads in the vaccinated fish compared to the sham-injected controls. Additionally, IPNV VP2 capsid protein secretory expression by *L. casei* was able to elicit a strong antibody response to provide protection against IPNV challenge (Min et al. 2012). In a similar study, Zhao et al. (2012) successfully expressed IPNV VP2–VP3 fusion protein in *L. casei* with natural antigenicity; the recombinant probiotics were capable of inducing antibodies against natural IPNV with significant reductions in viral loads in inoculated rainbow trout compared to the sham-injected controls. These reports suggest the usefulness of probiotic-based vaccines in inducing protective immunity in fish.

In a more recent study, rainbow trout was immunized orally with a recombinant *L. lactis* NZ3900 expressing the G gene of viral haemorrhagic septicaemia virus (VHSV) (Naderi-Samani et al. 2020). The vaccinated fish showed significantly higher relative expression of IFN-1 and MX-1 genes in the head-kidney, elevated VHSV-specific antibody levels, significant RPS against virulent VHSV challenge, and a significant reduction in viral loads in the immunized fish compared to the controls. The results demonstrated the protective immunity and efficacy of recombinant *L. lactis* vaccine against VHSV in trout fry.

6 Conclusions

The current vaccine regimen in aquaculture is dominated by injectable vaccines. The costly and labour-intensive injection vaccination strategies have prompted the scientific community to look for mucosal vaccines, administration of which is more practical and affordable. However, there are challenges limiting the development of effective mucosal vaccines for use in fish. One of the key hindrances is the lack of effective adjuvants. A good number of studies have explored the adjuvant effects of probiotics in enhancing vaccine efficacy with promising results, especially in human and animal mucosal vaccine models. Research on mucosal vaccination and the use of probiotics as vaccine adjuvants have started to attract the attention of fish vaccinologists only in recent times. A few attempts have been made by scientists to explore the effectiveness of probiotics as adjuvants as well as vaccine vectors with promising outcomes. However, it remains for many more studies to be conducted to fully realize the potential of using probiotics as vaccine adjuvants. The fundamental mechanisms involved in the adjuvant effects of probiotics, dosage optimization, length and mode of administration under standardized water quality and feeding conditions are some of the challenges to be considered whilst using probiotics as adjuvant. In addition, the use of probiotics as recombinant tools expressing vaccine antigen/adjuvants is a promising alternative for the protection and treatment of infectious diseases in the aquaculture sector.

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The Potential Use of Functional Ingredients with Probiotics as Immunostimulants

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Abstract Aquaculture has increased dramatically over the last few decades to cover the food gap and meet the human need for high-quality protein. Nowadays, intensification, zero water exchange, artificial feeding, and fertilization have become common breeding practices in aquaculture to improve overall production. However, some major obstacles in these sectors occur often. Among them, the presence of opportunistic pathogens and adverse water quality are hindrances and associated with the cause of mortalities. The frequent use of antibiotics, chemotherapeutic agents, and vaccines to mitigate infectious diseases has been recently criticized for the risks to aquaculture and public health. Therefore, inexpensive and effective alternatives are ultimately needed to replace these antimicrobial compounds. This chapter explores the use of promising functional components in aquaculture to control pathogens and boost aquatic animal immunity. These compounds combat pathogens by activating cellular, humoral, and specific immunity of aquatic species. In addition, there is no doubt that there are compromises to quality and environmental stability, which reflect various public health concerns and economic losses.

Keywords Functional ingredients · Immune function · Probiotics · Prebiotics · Aquaculture management

1 Introduction

Aquaculture is one of the most promising and sustainable productive sectors providing people with high-quality animal protein with global overall production increasing to 82.1 million tonnes in 2020 (FAO 2020). Similar to other industries, aquaculture constantly requires new technologies to increase production yields. With

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the increasing demand for aquatic animals, there has been a change in aquaculture practices from extensive to intensive culture where stressors, such as overpopulation, frequent handling, transport, grading, and adverse water quality, are common (Deivasigamani and Subramanian 2016). Consequently, diseases occur more frequently due to the rapid expansion and especially in intensive and highly intensive culture practices (Chen et al. 2014). The indiscriminate use of antibiotics in aquaculture feeding to alleviate infectious diseases or to boost growth performance is common and has been criticized and banned in many countries due to the emergence and spread of drug-resistant pathogens, immunosuppressive effects, environmental degradation, and chemical residues in the tissues of aquatic animals, which may well be dangerous to public health (Dawood et al. 2018).

In view of current limitations in the use of antibiotics, there is an urgent need to evaluate other possible alternatives for disease control. Functional ingredients, such as bioactive compounds, polysaccharides, prebiotics, synbiotics, complex carbohydrates, nutritional factors, herbs, hormones, and cytokines, are potential substitutes to antibiotics and are generally used in aquatic feeds to effectively enhance growth, the immune response, and control of various diseases in aquatic animals (Elayaraja et al. 2011; Ganguly et al. 2010; Sakai 1999).

Functional ingredients, also defined as immunostimulants, immunomodulators, adjuvants, or biological response modifiers, are non-toxic and non-pathogenic, and never produce unfavourable effects when applied to farmed aquatic organisms (Buchmann 2014; Mohapatra et al. 2013). Currently, the potential application of functional ingredients for applied medical research and specifically in aquaculture is of great interest and has been considered as a novel alternative remedy for controlling emerging serious diseases (Song et al. 2014). The biological activities of these compounds are influenced by some physicochemical parameters, including molecular weight and formula, solubility, elemental component, and polymer charge (Bohn and BeMiller 1995).

The prospective use of functional ingredients as an integral part of management and healthcare systems has found its way into a wide range of aquatic species, including fish (Anderson 1992; Bricknell and Dalmo 2005; Mehana et al. 2015), shrimp, and crustaceans (Apines-Amar and Amar 2015; Das et al. 2006). Functional ingredients not only improve the growth and survival rate of the aquatic species, but also strengthen the immune response and increase the capacity for disease resistance (Raa 2000).

Much evidence of exaggerated immune responses after administration of such ingredients has been reported. In fish, functional ingredients coordinate cellular and humoral immunity by stimulating the secretion of cytokines, improving the phagocytic capacity of neutrophils and lymphocytes, and eliciting antibodies and complement responses (Sahoo and Mukherjee 2001; Wang et al. 2017). The mechanism of action of these ingredients and their activation in the immune systems of crustaceans are different. The compounds opsonize the phagocytosis of threatening pathogens via mediating signal recognition and phagocytosis, boosting haemolymph bacteriocidal and antiseptic properties, and enhancing the prophenoloxidase system (Bondad-Reantaso et al. 2005; Castex et al. 2010; Smith et al. 2003).

Over the past few decades, the use of new potential functional supplements in the aquaculture sector has resulted in a variety of benefits that provide not only basic nutrition but also good health and longevity. Phytobiotics, known as plant-derived products, are the best of these new supplements. The derivatives are added to the diet to improve growth performance and enhance animal productivity (Cristea et al. 2012). Phytobiotics are prepared from leaves, roots, tubers or herbs, spices, and fruits of plants. The preparations are commercially available in solid, dry, and ground forms or as juices (as essential oils). In simple terms, phytobiotics are products of plant origin; products, such as thyme, oregano, turmeric, and garlic, are gaining extensive interest among researchers and aquaculture producers. Recently, several researchers have studied the role of some herbal plants and their extracts as potential therapeutic agents in aquaculture because of the production of bioactive substances with antimicrobial and antioxidant properties, and include Sabinene, Eugenol, Capsaicin, Zingerone, Piperine, Allicin, Cineole, Carvacrol, Thymol, Menthol, Azadirachtin, and Salannin (Altemimi et al. 2017; Jana et al. 2018; Mabrok and Wahdan 2018).

2 Classification

The use of functional ingredients has become available in aquaculture; several definitions have been proposed recently. These ingredients are simply defined as natural, safe, and eco-friendly compounds that mitigate the host's immune responses and increase its resistance against diseases especially those caused by pathogens (Bricknell and Dalmo 2005). Functional ingredients are classified according to their mode of action, their origin, and route of administration into the following: natural derivatives and synthetic commercial products (Fig. 1). Lists of some commercially available immunostimulants, their active ingredients, mode of action, route of administration, and recommended dosage are fully elucidated in the previous publications (Barman et al. 2013; Ismail et al. 2019).

What are phytobiotics? They are defined as natural bioactive compounds of plants and include herbs, spices, essential oils, and oleoresins (Papatsiros et al. 2009). Herbs, spices, and their extracts have been known from ancient times for their specific aroma and various medicinal properties (Greathead 2003). Many plant extracts contain carbohydrates; most of these are heteroglycans composed of hexoses and pentoses and methylated uronic acids (Delzenne and Roberfroid 1994), and exert growth-promoting effects (Xu et al. 2003). Prebiotic oligosaccharides may be obtained by direct extraction of natural plant oligosaccharides, controlled hydrolysis of plant polysaccharides, and enzymatic synthesis (Grizard and Barthomeuf 1999). Partial enzymatic hydrolysis of inulins and fructans produces fructo-oligosaccharides, which are fermented to short-chain volatile fatty acids in the distal intestine, and may enrich the growth of favourable bacteria. Arabinogalactans and fucogalactoxyglucans from the acacia tree have immuno-modulating effects and stimulate macrophages to secrete tumour necrosis factor (TNF) (Wagner and Jordan

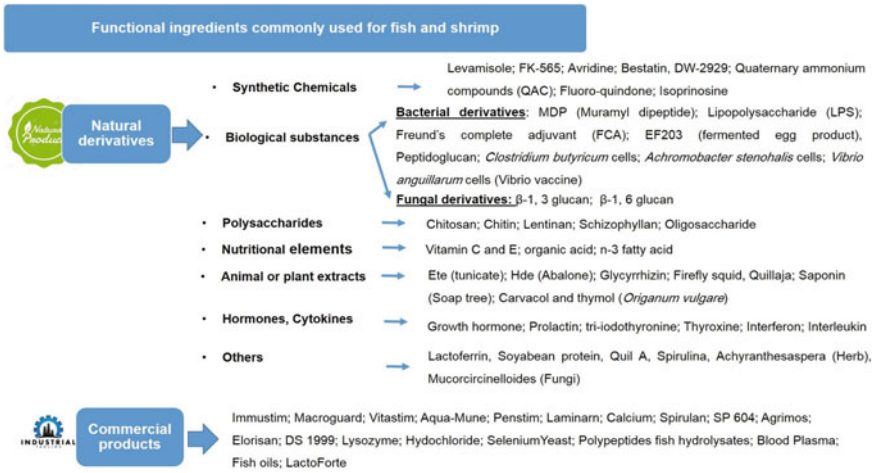

Fig. 1. Type of functional ingredients commonly used for fish and shrimp culture

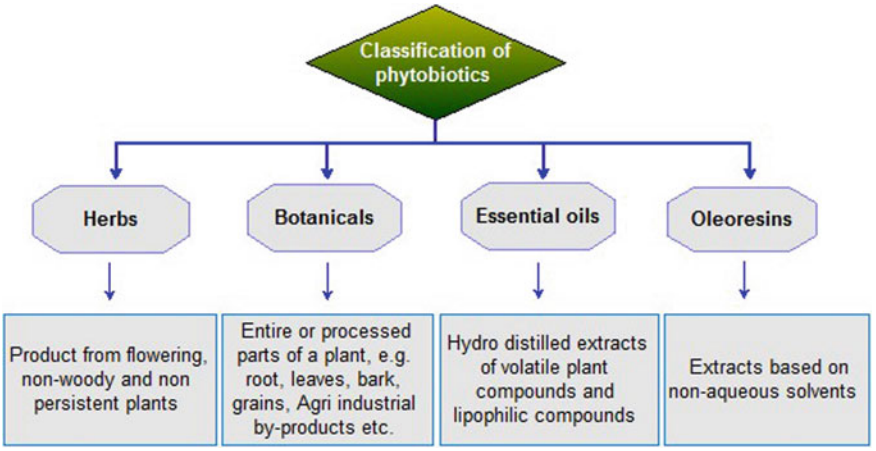

Fig. 2. Classification of phytobiotics commonly used in aquaculture

1988). Overall, the classification of phytobiotics is based on their nature and methods of extraction (Fig. 2).

3 Mode of Action

The way functional ingredients work is to improve growth performance and survival rate as well as strengthening the immune system of aquatic organisms to boost

the level of immunity/protection against invading pathogens (Bricknell and Dalmo 2005). Functional ingredients embrace a group of bioactive and synthetic components that boost the non-specific, cellular, and humoral defence mechanisms in fish (Maqsood et al. 2011). The evolved ingredients may be synthetized (chemical and drug) or extracted naturally and administered alone or synergistically to fortify the innate immunity as well as heightening a specific immune response of the host (Mehana et al. 2015). Studies on immunostimulants are being intensified mainly in the field of biomedical sciences and cancer research; however, their potential application alone or as an adjunct to vaccines has recently developed as one of the most promising approaches for preventing or controlling fish diseases (Maqsood et al. 2011).

Although studies briefly elucidate the mode of action of some functional ingredients, their approach is very diverse or poorly understood as it relies mainly on the type of ingredients, route of administration, dose, and period of exposure. The basic measures adopted for administering functional ingredients in aquaculture are by injection, immersion, and oral uptake; the latter route is the most practical way to deliver the ingredients, but their prolonged effects remain enigmatic (Dawood et al. 2018). Interestingly, some studies have shown that injection or immersion of functional ingredients can boost host immunity by activating leukocytes and increasing their resistance to invasive pathogens (Anderson 1992; Barman et al. 2013; Jeney and Anderson 1993a; Kono and Sakai 2001; Sakai 1999). However, both methods are labour-intensive, time-consuming, and impractical in intensive culture and seed fish farming systems (Soto et al. 2015). Moreover, the oral method is the only approach economically suited for an intensive culture system; it is not onerous, and it allows for mass administration regardless of the size and density of the fish (Galindo-Villegas and Hosokawa 2004).

Functional ingredients have received much attention and have been claimed to be successful in providing improved protection to fish and shellfish under experimental or farm conditions (Sahoo 2007). The ingredients comprise several categories; each has an independent mode of action (Table 1). The main responses in aquatic organisms treated with functional ingredients are summarized in Fig. 3.

4 Potential Application

The use of functional ingredients in aquaculture has opened a new horizon in protecting fish health. It activates the immune system of aquatic animals and enhances their capacity for disease resistance by cellular and humoral mediated immunity. The fish immune system is responsible for destroying micro-organisms through acquired, innate, humoral, and cellular processes that interact to prevent disease outbreaks (Biller-Takahashi and Urbinati 2014). Immunostimulants comprise a group of synthetic chemicals (levamisole, FK-565—isolated from *Streptomyces olivaceogriseus* cultures), biological substance (bacterial derivatives, polysaccharides, animal and plant extract), nutritional factors (vitamins C and E), hormones

Table 1 Mode of action, dosage, and administration routes of different functional ingredients commonly used for aquatic animal species

Categories	Examples	Mode of action			Route of administration	References
		Growth performance and feed utilization	Immunological, haematological, and physiological responses	Antimicrobial activity and disease resistance		
Synthetic chemicals	Levamisole; FK-565; avridine; bestatin; DW-2929; quaternary ammonium compounds (QAC); fluoro-quindone; isoprinosine	–	+++	++	Oral/water routine, bathing, and feed additives	Findlay and Munday (2000), Jeney and Anderson (1993b), Morrison et al. (2001), Shahbazi and Bolhassani (2016), Symoens (1977)
Probiotics	*Lactobacillus* spp.*; Lactococcus* spp.*; Bacillus* spp.*; Enterococcus* spp.*; Leuconostoc* spp.*; Aeromonas* spp.*; Carnobacterium* spp.*; Micrococcus* spp.*; Pediococcus* spp.*; Saccharomyces* spp.*; Vibrio* spp.*; Pseudomonas* spp.*; Methylococcus* spp.*; Rhodococcus* spp.*; Microbacterium* spp.*; Sphingopyxis* spp.*; Leucobacter* spp.*; Staphylococcus* spp.	+++	+++	+++	Oral/water routine and feed additives	Aguirre-Guzmán et al. (2012), Dawood et al. (2019), IRTA (2015), Merrifield et al. (2010), Pérez-Sánchez et al. (2014), Ringø and Song (2016)

(continued)

Table 1 (continued)

Categories	Examples	Mode of action			Route of administration	References
		Growth performance and feed utilization	Immunological, haematological, and physiological responses	Antimicrobial activity and disease resistance		
Prebiotics	Glucan; mannan oligosaccharide (MOS); fructo-oligosaccharide (FOS); yeast cell wall (YCW); lipopolysaccharide (LPS); peptidoglycan; inulin; galactooligosaccharides (GOS); arabinoxylan oligosaccharides (AXOS); chitooligosaccharide (COS); inactivated yeast bacteria; xylooligosaccharide (XOS); levan	++	+++	++	Oral/water routine and feed additives	Dawood and Koshio (2016), Dawood et al. (2018), Ganguly et al. (2013), Iwashita et al. (2015), Merrifield and Zhou (2011), Yousefian and Amiri (2009)
Synbiotics	Mixture of probiotics and prebiotics	++++	++++	++++	Oral/water routine and feed additives	Abid et al. (2013), Cerezuela et al. (2011), Dawood and Koshio (2016), Ringø and Song (2016), Torrecillas et al. (2018), Sewaka et al. (2019)

(continued)

Table 1 (continued)

Categories	Examples	Mode of action			Route of administration	References
		Growth performance and feed utilization	Immunological, haematological, and physiological responses	Antimicrobial activity and disease resistance		
Herbal extract and medicinal plants	*Achyranthes* derivatives; soybean derivatives; propolis; *Allium* derivatives; *Thymus vulgaris* derivatives; *Zingiber officinale*; *Urtica dioica; Viscum album; Magnifera indica; Origanum vulgare;* carvacrol*;* thymol; *Ocimum sanctum; Sanguinaria canadensis; Trigonella* derivatives; *Withania somnifera; Echinacea; Uncaria tomentosa; Mentha piperita*	+	++	++	Feed additives	Citarasu (2010), Hwang et al. (2013), Mabrok and Wahdan (2018), Reverter et al. (2017), Van Hai (2015), Vaseeharan and Thaya (2014)

(continued)

Table 1 (continued)

Categories	Examples	Mode of action			Route of administration	References
		Growth performance and feed utilization	Immunological, haematological, and physiological responses	Antimicrobial activity and disease resistance		
Animal by-products	Yucca extract; lactoferrin; chito-oligosaccharides; IgY egg powders; fish protein hydrolysates (FPH)	++++	++	++	Feed additives	Adegbeye et al. (2019), Esmaeili et al. (2019), Kim et al. (2004), Siddik et al. (2020), Stamer (2015)
Immunostimulants and nutritional factors	Vitamins (Vit C, Vit E, Vit B6 and B5); Ergosan algae (*Laminaria digitate,* Spirulina, and *Ascophyllum nodosum*); fucoidan minerals (chromium, activated charcoal); amino acids and derivatives (methionine, tryptophan, arginine, and/or glutamine); peptides (apidaecin); enzymes (amylase enzyme, N-acyl homoserine lactonase)	++	+++	++	Feed additives	Alishahi and Aïder (2012), Chanda et al. (2015), Costas et al. (2011), Dawood et al. (2018), Jiménez-Fernández et al. (2014), Kitikiew et al. (2013), Machado et al. (2015)

(continued)

Table 1 (continued)

Categories	Examples	Mode of action			Route of administration	References
		Growth performance and feed utilization	Immunological, haematological, and physiological responses	Antimicrobial activity and disease resistance		
Hormones and cytokines	Growth hormone; prolactin; tri-iodothyronine; thyroxine; interferon; interleukin	+	+++	++	Feed additives and injection	Barman et al. (2013), Mehana et al. (2015), Sakai (1999)
Nucleotides		++	+++	+	Feed additives	Dawood et al. (2018), Fegan (2006), Hossain et al. (2016), Li and Gatlin III (2006)
Organic acids or acidifiers	Acidic calcium sulphate; highly unsaturated fatty acid (HUFA); silage oil	+	+	+	Feed additives	Bai et al. (2015), Goosen et al. (2014), Renaud et al. (1991), Weimin (2010)

++++, exaggerated response; +++, highly significant; ++, significant; +, mild or possible; −, doubtful. These symbols are given based on the previous study of Dawood et al. (2018).

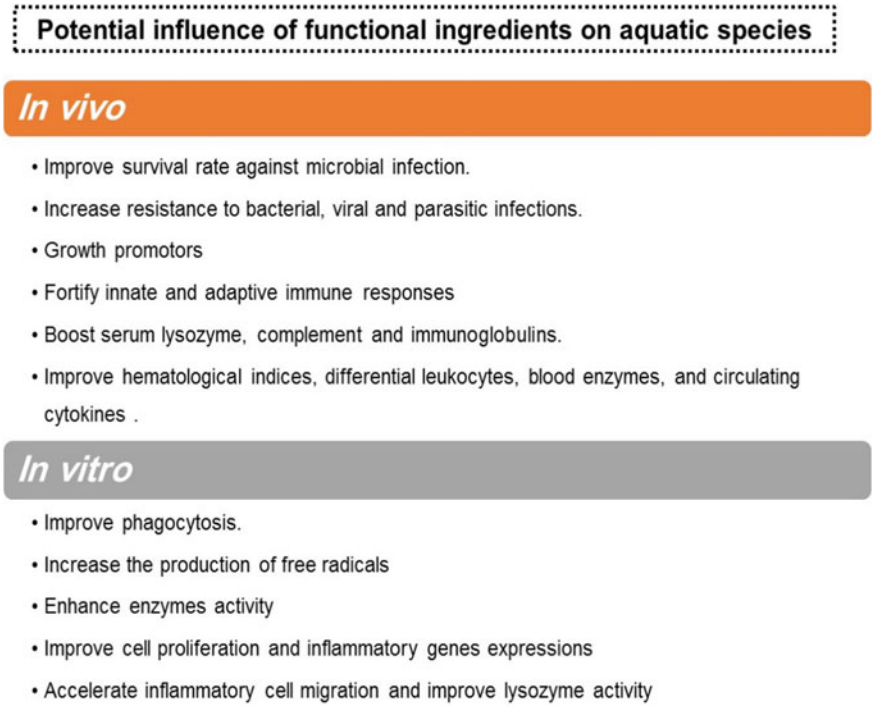

Fig. 3. Potential influence of functional ingredients on health status and immune response of aquatic species (in vivo and in vitro study)

(prolactin and growth hormone), and cytokines (polypeptides and glycoprotein that enhance the unspecified cellular and humoral defence mechanisms (Fujimoto et al. 2013; Sado et al. 2013). Foremost, functional ingredients offer many beneficial and vital effects in fish and shellfish culture systems (Fig. 4).

5 Synthetic Chemicals

Over the past few decades, the aquaculture sectors have used many synthetic chemical compounds, such as benzalkonium chloride, malachite green, and levamisole, to boost the immune system of fish, and to prevent and/or control microbial diseases (Idowu and Sogbesan 2017). A list of the synthetic chemicals commonly used in aquaculture sectors is included in Table 2.

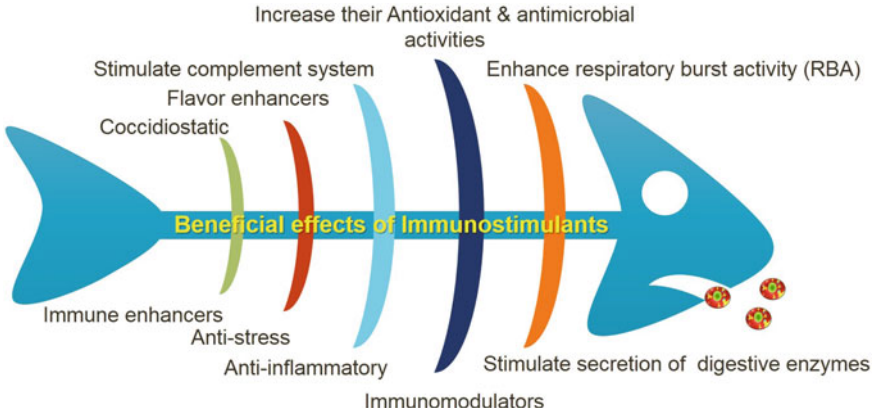

Fig. 4. Beneficial effects of active ingredients in fish and shellfish culture systems

6 Levamisole

Levamisole is an immunomodulatory and anthelminthic compound that is commonly used to treat parasitic, viral, and bacterial infections in humans and animals. Alves et al. (2019) reported on the use of levamisole to control and treat monogenean infections in fish culture. The compound enhances mainly phagocytic activity, the NBT reaction, and increases antibody-producing cells in fish. Oral administration of levamisole increased the leukocyte count and serum lysozyme activities, and decreased the phagocytic index of phagocytic cells (Siwicki 1989). However, after the administration of this compound to rainbow trout, differences were not observed in the levels of haematocrit, leucocrit, or immunoglobulin (İSPİR and Dörücü 2005). Researchers recommend early use of levamisole as an immunostimulant in fish (Findlay et al. 2000; Alves et al. 2019). It has been observed that rainbow trout and *C. macropomum* exposed to a bath treatment containing 5, 10, 25 μg/mL and 125 mg/L of levamisole for 2 h showed resistant to monogenean infestations and *Y. ruckeri* (Ispir 2009).

7 Bacterial Derivatives

The use of bacterial derivatives in aquaculture practices may have beneficial effects. Several promising biological response modifiers have been examined in fish either in vivo or in vitro (Nayak et al. 2007; Panigrahi et al. 2004). Earlier researchers have studied extensively the effectiveness of bio-derivative agent administration in several fish species and included β-glucan, muramyl dipeptide, chitosan, ectoine, trace minerals, vitamins or their additives, as well as various products derived from

Table 2 Synthetic chemicals used in aquaculture

Chemical	Use	Dosage	Application
Levamisole	Against parasitic, viral, and bacterial infections	5, 10, 25 µg/ml and 125 mg/L	Water
Acriflavine	Against bacteria, fungi, protozoa	5 mg/l for 5 days	Water
Albendazole	Against bacteria, parasitic infection	500, 100, 1500, and 2000 mg/L	Water
Some potential antibiotics	Against bacteria	Varies according to drug	Water, feed, injection
Benzalkonium chloride	Against bacteria	2 mg/l (active ingredient) for 60 min for 3 days	Water
Ivermectin	Against bacteria, parasitic infection	200, 250, 300, and 350 mg/L	Water
Copper sulphate	To control algae, protozoa, flukes, fungi	Harden water to above 170 mg/l then add 0.1 copper sulphate/l for 10–20 min	Water
Formalin	Against protozoa, flukes	0.125–0.250 mg/l for 60 min. 0.015–0.025 mg/l for several days	Water
Hydrogen peroxide (H_2O_2)	Against protozoa, fungi on eggs	0.10 ml of 3% H_2O_2/l for 10–15 min (every other day)	Water
Malachite green	Against fungus, protozoa, bacteria, trichodina	0.10 mg/l for 12 days, repeat with dosage each on days 3, 6, and 9	Water
MS-222 (tricaine)	Anaesthetic	10 mg/l during transport	Water
potassium permanganate ($KMnO_4$)	To control bacteria, protozoa, trichodina, flukes, lice, fungi,	2 mg/l on day 1, 1 mg/l each on day 2, 3, 4, and 5 OR 5 mg/l as a single treatment	Water
Praziquantel	Against flukes	2 mg/l for several days	Water

plants and animals. These workers found that bio-derivatives are effective in stimulating or modulating both specific and non-specific defence mechanisms and offer protection against viral and bacterial diseases in fish (Kodama et al. 1993; Siwicki et al. 1994; Wang et al. 2017).

8 Muramyl Dipeptide

Muramyl dipeptide (MDP) (N-acetylmuramyl-L-alanyl-D-isoglutamine) is an immunostimulating synthetic polypeptide containing N-acetyl muramic acid attached to the short-chain amino acid of L-Ala-D-isoGln derived from *Mycobacterium.* Some researchers have revealed that the administration of MDP-lysine in rainbow trout via intraperitoneal injection led to increases in phagocytic activities, respiratory burst, and leukocyte migration activities in the kidneys as well as resistance to disease (Žunić Zvizdić et al. 2012; Maharana et al. 2013).

9 Glucan

Among the group of active ingredients, β-glucans have been widely used as IS in aquatic animal species. β-glucans contain a group of β-D-glucose polysaccharides that occur naturally in the cell walls of cereals, bacteria, algae, and mushrooms/fungi (Dawood et al. 2015, 2016). Generally, these compounds enhance anti-tumour, antimicrobial, antiviral, and anti-parasite properties in fish and shellfish (Caipang et al. 2012; Hardy and Halver 2002). The addition of β-glucan in farmed aquatic species enhances various immune functions including haematopoiesis, production of lytic proteins, namely lysozyme and complement system proteins, and promoting phagocytosis activity (Soltanian et al. 2009).

Glucans are indigestible substances that allow specific changes in the composition and/or function of the intestinal microbes, which positively affects feed utilization and fish growth performance (Kühlwein et al. 2014; Song et al. 2014). Also, glucans promote growth and feed conversion along with enhanced health status and growth performance among different cultured species, including snapper, *Pagrus auratus* (Cook et al. 2003); rohu, *Labeo rohita* (Misra et al. 2006); sea cucumber, *Apostichopus japonicas* (Gu et al. 2011); and koi carp, *Cyprinus carpio* koi (Lin et al. 2011). Additionally, when glucans are taken orally, they exert potential antimicrobial effects against a variety of bacterial pathogens, including *Aeromonas hydrophila* and *Pseudomonas fluorescens* (Brogden et al. 2012; Ismail et al. 2019; Selvaraj et al. 2005).

10 Freund's Complete Adjuvant (FCA)

FCA is one of the most potent classical oil adjutants that boosts the immune response on both the humoral and cellular levels (Jiao et al. 2010) at a reasonable price (Abdy et al. 2016; Raa 1996). FCA serves as a broad-spectrum immunoregulator that can activate immunocytes, regulate the release of cytokines, promote the generation of antibody, and enhance the immune function of leucocytes (Pavan et al. 2016).

FCA improves immune responses, enhances the effectiveness of vaccine in fish, and provides protection against bacterial pathogens, including *A. salmonicida*, *A. hydrophila,* and *Vibrio ordalii* (Paterson and Fryer 1974).

11 Whole Cell Inactivated Vaccines (=Bacterins)

Whole cell, inactivated vaccines are commercially available, cost effective, and authorized for use in the aquaculture industry to control diseases including enteric redmouth and vibriosis. These vaccines have gained widespread use in aquaculture and are capable of inducing a protective immune response, increasing disease resistance of the host. Furthermore, these vaccines have been used widely to reduce antibiotic dependence and the severity of losses incurred by diseases (Bondad-Reantaso et al. 2005; Grisez and Tan 2005). These vaccines improve fish health, reduce disease outbreaks, and provide long-lasting protection from diseases. Moreover, there is not any evidence that the vaccines contain any harmful residues that could damage the recipient hosts or the environment (Ina-Salwany et al. 2019; Sudhagar et al. 2016).

12 Whole Cell Inactivated Vibrio Vaccines

The chemically inactivated whole cell *Vibrio anguillarum* vaccine is one of the most successful vaccines for raising fish and shellfish, and has been administered by intraperitoneal injection, orally, and by immersion methods (Sakai 1999). There is evidence of the vaccine conferring cross-protection in rainbow trout against *Aeromonas salmonicida* (Norqvist et al. 1989). Moreover, Horne et al. (1995) reported that the vaccine enhanced the immune system and increased the dynamic of haemocyte migration in black tiger shrimp.

13 *Clostridium butyricum* Cells

Clostridium butyricum cells enhance the disease resistant against vibriosis in rainbow trout via oral administration and improve leukocyte activation, phagocytosis, and super-anion production (Sakai et al. 1991). Also, they may stimulate macrophages and NK cells, and enhance further protection against *Candida* infection (Hour-Young et al. 1987).

14 Polysaccharides

Polysaccharides are present in plants, animals, and micro-organisms, and comprise long-chain polymeric carbohydrates composed of monosaccharide units bound together by glycosidic linkages (Mohan et al. 2019). Chitosan is a linear polysaccharide containing β-linked D-glucosamine N-acetyl-D-glucosamine, which are both derived from the hard-outer skeleton of shellfish, including crab, lobster, shrimp, and the cell walls of some fungi (Sakai 1999). Chitin and chitosan polysaccharides enhanced the immune activity in fish and shellfish, particularly enhancing macrophage activity and increasing resistance to pathogens. The compounds improved the absolute number of blood cells, respiratory burst activity, phagocytosis, and the survival rate in *Litopenaeus vannamei* after challenge with *V. alginolyticus* (Wang and Chen 2005). Furthermore, use of chitosan led to increased protection against *A. salmonicida* infection when administrated by injection or immersion to brook trout, *Salvelinus fontinalis,* and rainbow trout, *O. mykiss* (Anderson et al. 1995; Kawakami et al. 1998).

15 Extracts from Marine Invertebrates

Extracts derived from some marine invertebrates have special immune stimulating effects. For example, *Ecteinascidia turbinate* (Ete) from a tunicate and the glucoprotein fraction of an aqueous extract from abalone (*Haliotis discus* hannai (Hde)) enhanced the killing of tumour cells, inhibited tumour growth *in vivo* (Kawakami et al. 1998), enhanced the activity of phagocytes, NK cells, and increased eel survival against bacterial infection by *A. hydrophila* (Sakai 1999). Furthermore, greater survival with enhanced phagocytic activity was reported when Hde was injected into rainbow trout challenged with *V. anguillarum* (Sakai et al. 1991).

16 Algal Derivatives

Algae exert an important role in aquaculture. Currently, microalgae are being used worldwide as an alternative source of protein to replace fishmeal. Algae are naturally rich in carbohydrate polymers, such as agarose, alginate, and carrageenan, and produce a great variety of metabolic compounds, pigments, and oils that other macro-organisms (i.e. farmed fish) cannot produce (Plaza et al. 2008).

It has been suggested that the inclusion of algae derivatives as an ingredient in diets for fish shrimp and large oysters could improve their growth performance and survival rates (Battu et al. 2011). Some algae derivatives are available commercially, for example, AQUAVAC Ergosan, which contain high nutritional values of alginate and anionic polysaccharide from brown algae and seaweeds, and have been shown

to possess excellent immunostimulatory properties in fish (Huttenhuis et al. 2006). Futerpenol® is another algae product and contains fucoidans (=polysaccharides) and labdane diterpenes. The dietary incorporation of Futerpenol® induced in vitro expression of IL-12 and IFN-I in SHK-I cell line, and provided a high degree of resistance to *Piscirickettsia salmonis* in rainbow trout (Hernández et al. 2016).

17 Future Perspectives

Aquaculture has expanded considerably in the years after the Second World War, but issues have developed concerning food security. Yet, the overriding goal of aquaculture is to replace and indeed supplement wild caught production as the seas become overfished. It is most likely that aquaculture will be the dominant source of high-quality protein for many of the world's 9 billion people by 2050 (Hauton et al. 2015). Nevertheless, disease is an ongoing issue and is responsible for heavy losses to production in many countries. The dominance of antibiotics and other antimicrobial compounds for disease control—of mostly bacterial diseases—has been gradually reduced as issues of tissues residues, and the presence and spread of antibiotic resistance genes come to the fore. Vaccination has been regarded as a primary prophylactic tool, but too few vaccines are available commercially. Immunostimulants are a potentially promising alternative for disease control in aquaculture. To date, numerous products have been shown to be successful in disease control strategies and include β-glucan, polysaccharides, FCA, and algal derivatives. In general, food additives are widespread support as alternative approaches improve the health, immune status, and productivity of farmed aquatic animals; the outcome is increased net economic income. However, there are some ongoing issues with these compounds notably concerning the possible presence of contaminants.

For the future, the active components of plant or microbial products need to be researched with a view to purifying/synthesizing the bioactive components. This approach could help reduce waste involving the excessive use of whole plants when key component compounds would suffice the needs of aquaculture. Furthermore, more research is needed to determine more fully the precise role of these bioactive molecules in aquatic animals. Then, there are issues about determining the optimum dose(s) and duration of administration of the bioactive compounds. It is unclear how long the beneficial effect lasts after the cessation of application. Yet, there is optimism that these compounds will be successfully integrated into aquacultural procedures, and together with good management and hygiene will contribute to less disease, better immunity and feed conversion, and increased production. Overall, improvements will benefit consumers.

18 Conclusions

Disease outbreaks are a constraining factor that impedes the development of aquaculture. Like terrestrial mammals—including humans, fish depend on innate and adaptive immunity for protection against pathogens. These immune mechanisms may be triggered using bioactive compounds, such as found in plants and microorganisms, that exert immunomodulatory activity. The compounds, which have been described above, have tremendous beneficial prophylactic effects on the recipient aquatic animals. These compounds are functional alternatives to chemotherapeutants because they are safer to use, and in many cases demonstrate a wider range of effectiveness than achieved with vaccination. It is anticipated that functional ingredients will be an important part of disease control strategies in future and contribute to making aquaculture sustainable. In turn, this will contribute to better economic growth for the aquaculture industry and lead to increasing employment and job security especially in rural area.

19 Suggestions for Further Work

- Research is needed to determine the nature of the beneficial bioactive components in functional feed ingredients.
- More work is needed to determine the optimal doses for the feed supplements.
- The optimum duration of administration should be established—should this be continuous or by pulses? How long should a pulse last?
- How long does the beneficial effect last after cessation of administration?

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Modes of Action of Probiotics

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Abstract In recent years, technological advances in the microbiology and omics and bioinformatics fields have greatly expanded our understanding of the mechanisms of action of probiotics. However, probiotics seem to work in a strain and host species-dependent fashion, and only a small amount of information on the modes of action of beneficial bacteria used for aquatic animals is available. In this chapter, the mechanisms of action of probiotics will be reviewed; this will include traditional effects, including competitive exclusion of pathogens, increased enzymatic activity, and production of volatile fatty acids, as well as recent findings, such as modulation of immune responses at the molecular level, upregulation of low molecular weight metabolites, and bidirectional communication between the brain and gut. Further research is needed to elucidate the precise molecular mechanisms of action of probiotics.

Keywords Molecular mechanisms · Bidirectional communication · Immune response · Competitive exclusion · Enzymatic activity · Volatile fatty acids

1 Introduction

A true probiotic should preferably be of host-derived origin, safe, free of vectors that are able to transfer resistance to antibiotics and free of virulence or toxic factors (Plaza-Diaz et al. 2019). The use of host-associated probiotics has recently garnered

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a great deal of attention because such compounds are considered to have optimal benefits in similar natural habitats (Lazado et al. 2015; Nguyen et al. 2017). Naturally, a probiotic should have the capacity to survive under the intestinal conditions (including acidic pH, enzymes, and bile salts) of the host animal from which it originated. In addition, a probiotic should exhibit antagonism against pathogens and stimulation of the immune system and ultimately must have demonstrable beneficial effects on the host (Plaza-Diaz et al. 2019). Recently, the definition of "probiotics" has been extended to include novel functional agents that have the potential to repair gut dysbiosis in aquatic organisms. Dysbiosis is defined as a pathological condition in which the microbiota are harmful to the host; probiotics have been reported as a "corrective tool" for microbial manipulation in animals, including fish (Brugman et al. 2018). This means that probiotics can reverse the activity of pathogens that cause dysbiosis through factors such as (1) qualitative and quantitative changes in the populations of pathogenic microorganisms in the gut, (2) changes in the metabolic activities of the gut microbiota, and (3) changes in the concentrations of common gut–microbiota (La Fata et al. 2018).

Although several studies have looked at probiotics with the ability to stimulate appetite, improve digestibility, and enhance the host immune system (Balcázar et al. 2006; Gómez and Balcázar 2008), those studies did not always address the mode of action of probiotic bacteria. Though probiotics are currently used to treat dysbiosis, their ability to restore microbial diversity and alter the perturbed intestinal microbiota through specific mechanisms of action has not been completely elucidated. In this review, to promote an understanding of the role of probiotics in aquaculture, the following mechanisms are reviewed based on recent scientific articles: (I) competitive exclusion of pathogens, (II) enzymatic activity and production of volatile fatty acids, (III) modulation of immune responses, and (IV) interaction with the brain–gut axis.

2 Competitive Exclusion of Pathogens

The exact mechanisms underlying competitive exclusion of pathogens by probiotics remain unknown. The main proposed mechanisms are reduction in luminal pH, competition for nutritional resources, competition for adhesion sites and production of antimicrobial substances (e.g., bacteriocins) (Plaza-Diaz et al. 2019). Competitive exclusion occurs when co-occurring bacterial species in the same ecological niche compete for limited resources, i.e., nutrients and space, through two competitive strategies: exploitation and interference competition (Knipe et al. 2020). Also, the ability of probiotics to adhere to host cells and co-aggregate with pathogens is crucial to exclusion of potential pathogens in the gut (Monteagudo-Mera et al. 2019). Figure 1 summarizes the potential mechanisms of competitive exclusion of pathogens in fish.

Exploitation competition. Exploitation competition is an indirect mechanism characterized by restriction of the nutrient supply to other microbes. The existence of any microbial population depends on its ability to compete for nutrients and available

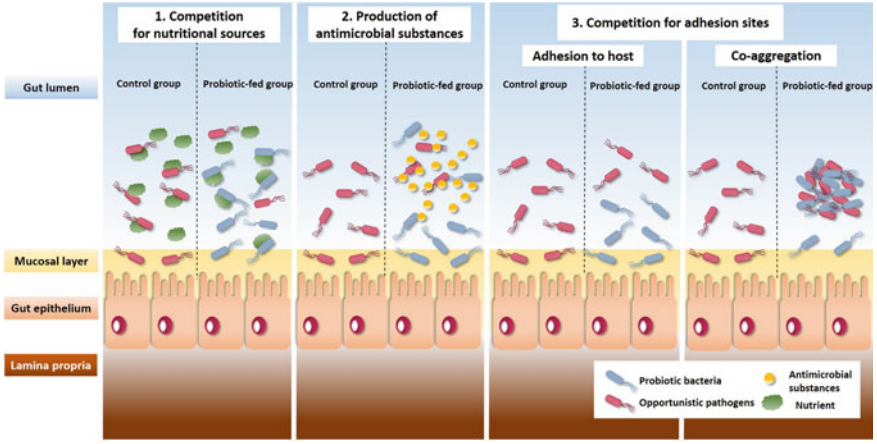

Fig. 1 Some of the mechanisms through which probiotics positively influence fish health. There are three types of competitive exclusion: (1) Exploitation competition: Probiotics rival pathogens for nutritional sources or available energy in the same environment; (2) interference competition: Probiotics directly affect pathogens by producing bacteriocins to stimulating the colonization of preventing the proliferation of selected pathogens; (3) competition for adhesion sites: Probiotics and pathogens compete in space and adhesion sites (left), and probiotics attach to pathogens via specific molecules, then inhibit the infections of pathogens (right)

energy with other microorganisms in the same environment (Verschuere et al. 2000). Numerous studies in mammals have shown that competition for nutrients in the gut appears to occur primarily between metabolically related bacteria. For instance, elements of the microbiota, including commensal *Escherichia coli* strains, limit the supply of nutrients, carbohydrates, and amino acids that would otherwise be available for the growth of pathogens such as *Clostridium difficile*, enterohaemorrhagic *E. coli* and *Salmonella enterica* serovar Typhimurium, and *Citrobacter rodentium* (Wilson and Perini 1988; Momose et al. 2008; Maltby et al. 2013; Kamada et al. 2012; Pickard et al. 2014). Also, iron is an essential micronutrient for most intestinal pathogenic and commensal bacteria (Vazquez-Gutierrez et al. 2015). In most pathogenic bacteria, iron sequestration is associated with cellular replication and persistence and is thus involved in pathogenesis (Nairz et al. 2010). The molecules involved in iron sequestration include proteases and iron-chelating siderophores that access insoluble iron. Hence, siderophore-producing probiotic strains can compete for iron in the intestinal tract, which is an iron-poor environment, thus making that iron unavailable for the proliferation and colonization of pathogens (Gram et al. 1999; Smith and Davey 1993; Tinh et al. 2008). In a genomic analysis of *Lactococcus lactis* WFLU12, Nguyen and colleagues (2018b) demonstrated that this probiotic possesses genes involved in salvage NAD synthesis, a process which synthesizes the essential cofactor NAD from nicotinic acid in the environment; thus, supplementation with this probiotic may limit the availability of nicotinic acid for pathogenic species, including streptococci and staphylococci, that only operate the salvage NAD pathway (Nguyen et al. 2018a,

2018b; Sorci et al. 2013). However, the pathogen can become resistant to the competitive mechanism, at which point it may evoke a new (or opportunistic) pathogenic invasion. Thus, tailoring of treatments so that they contain species, or strains of probiotics, that can competitively exclude specific pathogens using multiple mechanisms will increase the likelihood of successful pathogen exclusion. For example, Nguyen and colleagues (2017) showed that colonization and invasion of *Streptococcus parauberis* in the gut and epithelia of olive flounder (*Paralichthys olivaceus*) can be efficiently interrupted by *Lc. lactis* WFLU12. Examinations of natural infection in pilot- or large-scale experiments showed that some mechanisms of action of probiotic strain WFLU12 (e.g., antimicrobial secretion, stimulation of immune parameters) are exploited when this strain acts to inhibit streptococcosis (and/or other fish disease) and participates in growth performance in fish (Nguyen et al. 2017).

Interference competition. Interference competition is a direct method characterized by inhibiting colonization and proliferation of other microbes through precolonization and the production of antimicrobial compounds (Knipe et al. 2020). In vitro antagonism against aquatic pathogens is an important selection criterion for candidate probiotics. Some bacteria produce antimicrobial peptides known as bacteriocins, especially in the digestive tract, and might be responsible for stimulating the colonization of potential pathogenic bacteria or preventing the proliferation of selected pathogens (Austin, 2002). Bacteriocins are small cationic molecules composed of ∼30–60 amino acids (Meade et al. 2020). These molecules act at bacterial cytoplasmic membranes and target energized membrane vesicles to disrupt the proton motive force. The in vitro and in vivo antagonistic activities of probiotic strain WFLU12 against fish pathogens (Nguyen et al. 2017) were elucidated by studying its genome, which harbors various genes encoding for antagonist products such as the complete nisin gene cluster as well as genes encoding lysozyme and colicin V (Nguyen et al. 2018a). In addition, short-chain fatty acids (SFCAs), a main product of probiotic cells, increase the intracellular concentration of protons after entering and dissociating in the more alkaline cytoplasm of bacterial cells; thus, SFCAs may inhibit pathogenic cells that have to expend energy maintaining an optimal intracellular pH (Defoirdt et al. 2007; Nguyen et al. 2018b). Table 1 shows the various antimicrobial compounds that are responsible for the antagonistic activity of probiotics against bacterial fish pathogens.

Competition for adhesion sites. Disease can be precluded by restricting colonization of etiological agents in the gastrointestinal tract (GIT) and preventing such agents from reaching their target organs. Adhesion ability to the host is a classic selection criterion for potential probiotic bacteria, as adhesion can result in transient colonization that protects the host from pathogens through the two competitive strategies discussed above as well as by competing for host cell-binding sites (Monteagudo-Mera et al. 2019). Lara-Flores and colleagues (2009) tested the adhesion potency of several microorganisms in vitro and in vivo. Those authors determined that a successful probiotic can antagonize pathogenic bacteria through its ability to bind to the mucosa, and that this characteristic is strongly associated with competition for necessary nutrients and space. Also, Zhou and colleagues (2010) evaluated the

Table 1 Probiotic strains showing antagonistic activity against bacterial fish pathogens

Bacterial strains	Inhibitory compounds	Antagonistic activity against	Reference
Lactococcus lactis MM1 and MM4	Hydrogen peroxide and bacteriocin-like substances	*Vibrio metschnikovi*, *V. harveyi,* and *Staphylococcus aureus*	Yang et al. 2010
Lactococcus lactis WFLU12	Genes encoding for nisin and colicin V*	*S. Iniae*, *S. parauberis*, *A. samonicida, V. anguilarium*, and *V. ichthyoenteri*	Nguyen et al. 2017
Bacillus amyloliquefaciens M1	Lipopetide N3	*Vibrio harveyi, V. anguillarum, V. parahaemolyticus, V. vulnificus, V. salmonicida, Shewanella aquimarina, V. fischeri, V. splendidus, V. septicus, and V. ichthyoenteri*	Xu et al. 2014
Bacillus pumilus H2	Amicoumacin A	29 *Vibrio* strains	Gao et al. (2017a)
Bacillus velezensis V4	Anti-*A. salmonicida* compounds belonging to the iturin, macrolactin, and difficidin groups	*Aeromonas salmonicida*	Gao et al. (2017b)
Clostridium butyricum	Organic acids	*Salmonella enteritidis* and *V. parahaemolyticus*	Gao et al. 2013

* The strain WFLU2 and its products were demonstrated to inhibit fish pathogens when the strain was treated by heat at 65 °C for 30 min or 100 °C for 30 min

GIT adhesion property of ten *Lactobacillus* strains with similar antibacterial activities in vitro and found that the highly adhesive strain *Lactobacillus plantarum* JCM 1149 ^{T} conferred stronger resistance against *Aeromonas hydrophila* infection in zebrafish compared with the less-adhesive strain *Lactobacillus acidophilus* JCM 1132 ^{T}. These results highlight GIT adhesion (adhesion to mucosa) as a favorable criterion in the selection of dietary probiotics. The adhesion ability of probiotics includes not only attachment of bacterial cells to the host intestinal tract but also attachment to other bacterial cells of different species (co-aggregation) or the same species (auto-aggregation). This ability can be used for preliminary screening in order to identify potential probiotic bacteria (Collado et al. 2008; Monteagudo-Mera et al. 2019). Co-aggregation of probiotics with pathogens can inhibit the biofilm formation frequently seen in infectious states (Matsubara et al. 2016). Campana and colleagues (2017) demonstrated that lactic acid bacteria (LAB) strains may protect the intestinal epithelium from human intestinal pathogens through co-aggregation with pathogens and adherence and interference mechanisms.

3 Enzymatic Activity and Production of Volatile Fatty Acids

Enzymatic activities in GIT. The other main mechanisms of action of probiotics are stimulation of host digestion and growth performance. Host digestion can be modulated by secretion of probiotic enzymes into the gut lumen, which promotes the overall hydrolytic capacity in the small intestine and fermentation in the colon (Francavilla et al. 2017; Martínez Cruz et al. 2012). Increased enzymatic activity in the GIT increases food consumption, digestive potency, and overall host performance (Cerezuela et al. 2011). Many studies have shown that probiotic bacteria can enhance protease, amylase, lipase, and cellulase activity in the guts of probiotic-fed animals (e.g., Suzer et al. 2008; Zokaeifar et al. 2012; Ziaei-Nejad et al. 2006). Ringø and colleagues (2016) reported that several *Bacillus* spp. can significantly promote the host's growth performance, which may be attributable to the activity of digestive enzymes produced by the probiotic bacteria. Wang and colleagues (2020) found that host-associated mixed probiotic bacteria induced the production of digestive enzymes in the gut of tiger shrimp (*Penaeus monodon*). Higher intestinal digestive enzyme activity and higher growth rate were found in probiotic-fed silver pomfret (*Pampas argenteus*) (Gao et al. 2016), suggesting better utilization and digestion of dietary nutrients. Also, probiotics can induce host digestive protease and peptidase activity, and some can release exoenzymes involved in the digestion of proteins (Wang and Ji 2019), which are the main ingredients in manufactured fish feed. Nguyen and colleagues (2017) showed that the activity of some enzymes, specifically phosphohydrolase and glycosidase, increased significantly in the gut mucus of probiotic-fed fish (*L. lactis* strain WFLU12) and resulted in significant increases in specific growth rate and feed conversion ratio, indicating that the probiotic strain enhanced dietary energy extraction and metabolism.

Many studies have suggested that specific probiotics can improve the absorption of small peptides and amino acids by improving the absorption ability of the epithelium and enhancing transport (Wang and Ji 2019). It might be because short chain fatty acids (SCFAs) produced by probiotic bacteria can stimulate proliferation and permeability of epithelial cells (Hague et al. 1996; Singh et al. 1997). Also, SCFAs have the potential to increase the absorptive surface area of intestinal microvilli (Wang and Ji 2019). Keller and colleagues (2017) showed that the probiotic *Bacillus coagulans* GBI-30 can increase the digestion and uptake of three alimentary plant proteins in the gut. Stecker and colleagues (2020) demonstrated that this same probiotic strain may allow for higher amounts of amino acids to be absorbed into the blood, which is important for aquaculture as it allows farmers to reduce the dose of proteins given to the fish. Ingestion of multiple species of probiotics, including *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus reuteri,* and *Pediococcus acidilactici,* for 8 weeks induced a significant increase in microvilli density and length in the gut of fish, suggesting that the absorptive surface area was significantly enlarged by the probiotic treatment (Standen et al. 2015; Wang and Ji 2019).

Fish growth requires some species-specific metabolic pathways which are found to be limited or even completely absent in fish due to a lack of host enzymes. For example, fish are limited in their ability to synthesize arginine de novo (Li et al. 2009) and lack the ability to synthesize nucleotides in intestinal cells (Quan 1992); also, the olive flounder has a weak capacity for taurine biosynthesis (Wang et al. 2016). Using CE-TOFMS analysis of fluid samples harvested from fish fed with the probiotic WFLU12, Nguyen and colleagues (2018b) showed that 53 metabolites from the intestinal luminal metabolome and five metabolites from the serum metabolome were present in significantly higher concentrations in the probiotic-fed group than the control group. The concentrations of metabolites such as citrulline, tricarboxylic acid cycle (TCA) intermediates, SFCAs, vitamins, and taurine were significantly higher in the probiotic-fed group than the control group (Nguyen et al. 2018b). These results are depicted in Fig. 2. The probiotic strain WFLU12 possesses genes encoding enzymes that help produce these metabolites. KEGG analysis of the *Lc. lactis* genome showed that it possesses genes encoding enzymes linked to the biosynthesis of serine/sulfate and conversion of storage compound of SCFAs to diacetate, which may be reconverted to acetyl-CoA to produce energy (Nguyen et al. 2018b). Various genes involved in the bioconversion of vitamins have been reported in several studies of *Lc. lactis* strains (e.g., Burgess et al. 2004; Nguyen et al. 2018b; Shimizu-Kadota et al. 2013). Thus, probiotics may produce enzymes involved in

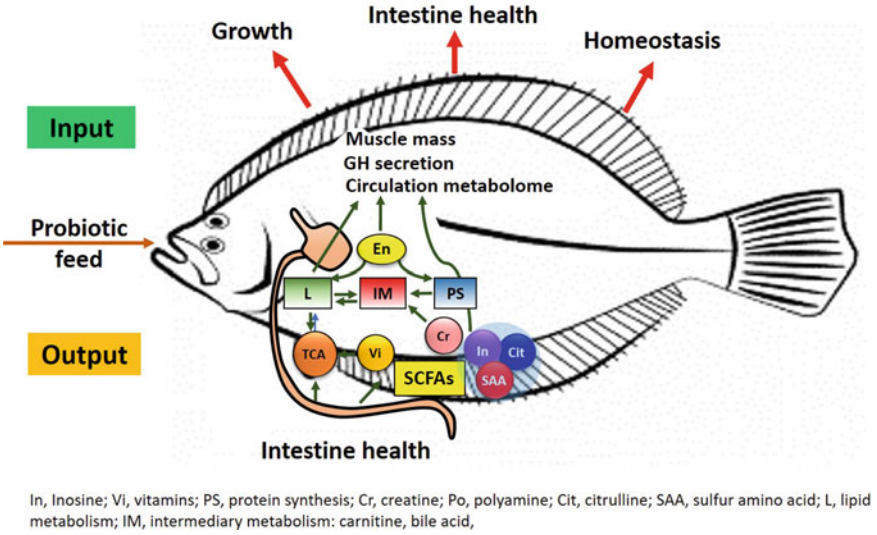

Fig. 2 Proposed systems model of nutrient pathways and growth in probiotic-fed fish. The input variables include probiotic-mixed pellet, the system variables include amino acids, proteins, and tricarboxylic acid cycle intermediates, and the outputs include growth, homeostasis and intestinal health. In, inosine; Vi, vitamins; PS, protein synthesis; Cr, creatine; Po, polyamine; Cit, citrulline; SAA, sulfur amino acid; L, lipid metabolism; IM, intermediary metabolism (i.e., carnitine, bile acid). (Extracted from data published in our previous study, Nguyen et al. 2018b)

the synthesis of important organic compounds (e.g., vitamins) that are essential for optimal growth and health of animals including fish. A weight gain-associated mechanism of probiotics has attracted attention in recent years. Drissi and colleagues (2014) showed that *Lactobacillus* encodes ubiquitous enzymes contributing to host weight gain, such as thiolase I (EC 2.3.1.16) in the β-oxidation pathway of fatty acid degradation and thiolase II (EC 2.3.1.9) in various biosynthetic pathways such as poly β-hydroxybutyric acid synthesis and steroid biogenesis. Likewise, the fish gut probiotic strain WFLU12 harbors both thiolase I and II genes (Nguyen et al. 2018a) and causes an increase in the poly-β-hydroxybutyric acid and β-oxidation pathway (e.g., level of carnitine) (Nguyen et al. 2018b). This evidence supports the notion that probiotics can increase the levels of various metabolites related to fish growth. Taken together, the evidence suggests that probiotics can generate and also induce digestive enzymes in the host gut, which in turn regulate the synthesis of specific essential nutrients such as amino acids, fatty acids, and vitamins, leading to enhanced digestion and absorption of nutrients and increased growth.

Short-chain fatty acids (SCFAs). Numerous studies have demonstrated that volatile fatty acids (also called short-chain fatty acids, SCFAs) are essential for the health and well-being of the host when present in sufficient quantities (LeBlanc et al. 2017; Plaza-Diaz et al. 2019) and can improve growth performance and health status in several fish and shellfish species (Hoseinifar et al. 2017). SCFAs include carboxylic acids (with aliphatic tails of 1–6 carbons), as well as acetate (C2), propionate (C3), and butyrate (C4), which are the most abundant molecules generated by anaerobic fermentation of carbohydrates in the intestine (Tremaroli and Bäckhed 2012). SCFAs provide the major energy sources of intestinal cells (den Besten et al. 2013). As such, SFCAs play an essential role in the physiology and metabolism of the digestive tract. They also affect peripheral tissues via interactions with SCFA receptors. SCFAs are the most important metabolites generated by intestinal bacteria including probiotics (Fig. 2).

SCFAs are also involved in gut–brain communication and brain function, both directly and indirectly (Silva et al. 2020; Plaza-Diaz et al. 2019). In fact, it has been demonstrated that 70% of the energy obtained by intestinal epithelial cells is derived from butyrate, which is mainly produced by gut bacteria (Serpa et al. 2010). Also, SCFAs play a very important role in maintaining intestinal and immune homeostasis in the host (LeBlanc et al. 2017; Markowiak-Kopeć and Śliżewska 2010). A number of studies have shown that probiotic LAB can produce SCFAs through fermentation of carbohydrate end products (Table 2); for instance, acetate and lactate are generated by bifidobacteria, and propionate and butyrate are generated by *Lactobacillus salivarius* and *Lactobacillus agilis* (Macfarlane and Macfarlane 2003; Meimandipour et al. 2010). Among SCFAs, butyric acid has received particular attention for its beneficial effects on the health of the intestinal tract and peripheral tissues in humans and animals, including fish (Liu et al. 2014; Robles et al. 2013; Rimoldi et al. 2018). A previous study showed that host-derived probiotics, particularly *Alcaligenes* sp., enhanced nutrient utilization and metabolism by increasing the gut surface area and volatile SCFA production as well as adjusting the gut–microbiota balance in the Malaysian mahseer (*Tor tambroides*) (Asaduzzaman et al. 2018). Previous study

Table 2 Volatile short-chain fatty acids produced by probiotic strains used for aquatic animals

Microorganism/s	Origin	Compounds	Fish used for trials	References
Alcaligenes sp. AFG22	The gastrointestinal tract of adult Malaysian mahseer *Tor tambroides*	Acetate and butyrate	Malaysian mahseer (*Tor tambroides*)	Asaduzzaman et al. (2018)
Lactococcus lactis strain WFLU12	Wild olive flounder gut (Kim and Kim, 2013)	Derive of acetate: 3-hydroxybutyric acid and acetoacetic acid	Olive flounder, (*Paralichthys olivaceus*)	Nguyen et al. (2018)
Enterococcus faecalis	Unknown	Propionic and butyric acid	Javanese carp (*Puntius gonionotus* Bleeker 1850)	Allameh et al. (2017)
Clostridium butyricum	Unknown	Propionic acid and butyric acid	Kuruma shrimp (*Marsupenaeus japonicas*)	Duan et al. (2018)
Hindgut microbiome of grass carp	Unknown	Acetate, butyrate and propionate	Grass carp (*Ctenopharyngodon idellus*)	Hao et al. (2017)

(Nguyen et al. 2018b) on the effect of host-derived probiotic strain WFLU12 in olive floundersss showed that the levels of storage compounds for acetate production (including 3-hydroxybutyric acid and acetoacetic acid) are increased in the gut and serum of probiotic-fed fish which may aid in nutrition and ATP production in cells lining the intestine.

4 Modulation of the Immune Response

The gut of fish is a multifunctional organ with diverse physiological and defensive functions (Grosell et al. 2010). The GIT microbiota plays an essential role in the growth and maturation of gut-associated lymphoid tissue (GALT), which in turn mediates several host immune functions (Kuebutornye et al. 2019). Immune-related structures in the GIT consist of the intestinal epithelial barrier, which is populated with intraepithelial lymphocytes, the lamina propria, which is populated with lymphocytes and innate cells, and GALT (Peterson and Artis 2014). Modulation of immune responses is one of the mechanisms underlying the beneficial effects of probiotics on host health. Most early studies in fish dealt with the development-promoting and anti-disease potency of probiotic bacteria. In recent studies, however, much attention has been paid to the immune-modulating effects of probiotics in the piscine gut immune system (e.g., Picchietti et al. 2009; Salinas et al. 2008). As an important constituent of the mucosal immune system, the GALT of teleost fish

constructs a local immune system to deal with various microbes including pathogens entering through the GIT lumen.

The fish GIT is continuously challenged with various types of microorganisms, including bacteria, viruses, parasites, and fungi. The mucosal layer has several functions, including creation of a physical and chemical barrier, osmoregulation, lubrication, and nutrient uptake and digestion (Bakke et al. 2010; Ellis 2001). Teleost mucosal secretions comprise water (~95%), glycoproteins (mainly mucins, see below) (~5%), and antimicrobial substances including immunoglobulins (Gomez et al. 2013; Salinas et al. 2008). Goblet cells in the GIT secrete high-molecular weight glycoproteins called mucins. These mucins have a high negative surface charge and a large hydration capacity, act as the main structural component of the mucus layer, and give rise to its polymeric, viscoelastic, and protective properties (Dharmani et al. 2009). Mucins are subdivided into secretory and membrane-bound forms depending on their structure and location (Dharmani et al. 2009; Pérez-Sánchez et al. 2013). Secreted mucins are large and highly O-glycoslated glycoproteins that assemble into oligomers to grant mucus its viscosity (Thornton et al. 2008). Membrane-bound mucins are located on the surface of epithelial cells, providing an additional layer of defense (Lang et al. 2007). The secretion of mucins by goblet cells in the lumen is unregulated, continuously occurs at a low level to cover the epithelium under normal physiological conditions, and is accelerated in response to external stimuli (Dharmani et al. 2009).

The main defined function of mucins is to protect epithelial cells from pathogens by limiting pathogen invasion through steric hindrance as well as to provide a physical–chemical barrier that prevents pathogen adherence, colonization, toxin release, and invasion (reviewed by Hasan and Banerjee 2020; Paone and Cani 2020). The presence of a healthy microbiota results in higher baseline expression of a mucin gene, *Muc*2, encoding a secreted mucin (MUC2), but not the membrane-bound mucins (MUC1, 3 and 4) (Bergström et al. 2012). In addition, mice mono-colonized with probiotic strains *Lb. acidophilus* NCFM or *E. coli* Nissle 1917 showed upregulation of *Muc*2 (Bergström et al. 2012; Schroeder 2019). These indicate that particular microbial members can stimulate *Muc*2 expression or that a potential metabolic interaction between microbial species is required to produce the *Muc*2-inducing signal (Schroeder 2019). Desai and colleagues (2016) showed that, during chronic or intermittent dietary fiber deficiency, the gut–microbiota resort to consumption of host-secreted mucus glycoproteins as nutrient sources, leading to erosion of the colonic mucus barrier and promoting greater epithelial access and lethal colitis by the mucosal pathogen *Citrobacter rodentium.* Notably, a previous study demonstrated that mice genetically lacking the dominant colonic mucin glycoprotein (MUC2), but not wild-type mice, develop lethal colitis following infection with *C. rodentium* (Bergstrom et al. 2010), highlighting the notion that the mucus layer acts an important initial barrier to pathogens. In fish, mucin gene expression in naïve pathogen-free gilthead sea bream was confirmed in a previous study (Pérez-Sánchez et al. 2013), which showed *Muc19* expression mostly in the esophagus and *Muc13* expressing along the entire intestinal tract. Expression of these mucin genes is transcriptionally regulated by dietary and pathogenic factors. Comparatively, however, little is known

about mucin production in aquatic animals fed with probiotics. Changes in mucin gene expression may reflect the intensity and progression of an infection, and mucins are believed to be reliable markers of fish intestinal health with prognostic and diagnostic value (Pérez-Sánchez et al. 2013). Fish fed a diet supplemented with butyrate reportedly benefit in terms of intestinal function and integrity (e.g., liver-expressed mucin 2 in a study of grass carp by Tian and colleagues (2017); high abundance of mucins in fish fed with butyrate compared to control fish in a study by Piazzon and colleagues (2017)). Further investigations are required to explore whether host production of mucin can be induced by probiotic supplementation.

Toll-like receptors (TLRs) are transmembrane proteins expressed on numerous non-immune and immune cells, including epithelial cells. TLRs are well-studied pattern recognition receptors that can recognize microbial components (e.g., LPS, peptidoglycans, nucleic acids, or flagella) (Hoseinifar et al. 2018). Probiotics may be involved in suppression of gut inflammation (Azimirad et al. 2016; Modanloo et al. 2017; Plaza-Diaz et al. 2019) via down-regulation of TLR expression, and can produce metabolites that may stimulate cytokines via stimulation of NF-κB signaling in enterocytes (Galindo-Villegas et al. 2012; Kanther et al. 2011; Xia et al. 2019). The known interactions of probiotics with TLRs are summarized in Fig. 3. In this regard, the cell wall components of the viable probiotic *Psychrobacter* sp. SE6 may be able to signal through attachment to TLR2 and TLR5 in combination with adaptor MyD88, thus activating intestinal mucosal immunity and enhancing expression of antibacterial epinecidin-1 and IgM in orange-spotted grouper (*Epinephelus coioides*) (Sun et al. 2014). Also, addition of a high concentration of the *Lc. lactis* subsp. *lactis* JCM5805 to the water continuously for 15 days was shown to induce upregulation of IFNα via the TLR7/TLR9-Myd88 pathway, thus enhancing disease resistance larvae of Nile tilapia (*Oreochromis niloticus*) (Xia et al. 2019). Inhibition of TLR2 boosts cytokine secretion, and TLR activation has an essential role in increasing transepithelial defense against invading microbes (Plaza-Diaz et al. 2019). In a zebrafish model, a commercial diet mixed with multiple probiotic strains can induce upregulation of Myd88, TLR1, TLR2, TLR3, and TLR9 (as well as protein levels) in the adult fish gut after 30 days of feeding (Gioacchini et al. 2017). Single-strain feeding of *Bacillus amyloliquefaciens* also increased expression of TLR1, TLR3, and TLR4 in the gut of adult fish after 30 days of feeding (Lin et al. 2019). Treatment with *Lactobacillus casei* BL23 and exopolysaccharide-protein complex increased TLR1 and TLR2 expression (Qin et al. 2017). A recent study (Guo et al. 2020) also showed that giving turbot (*Scophthalmus maximus*) feed supplemented with *Leuconostoc mesenteroides* HY2 strain (1.0×10^5 CFU/ml, 4 weeks) led to upregulation of TLR3 expression levels in various organs including the intestine. Numerous studies have shown that probiotics modulate gene regulation in a strain-specific fashion. Probiotic-treated fish show increased expression of innate immune-related genes (IL-1β, IL-6, IL21, TNF-α, and TLR1,3, and 4) and have higher survival rates compared to control fish following challenge with *A. hydrophila* and *Streptococcus agalactiae* (Lin et al. 2019). As a consequence of increased TLR1, TLR2, TLR3, and TLR9 expression in the gut of zebrafish, treatment with a probiotic mixture containing eight different strains increased IL-1β, TNFα, Myd88, IL-10, Casp1, NOS2A, TGFβ1A, and NF-κB

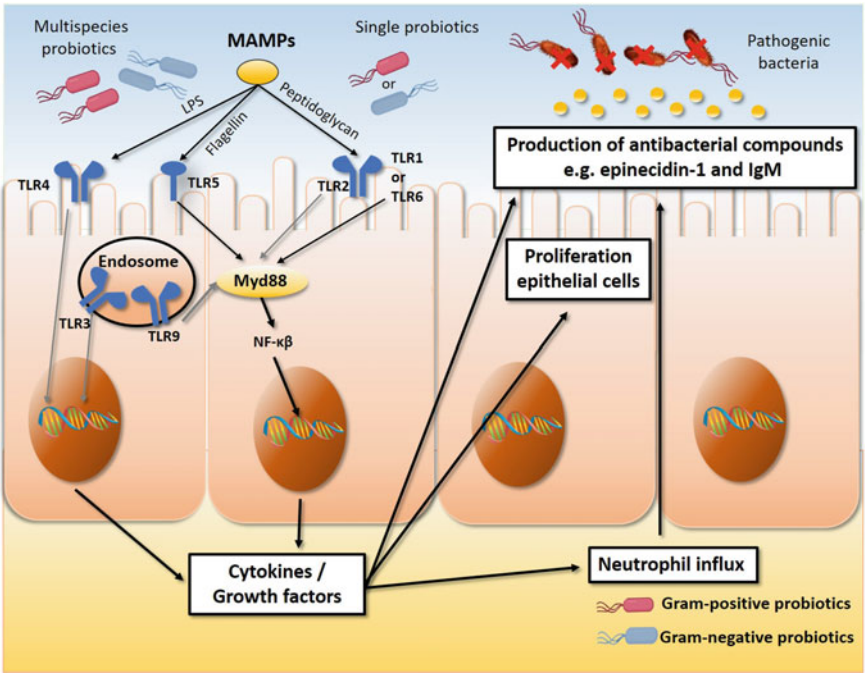

Fig. 3 Immunomodulatory molecular pathways related to the microbe–host interaction in the epithelium of aquatic species. Probiotics involve in suppressing gut inflammation via the down-regulation of TLRs expression. TLRs recognize microbial components (LPS, peptidoglycan, etc.) and activate immune cell responses which activate the Myd88 pathway and stimulate cytokines secretion via stimulation of NF-κβ pathway, thus enhance the expression of antibacterial compounds (e.g., epinecidin-1, IgM)

levels (Gioacchini et al. 2017). Also, *Lb. casei* BL23 upregulated IL-1β, C3a, and IL-10 expression in the zebrafish gut, while *Lactobacillus fermentum* NA4 led to an increase in IL-10 expression, but a decrease in IL-1β and TNF-α expression after chemically induced inflammation (Aoudia et al. 2016; Qin et al. 2017). Furthermore, significant induction of several cytokines (IL-1β, IL-8, and TNF-α) in the head–kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*) following treatment with *Carnobacterium maltaromaticum* and *Carnobacterium divergens* highlight the immune-stimulatory effect of probiotics and the potential participation of these molecules in anti-inflammatory responses (Kim and Austin 2006). On the other hand, Picchietti and colleagues (2009) observed down-regulation of cyclooxygenase 2 (COX2) transcripts along with IL-10 and TGF-β genes following treatment with *Lactobacillus delbrueckii* provided as a live carrier in European seabass (*Dicentrarchus labrax*). As above, a large number of studies show that administration of probiotics in fish induces changes in immune-related gene expression. However, such gene expression may reflect a balance in the host during an immune response: specific and strong enough to defend against potential pathogens, but not so strong as

to destroy commensal and probiotic bacteria (Kelly and Salinas 2017; López Nadal et al. 2020).

A number of studies demonstrated that different probiotic strains show adjuvant efficacy for different types of vaccines (e.g., Taylor et al. 2006; Boge et al. 2009; Rizzardini et al. 2012). Although the adjuvant effect is not yet fully understood, it likely involves complex mechanisms by which the microbiome impacts immune cell development and differentiation. Also, intestinal microbiome dysbiosis in chronic intestinal inflammatory states compromises the effectiveness of vaccination. Probiotics may be able to improve vaccine efficacy by promoting homeostasis of the intestinal microbiome (Vitetta et al. 2017). As an exciting new approach in aquatic animals, probiotics have been defined as novel mucosal adjuvants to enhance vaccine immune responses (reviewed in Soltani et al. 2019). Studies on the role of probiotics as adjuvants in fish are limited, but the results of experiments using probiotics in combination with antigen vaccines have led to approval on the basis of current adjuvant requirements. Also, application of probiotic adjuvants in aquatic prophylaxis has enormous potential given that the most difficult aspect of vaccination is always immunization of younger/smaller fish (Kaattari and Piganelli 1997).

Two strategies for probiotic adjuvant use have been attempted in fish, including co-administration during vaccination periods and co-expression in a probiotic strain. Tilapia with immunity induced by an *A. hydrophila* vaccine that were then fed to *Lactobacillus sporogenes* at various concentrations showed increased activation of neutrophils and lymphocytes in comparison with the vaccine-only group (Venkatalakshmi and Ebanasar 2015). Furthermore, Aly and colleagues (2016) fed *A. hydrophila*-vaccinated tilapia a mixture of *B. subtilis, Saccharomyces cerevisiae, Lb. acidophilus,* and *Aspergillus oryzae* for five months and observed a dramatic boost in antibody titer and survival of the challenge infection (Aly et al. 2016). Demonstrating the advantages of live vaccines, which mimic the properties of pathogen/antigen adjuvants, an oral vaccine formed by *Lb. plantarum* co-expressing a glycoprotein from spring viremia of carp virus and ORF81 protein from koi herpes virus might induce protection in both common carp (*Cyprinus carpio*) and koi carp (*Cyprinus rubrofuscus*), as exhibited by higher anti-virus IgM levels and increased survival rates (71% in vaccinated carp and 53% in vaccinated koi carp) compared to controls (15–22%) over a 65-day vaccination period (Cui et al. 2015). Likewise, a recombinant *Lc. lactis* vaccine with the SiMA antigen of *Streptococcus iniae* produced an increase in antibody titer, CD4 cells, CD8 cells, and survival rates in the olive flounder (Kim et al. 2016).

5 The Brain–Gut–Microbiota Axis

There is a complex communication system between the GIT, gut microbiota, and brain called the microbiota–gut–brain axis (Powell et al. 2017). A review by Powell et al. (2017) has shown that communication between the brain and gut is not a one way, but a bidirectional highway through which reciprocal signals, including neural,

metabolic, endocrine and immune mediator signals, are exchanged between the two organs to coordinate function. Reconstitution of germ-free (GF) animals with probiotic bacteria, such as *Bifidobacterium infantis*, suppressed the stress response, whereas monoassociation with enteropathogenic *E. coli* (EPEC) amplified the stress response, demonstrating that qualitative compositional changes with individual species have profoundly different effects (Sudo et al. 2004). Also, gut–microbiota are associated with motor activity, memory, and social functioning, as GF animals exhibit deficits in those functions (Diaz Heijtz et al. 2011; Gareau et al. 2011; Desbonnet et al. 2014). Indeed, disruption of gut microbiota (dysbiosis) has been linked to various clinical disorders such as obesity, irritable bowel syndrome, schizophrenia and depressive disorders (Sherwin et al. 2016; Thursby and Juge 2017; van de Guchte et al. 2018). GF rodents transplanted with fecal microbiota of patients having depressive disorders developed depressive-like behaviors, while those transplanted with normal microbiota did not (Kelly et al. 2016; Zheng et al. 2016). In addition, fish with disrupted microbiota following antibiotic administration showed abnormal locomotive activities, which might affect their feeding behavior and foraging (Phelps et al. 2017; Butt and Volkoff 2019).

The gut microbiota and appetite system are highly associated with each other. Energy metabolism and microbial metabolites may serve as potential mechanisms (Han et al. 2021). GF mice lacking a gut–microbiota are leaner than normal mice even when they consume more calories (Duca et al. 2012). These mice have lower levels of hunger hormones such as leptin and ghrelin, indicating that gut–microbiota is involved in the regulation of appetite and metabolism (Duca et al. 2012; Fetissov 2017). Administration of probiotic *Lactobacillus rhamnosus* IMC 501 resulted in increased gene expression level of neuropeptide Y, Agouti-related protein (AgRP) and ghrelin but decreased leptin expression, along with improved growth performance and feed intake in Nile tilapia larvae (Giorgia et al. 2018). Flounder fed a diet supplemented with *Bacillus clausii* displaying increased weight gain, feed effectiveness, and developmental performance compared to fish fed a control diet (Ye et al. 2011). Also, fish that displayed vigorous appetites when fed to *Lc. lactis* WFLU12 grew faster than fish in the control group (Nguyen et al. 2017). However, treatment with *Lb. rhamnosus* IMC 501 reduced appetite and glucose levels in zebrafish (Falcinelli et al. 2016). These findings suggest that probiotic strains can modulate gut–microbiota and appetite in a strain-specific and host-specific manner.

The most obvious scenario underlying how the gut microbiota can interact with the nervous system would be through modulation of host neuroactive molecules, including neurotransmitters, neurotrophic elements, and neuromodulators, thereby affecting gastrointestinal (GI) motility, function, and hormone release as well as feeding behavior (Strandwitz 2018; Butt and Volkoff 2019). For instance, concentrations of tryptophan (precursor of serotonin), tyrosine (precursor of dopamine and noradrenaline), and glutamine (involved in metabolism of γ-aminobutyric acid and glutamate) in brain were significantly lower in GF mice than in normal mice, providing an evidence of gut microbiota in metabolism of neuroactive molecules (Matsumoto et al. 2013; Kawase et al. 2017). In previous studies, treatment with

probiotics promoted intestinal peristalsis and alleviated stress-induced depression-like behavior via modulation of serotonin (Huawei et al. 2019; Lu et al. 2019). Also, administration of probiotic *Lb. rhamnosus* IMC 501 in zebrafish induced increased gene expression of brain-derived neurotrophic factor (BDNF) and genes involved in serotonin metabolism and more uniform schooling behavior (Borrelli et al. 2016). Serotonin biosynthesis occurs majorly in enterochromaffin cell in the lumen of the digestive tract. It is stimulated by SCFAs derived from intestinal microbial fermentation (Dalile et al. 2019). Administration of probiotic bacteria could elevate the concentration of SCFAs in GIT tracts of various animal models including *Lc. lactis* WFLU12 in flounder (Nguyen et al. 2018b; Cheng et al. 2021). Our transcriptome study (unpublished data) found significant upregulation of serotonin (5-hydroxytryptamine) receptor gene (=>170-fold change) in the liver of olive flounder given feed supplemented with *Lc. lactis* WFLU12 compared to that in the control group, suggesting that the probiotic strain could mediate hormone release.

6 Conclusions and Suggestions for Future Work

Interest in the use of probiotics as an aid to manipulate fish health and welfare is growing. The mechanisms of action of probiotics (Fig. 4) include colonization and normalization of perturbed intestinal microbial communities (amelioration of dysbiosis); competitive exclusion of pathogens and bacteriocin production; modulation of enzymatic activities related to nutrient metabolism; and production of SCFAs, which play a role in the maintenance of energy homeostasis and regulation of functionality in peripheral tissues. However, the availability of molecular data on these mechanisms remains limited in aquatic animals. Modern approaches (e.g., transriptome and metabolome analysis) can hold great potential in revealing co-regulatory networks that govern <>probiotics–microbiome and probiotics–host interactions. For example, metabolomics analysis conducted by Nguyen and collegeaues (2018b) have aided in gaining a deeper understanding of the mechanisms of action of probiotics in fish and the ways in which probiotic cells can contribute to host health. Specific metabolites obtained from this analysis may be used for the development of new ingredients for fish diets. Also, metabolites play an important role in the gut–microbiome–brain interactions in fish. Aforementioned mechanisms of probiotics can be included in future studies to demonstrate the effectiveness of probiotics for the use in fish.

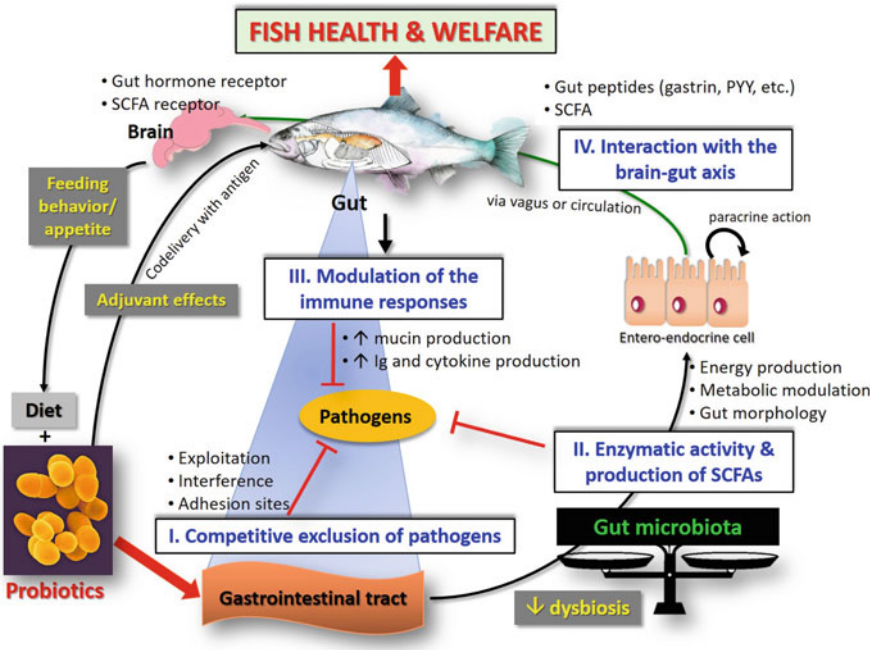

Fig. 4 Overview: Probiotics increase fish health and growth through four main mechanisms. (I) Probiotics show three strategies to compete with pathogens: exploitation competition, interference competition, and adhesion sites. Also, fish are protected from pathogens through immune system modulated ability of probiotics and the production of bacteriocin and SCFAs. (II) Enzymatic activity and production of SCFAs can be enhanced by probiotics, thus influence energy production, metabolic modulation, and gut morphology. Therefore, the host digestion, growth performance, and health status are stimulated. (III) Probiotics involve in modulating immune responses by increasing production of mucin, cytokine, and Ig. (IV) Probiotics release metabolites which can stimulate the enteroendocrine cells secrete gut peptides, thus impacting brain feeding centers and then modifying feeding behavior and appetite

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Factors Influencing the Efficacy of Probiotics

Elijah Nya

Abstract Oral application of live bacteria to aquaculture species offers interesting potential benefits and numerous publications have documented the facts. Many probiotic bacteria do not survive the extreme pressures of feed handling and/or the milling process and the heat of the gastrointestinal tract (GIT) of the host animal. The general public considers that *Lactobacillus* spp. are the most common group of probiotics. Studies conclude that when probiotics are fed directly to fish or shrimp, measurable and reproducible benefits are produced particularly those connected with improved health and resistance to infectious diseases. However, the concept of a probiotic is one of a feed supplement that acts in the digestive tract by inhibiting potentially harmful organisms, i.e. competitive exclusion. In terms of aquatic species, research does not always support the notion of the probiotics binding to and colonizing the gastrointestinal tract. Indeed, there is a wealth of information suggesting a role as non-specific immunostimulants, stimulating protective immunity by acting on the non-adaptive or innate component of the immune system. These activities and modes of action do not necessarily require the presence of viable cells. This narrative will explore the available information regarding the efficacy of probiotics, various factors influencing their stability in culture, the longevity of viable cells in/on commercial diets; and the effect of dosage, condition of the host, and physico-chemical factors associated with the rearing environment.

Keywords Probiotics · Efficacy · Shelf stability · Longevity · Probiotic profile

1 Introduction

The addition of live bacteria into the diets of aquatic species, such as fish and shrimps, has been demonstrated repeatedly to be beneficial in terms of nutrition, i.e. growth

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benefits, and improved health (e.g. Dawood et al. 2019; Landsman et al. 2019; Moonsamy et al. 2020). Some conditions or factors that guarantee the efficacy or effectiveness and workability of probiotic supplemented diets in aquaculture have recently attracted concern amongst stakeholders in the industry. These conditions could be regarded as indispensable in the application process of probiotics, and include:

- the inherent factors influencing the stability of cultures,
- longevity of the number of viable cells in the diets,
- improved digestibility and nutrient absorption of the feed (Mensah 1997; Duggan 2002; Olmos et al. 2020).

However, the addition of live bacteria into the diets has been shown to be problematic. Achieving the shelf stability of live bacterial cultures in a dried form or in dry commercial diet is challenging. With this in mind, it is necessary to explore the best possible way of dispensing stable bacterial products to aquatic hosts. The difficulties associated with growth, longevity of the numbers and viability of cells in the diets may be readily anticipated. For example, the addition of a few million colony forming units (CFU) of probiotic bacteria to dry commercial feed is likely to be elusive or vague with respect to in vivo outcome of the actual number of viable cells and their survival/stability in the product. On the other hand, adding probiotic (as live bacteria) cultures directly to the rearing water in the aquaculture facility promises deliveries of many more bacterial cells to the bottom where the organic materials accumulate (Landsman et al. 2019). However, this may be the best way of dispensing stable bacterial products to fish in pond water or cages, but this would entail the concept of targeted delivery. The concept is scientifically valid, but only regarding the administration of specific bacteria that have been chosen exclusively on the basis of their ability, proliferation capacity and delivery mechanisms. This has been demonstrated in hatchery tanks, where a significant reduction in vibrio populations was noted, possibly as an outcome of the competition of the probiotic bacterial cells for nutrients with the vibrios in the system, i.e. the hatchery environment (Soundarapandian and Babu 2010).

Many different types of bacteria (and fungi) putatively serving probiotic functions are available for sale in markets. Commonly available products include those containing *Lactobacillus*, *Bacillus*, *Nitrosomonas*, *Nitrobacter*, *Saccharomyces*, photosynthetic bacteria such as purple sulphur bacteria and *Pseudomonas* species. Each of these organisms has specific properties and for many of them it precludes them being efficacious and viable in a commercial dried diet or in meaningful numbers in a commercial liquid product. It therefore becomes very important to look at some of these factors and others that influence the efficacy of probiotic bacteria used in aquaculture (see https://www.bioremediationaquaculture.com/uploads/5/3/7/2/5372499/_aquapro_f.pdf).

2 Impact of Temperature on Probiotics

The temperature at which probiotic organisms exist in feeds and grow during storage is important in probiotic supplementation, and especially where fermentation is required. Temperatures obtained during fermentation are important because of the effect on the efficacy of the probiotics and their viability in feeds (see Brzozowski 2019). The optimum temperature for growth of many probiotics is between 37 °C and 43 °C (Boylston et al. 2004; Lee and Salminen 2009), although culturing of some organisms may be achieved at > 45 °C. For example, the optimum growth for *Bifidobacterium* is 37–41 °C (Korbekandi et al. 2011; see Brzozowski 2019). *Bifidobacterium* isolates, i.e. *Bifidobacterium longum (infantis)*, *B. breve*, *B. bifidum* and *B. adolescentis*, which have been obtained from the infant intestinal tract, show an optimal growth at 36–38 °*C*; *B. animalis* and *B. lactis* may be cultured at 41–43 °C (Crittenden 2004; Lee and Salminen 2009; Brzozowski 2019).

Temperature is also an important factor affecting the survival of probiotic cells in feeds during periods of storage. Probiotic supplemented feed should inevitably be refrigerated at 4–5ºC. It has been well established that the storage temperature of probiotic supplemented feeds affects the viability of the microbial cells by the deleterious effect on the cell membrane. The metabolites formed during this period between indigenous organisms and probiotics in fermented products is critical (Brzozowski 2019). For example, Mortazavian et al. (2011a, 2011b) determined that storage of *Lactobacillus acidophilus* LA-5 and *B. lactis* BB-12 at 2 °C for 20 days resulted in a high level of viability of the former whereas the latter survived better at 8 °C (Mortazavian and Sohrabvandi 2006). Furthermore, low resistance probiotic cells stored at low temperatures, such as 2 °C or less, have been found to survive well (Korbekandi et al. 2011).

The handling temperatures during feed processing of above 45–50 °C have been shown to be detrimental to probiotic bacteria. Therefore, in feed manufacturing to prevent deterioration in the numbers of viable cells, it is imperative to add probiotics after the heating/cooling/pasteurizing stages (Lee and Salminen 2009). It is regarded by some experts that the higher the temperature, the shorter the time of exposure required in order to prevent deterioration.

Freezing temperatures may affect the efficacy of probiotic cells by influencing their viability in the finished feed products. During the freezing process, probiotic cells may well be damaged irreparably by ice crystals formed in the external environment thereby damaging the cell membranes. Furthermore, freezing causes temperature shock and injury to the cells; mechanical stress within the cells can lead to cell death. Invariably, probiotic bacteria are subjected to chemical stress during freeze-thawing of the frozen products, which may well cause the microorganisms to lose viability. Furthermore, the probiotic cells may be exposed to osmotic effects and high concentrations of other factors, such as hydrogen ions (pH), organic acids, oxygen and melting media constituents have been found to exhibit a crucial effect on viability loss of the probionts (Jay et al. 2005). All these mechanisms cause diminution in the metabolic processes of the probiotic cells vital for being efficacious in their mode of

actions (Davies and Obafemi 1985; Jay et al. 2005; Gill 2006). Conversely, Mohammadi and Mortazavian (2011) recommended the immediate freezing of feed products after seeding with the probiotic because this approach contributes to safeguarding and preservation of the populations of these beneficial microorganisms in the product.

3 Influence of Dosage

The reasons for specifying dosages of probiotic is often arbitrary with researchers using a stated number of cells in the supplemented feeds, but without giving any substantive reasons to support the number. The dose may well have been decided for historical reasons, i.e. the number of cells added to feed follows previous works. Certainly, the response of animals to different dietary probiotic dosages has been observed previously (Panigrahi et al. 2004; Bagheri et al. 2008). Research has determined that administrations of probiotics may induce immunosuppression of enhance the immune response depending on the precise dosage used (Sakai 1999). For example, a dietary feed supplemented with *L. lactis* at 10^8 CFU/g was shown to be efficacious improving the growth rate of Japanese flounder fingerlings with accompanying enhancement in lysozyme, antiprotease, serum peroxidase and blood respiratory burst activities (Heo et al. 2013). Furthermore, the application of *Bacillus subtilis* and *B. licheniformis* in diets at 10^9 CFU/g led to an improvement in specific growth rate (SGR), weight gain, feed conversion ratio (FCR) and protein efficiency ratio (PER) of rainbow trout fry (Bagheri et al. 2008). In a study conducted by Liu et al. (2013), a dietary product supplemented with *L. brevis* at 10^9 cells/g conferred protection in hybrid tilapia *Oreochromis niloticus* × *Oreochromis aureus* against challenge with *Aeromonas hydrophila.*

In other studies, a total of 10^5 CFU/ml has been regarded as appropriate probiotic dosage (Guo et al. 2009; Zhou et al. 2009). At this dose, probiotic use led to good protection and cellular and innate immunostimulation (Salinas et al. 2006). Interestingly, a higher dose did not lead to a greater level of protection after challenge (Perez-Sanchez et al. 2013). Recommended dosages need to depend on the species of probiotic used, the size and physiological status of the aquatic animal, and the rearing conditions (Merrifield et al. 2010).

4 Effect of Duration of Administering Probiotics

The time span of administering probiotics is an important factor influencing their efficacy. Studies have assessed various durations of administration from 6 days (Joborn et al. 1997), 28 days (Landsman et al. 2019), more than 5 months (Aubin et al. 2005) and up to 8 months (Aly et al. 2008a). There has been a realisation that prolonged administration of probiotics may result in negative impacts by inducing

immunosuppressive responses (Sakai 1999). Furthermore, probiotics were ineffective in colonizing cultured rotifers after feeding for only 3 days (Qi et al. 2009). Studies have revealed that short-term supplementation with probiotics of 1–3 weeks led to clearly demonstrable beneficial effects to the host in terms of growth and health improvements (Robertson et al. 2000; Kim and Austin 2006; Balcazar et al. 2007). This assertion was confirmed by Brunt and Austin (2005) and Brunt et al. (2007) in their studies showing that short-term feeding with probiotic supplemented food was effective for disease control. Similarly, Wu et al. (2015) observed that after feeding with probiotics for 28 days, the cumulative mortality of grass carp (*Ctenopharyngodon idellus*) was reduced following challenge with *Aeromonas hydrophila*. Therefore, it is clear that adequate dietary supplementation with probiotics may definitely provide health benefits in terms of protection against infectious diseases. Whereas data emanating from long-term application of probiotics do not always reveal benefit to the host. Aubin et al. (2005) compared probiotic recovery levels from rainbow trout (*Oncorhynchus mykiss*) over time and discovered that the levels were higher after 20 days than after 150 days.

The frequency of administering probiotic supplemented diets exerts a significant role in influencing efficacy. For example, a daily administration of probiotics has been regarded as more efficacious than application every other day (Guo et al. 2009). Furthermore, regimes that involve administration of probiotic supplemented diets for short periods (= a pulse) alternating with unsupplemented diets may well be beneficial to the host (Bricknell and Dalmo 2005), providing various health benefits, including immunomodulation (Balcazar et al. 2007).

5 The Advantages of Using Single or Multiple Cultures in Probiotic Preparations

Probiotics may be administered as single or multiple cultures (Havenaar et al. 1992; Gatesoupe 2002; Salinas et al. 2005; Meidong et al. 2017; Thurlow et al. 2019; Emam et al. 2020; Kanpiengjai et al. 2020; Moonsamy et al. 2020). Many research articles have focused on the use of single cultures rather than multiple combinations of probiotics. Generally, this has led to a dearth of knowledge on whether two or more combinations of probiotic cultures could be advantageous to aquaculture (Hai 2015). Certainly, some work has reported the benefit of mixed culture rather than single culture preparations of probiotics (e.g. Verschuere et al. 2000) in terms of enhanced protection against challenge with pathogenic microorganisms (Timmermans et al. 2004; Kesarcodi-Watson et al. 2012; Hai 2015). For example:

- a combination with *Roseobacter* led to increased survival of scallop larvae (Ruiz-Ponte et al. 1999; Hai 2015).
- *B. subtilis* and *Lactobacillus acidophilus* together led to increased haematocrit and serum bactericidal activities in Nile tilapia compared to fish group fed with a single culture of probiotic (Aly et al. 2008b).

- use of a preparation containing both *Pediococcus pentosaceus* and *Staphylococcus hemolyticus* led to a reduction in the level of white spot virus (WSV) in white leg shrimp, *Litopenaeus vannamei* (Leyva-Madrigal et al. 2011).
- a mixture of probiotics containing *Lactococcus lactis* and *Lactobacillus plantarum* conferred protection in Japanese flounder when challenged experimentally with *Streptococcus iniae* (Beck et al. 2015).

Moreover, Jha et al. (2015) reported the beneficial effects of using multi-strain probiotic combinations on the growth and survival of rohu (*Labeo rohita*) at the early stages, i.e. at hatch and in fry, but not with older fish. Combinations of probiotics with either prebiotics, immunostimulants or natural plant products have been considered for use in aquaculture with promising outcomes (Salminen et al. 1998; Hai and Fotedar 2009; Hai 2015; Hindu et al. 2019). This has led to the concept of synbiotics; a combination of probiotics and prebiotics application of which is based on the principle of competitive advantage over endogenous populations, resulting in an enhancement in the survival, adhesion and colonization of the live probiotic dietary supplement in the GIT of the host animal (Gibson and Roberfroid 1995). Synbiotic feeding of *Enterococcus faecalis* and mannan oligosaccharide (MOS) was reported to lead to better food conversion ratio compared to the components used singly (Rodriguez-Estrada et al. 2009). Similarly, a combination of *Bacillus* sp. and MOS enabled better growth and survival and more stress tolerance to low salinity in European lobster (*Homarus gammarus*) (Daniels et al. 2015). Clearly, the applications of probiotics and prebiotics are beneficial to enhance the survival of aquatic animals (Decamp and Moriarty 2007; Daniels et al. 2015).

6 Effect of pH and Titratable Acidity on the Efficacy of Probiotics

pH and titratable acidity of probiotic products do noticeably affect their efficacy (Mortazavian et al. 2010). Many studies have demonstrated that acidic pH inhibits the growth and stability of probiotics in fermented products (see Brzozowski 2019). Also, hydrogen ions damage the membrane of probiotic cells thereby disrupting the food transfer pathway through cell membranes leading to cell starvation (Mortazavian and Sohrabvandi 2006; Brzozowski 2019). Extremely low ranges of pH in fermented milk have been shown to result in heightened concentration of undissociated organic acids and as such enhances their bacteriocidal effects (Brzozowski 2019). The deleterious effect of pH occurs of the lipophilic nature of organic acids; their distribution within microbial cells, together with the intracellular pH, may give rise to disturbances phenomenon in cell metabolism (Korbekandi et al. 2011). However, De Vuyst (2000) observed that the optimum pH for growth of probiotic bacteria, especially *Lactobacillus acidophilus*, is from pH 5.5–6.0, whereas for *Bifidobacterium*, the range is pH 6.0–7.0. Moreover, in fermented food products, lactobacilli have been shown to grow and survive in pH values between 3.7 and 4.3 (Boylston et al. 2004).

Nevertheless, *Bifidobacterium* tend to be less tolerant to acid conditions particularly at pH levels below 4.6 (Boylston et al. 2004; Lee and Salminen 2009; Ross et al. 2005; Brzozowski 2019). Furthermore, the ability of *Bifidobacterium* spp. to tolerate acidic pHs is strain-specific. The best survival of *Bifidobacterium* was observed with *B. longum* in bile salt acidic conditions and *B. lactis* in fermented milk (Korbekandi et al. 2011; Tamime et al. 2005; Brzozowski 2019). The survival of probiotic cells in acidic pH (pH 3.5–4.5) beverages, such as fruit juices, possesses a significant challenge. Shah (2001) reported that the viability of probiotic cells and their efficacy was strain-specific, and depended on the characteristics of the substrate for growth, the oxygen content and the final acidity (the concentration of lactic acid and acetic acid of the product). Similarly, Sheehan et al. (2007) observed that upon adding *Bifidobacterium* and *Lactobacillus* to orange juice, pineapple and cranberry juice there was a significance difference in acid resistance. Also, it was noted that all the probiotic strains survived for longer periods in orange and pineapple juices compared to cranberry juice (Rivera-Espinoza and Gallardo-Navarro 2010). In particular, *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei* were tolerant to these pH variations, and as such may be better suited for use in aquaculture feed products.

7 Longevity of Viable Probiotic Cells on Dry Commercial Diets

The longevity of viable probiotic bacterial cells in feed is strain-dependent, and reflects the composition of the food, its method of preparation, and the storage conditions. The interactions between probiotic bacteria and feed constituents may exert a great effect on survival. The interactions between probiotic cells and feed components may well be synergetic or harmful to the stability and longevity of the microbial cells (Lee and Salminen 2009; Mattila-Sandholm et al. 2002; Brzozowski 2019). Clearly, feed handling or processing affects the longevity and stability of the probiotics. For example, pulverized feeds are thought to have a much longer shelf life at ambient temperature. This is in contrast to pelleted feeds with varying moisture contents. Feed handling or processing involving drying of the ingredients is routinely carried out by lyophilisation, spray, microwave or vacuum drying. It is apparent that spray drying is very cost effective compared with the alternatives. However, spray drying does not ensure the longevity and stability of cells, and could lead to loss of viability due to high temperature during preparation, dehydration, osmotic pressure and a gradual increase in adverse compounds during the drying process (see Brzozowski 2019). Furthermore, dissolved oxygen concentration could increase in dried products which may be toxic especially in *Bifidobacterium* (Korbekandi et al. 2011; Rybka and Kailasapathy 1997). The most critical parameters affecting the survival of probiotic cells during spray drying are the air pressure and temperature (Champagne and Møllgaard 2008; Brzozowski 2019). Lyophilisation has been regarded as the most

suitable method for maintaining the longevity of bacterial cells, and is routinely used long-term survival of stock cultures, which in term are used for preparing starter cultures.

8 Effects of the Addition of Prebiotics

Prebiotics, which are described as "selectively fermentable fibres or roughages" have been reported to enhance the efficacy of probiotics by promoting specific changes in the composition and activity of the gastrointestinal microflora of the host animal (Roberfroid 2007). Thus, prebiotics confer health benefits on the host and stimulate the growth of beneficial microflora, specifically intestinal lactobacilli and *Bifidobacterium*. Prebiotics include bananas, beans, eggplants, garlic, honey, onions, plantains, strawberries and dark leafy green vegetables. They contain functional prebiotic fibres, such as inulin and fructo-oligosaccharide (Zdunczyk 2004; Buttriss and Stokes 2008; Gibson 2008). Prebiotic tasks may be accomplished with fermentation of prebiotic fibres in the digestive tract during which short chain fatty acids (SCFA) are produced. SCFA provide nourishment for the gastrointestinal microbiota, and enable the colonization of beneficial organisms by enhancing their survival and metabolic growth (de Vres and Schrezenmeir 2008; Buttriss and Stokes 2008).

The compatibility of prebiotics with probiotics in feed formulations may have a significant impact on the efficacy and survival of the beneficial bacteria. Interactions between pre- and probiotics may be synergistic or anti-synergistic; this may be protective, neutral or detrimental to the stability and survival of the probiotics (Lee and Salminen 2009; Mattila-Sandholm et al. 2002). This gives rise to the term "synbiotics" in aquaculture.

The use of synbiotics, which is a term used to describe products that contain both prebiotics and probiotics, is currently described in detail elsewhere in this book. These symbiotic products include fructo-oligosaccharides and galacto-oligosaccharides, which may impact on the viability of probiotics in food products and in the GIT (Mizota 1996; Gibson et al. 2004; Rycroft et al. 2001; Mohammadi et al. 2011). The addition of prebiotics can modify the feed matrix making it more protective. This has been demonstrated with the use of cheeses, where anaerobiosis, high fat content and buffering capacity of the matrix helps to protect the probiotic cells (especially *Bifidobacterium*) in the product and during passage through the GIT (Boylston et al. 2004; Lee and Salminen 2009; Brzozowski 2019). Increasing the buffering capacity stimulates multiplication and efficacious activity of probiotic cultures. Also, it results in greater viability of probiotic cells in the GIT because of the high pH values.

9 Influence of Feed Additives on the Efficacy of Probiotics

Feed additives have been shown to significantly influence the growth, viability and efficacy of probiotic bacteria, including *Lactobacillus acidophilus*, *L. casei*, *L. paracasei*, *L. rhamnosus* and *Bifidobacterium* (Vinderola et al. 2002).

From Brzozowski (2019), it is apparent that these additives include:

- NaCl and KCl,
- sucrose and lactose,
- the sweeteners acesulfame and aspartame,
- the aromatic compounds diacetyl, acetaldehyde and acetoin,
- natural colourings, i.e. zeaxanthin and lutein,
- flavourings, i.e. strawberry, vanilla, peach and banana essences,
- colouring compounds, i.e. strawberry, vanilla and peach,
- nisin, which is a polypeptide-type inhibitory agent from *Lactococcus lactis* with activity directed towards endospore forming bacteria and is often used as a preservative,
- natamycin,
- lysozyme,
- nitrate (Vinderola et al. 2002).

There are reports of elevated levels of additives inhibiting the efficacy of probiotics (Arihara et al. 1998; Boylston et al. 2004; Kourkoutas et al. 2006; Lee and Salminen 2009; Brzozowski 2019). Similar effects have been observed with starter cultures, such as L. *delbrueckii*, *L. bulgaricus*, *Lactococcus lactis*, *S. thermophilus* and *Saccharomyces* sp., which have been used for fermented and nonfermented products (Vinderola et al. 2002). Furthermore, the addition of growth promoters have been regarded as effective for achieving a dramatic and significant impact on the efficacy of probiotics (Mohammadi et al. 2011). These growth promoters used as additives, supplement or in fortification of feed products include casein, whey protein hydrolysates, L-cysteine, yeast extract, glucose, vitamins, minerals and antioxidant (see Brzozowski 2019). For example, the use of L-cysteine, tryptone, whey protein concentrate, and casein hydrolysate as additives improves the efficacy of probiotic bacteria (especially *L. acidophilus* and bifidobacteria) by the provision of growth factors to these cultures as they lack proteolytic activity (Dave and Shah 1998). Also, proteinaceous products have been known to enhance probiotic survival because:

i. they constitute nutritional value to the probiotic bacteria,
ii. they cause a reduction in redox potential and increase the buffering ability of the media, which leads to a small decrease in pH (Dave and Shah 1998; Mortazavian et al. 2010).

The effects of proteinaceous additives on efficacy or survival of probiotics reflects other factors, including the nature/type of the probiotic cultures, specifics of the feed constituents, inoculation conditions and product formulations. Research has found that that casein and whey protein hydrolysate enhance the rate of acidification of

the feed products, and reduced the growth rate of probiotic cells during storage (especially *L. acidophilus* and *L. rhamnosus*) (Lucas et al. 2004; Brzozowski 2019).

10 Influence of Strain Type on the Efficacy of Probiotics

This is one of the major factors influencing the efficacy of probiotic bacteria in aquaculture. The first step in the choice of the most appropriate probiotic strain(s) for incorporation into a feed application is to carefully identify the strain with attributes that will withstand the feed handling and heat of manufacturing processes. Secondly, the compatibility with the feed matrix whilst not neglecting the adaptation to storage conditions is critical. Generally, for improved efficacy, the selection of probiotic strains used in aquaculture feeds should be based on criteria of resistance to extreme pressures of the feed milling and handling processes, heat tolerance in the GIT of the host animal and compatibility with the product substrates (Korbekandi et al. 2011). However, these criteria are often strain-dependent.

Many workers have shown that the efficacy, viability and longevity of bacteria in the feed matrix and the internal harsh conditions in feed products, such as pH, titratable acidity, oxygen toxicity and storage temperatures, such as freezing and low temperatures, are species- and/or strain-specific (Ravula and Shah 1998; Tamim et al. 2005; Takahashi et al. 2007). Therefore, the preferred probiotic bacteria are those that are able to maintain their survival rate and shelf stability during commercial feed processing and during the storage period. Also, there is a need for the probiotic to maintain a high survival rate during transition through the GIT (Talwalker and Kailasapathy 2004). The organoleptic characteristics of the final products should be considered during the selection of probiotic strains. This aspect will definitely have a major impact on the consumption pattern of the host aquatic species. Some studies have shown that sensory characteristic (flavour) is the first indicator with respect to the choice of feed (Mohammadi and Mortazavian 2011). Fish and other aquatic species may not consume feed with unpalatable flavour especially when the added probiotic strain is detected to be responsible for the poor taste of the feed products. This consideration is in addition to the health advantage of the products. Therefore, good aroma and taste profiles of the probiotic is an important factor in the selection and incorporation of cultures into aquaculture feeds. Another point for consideration is the metabolic activity of the probiotic cultures which may result in the production of compounds with negative impact on the taste of the product. An example includes acetic acid, which is produced by *Bifidobacterium* during fermentation and storage.

11 Effect of the Viability of Probiotics

The survival of probiotics influence the actual number of viable and active cells in the formulated feed products at the time of consumption. This is a critical aspect in terms

of the benefit of the supplemented feed for the farmed aquatic species (Korbekandi et al. 2011; Tamime et al. 2005).

Many factors affect the viability of probiotics in feed especially during production and storage. Amir et al. (2012) outlined important factors including pH, titratable acidity, molecular oxygen, redox potential, hydrogen peroxide, bacteriocins, short chain fatty acids, flavouring agents, microbial competitions, packaging materials and packaging conditions and micro-encapsulation.

In order to have a positive effect on the intestinal tract of the host, the probiotics must:

i. resist the effects of heat of during the manufacturing process.
ii. remain viable during the storage period of the commercial feed products.
iii. must be able to survive and remain active during passage through the digestive system. This may involve adherence to and multiplication (= colonization) on the lining of the digestive tract.
iv. exhibit beneficial properties, including immunomodulatory, antimicrobial, antifungal and/or antioxidant effects in the host (El-Arab et al. 2006).

The literature suggests that probiotics are often administered at a dose of 10^6–10^9 viable CFU/ mL or viable CFU/g. These levels have been regarded commonly as satisfactory for achieving benefit to the hosts, although there is not any agreed dose (Karimi et al. 2011; Mohammadi et al. 2011; Vinderola et al. 2000). Of course, there is the issue about whether or not viable probiotic cells remain in the feed during storage and administration to the aquatic animals. Passage through the GIT will be challenging for the probiotics as they will need to compete with the resident, diverse microflora and survive the harsh condition therein (Nya 2015). Specifically, the probiotics need to tolerate:

i. the pH of the stomach,
ii. the harsh acid conditions in stomach and the bile substances in the duodenum,
iii. the bile salts and gastro-enzymes in the small intestine,
iv. the salivary lysozyme in the buccal cavity,
v. the temperatures in the colonic environment,
vi. competitions with other microorganisms in the GIT

The survival of probiotics during passage through the GIT is critical for achieving efficacy, and has been comparatively ignored by researchers. Nevertheless, Rochet et al. (2008) did not witness any differences in the faecal content of *Bacillus animalis* when 6×10^{10}–2×10^{11} CFU/ g were administered in fermented milk products or in freeze-dried form. However, the food matrix was considered to significantly improve the survival of *L. plantarum* (Klingberg and Budde 2006). This was confirmed in another study with *L. rhamnosus* when doses of 6×10^9 CFU and $1–2 \times 10^9$ CFU were utilized (Saxelin et al. 2010). The other barriers that probiotic cells need to survive are the highly acidic pH values of the stomach, i.e. pH 1 to 3, and the average exposure time of 90 min. Progression into the duodenum leads to a higher pH value of 6–6.5. However, there is input of bile salts from the gallbladder leading to a rise in concentration from 1.5 to 2% during the first 60-min of digestion, and

subsequently decreasing to 0.3% w/v (Noriega et al. 2004; Brzozowski 2019). The time of residency in the small intestine fluctuates between 2.5 and 3 h until 50% digestion. Then, transition through the colon may take up to 40 h (Camilleri et al. 1989).

The viability and survival potential of probiotics is strain-dependent. The evidence suggests that generally *Bifidobacterium* are less tolerant to acidity than the lactobacilli; conversely the former appear to be more tolerant to bile salts (Lee and Salminen 2009). *Bifidobacterium* are intrinsically resistant to gastric acid conditions of pH 2.0, and are tolerant to high concentrations of bile salts and NaCl. Previously, the cross-resistance between acidity and bile salts has been recorded in bile-adapted cultures (Noriega et al. 2004).

12 Impact of Health Status of the Gastrointestinal Tract of the Host

The health status of the GIT is crucial when considering the efficacy of probiotics used in aquaculture. There are two main ways of maintaining a good and healthy GIT, which will influence the efficacy of probiotics:

i. by monitoring the population of viable probiotic bacteria within the host and by ensuring the ingestion of appropriate numbers of viable cells.
ii. by ensuring adequate nutrition and avoiding stress that may lead to a decrease in viable probiotics population in vivo.

Probiotic containing products are regarded as an important group of "functional foods". Since every fish species is unique in terms of its nutritional needs, chemical and biological makeup, the viability of probiotic bacteria in the GITs and in feed products before and at the time of consumption is most critical because it determines the efficacy and health benefits (Amir et al. 2010). The GIT of aquatic animals is more humid than that of terrestrial vertebrates and as such is highly sensitive to dietary changes (Amir et al. 2010). In aquaculture, it has been shown that many factors affect the gut microbiota causing remarkable alteration in the condition of the GITs. These factors include dietary lipid sources and polyunsaturated fatty acids (Nya 2018), protein sources such as soybean meal, fish meal and other meal products (Nya 2018; Atefeh et al. 2012), nutraceuticals such as prebiotics, symbiotics and immunostimulants (Nya 2018; Bidhan et al. 2014), and antibiotics (Jinendiran et al. 2019; Olmos et al. 2020). Colonization of the GIT surface as a result of dietary manipulation leading to in the indigenous gut microbial populations may have consequences for probiotics and their efficacy.

13 Impact of Genetic Makeup on the Efficacy of Probiotics

The genetic makeup of the host has a direct impact on the efficacy of probiotics including tolerance to pathogens. Species or strains of aquatic animals that are more susceptible to specific diseases may be better suited for environments in which those pathogens do not cause problems (Kathy et al. 2000). Conversely, stocks that are more resistant would be better suited to areas where those diseases occur. For example, shrimp *Litopenaeus stylirostris* has been introduced into areas where the Taura Syndrome Virus (TSV) seriously affected the cultivation of *L. vannamei* (Stuck and Overstreet 1994). It is also important to emphasize that there may be differences in susceptibility between different ages or stages of the aquatic animals. Low levels of pathogen may be sufficient to induce diseases in early life stages (e.g. larval) of animals than in the later stages (e.g. juvenile or grown-out). This resulted in minimizing exposure to pathogens in the early stages by means of probiotic treated feeds (Stuck and Overstreet 1994). Thus, disease resistance is achieved by probiotics in aquaculture. However, it is conceded that some probiotics that appear to work well in laboratory condition do not necessarily do so in commercial sites.

14 Effect of Packaging Materials on Probiotics

Packaging materials used for probiotic feed products influences the oxygen permeability into the product, and as such affects the efficacy of probiotic cells, most especially *L. acidophilus, Bifidobacterium* and other probionts. Most packaging materials used for aquaculture feeds including glass and plastics that are known to positively influence the survival and efficacy of probiotic bacteria (Korbekandi et al. 2011). Also, the temperature and relative humidity are key factors affecting oxygen permeability. Arguably, oxygen permeability influences the efficacy of probiotics. Economic aspects need to be considered because the price of packaging materials and their associated machinery will influence the price of the products. It is desirable to use low cost packing materials with reduced oxygen permeability to prevent oxidative damage to probiotic cells and vital feed ingredients.

15 Effect of the Farming Environment and Rearing Conditions

Ideally, aquaculture sites need to have access to high quality, clean water and to be sited away from agricultural run-off, i.e. the water supply needs to be devoid of pollutants (Bayne 1975). Water treatment involving filtration through 150–200 μm filters can minimize and lessen the impact of environmental stressors including pathogens, such as *Aeromonas* spp., *Vibrio* spp. and WSV. Certainly, filters need to be maintained

and checked regularly to ensure durability and integrity. It is argued that the major stressors in aquaculture are environmental factors, such as the presence of chemical and biological pollutants, and suboptimum temperatures as well as poor husbandry techniques, including overcrowding, overfeeding, insufficient oxygen levels and the resulting inadequate hygiene (Charmantier and Soyez 1994; Martinez-Palacios et al. 1996; Moullac et al. 1997). These exacerbate the need for effective disease control measures, of which the use of probiotics is a topical example. Could oxygen levels impact on the effectiveness of probiotics? The answer is that molecular oxygen could be injurious to some probiotics by inhibiting growth and survival. Nevertheless, the level of sensitivity to oxygen varies considerably amongst various organisms (Kawasaki et al. 2006). For example, lactobacilli, which are considered to be facultative anaerobes/micro-aerophilic, are more tolerant of oxygen than *Bifidobacterium* (Lee and Salminen 2009). Oxygen affects probiotic cultures as follows:

- It may be harmful/toxic to some microorganisms.
- Some cultures, e.g. *L. delbrueckii* subsp. *bulgaricus* in the presence of oxygen produce peroxide, which is toxic to bacterial cells.
- Free radicals, which result from the oxidation of key cellular components, for example fats, are harmful/ to probiotics (Korbekandi et al. 2011; Tamime et al. 2005).

Furthermore, oxygen may impair some metabolic activities, and impact negatively on many physiological functions, including moulting and the immune response of the host (Allan and Maquire 1991; Moullac and Haffner 2000). It is clear that many environmental conditions affect the immune response leaving animals more susceptible to disease as the consequence of poor farming and husbandry techniques. These issues could impact negatively on the efficacy of probiotics (Dunier and Siwicki 1993; Moullac and Haffner 2000). In short, many environmental stressors have been identified that impact directly or indirectly on the efficacy of probiotics used in aquaculture (Table 1).

The question to be resolved is what of environmental stressors could be considered as normal and acceptable in fish husbandry. The answers do not necessary stem from laboratory studies, which do not reflect the complexity of the farming environment. A stressor may well be problematic in one set of environmental conditions but might not be such an issue in another. This precludes the situation where various environmental stressors interact compounding an already serious situation. Healthy animals in ideal situations may not be adversely affected by a few stressors but with

Table 1 Some of the stressors impacting aquaculture operations that affect probiotic efficacy

1. Ammonia	2. Insufficient oxygen	3. Elevated CO_2	4. Salinity
5. Nitrites	6. Poor nutrition	7. Overcrowding	8. Changes in pH
9. Changes in temperature	10. Moulting phase	11. Heavy metals	12. Toxicity
13. Suspended solid	14. Infectious agent	15. Parasitic infestation	16. Diseases

complex polluted environments in which the farmed animals are immunocompromised the outcome may well be critical. In the years after the end of the Second World War, antibiotics and other antimicrobial compounds dominated in disease control strategies especially where bacterial pathogens were suspected. Clearly, the use of antibiotics influences the development and spread of antimicrobial-resistance genes including within aquaculture facilities (Sun et al. 2016). This could impact on the use of probiotics, which could gain antibiotic-resistance genes or be inhibited by the presence of antimicrobial compounds in and around the recipient host animals. In turn, this could have consequences for aquaculture staff and the wider human community (Watterson et al. 2012; Ali et al. 2016; Phu et al. 2016; Sumon et al. 2016; Resende et al. 2017).

16 Conclusions and Suggestions for Further Study

There is an extensive literature demonstrating the benefit of probiotics in aquaculture, including roles in improving nutrition/growth and health/immunomodulation. However, there are concerns about who should be considered to prepare the probiotic cultures and when they should be used in aquaculture. The most convenient scenario for aquaculturists would be for feed manufacturers to include probiotics during the manufacturing process. However, this should be done as close to the end process as possible, avoiding possible damage during heating and pressure stages (= extrusion/pelleting). Yet, it is uncertain how long viable probiotic cells would survive. Specifically, would the microorganism survive in appropriate numbers throughout the shelf life of the product? Clearly, endospore formers would have a distinct advantage with their ability to survive for long periods in adverse conditions. However, it is unclear who has the responsibility of verifying the presence of probiotic in the stated quantity of cells in the feed throughout the shelf life. The alternative would be for the probiotic to be added on the aquaculture site immediately before use. Whereas there is some evidence that probiotics are produced by fermentation on some aquaculture sites, this is not to be encouraged as there could be issues with contamination and the introduction of potentially harmful organisms, i.e. putative pathogens. If the probiotic is produced elsewhere and shipped to the aquaculture sites then there are questions about shelf life, i.e. how long would the probiotic cells remain in a useful form. The use of lyophilised preparations are also advantageous. The choice of probiotic is crucial to ensure its usefulness in aquaculture settings in terms of survival during passage through the digestive tract of the host. In the case of the direct addition of probiotic to water in the aquaculture facility, it is relevant to ensure that the culture survives and is not antagonized by members of the resident aquatic microflora. What is the ideal dosage and the duration of application? Not all studies adequately address this issue. The commonly used dosages range from 10^6–10^8 CFU/g of feed, but it is unclear if application should be continuous, by short periods of 1–2 weeks or by pulses. Researchers have used single and/or multiple isolates in probiotic preparations with or without the presence of prebiotics, and

there are advantages and disadvantages to all these approaches. For example, it is conceivable that one or more isolates could be inhibited in multi-culture preparations.

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Quality, Safety and Regulatory Issues of Probiotics

E. Awad, A. von Wright, and B. Austin

Abstract Consideration needs to be given to quality issues for commercial probiotics. There are safety concerns with the use of some organisms, notably Gram-negative bacteria, e.g. *Aeromonas,* from genera associated with diseases of aquatic animals. The legal standing of probiotics needs clarification, namely are they medicinal substances or food additives? Regulation governing the use of probiotics has been developed in some countries, e.g. the European Union, but not so in many other countries in which aquaculture is an important industry.

Keywords Quality control · Safety issues · Legal status · Regulations

1 Criteria for Probiotics

An effective candidate probiotic that can increase the performance and health of aquatic animals should fulfil all of several criteria. However, previous investigations have been concerned often with the favourable characteristics of potential probionts rather than considering safety and efficacy criteria for their application in aquaculture (Balcázar et al. 2006; Gómez and Balcázar 2008; Merrifield et al. 2010; Banerjee et al. 2007a; Sayes et al. 2018). Certainly, most investigations have shed light on basic criteria that should be considered and include:

1. Safety. This is considered as the most important criterion for any probiotic insofar as the culture should be safe for use in aquatic animals, to human

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beings as consumers and to the natural environment. It is necessary to ensure that probiotics do not contain [or on the basis of risk, are most unlikely to acquire] virulence or antibiotic resistance genes, particularly via plasmids or bacteriophages.

2. Adaptability. Many studies have focused on the ability of bacterial cultures to colonize the digestive tract of aquatic organisms. This would include adherence to and multiplication in the digestive tract in the case of orally administered probiotics, and survival in the aquatic environment if the waterborne route is used.

3. Function. The probiotic should confer advantages to the host, including improvements in growth performance, immunomodulation, resistance to diseases and/or general improvements in overall health. When probiotics are to be used directly into the water, it would be expected that there should be improvements to water quality.

4. Convenience. It is important that probiotics should be capable of being stored and administered easily (Wang et al. 2019).

These criteria will be considered in more detail below:

1. **Absence of pathogenicity**

It is most important to ensure that probiotics are safe and are not harmful/pathogenic to the aquatic organisms and for human beings, i.e. the consumers of the aquatic species (Merrifield et al. 2010; Balcázar et al. 2006; Zokaeifar et al. 2012). The pathogenic nature of the bacterial culture will reflect the toxin-producing capability, which will differ from one strain to another. The perceived worry is that the probiotic culture, particularly if it is a Gram-negative organism from a genus associated with disease, e.g. *Aeromonas* and *Vibrio,* could acquire and express virulence genes. However, this event has not ever been associated with any probiotic. Nevertheless, the concern persists. For example, the fish pathogenic organism, *Aeromonas hydrophila,* has been responsible for large-scale mortalities in aquaculture (Beaz-Hidalgo and Figueras 2013; Harikrishnan and Balasundaram 2005). Yet, a culture of *A. hydrophila* has been used as a probiotic in aquaculture (Gunasekara et al. 2010). As another example, *Vibrio* spp. have been recognized to infect larvae leading to significant mortalities. However, some researchers have considered that *Vibrio* spp. are not true pathogens, but under intensive aquaculture conditions may acquire virulence genes leading to pathogenicity (Thompson 2004). Another representative, *V. alginolyticus,* which has been recognized as an aquatic animal pathogen, has been adopted as a probiotic with antibacterial activity against *Aeromonas salmonicida, V. anguillarum* and *V. ordalii* (Austin et al. 1995), and has been used successfully in an Ecuadorian shrimp farm to control disease caused by *V. harveyi* (B. Austin, personal observation). Many in vitro tests have been used to check the bio-safety of candidate probiotics

and include the demonstration of haemolytic activity and mannitol utilization. Moreover, feeding experiments with putative probiotics may be used to confirm the absence of pathogenic activity in fish, particularly when dosed at use and higher levels (Sayes et al. 2018; Banerjee et al. 2007b).

2. **Antibiotic resistance genes**

The emergence and spread of antibiotic resistance genes in bacteria is a great threat to animal and human safety. Thus, the European Food Safety Authority (EFSA) established Qualified Presumption of Safety (QPS,) which states "the nature of any antibiotic resistance in a candidate micro-organism should be determined prior to approval as probiotic" (Authority 2008). Furthermore, the presence of antibiotic resistance genes is related to either intrinsic resistance, acquired as a result of a chromosomal mutation(s) or acquired by horizontal gene transfer (Gueimonde et al. 2013). The successful bacterial probiotic candidate should not contain plasmid-encoded antibiotic resistance genes (Gueimonde et al. 2013; Merrifield et al. 2010) or have negligible possibility of acquiring them. In fact, bacteria react very fast to the presence of antibiotics because of their high mutation characteristic, which leads to gene transfer from one species to another through lateral gene transfer. Therefore, investigations including the determination of antibiotic sensitivity and the PCR detection of multi-drug resistant genes should be carried out before being approved for use in aquaculture as probiotics (Banerjee and Ray 2017).

3. **Antagonistic activity**

Antagonistic activity is a common phenomenon in bacteria, which is used to combat harmful organisms and to facilitate the uptake of nutrients. Bacterial antagonists exert their effect by:

(A) Competitive exclusion

(B) The ability to produce inhibitory metabolites.

(A) Competitive exclusion

The first requirement of a pathogenic organism is the need to attach to mucosal cell layers of the gastrointestinal tract in order to develop disease. Conversely, probiotic bacteria compete with pathogenic bacteria for the binding sites on mucosal cell layers or sometimes bind directly to the pathogen, thereby reducing the virulence activity (Adams 2010). This is known as competitive exclusion or competition for adhesion sites. Consequently, the ability of microorganisms to compete for binding sites is regarded as a desirable criterion in the selection of potential probiotics (Balcázar et al. 2006). Furthermore, the mode of attachment of probiotics may be nonspecific because of the physicochemical agents or specificity due to the adhesion of bacterial cells on the surface of pathogens and receptor molecules on the epithelial cells (Lazado et al. 2015).

(B) Ability to produce inhibitory metabolites

Bacterial probiotics have the ability to produce inhibitory metabolites that inhibit the reproduction or the activity of pathogens through the production of broad-spectrum small peptides to larger proteins, i.e. bacteriocins or lysozyme, proteases and/or hydrogen peroxide (Nates 2015; Irianto and Austin 2002a; Balcázar et al. 2006). Alternatively, the inhibitory effect may result from the production of antimicrobial proteolytic enzymes, such as aminopeptidase, trypsin-like serine protease and enzymes that are reactive against substrates for cathepsin and caspase 1-like proteases (Richards et al. 2017).

Several investigations have been conducted to evaluate the antagonistic activity of probiotics in vitro using assays, such as the well diffusion disc assay and double-layered molten agar assay (Balcázar et al. 2007; Mukherjee and Ghosh 2016). An in vitro test is the first step in evaluating probiotics and demonstrates the effectiveness at inhibiting pathogens. However, an in vitro test does not necessarily reflect effectiveness in aquatic animals. Therefore, after administration of a putative probiotic, the aquatic animals should be challenged with one or more pathogens. Ideally, probiotics should be evaluated in a range of different aquatic animal species to determine effectiveness and to ensure that there are not any deleterious effects in any single host (Banerjee and Ray 2017). The putative probiotic candidates should show antagonistic activity against a wide range of pathogens of aquatic animals.

4. **Tolerance to pH and bile salts**

Most probiotics are administered orally and in a viable form; a problem may well reflect harmful effects in the digestive tract because of fluctuating pH levels. Generally, the environment in the digestive tract is favourable for endosymbionts (Ray et al. 2012). In addition, the bile secreted different types of compounds (= bile salts) during metabolism, which may impact on the putative probiotic cells (Buchinger et al. 2014; Nates 2015). Thus, a successful probiotic should have the capability of tolerating a wide range of pH values as well as a high concentration of bile salts.

5. **Colonization ability**

Gut bacteria have been grouped according to the colonization property on epithelial mucosal surfaces, as autochthonous and allochthonous. Allochthonous organisms are regarded as free-living associated with the digesta. Conversely, autochthonous bacteria are able to colonize mucosal surfaces of the digestive tract (Nayak 2010; Egerton et al. 2018). With regard to choosing the best probiotic candidates, autochthonous bacteria are preferred. It is argued that the adhesion property of bacteria on gut mucosal surfaces will improve the health status (nutrition, growth, reproduction and immunity)

of the host (Carnevali et al. 2017). Scanning (SEM) and transmission electron microscopy (TEM), fluorescence microscopy and confocal microscopy are advanced instruments that have been used to examine the colonization ability of bacteria. These approaches complement other laboratory techniques, namely PCR and qPCR (Mukherjee and Ghosh 2016; Banerjee et al. 2016).

6. **Production of extracellular enzymes**

The ability of probiotic bacteria to produce extracellular enzymes, such as proteases, amylases, cellulases, phytases, chitinases and lipases, is an effective criterion for choosing probiotics especially for use in aquaculture. Fish do not produce any vitamins but obtain them through the presence of endosymbionts (Banerjee and Ray. 2017). Therefore, many probiotics influence the nutrition of the host, either by aiding the digestion of food particles and/or the supply of essential micronutrients (LeBlanc et al. 2017). For example in fish, *Bacillus cereus*, which was isolated from the intestine of *Mugil cephalus,* demonstrated pronounced extracellular protease activity in vitro (Esakkiraj et al. 2009). Also, feeding kuruma shrimp (*Marsupenaeus japonicus*) with dietary *Clostridium butyricum* for 56 days led to significantly increased activities of intestinal pepsin, 5-hydroxytryptamine, amylase and lipase. Furthermore, a high level of short-chain fatty acids and body crude protein was observed, which suggested a role for probiotics in promoting intestinal digestion, metabolic capacities and growth performance (Duan et al. 2018). Therefore on the basis of these studies, it is reasoned that candidate probiotics should have the ability to supply enzymes and vitamins for improving growth performance and general health for aquatic animals. Here, the assumption is that the mode of action is competitive exclusion in which the probiotic colonizes the digestive tract. However, probiotics have been credited with other positive effects on the host, as will be discussed later.

7. **Indigenous in nature**

A successful candidate probiotic should be able to colonize and multiply in the digestive tract of the aquatic animal. A poor selection could lead to undesirable effects in the host. Workers have reported that some commercial probiotics are relatively ineffective because they are unable to colonize or remain viable at optimum concentrations in the digestive tract (Abraham et al. 2008). Furthermore, using indigenous strains as probiotics could be considered as preferable because they are part of the natural microbiota and should not have any adverse effects (Boutin et al. 2013). Thus, indigenous organisms, which have been derived from the aquatic animal should have distinct advantages over exogenous sources (Banerjee and Ray 2017; Merrifield et al. 2010). Certainly, studies have shown the success of probiotics derived from the digestive tract of mature fish when applied to immature fish, i.e. larvae, of the same species (Gildberg et al. 1997; Gram et al. 1999; Gomez-Gil et al. 2000). Interestingly, some authors have preferred the use of indigenous bacteria on the assumption that immune cells do not attack them but rather consider them the

micro-organisms of the normal internal population of the host (Salinas et al. 2006).

8. **Synergistic effect of multi-species**

Studies have pointed to the effectiveness of using more than one species of probiotics in vitro and in vivo (Nayak 2010; Timmerman et al. 2004). Authors postulated that multiple species of probiotics are more bioactive and consistent compared to monospecies because of the beneficial, i.e. synergistic, properties of the mixture (Timmerman et al. 2004). This synergistic effect of multiple species of probiotics exerted a profound influence on the development of innate immunity in fish. For example, Nile tilapia, *Oreochromis niloticus,* showed significantly higher respiratory burst and lysozyme activities in groups fed with a mixture of probiotics, i.e. *Bacillus subtilis* and *Lactobacillus acidophilus*, compared with groups fed with individual cultures (Aly et al. 2008). Using food supplemented with a combination of two probiotics, *B. subtilis* and *L. delbrueckii,* seabream developed significantly increased levels of complement, phagocytic and peroxidase activities and immunoglobulin, i.e. IgM, levels. Conversely, fish receiving individual bacterial cultures failed to develop any of these activities or immunoglobulin levels (Salinas et al. 2008). Certainly, the synergistic effects in multi-species combination of probiotics will depend on the component micro-organisms. Combinations do not always work better than single preparations. For example, Pdp11 and 51M6 are two probiotics belonging to the family *Vibrionaceae*, which did not demonstrate immunomodulation in rainbow trout, *Oncorhynchus mykiss,* when used together but did so when administered individually (Choi and Yoon 2008).

2 Safety Issues

The general principle of the safety assessment of feed additives is that the target animal safety, consumer safety, user safety and environmental safety have to be demonstrated, and this applies also to animal probiotics, unless the micro-organism is a QPS species. For QPS micro-organisms, only the user safety has to be established.

To date, many probiotics have demonstrated their effectiveness for use in aquaculture. Laboratory studies have led to improved growth and appetite, better health and resistance against challenge with pathogens (Brunt et al. 2007; Duan et al. 2018; Balcázar et al. 2006). However, it is important to consider safety aspects particularly before widespread use in aquaculture, which will inevitably involve the release of high numbers of microbial cells into the receiving environment from food and directly via the waterborne route of administration. Clearly, there should not be any adverse effects to the host, human consumers and the aquatic environment. In practice, proponents of probiotic need to demonstrate that the cultures remain authentic, i.e. that during the research and production cycles the cultures remain the same and that contaminants are not unwittingly used. During the research and production phases, the lack of harmful effects is to be verified at the use and 2–10 times

the recommended dose, as appropriate. Modern techniques, such as cell culturing, have been used to evaluate the safety of probiotics and guarantee that health benefits occur in aquatic animals. For example, the epithelial cells of Nile tilapia have been isolated and cultured as primary cell cultures as a model to evaluate the effect of probiotic *Rhodopseudomonas palustris* PSB0201 through morphological characters, cell viability, viability and permeability (Wang and Xu 2007).

Lactic acid bacteria have been used for a long time as probiotics for agricultural, aquacultural and human use without any reported harmful effects (Salminen et al. 1998; Ringø and Gatesoupe 1998). Certainly, the group is infrequently associated with fish diseases (Austin and Austin 2007), but generally their use as probiotics has been largely beneficial. To date, there has not been any safety concern connected with intrinsic type of antibiotic resistance. The occasional occurrence of plasmid-associated antibiotic resistance creates serious problems related to the ability of transferring resistance factors to other more harmful species and genera (Salminen et al. 1998).

Issues concerning plasmid-mediated antibiotic resistance have rarely been considered by the wider international aquaculture community. This is a pity as resistance could be transferred to other organisms of concern to terrestrial and aquatic animal pathology. However, there is some evidence that the development and commercialization of probiotics are beginning to consider the issue of antibiotic resistance (Courvalin 2006). It is argued that prospective probiotics should not possess transferable resistance factors to common classes of antibiotics, such as tetracyclines, quinolones and macrolides. Clearly, assurances are needed that probiotics will not transfer antibiotic resistance and/or virulence genes (Moubareck et al. 2005). Evaluations are needed to ensure that the end-products destined for aquaculture do not lead to any adverse effects (Wang et al. 2008).

3 The Regulatory Status of Probiotics

Many studies on the effects of probiotics on aquatic animals have confirmed improved growth performance and/or reduction in mortality or enhancement in resistance against pathogens (Irianto and Austin 2002b; Sharifuzzaman et al. 2011). However, it is apparent that the beneficial effects of probiotics will reflect the dose, time of exposure, external stresses such as water temperature and quality, and inherent features of the receiving aquatic animal species (Bagheri et al. 2008; Merrifield et al. 2010). Certainly, only a minority of the probiotics described in publications reach commercialization. The transition from scientific research to industrial scale applications is subject to many rules, which may differ from country to country. Within the European Union (EU), any field trial including feed additives needs to be approved initially by national agencies with responsibility for food safety before commercialization. Such field trials are essential to determine the efficacy of probiotics at the farm level and subsequently to demonstrate the financial viability of the products in aquaculture.

The progression of research to scale up of the commercial product is subject to many obstacles. As an example, the useful characteristics seen in small-scale culture may be lost in larger batches. This could reflect the switch-off of useful genes during large-scale production or that the beneficial organisms are effectively outcompeted by variants more suited to growth in larger volumes. Moreover, procedures adopted in the laboratory are unlikely to be replicated exactly during commercialization (Crittenden 2009). Will the media, incubation and cell processing conditions remain the same? Extraction of cells from large volumes will undoubtedly lead to greater stresses caused by filtration or centrifugation (heat and sheer stresses) (Crittenden 2009). One answer is to gradually scale up production from the laboratory to commercial quantities so that changes to the cells as a result of scale-up procedures may be monitored, and where appropriate, corrective action taken (Fenster et al. 2019). Then, there is the matter of how the probiotic will be presented to aquaculture—will it be incorporated into feed or made available as a suspension, paste or lyophilized material? Whereas laboratory studies may incorporate a broth or saline suspension of the probiotic (maybe with the addition of oil to facilitate binding) onto the surface of small quantities of feed (e.g. Irianto and Austin 2002b), this is highly unlikely to happen in industry, which deals with large quantities of feed. If the probiotic is incorporated into the feed during production, it would be necessary to determine the effect of processing—including heat—on viability of the microbial cells. Then, there is the issue of determining the effective life of the product, and that will be influenced by storage conditions. To overcome some of these problems, methods have been developed to protect the viability of the probiotic with a physical barrier against adverse environmental conditions that could occur during manufacture and storage. For example, micro-encapsulation in a polymeric matrix has a positive effect on the stability of the organisms during production and passage through the digestive system of the recipient animal (Zigger 2005; Londoño et al. 2017; Kailasapathy, 2002). Also, consideration has been given to spraying the probiotic suspension onto the feed followed by drying to achieve a specified level of moisture for storage; here, the endospore forming *Bacillus* would have a distinct advantage over many vegetative cells (Londoño et al. 2017). Regardless of method used, it is necessary to verify the authenticity, viability and effectiveness of probiotics during manufacture and storage (Qi et al. 2009).

Ultimately, a probiotic must satisfy both national and in certain cases international regulations in order to receive authorization and access to market. While there is an extensive regulatory framework for human probiotics, animal probiotics have received relatively little attention, with the notable exception of the EU, which has probably the most stringent regulatory approach as outlined in the Regulation (EC) No 1831/2003 of the European Parliament and Council. According to this Regulation, the European Food Safety Authority (EFSA) has a central role in the safety and efficacy assessment of feed additives, including live micro-organisms used as animal probiotics. Accordingly, EFSA has published several guidance documents to help the applicants to prepare their notification dossiers. In practice, the EFSA requirements have become a kind of universal standard for probiotic producers all over the world and therefore merit a closer look in the following paragraphs.

The general principle of the safety assessment of feed additives is that the target animal safety, consumer safety, user safety and environmental safety have to be demonstrated, and this applies also to animal probiotics, unless the micro-organism is a QPS species. For QPS micro-organisms, only the user safety has to be established.

The first step of the assessment of an animal probiotic is its proper characterization/identification. According to the current EFSA guidance (EFSA 2018), the micro-organism has to be unequivocally identified. This should be verified both by phenotyping and by bioinformatic analysis of the whole genome sequence (WGS). Also, there must be an absence of transmissible antibiotic resistance. Accordingly, WGS is demanded for all bacteria and yeast strains notified to EFSA. WGS analysis is also used to verify the absence of toxin genes or other virulence factors. Yet not so long ago, identification of probiotics reflected only phenotypic characterization, i.e. micro- and colonial morphology, growth requirements and biochemical characteristics and serology analysis (McCartney 2002). The accuracy of these approaches may now be questioned, but much of the literature published prior to 2000 relied on these conventional approaches to taxonomy. However, the subsequent adoption of nucleic acid-based techniques, such as 16S rRNA gene sequencing, oligonucleotide probes and WGS, has led to more reliable identification of probiotics. The accuracy of the outputs is important for gaining consumer confidence in product labelling and for safety considerations (Yeung et al. 2002). Techniques have continued to evolve, and newer approaches, such as Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) and PCR denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE), are fast and accurate (Qi et al. 2009). The fluorescence in situ hybridization (FISH) technique with rRNA target probes permits in situ identification of single microbial cells. This technique is built on the hybridization of synthetic oligonucleotide probes to specific regions within the bacterial ribosome without the need for cultivation (Qi et al. 2009).

For non-QPS micro-organisms, the target animal safety is basically demonstrated by a tolerance test in which the animal is exposed to an overdose of the additive for a relevant time period. Alternatively, a thorough literature survey or extrapolation from toxicological data (if available) can be applied in certain cases. EFSA has published practical guidance on the performance of tolerance trials for different animal categories, including aquatic species (EFSA 2017a). Regarding the extrapolation from one species to another, the guidance states: “If the application is for all fish, then tolerance studies should be submitted in a salmonid (salmon or trout) and another species (e.g. carp, sea bream or sea bass). If the application includes crustaceans, then an additional study in shrimp would be required”.

There are also EFSA guidances for the establishment of consumer safety (EFSA 2017b) and for worker/user (EFSA 2012). For the consumer safety, both genotoxicity studies and a 90-day rodent feeding trial may be required for microbiological feed additives to rule out the possibility of unknown metabolites contaminating animal products. The emphasis on worker safety is on respiratory toxicity, and skin or eye irritation (unless these risks have been minimized by a proper formulation). Microbiological additives are always considered as respiratory sensitizers, and appropriate

risk management measures are recommended. It is essential that the final formulation of the additive is used in the tests required for the establishment of worker safety.

Regarding environmental safety, the general approach of EFSA has been that microbiological additives are not an environmental concern, unless their use would significantly increase the presence of the micro-organism in question in the receiving environment. This applies to conventional micro-organisms. If a genetically modified microbiological feed additive were to be introduced into the market, it should satisfy both the special safety requirements of genetically modified micro-organisms (GMMs) and the especially stringent environmental safety criteria defined in the EFSA guidance on GMMs (EFSA 2011).

4 Quality Control

Although different, quality control and quality assurance are used for the same purpose of producing quality product. Quality assurance has the responsibility of preserving quality systems within a facility so that product defects and faults may be minimized. Quality control is the actual testing of raw materials, processes, intermediate and end-product samples, and includes a variety of tests. Indeed end-product testing is considered as the primary goal for quality control. Thus, quality control laboratories operate to the highest standards, such as Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) (Sanders et al. 2016). GLP should minimize the risk of cross-contamination by understanding all the steps used in production, and the composition and handling of the end-product. Attention needs to be focused on personal hygiene, the availability of proper personal protective equipment, product flow through the laboratory, sanitation procedures and thorough documentation (Fenster et al. 2019). The accuracy of equipment and the methods need to be validated in order to ensure the accuracy of test results. Control points and hazard analyses need to be used to define critical control points and to set up acceptable protocols in order to reduce risk.

5 Recommendations

- There needs to be internationally agreed clarification about whether probiotics should be regarded as feed additives or medicinal products, insofar as their standing will impact on the procedures needed to be adopted for their use in aquaculture.
- Probiotics should not be chosen from taxa, i.e. genera and species, known to contain pathogens of aquatic or terrestrial animals to minimize any risk of the acquisition and spread of virulence determinants.

- Safety data are needed to verify that the probiotics are safe to use in aquaculture. This means that it is essential to demonstrate that the probiotic cultures do not cause disease or other harmful effects in the host.
- Probiotics need to be acceptable to the host in whatever vehicle is designated for their use.
- Quality data need to prove that the probiotic numbers do not deteriorate during the life of the products. Specifically, the numbers of live cells need to remain within clearly stated boundaries.

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Conclusions

B. Austin and S. M. Sharifuzzaman

Abstract Although a diverse range of microbial taxa has been reported as potential probiotics, there is concern about the use of Gram-negative bacteria particularly as they are often from groups that have been associated with pathogenicity. However, there is not any published or anecdotal evidence for the acquisition of putative virulence genes by the Gram-negative bacterial probiotics. There are ongoing issues in numerous countries concerning the legal status of probiotics, i.e., are they feed additives or veterinary medicines? There are information gaps that need to be filled, including the precise mode of action and the longevity of effectiveness after the cessation of application.

Keywords Virulence gene · Pathogenicity · Immunostimulant · Oral vaccine · Future needs

In contrast to human and terrestrial animal use, a greater range of microbial species has been evaluated for use as probiotics in aquaculture. Moreover, the list of putative probionts continues to grow. Yet, there are concerns about the inclusion of organisms from taxa considered as potential pathogens of aquatic animal species. What is the possibility that a probiotic culture of, for example, *Aeromonas*, could acquire virulence genes? Thankfully, there is not any published or anecdotal evidence that this situation has ever arisen, but the possibility remains however unlikely. Thus to reduce any potential risk to the host, it would be preferable to choose candidate probiotics from groups that are less likely to be pathogenic. This is where the Gram-positive lactic acid bacteria have a distinct advantage. Of course, the pathogenicity issue is removed if the probiotics are inactivated and enter paraprobiotics. This raises the question about whether scientists check the viability of their probiotic preparations. Scrutiny of the literature would suggest that not all probiotic preparations

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are of confirmed viability especially if vegetative cells rather than endospores are used. However, if deliberately inactivated preparations, i.e., paraprobiotics, are to be adopted, then it would be appropriate to enquire about whether they should be regarded as probiotics [= paraprobiotics], immunostimulants or heterologous oral vaccine candidates especially if the mode of action is immunomodulation. Licensing authorities have a real dilemma to resolve.

For the future, work is needed to clarify/research the following points:

Further studies

1. The majority of probiotics used in aquaculture are derived from the host, notably the digestive tract. The issue concerns the criteria used to choose potentially useful microbial cultures, with emphasis placed on antimicrobial activity. This approach is of questionable relevance where the mode of action is immunomodulation. Consequently, research is needed to develop appropriate and relevant methods for identifying potential probiotics.
2. Should probiotics be fed continuously or for a finite period of one or more weeks? The optimum duration of application needs to be researched and may reflect the nature of the species, age and environment of the host.
3. How long does the beneficial effect last after the cessation of application? Is there evidence of memory, and if so for how long? This is likely to occur when the mode of action is immunomodulation.
4. What is the mode of action? Does it depend on the host and the nature of the probiotic or does it reflect a combination of all, most or only some of the different possibilities, including competitive exclusion and immunomodulation?
5. More work is needed to determine the components of the probiotics that are responsible for the beneficial effects. It would not be unreasonable to evaluate the effectiveness of subcellular components where these are shown to be beneficial to the host.
6. Should probiotics be applied as single or multiple cultures? If multiple cultures are to be used, it is essential to verify whether or not there is any indication of antagonism among the components. For example, could the presence of some organisms adversely affect the viability of others?
7. More work is needed to address the issues regarding the use of probiotics in combination with prebiotics, oral vaccines, non-specific immunostimulants and/or other functional foods.
8. What is the period of effectiveness of a probiotic culture? Should a new starter stock be used whenever probiotics are prepared or could a bench culture/subculture be used multiple times while retaining its effectiveness? This aspect has been largely ignored although there is anecdotal evidence from Ecuador that probiotic cultures need to be replaced every few weeks.
9. Do probiotics really need to be viable or will inactivated preparations suffice, i.e., paraprobiotics, in which case there could be an overlap and confusion

between heterologous oral vaccines and probiotics? There needs to be discussion over licensing/registration as to whether inactivated probiotics should be treated as feed additives or veterinary medicines.

10. When should probiotics be used during the life cycle of the host? Specifically, should probiotics be used continuously, at specific stages—for example, fry and fingerlings, and/or for special reasons, such as after the use of inhibitory compounds? Here, the probiotics could be instrumental at influencing re-colonization of the digestive tract.
11. How should probiotics be marketed? Should they be incorporated in feed before sale to aquaculture? If so, attention needs to be focused on how the probiotics should be used—applied to the already prepared feed as a coating, if so in what medium [saline, oil or gelling agent for example]? This would lead into consideration of the shelf life of the product as certain probiotics are not capable of surviving during pellet manufacturing process carried out under high-temperature and -pressure conditions. Alternatively, the probiotic could be provided as a suspension or lyophilized preparation for incorporation on the aquaculture facility. Again, the shelf life of the product would need to be ascertained. As a follow on, there needs to be clarity/advice about how the probiotic should be stored in the aquaculture facility—Would refrigeration be needed, or would room temperature suffice? Endospore forming *Bacillus* would be expected to be hardy and long lived, not so some Gram-negative bacterial taxa.
12. More attention needs to be devoted to determining the precise identity of the probiotic and to verifying the authenticity of the cultures throughout their life in the finished product. Contamination does occur, and workers need to be watchful to ensure that the useful culture is not replaced by a contaminant.
13. When it comes to grow-out ponds, is combined application of probiotics through feed and water better? Or should farmers select either of the delivery methods? Data on actual field trials are limited, and thus, extensive evaluation of probiotics under different farming conditions and changing environments is necessary to produce concrete, evidence-based examples on the efficacy of probiotics in pond system.
14. Is addition of probiotics to biofloc culture system a viable option? It is anticipated that the presence of heterotrophic bacteria, including other beneficial bacteria, in biofloc can play role in microbial balance of the microbiome and help improve the rearing water quality. A balanced microbiome tends to control or inhibit proliferation of microbial pathogens in the system. Therefore, it warrants further investigation whether supplementation of probiotics into the culture water of biofloc system or to the feed alone, or combination of both is necessary.

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