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Review

Application of Probiotics for Environmentally Friendly and Sustainable Aquaculture: A Review

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Abstract: Aquaculture plays an important role in food production for the world population and at the same time for the livelihood of the most needed globally. The concerns about sustainability and ecological health are growing in this extremely diversified sector just like in the whole agriculture industry. The use of probiotics in aquaculture already has a long history and has served from the beginning the goals of more sustainable production; however, the expansion of intensive systems along with global climate change produces new challenges. The present work aims to provide an overview of the most relevant literature. Firstly, the microbiome of aquatic animals and its functioning is surveyed followed by the aims and methods of probiotic application. The screening and testing of novel probiotics are also assessed as well as the scientific and technical novelties in probiotics research. The mainstream development in probiotic research aims to serve the sustainability of aquaculture in all respects including traditional animal health, feed efficiency, and environmental issues. New state-of-the-art techniques may lead to a future paradigm change in aquaculture under the aegis of the Blue Revolution.

Keywords: probiotics; aquaculture; microbiome; metagenomics; sustainability

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Citation: Hancz, C. Application of Probiotics for Environmentally Friendly and Sustainable Aquaculture: A Review. *Sustainability* **2022**, *14*, 15479. https://doi.org/10.3390/su142215479

Academic Editor: Helvi Heinonen-Tanski

Received: 27 September 2022
Accepted: 17 November 2022
Published: 21 November 2022

1. Introduction

Aquaculture is one of the world's largest and most rapidly developing food production sectors; it is practiced with a wide variety of intensities and techniques, culturing—besides plants—a plethora of vertebrate and invertebrate species in the sea and freshwater. It contributes significantly to the livelihood and food of the ever-increasing world population [1–3]. The Blue Food Assessment, a collaboration involving more than 100 researchers, systematically assessed how aquatic food contributes to global food security, providing protein and other valuable nutrients for more than 3.2 billion people [4]. "Blue food" is a rich source of the omega-3 long-chain polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), as well as vitamins A and B_{12} [5].

Since the aquaculture industry has been gradually shifting to crop-based feed ingredients that fundamentally link aquaculture production to terrestrial agriculture, multidisciplinary research is needed to study the ecological and environmental health implications [6]. However, the intensification of aquaculture practices has increased the stress for both the aquatic animals and the environment [7,8]. Various chemicals and antibiotics have been applied [9–11] that cause serious problems and indirectly affect human health and even directly by producing antibiotic-resistant bacterium strains. It was long ago that the European Union stated in Regulation (EC) No. 1831/2003 that "Antibiotics, other than coccidiostats or histomonostats, shall not be authorized as feed additives", which prompted researchers to seek for alternatives to reduce the abuse of antibiotics, not only in Europe, and one of the most promising was the group of probiotics. Ensuring animals' good health and well-being remains a cornerstone in all aquaculture systems. To achieve this goal, probiotics offer a sustainable and eco-friendly alternative by replacing antibiotics and synthetic chemicals [12]. Probiotics'

beneficial role in aquaculture is diverse but perhaps the best-known effect of them is the amelioration of feed utilization of the host, easily measurable by the feed conversion ratio (FCR). Lowering the FCR is crucial from an economic point of view but is also important to decrease the load on the environment. Fry et al. [6] discuss the limitations of simply using FCR to evaluate the effectiveness of feed utilization concluding that using multiple measures to compare the efficiency of various types of food including nutrient and calorie retention is advisable. Environmental footprint measures including resource use, greenhouse gas emissions, biodiversity loss, and water pollution have also to be considered. Avadí et al. [13] compared the environmental performance of different types of feed use by applying the Life Cycle Assessment (LCA), which provided a more complex picture than the simple consideration of FCR. The consideration of the environmental footprint of given production technologies is also a growing concern in aquaculture [14]. The Blue Growth Strategy of the European Union is turning out to be a global movement [15] and is in accordance with the United Nations Sustainable Development Goals set in 2015 [16].

Probiotics are relatively simple to define correctly: "a product containing microbiota in a quantity capable of producing a putative beneficial effect" [16]. As will be discussed later, this effect is mainly achieved by influencing the microbiome of the intestinal tract both in human medicine and animal husbandry. A wider definition was provided by Verschuere et al. [17] suggesting that: "it is a microbial supplement with living microorganisms, with beneficial effects on the host, by modifying its microbial community associated with the host or its cultivation environment, by ensuring improved utilization of the artificial feed or its nutritional value, enhancing the host response toward diseases and by improving its vigor in general". Probiotics are the functional feed additives group derived from different sources and include prebiotics, probiotics, seaweeds, mushrooms, microalgae, enzymes, organic acids, mycotoxin binders, phytobiotic compounds, and yeasts [18].

El-Saadony et al. [18] also collected several works from the literature defining probiotics. Felis et al. [19] provide an excellent summary of probiotics taxonomy that includes fungi. Ran et al. [20] found that both live and heat-inactivated baker's yeast had beneficial effects on Nile tilapia while live yeast showed advantages as a dietary supplement. Abdalkareem et al. [21] confirmed the probiotic role of the unicellular fungi Aspergillus niger in common carp since it improved growth, immunity, digestion, and fish hematology.

Probiotics are mostly living microbial cells, although heat-inactivated versions have also been proven to have benefits for the host. These beneficial microbes play a pivotal role in regulating health conditions directly and by helping the immune system, growth performance, and feed utilization of animals. Their use in aquafeeds was the first area of intense development that continues until now. However, the application of probiotics offers effective new and sustainable ways of maintaining good water quality and even increasing the biomass of natural food organisms in different pond cultures [22] as will be discussed later. In aquaculture, the use of probiotics is similar to that of terrestrial animals, but there are also significant differences due to the different environments. The more direct link between aquatic animals and their environment has even led to a broader interpretation of the concept of probiotics, including the environment [17]. This indicates the 109 exposures to aquatic pathogenic microbes (*Vibrio* sp., *Plesiomonas shigelloides*, *Aeromonas* sp.), which cause most mortality in cultivated crustacean and fish species and are also important from a human perspective as they can cause food poisoning. Limbu et al. [23] analyzed the systemic effects of antibiotics in cultured fish and their potential human health risk. In the spirit of this more holistic approach, Infante-Villamil et al. [24] stress the importance of maintaining a high diversity level in the microbiome of aquaculture animals (vertebrate and invertebrate).

Probiotics used in aquaculture constitute a significant part of a huge market of global probiotics in animal feed. This market was valued at USD 4.4 billion in 2020 and is estimated to reach USD 7.3 billion by 2026. The demand is projected to remain high due to the increasing awareness of their benefits [25]. This sound background certainly fosters scientific research and product development, too.

Developments in microbiome science have certainly opened new frontiers of research for probiotics, prebiotics, and postbiotics not only in human healthcare but also in aquaculture. Novel products and applications may significantly change the profile of good practice in many fields and even portend a new era in sustainable development. It is a difficult task to provide an overall summary of the recently published literature on this field—an ever-growing mass of information—where even excellent reviews can be found in great numbers such as [12,18,24,26–38], not mentioning some earlier ones.

The present work aims to offer an overview of the subject, focusing on the points of view articulated in the title.

2. The Microbiome of Aquatic Animals and Its Functioning

2.1. Main Characteristics of the Aquatic Microbiome

Natural water bodies and aquatic organisms living in them are occupied by a galaxy of microorganisms. The interaction between the microbes of the environment and the vertebrate and invertebrate organisms is constant. The interactions with positive or negative effects among microbial species in the gut of the animals also play an important role that profoundly affects health and vitality. The exogenous microbes also enter the host organism through the skin and gills that harbor symbiotic, commensal, and pathogenic bacterial communities thereafter. Protective mucous covers the surfaces that, with its resident microbiome, can protect against deleterious microbes [39]. The persistent microbiome provides the host with both immunogenic and metabolic integrity and functionality [37].

The main site of host–microbe interactions, however, is the gastrointestinal tract (GIT) and its colonization and functioning have major importance [37,40]. The concept and role of the core microbiome resulting from the coevolution of host and microbiota are discussed by Wuertz et al. [37]. The GIT of fish is populated by a bacterial load of about 108 bacterial cells per gram, which represent approximately 500 mainly aerobic or facultative anaerobe microbes [41]. Ringø et al. [42], for example, summarize results obtained using lactic acid bacteria and bacilli (LAB) in the aquaculture of crustaceans and fish listing in 14 papers published only between 2017 and 2019 that discuss the beneficial effects of *Lactobacillus* and *Enterococcus* species. The oxygen content in the fish gut is higher than in the human gut, which can explain the low abundance of anaerobic bacteria. *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* comprise the dominant proportion of the gut microbiota in most fish [1,43]. Herbivores' guts have the most diversified microbiomes because the digestion of cellulose needs bacteria such as *Clostridium*, *Leptotrichia*, or *Citrobacter* [44].

2.2. Probiotics' Role in Disease Control

Probiotics are used extensively in aquaculture for disease control, notably against bacterial diseases [45]. Probiotics' pathogen antagonism works in various ways. Probiotics can produce materials that prevent the reproduction of pathogens or kill them directly [46]. The most important postbiotics group is bacteriocins, which are proteinaceous or peptidic toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s). Although bacteriocins have been predominantly used as food preservatives, they are now receiving better attention as potential clinical antimicrobials and as potential immune-modulating agents [47–49]. Besides bacteriocins, the production of exoenzymes can also be an issue in the evaluation of potential probiotics [50]. Probiotics support the host organism's immune system and/or inactivate toxins produced by pathogens [51,52]. Probiotics compete with pathogens for nutrients and adhesion sites [53].

Bacterial probiotics play a key role both in the immune responses of the host animal and in the interaction between these responses and intestinal bacterial communities [54]. Montalban-Arques et al. [55] extensively describe the potential microbial strategies that improve the gut mucosal immunity in fish. According to them, the therapeutic approach mechanisms include a competitive exclusion for binding sites and translocation, enhanced barrier function by reversing the increased intestinal permeability, enhanced mucosal immunoglobulin IgT/Z response to enteral antigens, reduction in secretion of inflammatory mediators, stimulation of innate immune functions, stimulation of the release of antimicrobial peptides (AMPs) at the mucosal layer, enhancement of the availability of anti-inflammatory mediators by regulatory immune cells, production of metabolic health-enhancers such as SCFAs by non-digestible prebiotics, and diffusion of SCFAs through the enterocytes to improve mucosa barrier functions.

2.3. Probiotics for Enhancing Feed Utilization

Probiotic supplementation enhances feed utilization and weight gain in aquatic animals and stimulates the host's appetite and feed palatability by breaking down indigestible components, producing vitamins, and detoxifying poisonous compounds in the diet. Probiotics increase aquatic animals' resistance to stress caused by environmental and technological hazards [12,29]. Wang et al. [56] mention another important application of beneficial bacteria, namely serving alternative aquaculture feeds that provide micronutrients such as vitamins, fatty acids, and essential amino acids in addition to macronutrients to support the healthy growth of aquatic animals.

2.4. Concept of Synbiotics

Prebiotics play an important role in the effective functioning of probiotics, so it is justified to use the common name "synbiotics", which is frequently used in the medical and veterinary literature. Montalban-Arques et al. [55] explain the interactions during the preventive and curative probiotic treatments, stressing that adding a diet of exogenous microbial sources may increase fish health through a host–microbe positive loop. Commensal gut microbes might be modulated by dietary administration of target microbes, non-digestible elements, or a mix of both. The expected output should turn into preventive or curative strategies. The use of synbiotics is expected to restore the homeostatic stage, which is illustrated in Figure 1. The assessment of the selected approach might be quantified, modeled, or dissected using omics tools, germ-free models, and microbiome analyses.

Wuertz et al. [37] also profoundly discussed the probiotic action modes as illustrated and simplified below (Figure 2). Several reviews discuss in great detail all aspects involved in the beneficial effects of the probiotics mentioned above, providing lists of the microbe species responsible [27,32,37,41,57].

A recently published excellent review by Mougin and Joyce [38] provides an elucidating summary of disease etiology as a preliminary step toward the development of new prevention methods, underscoring the importance of the early identification of dysbiosis-associated biomarkers prior to any physical signs of the disease.

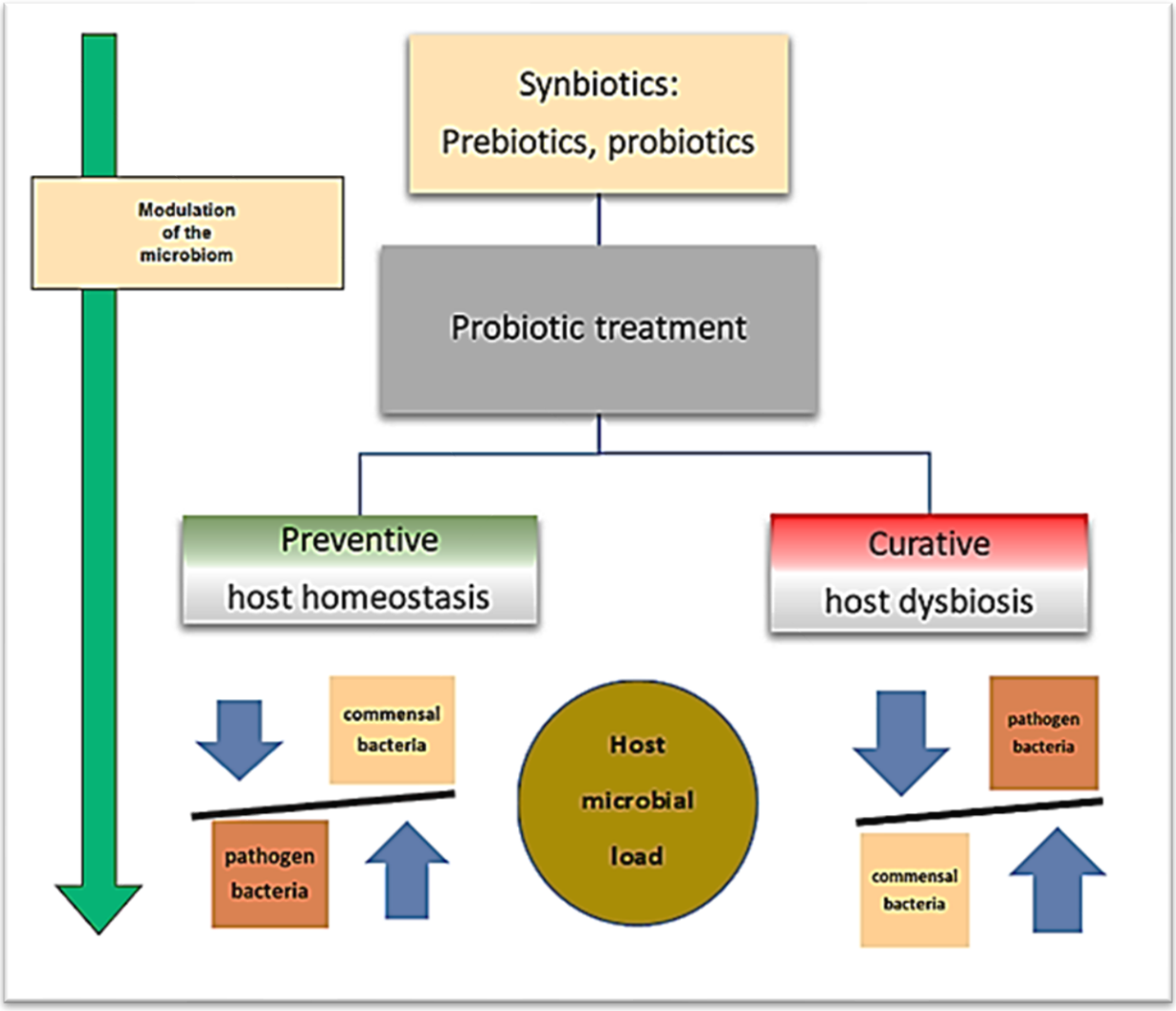

Figure 1. The action of synbiotics under health and dysbiosis (modified after Montalban-Arques et al. [55].

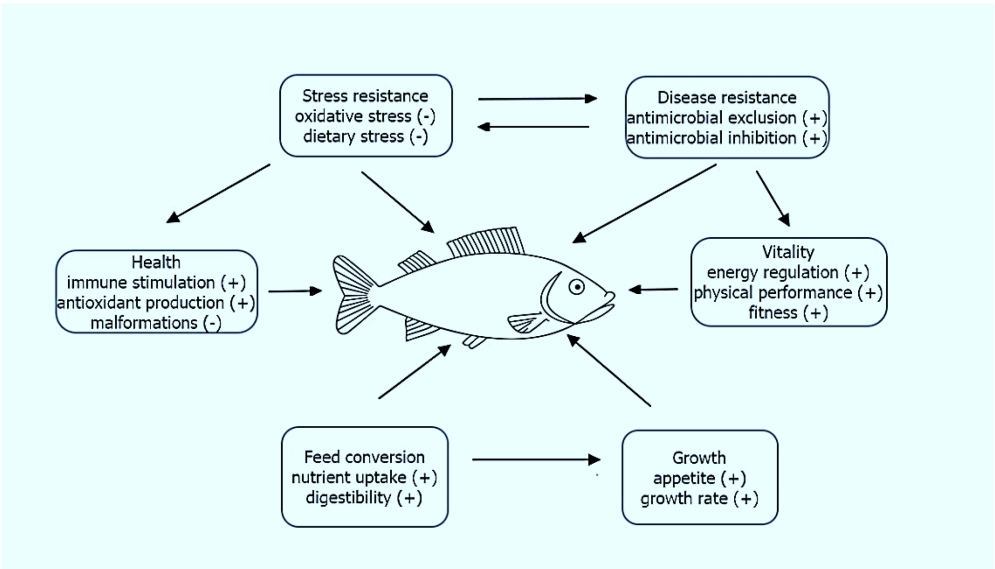

Figure 2. Probiotic modes of action (modified after Wuertz et al. [37]).

3. Aims and Methods of Probiotic Application

The main goals of using probiotics, similar to disease prevention and treatment, can be achieved via the same method in aquaculture. By adding them to the water, the following classification may be incoherent in some way. Moreover, different administration modes can be applied depending on factors such as probiotics used, supplementation form, vector of administration, dosage level, and duration of application [57]. However, Bidhan et al. [31] used a different logic for classifying probiotics that can also be justified.

3.1. Oral Administration Via Diet

Mixing probiotics into the feed is the most widely used method. Probiotics (and para- and postbiotics) can be applied in the feed or added to the tank or pond water to ensure protection against infection. It must be stressed that although the terms para- and postbiotics are not well defined yet, for the time being, the following definitions by Nataraj et al. [58] can be accepted. According to them, postbiotics are the complex mixture of metabolic products secreted by probiotics such as enzymes, proteins, short-chain fatty acids, vitamins, biosurfactants, amino acids, peptides, organic acids, etc. Parabiotics are the inactivated microbial cells of probiotics containing cell components such as peptidoglycans, surface proteins, etc. These have several advantages over probiotics such as availability in their pure form, ease in production and storage, and that they are even more likely to trigger only the targeted responses by specific ligand-receptor interactions. Vargas-Albores et al. [59] created an overview of the possible methods of therapeutic modulation of fish gut microbiota, focusing on probiotics but also including the use of phytogenics (derived from plants and their extracts) that also demonstrate beneficial effects on the gut microbiota of fish.

3.2. Bathing in Probiotics-Added Culture Water

Blending probiotics with water is also a viable option for disease prevention or treatment. Austin et al. [60] found that bathing in solutions of a probiotic strain of *Vibrio alginolyticus* effectively reduced Atlantic salmon diseases caused by *Aeromonas salmonicida, Vibrio anguillarum,* and *Vibrio ordalii*. According to Gram et al. [61], the inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2 proved effective when tested in vitro and in vivo with rainbow trout. The control of luminous *Vibrio* species in penaeid aquaculture ponds was successful using probiotic *Bacillus* that are able to produce antibiotics [62]. Spanggaard et al. [63] investigated the antibacterial properties of the indigenous microflora of rainbow trout (*Oncorhynchus mykiss Walbaum*) and found that a total of 1018 bacteria and yeasts can be isolated on tryptone soy agar (TSA) from the skin, gills, and intestine. Forty-five of these inhibited growths of the fish pathogenic bacterium *Vibrio anguillarum* in a diffusion assay. The antagonism was most prominent among *Pseudomonas* spp., as 66% of the antagonistic bacteria belonged to this genus, despite constituting only 15% of the total tested flora. The survival of rainbow trout infected with vibriosis was improved by 13–43% by six antagonistic strains out of nine, tested in vivo. All disease-protecting strains were pseudomonads, whereas two *Carnobacterium* spp. that were antagonistic in vitro well diffusion assays did not alter the accumulated mortality of the rainbow trout. They conclude that the addition of live bacterial cultures to fish-rearing water may improve the survival of the fish, however, the in vitro antagonism could not completely predict an in vivo effect. Gram et al. [64] came to a similar conclusion by studying the antagonistic effect of gut bacteria on pathogens in Atlantic salmon. Using *P. fluorescens* strain AH2, a strain showing strong in vitro inhibitory activity toward *A. salmonicida* but co-habitant infection by *A. salmonicida* in Atlantic salmon, did not result in any effect on furunculosis-related mortality. In the experiment of Mirbakhsh et al. [65], the larvae of white shrimp, *Litopenaeus vannamei,* were supplemented with the probiotic *Bacillus subtilis* IS02 in the rearing water to improve the survival rate. The probiotic was applied once every 3 days during the 21 days of breeding. It was found that the larval development stages and survival rate were significantly improved following the application of the 108 CFU mL^{-1} of the probiotic. The

total bacterial and presumptive *Vibrio* counts were numerically reduced and a considerable increase in the postlarvae vitality was observed.

3.3. Timing of Administration

Probiotic administration can be continuous or created at regular intervals. Most studies carried out continuous feeding of the host fish for a wide range of time, varying from 15 to 94 days [33]. There are very few results on the continuous application of probiotics so the important question of whether the probiotics can be permanent colonizers in the GIT cannot be answered yet.

3.4. The Application of Several Probiotics in Combination

Using multiple-strain products has the advantage of being active against a wider range of conditions and species [66,67]. Co-administrating probiotics with prebiotics and/or plant products are also widespread [68]. Dawood et al. [69] also used the term "synbiotics" for a product containing both probiotics and prebiotics and discussed the synergistic effect of prebiotics on live microorganisms. Recent attempts in fish feeding to apply fructooligosaccharide (FOS), mannan oligosaccharide (MOS), and inulin as a prebiotic, resulted in long-term health benefits and enhanced growth [42,69–71]. Hossain et al. [72] found that multi-species probiotics containing *Bacillus* spp. (1×10^9 CFU/mL) and *Lactobacillus* spp. (1×1011 CFU/mL) provided at concentrations of 0, 0.5, and 1.0 mL/L in water for 8 weeks enhanced the growth of Nile tilapia (*Oreochromis niloticus*) by upgrading gut, liver, and muscle health. Melo-Bolívar et al. [73] created a systematic review based on bibliographic searches in Scopus, Web of Science, and PubMed, evaluating 81 articles about multi-strain probiotics supplementation. Most of the articles showed different benefits, including enhancement of fish growth performance, immune response, and resistance against some pathogenic bacteria, such as *Aeromonas hydrophila, Streptococcus agalactiae*, and *Streptococcus iniae*; however, only 13 journal articles including a mono-strain probiotic as a control would allow direct comparison with a probiotic bacterial mixture to determine if the mixture offered higher benefits in comparison to a mono-strain probiotic and a control group without probiotic supplementation. They concluded that it is necessary to increase the number of studies on the potential use of strict and facultative autochthonous anaerobes as probiotics and since most of the studies in the journal articles examined were carried out on a laboratory scale, the results achieved on fish farms are highly needed.

3.5. Using Live Feed for Probiotics' Encapsulation

This method proved to be a viable and effective method since probiotics can even proliferate on the live feed. The enrichment of live feed such as rotifer [74], copepods [75], and *Artemia* [75–77] with probiotics proved to be a success. Gomez-Gil et al. [78] studied the effectiveness of encapsulating different *Vibrio* species in the nauplii of the brine shrimp (*Artemia franciscana*). The Artemia nauplii most effectively encapsulated a combination of *Pseudomonas synxantha* and *Pseudomonas aeruginosa* for *Penaeus latisulcatus* (Hai et al., 2010). In grouper, *Epinephelus coioides* larvae *Copepod* (*Pseudodiaptomus annandalei*) is a suitable vector of probiotics *Bacillus* spp. [75].

3.6. Improving Water Quality

Using probiotics for improving the quality of culture water is especially associated with *Bacillus* sp. because Gram-positive bacteria better convert organic matter back to CO_2 than Gram-negative bacteria. Dalmin et al. [79] found that high levels of Gram-positive bacteria can minimize the buildup of dissolved and particulate organic carbon and the use of *Bacillus* sp. improved water quality, survival, and growth rates, and the health status of juvenile *Penaeus monodon*. Findings by Hu et al. [80] showed that the core microbiome is involved in nitrite removal in shrimp culture ponds. Their results indicated that in eutrophic water of shrimp aquaculture, the core microbiome, including *Flavobacteriaceae* and *Rhodobacteraceae,* and other denitrifiers, play an important role in

nitrite cycling and maintaining the health of the aquaculture system. Bidhan et al. [31] discuss this subject of "water probiotics" in much more detail and group the literature found as "bioaugmentation", "bioremediation", and "bioreporters"; the latter one for groups of organisms whose behavior, growth, survival, and histopathological changes could be monitored to assess environmental changes. Jiang et al. [81] investigated the highly antagonistic *Aeromonas hydrophila* strain, *Bacillus methylotrophicus* WM-1, isolated from grass carp (*Ctenopharyngodon idella*). The grass carp were for 30 days at low (103 CFU/g feed), medium (105 CFU/g feed), and high concentrations (107 CFU/g feed). According to their findings, WM-1 is safe for water quality and culture objectives and has the effects of improving water quality and the immunity of grass carp. Hassan et al. [82] studied the effects of two probiotics on NH_3 degradation, as well as the magnetic field (21.56 m tesla) on the germination and proliferation of *Bacillus* spores. Additionally, the effect of these probiotics on water quality maintenance in *Litopenaeus vannamei* holding ponds was investigated. Overall, both probiotics were able to degrade NH_3 and the magnetic field was efficient to improve the germination and proliferation of *Bacillus* spores in vitro. Probiotics were also effective in reducing TVC and NH_3 levels by increasing dissolved oxygen and pH in pond water.

4. Screening and Testing of Novel Probiotics

Potential probiotics may be obtained from various sources such as the GIT of aquatic animals [83,84] and fish mucus [85] that are applied later as collected cultures [86] and commercial products [87,88]. The aquatic environment such as water or sediment also can serve as a source [87,89]. However, most of the results published until now provide information about probiotics originating from the above sources some papers are indicating that microbes of terrestrial origin also deserve attention [90].

Probiotics for aquatic animals should be tested as described in detail in the paper of El-Saadony et al. [18]. The in vitro trials have advantages over the in vivo ones in being simpler and cheaper. For the initial screening, the pathogen antagonism tests are considered suitable [91,92]. However, there are obvious limits in predicting the in vivo efficiency from the in vitro results as it was mentioned before [62–64]. Multi-parameters such as pathogenic antagonism, susceptibility to antibiotics, ability to produce lactic acid, and pH and bile salt tolerances were applied to select probiotics [92].

In the pathogenic inhibition methods, bacteriocins, siderophores, lysozymes, proteases, and hydrogen peroxides produced by probiotics are confronted with the in vivo culture of pathogenic bacteria [93]. The blood hemolysis is based on the fact that bacterial pathogens such as *Aeromonas* spp. and *Streptococcus* spp. normally found in the GIT of fish contain virulence genes (hemolysin and aerolysin) that hemolyze blood cells [94]. The hemolytic activity thereafter can be assessed using several blood types such as human, horse, sheep, fish, and shrimp hemolymphs [95].

Pathogenic microbes can develop specific resistance and/or multi-resistance against the inhibiting agents mentioned above. The resistance genes are inherited and can also be transferred to other bacterial species or strains using horizontal gene transfer. Microbial pathogens such as *E. coli*, *Enterococcus* spp. and *Salmonella* spp. have been found to have resistant genes [96]. Several other authors found that probiotic strains, such as *Bacillus* spp., showed resistance to penicillin and kanamycin and some LAB strains displayed multiple resistances against chloramphenicol, penicillin, kanamycin, and oxacillin [92,97]. Therefore, it was suggested that probiotics should be free of plasmid-encoded antibiotic-resistance genes.

Adhesion assays are also used to explore the potential of probiotics to adhere to fish mucus, epithelial cells, semi-solid media, hard substrate, gelatin, polystyrene, and bovine serum albumin. The adhesion has been evaluated in terms of bacterial adherence to solvents, hydrophobicity, or biofilm formation as is discussed in great detail by El-Saadony et al. [18] where conventional methods, such as the plate-count technique, a direct bacterial

count, optical density, or bacterial-labeled radioactivity, and auto-fluorescence monitoring, are mentioned, too.

The tolerance of probiotics to the harsh environment of the GIT is an important issue since digestive enzymes, pH variations, and bile salts may hinder its beneficial effects. Hlope et al. [98] found that the mucous cells in the GIT of Nile tilapia resisted the acidity well and tolerated pH values ranging from 1.58 to 5.0 in the stomach. The changes in pH ranging from 1 to 7.8 in the intestinal tract of fish are normally caused by pepsin activity, while the pH values higher than 7.8 may occur during the lipid activity. The Bacterial isolates tolerating low pH are important in probiotic selection. Wanka et al. [99] isolated and characterized native probiotics from the digestive system of three flatfish species and provided a viable strategy that can be adapted to other farmed fish species. Using 16S rRNA gene sequencing for 195 isolates, 89.7% of the Gram-negatives belonging to the Alpha- (1.0%), Beta- (4.1%), and *Gammaproteobacteria* (84.6%) were identified. The candidate probiotics were further characterized using in vitro assays of characterization such as inhibition of pathogens, degradation of plant-derived anti-nutrient (saponin), and the content of essential fatty acids (FA), and their precursors. The cost-effective method to coat feed pellets used provided high viability of the supplemented probiotics over 54 days of storage at 4 °C.

The in vitro testing of potential probiotics has to be followed by in vivo trials as perfectly demonstrated by Burbank et al. [100] who evaluated the efficacy of non-pathogenic probiotic bacterial strains to reduce mortality due to *Flavobacterium psychrophilum* infection in rainbow trout causing coldwater disease (CWD), also known as rainbow trout fry syndrome (RTFS). The candidate probiotics, previously demonstrating in vitro inhibition of *F. psychrophilum* and the potential to survive in the GI, were administered through an oil-dressed feed. Two of the ten probiotic strains showed a significant reduction in the mortality of the fish. Both were consistently reisolated from the GI tract proving their ability to persistently colonize the GI. The 16S rRNA sequences from the strains were distinct but very similar varieties of *Enterobacter amnigenus*. However, in some studies, probiotic cells have been injected intraperitoneally into the fish first, to test the mortality for evaluating the safety of potential probiotics before in vivo trials [101,102]. Hassan et al. [82] evaluated the inhibitory effect on pathogen growth and the decrease in concentrations of waste ions and found that based on 16S RNA sequence homology, the isolates were identified as *Bacillus subtilis, Bacillus cereus,* and *Bacillus licheniformis*, respectively.

The steps of the process leading to developing a commercial probiotic product are prominently described by Balcázar et al. [26]. Most of these steps were discussed above but the finishing phase following the challenge tests against pathogenic strains, namely the economic evaluation and registration procedures, are also important.

In the study of Lalloo et al. [103], natural isolates obtained from mud sediment and *Cyprinus carpio* were purified and assessed in vitro for efficacy based on the inhibition of growth of pathogenic *Aeromonas hydrophila* and the decrease in concentrations of ammonium, nitrite, nitrate, and phosphate ions. Based on suitability to predefined characteristics, three isolates were selected and evaluated in vitro in the presence of *Aer. hydrophila* and in a preliminary in vivo trial with *C. carpio*. These promising results also showed how multispecies probiotics can effectively serve multiple aims.

5. Scientific and Technical Novelties in Probiotics Research

5.1. Metagenomics

According to the definition of the National Human Genome Research Institute, metagenomics is the study of the structure and function of entire nucleotide sequences isolated and analyzed from all the organisms (typically microbes) in a bulk sample. It can shed light on the structure of the microbiome community and its changes, the isolation of different bacterial strains, and their functioning which is a key concern in a wide variety of investigations. The availability of gene-based molecular tools such as 16S rRNA and whole-genome sequencing, moreover next-generation sequencing (NGS) technology, including amplicon

and shot-gun approaches with bioinformatics skills, have enabled it to be possible to count and classify a commensal microbiome at the species level [104]. Yukgehnaish et al. [105] provide a detailed overview of factors influencing the gut microbiome and its physiological role in fish discussing also the role of prebiotics and the consequences of using antibiotics. Metagenomics offers effective means to understand the microbial diversity in aquaculture facilities by studying the hypervariable regions of 16S rDNA for prokaryotes and 18S for eukaryotes [106]. The main areas of microbiome research using metagenomics are summarized in Table 1.

Table 1. Main areas of using metagenomics in microbiome research.

Research Area	Methodology Used/Results	Source
***Evaluation of antibiotic resistance in bacterial communities*—** general reviews review on fish	Specific and multiplex PCR, real-time PCR, DNA sequencing, and hybridization-based techniques. 9 + 6 ARGs were detected by qPCR in the gut, mucosal skin, and gill filaments in four mariculture systems.	Schmieder and Edwards [107]; Zhang et al. [108] Zhang et al. [109]
Virus studies using metagenomic methods General reviews Aquaculture review (viral genomes and virus–host interactions)	Epifluorescent microscopy counts of virus-like particles (VLPs), transmission electron micrographs—on reclaimed water. In silico study, BLAST program and viral databases, Illumina Genome Analyzer—on human pathogens. More than 100 viral genomes have been sequenced and genetically characterized in aquaculture animals.	Rosário et al. [110] Bibby et al. [111] Zhang and Gui [112]
Evaluating seasonal dynamicsof bacterial communities	Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments, fluorescent in situ hybridization (FISH) to find a critical time period when phage therapy should be applied. 16S rDNA V4 hypervariable region to evaluate microbial community composition. The results suggested that seasonal shifts and wastewater pollution together shape the structures of the microbial communities.	Pereira et al. [113] Wang et al. [114]

Table 1. *Cont.*

Research Area	Methodology Used/Results	Source
Absolute quantification of priority bacteria	Digital PCR. The use of TaqMan® probe technology allowed for the development of multiplex assays capable of simultaneous quantification of priority bacteria.	Netzer et al. [115]
Characterization of probiotic strains: safety, DNA fingerprinting, and bacteriocinogenicity	Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) fingerprinting allowed the clustering of the *Pediococcus acidilactici* strains. Two bacteriocinogenic strains were identified.	Araújo et al. [116]

5.2. Applying State-of-the-Art Genetic and Biotechnological Technologies

Introducing the newest methods is an urgent need for global aquaculture to combat disease and parasitism that cause serious welfare, environmental, and economic concerns. Robinson et al. [117] discuss this issue in a holistic approach demonstrating case studies of sea lice infestations in salmonids and white spot syndrome in shrimp. They summarize the up-to-date genomic technologies with potential applications such as GWAS/genome-wide association studies, scRNA/Single-cell RNA sequencing, snRNA/Single-nuclei RNA sequencing, CHIA-PET, chromatin interaction analysis with paired-end-tag-sequencing, ATACseq/assay for transposase-accessible chromatin with high throughput sequencing/CHIPseq/chromatin immunoprecipitation sequencing, WGBS/whole-genome bisulfite sequencing, and RRBS/reduced representation bisulfite sequencing; these are adequate to explore the genetic basis of host resistance. The application of gene editing and the use of semiochemicals are also evaluated as well as vaccination, in their review quoting almost seven hundred references. The current state of knowledge of biotechnological approaches that help to better understand and engineer the fish microbiome and host–microbe interactions are discussed by Luna et al. [118]. Their overview includes important issues from research in the field of gnotobiotic fish, in vitro and ex vivo manipulation, and fecal material transplant.

6. Conclusions

1. Fish microbiome research is moving from its descriptive era toward more experimental and manipulative approaches. However, the effective integration of the descriptive studies with the newest ones that are based on methodologies such as in vitro gut simulators, synthetic microbial communities, and in vitro and in vivo systems is needed for improving production and significantly lowering the risks in production. A more complete understanding of compositional and functional alterations of the microbiome and their effects on health and safety remains an everlasting goal. The beneficial effects of this approach certainly can improve practices in areas such as novel feed design, fighting against antimicrobial resistance and transfer, and management of pathogens. Better knowledge of aquaculture species' microbiomes will contribute to implementing more sustainable aquaculture systems with lower environmental footprints.
2. Microbiome diversity is certainly correlated not only with numerous health issues of the animals but metabolism, growth, and reproduction. The developments in microbiome science have certainly opened new doors to research for probiotics, prebiotics, and postbiotics in aquaculture. Novel products and applications may significantly change the profile of good practice in many fields from disease prevention to water quality management and open a new "blue" era in sustainable development.

3. Much of the ongoing research aims to understand the relationship between disease and specific taxa of microbial species for optimizing the gut microbiota composition to mitigate disease. There is also a need for more research on microbiome composition, evaluating its diversity of various culture and ambient factors. The availability of gene-based molecular tools such as DNA sequencing and NGS technology associated with bioinformatics knowledge causes it to be possible to classify the commensal microbiome. The gut microbes that have been shown to have a positive effect on health can be used as probiotic candidates.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: This work was supported by the Ministry of Human Resources of Hungary (EFOP-3.6.3-VEKOP-16-2017-00005).

Conflicts of Interest: The author declares no conflict of interest.

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