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Review

Probiotics in Fish Nutrition—Long-Standing Household Remedy or Native Nutraceuticals?

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Abstract: Over the last decades, aquaculture production increased rapidly. The future development of the industry highly relies on the sustainable utilization of natural resources. The need for improving disease resistance, growth performance, food conversion, and product safety for human consumption has stimulated the application of probiotics in aquaculture. Probiotics increase growth and feed conversion, improve health status, raise disease resistance, decrease stress susceptibility, and improve general vigor. Currently, most probiotics still originate from terrestrial sources rather than fish. However, host-associated (autochthonous) probiotics are likely more persistent in the gastrointestinal tract of fish and may, therefore, exhibit longer-lasting effects on the host. Probiotic candidates are commonly screened in in vitro assays, but the transfer to in vivo assessment is often problematic. In conclusion, modulation of the host-associated microbiome by the use of complex probiotics is promising, but a solid understanding of the interactions involved is only in its infancy and requires further research. Probiotics could be used to explore novel ingredients such as chitin-rich insect meal, which cannot be digested by the fish host alone. Most importantly, probiotics offer the opportunity to improve stress and disease resistance, which is among the most pressing problems in aquaculture.

Keywords: probiotics; aquaculture; microbiome; living gut; species-specific bacteria; mode-of-action

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1. Introduction

In view of stagnating fishery landings reported over the past 50 years, only the rapidly developing aquaculture industry can meet the increasing per capita demand for fish worldwide. Over the past decades, global aquaculture production has nearly doubled every ten years, which reflects the fastest growth in the food-producing sector [1]. Undoubtedly, the sustainable utilization of scarce natural resources is a challenge for the future development of the industry. Among the obstacles for future expansion, fish nutrition and the management of fish diseases and health are among the most critical. Sustainable development of the industry requires advanced disease and health management because the aquatic environment renders fish particularly susceptible to ubiquitous pathogens [2]. However, the administration of drugs such as antibiotics is associated with human health concerns, and prophylactic alternatives are highly desirable.

Feeding costs represent 40–70% of expenditure in intensive fish farming [3], mainly attributed to the protein-rich ingredients. In the past, fishmeal was the main protein source in fish nutrition, but, nowadays, it has become a scarce, costly ingredient. As a consequence, but also with regard to the vulnerable status of several industrial species, such as the Peruvian anchoveta, alternative plant ingredients are used in the diets [4–6]. Unfortunately, plant-based ingredients can have several negative effects on fish nutrition that involve the antinutritional effects of secondary plant metabolites, suboptimal amino acid composition, as well as mineral imbalances, which, in turn, may impact health and immune status [7–9]. Such restraints can be remedied, at least partly, by improving the digestion of these feedstuffs by making use of probiotic supplements and adjusting the gut microbiota.

In 1907, Metchnikoff was the first to point out the positive role of bacteria in milk and yogurt products. He assumed that these beneficial bacteria replace harmful microbes and are, therefore, responsible for the prolonged life of Balkan farmers who consumed high quantities of these products. In 1953, Kollath introduced the term probiotics, originating from the Latin word pro and the Greek word bios "for life" [10]. Traditionally, probiotics have thus been regarded as bioactive food additives, especially living bacteria, which have a positive influence on digestion and, moreover, the microbiome of the gastrointestinal tract (GIT) in general [11]. Verschuere et al. [12] expanded this definition, stating that probiotics are live microbial additives that have a beneficial effect on the host by (1) modifying the host-associated microbial community, (2) ensuring improved use of the feed or enhancing its nutritional value, (3) enhancing the host response towards disease, and/or (4) improving the quality of its ambient environment. Merrifield et al. proposed a slightly modified definition for probiotics in aquaculture [13]. They defined that "a probiotic organism can be regarded as a live or dead component of a microbial cell, which is administered via the feed or to the rearing water, benefiting the host by improving disease resistance, health status, growth performance, feed utilization, stress response, or general vigor. This is achieved, at least in part, by improving the host's microbial balance or the microbial balance of the ambient environment".

Intensification of aquaculture production exacerbates health threats of infectious diseases, including those arising from immunosuppression by plant ingredients. Over the last two decades, disease management has addressed new vaccines, immunostimulants, and disinfection strategies; in particular, probiotics have a huge potential in today's disease management strategies. Administered probiotic strains can counteract the colonization of pathogens by competitive exclusion. This may involve either competition for binding sites, synthesis of antibacterial compounds, immune stimulation, or competition for nutrients [14]. Detailed reviews on the probiotic species and the respective fish host species have been provided elsewhere [14–19]

In this review, we summarize the current knowledge and recent findings in probiotic research. In particular, we address the concept of the core microbiome of the digestive system, discuss the utilization of host-associated, native (autochthonous) bacteria, and present modes of action that focus on the main site of host–microbe interaction, the gastrointestinal tract (GIT).

2. The "Living Gut" and Its Core Microbiome

A proverbial galaxy of living microorganisms colonizes every aquatic habitat and every aquatic organism. Constant confrontation and interaction with the microbes of the environment surrounding an individual are an unavoidable fate throughout its life, starting from an immature egg to the adult animal. The taxonomic composition of the ambient microbiota is, therefore, highly variable, depending on climatic and seasonal conditions, including environmental factors such as salinity or temperature [20–22]. Aquatic organisms, such as fish, experience this variability and face continuous microbial invasions. However, to maintain its basic intestinal functionality, the host also actively selects for symbiotic and commensal microorganisms. Therefore, the gut microbiota is influenced by both external factors and the selective pressure exerted by the host [23–26]. Compared to the surrounding environment, the microbial communities associated with an organism are regarded as relatively stable, particularly those in the GIT [27,28], which assures metabolic, nutritional, and immunologic functionality. Nevertheless, to a certain degree, variable chemical and physiological factors (e.g., pH, salinity, temperature) influence its composition dynamically [24,29–31]. Depending on the duration of their presence, either temporarily or permanently, microbes are referred to as transient or persistent.

Potential microbial colonization in teleosts starts at the exposed host surfaces and their apertures. In fish, these are primarily the skin, gills, and GIT, with mouth, pharynx, and intestinal compartments. These surfaces are covered with protective mucous, which, in association with a specialized resident microflora, usually prevents the penetration of deleterious microbes [32]. Often, the persistent microbes strongly associated with the intestinal membrane are symbiotic bacteria [33]. Indeed, persistent microbes in their entirety must provide the host with immunogenic and metabolic integrity and functionality. However, due to food intake and the large surface area, the GIT is the main entry site for exogenous microorganisms, symbiotic, commensal, and pathogenic.

The microbial interactions within the GIT drastically affect the development and performance of the host animal. It has been shown that the GIT crucially affects nutritional conversion, gut physiology, and immune and stress response [34–36]. The pioneering work of Rawls et al. [37,38] identified a core microbiome in zebrafish for the first time. Accordingly, striking similarities in the microbiome of wild and farmed fish exist, which implies a role for host selection of microbiota [39]. The concept of a core microbiome also suggests that the coevolution of the microbiota and the fish host is stronger than the possible influence of environmental bacteria. Several studies on gut bacterial community censuses agree that the fish GIT harbors a bacterial load of approximately 10^8 bacterial cells per gram [40–44], representing ~500 species that consist mainly of aerobic or facultative anaerobe microbes. Indeed, the oxygen content in the fish gut is higher than in the human gut, which, in part, explains the low abundance of anaerobic bacteria [45]. In many fish species, Bacteroidetes, Firmicutes, and Proteobacteria comprise the dominant proportion of the gut microbiota [46–48]. Among fish, herbivores harbor the highest diversity of the microbiome [49] because they require bacteria such as *Clostridium*, *Leptotrichia,* or *Citrobacter* to support the digestion of plant-derived cellulose [50]. Due to its compartmentalization and microstructuring, the GIT provides multiple habitats with varying pH and O_2 concentrations and, hence, realizes multiple ecological niches. This explains the huge species diversity observed. Recent studies imply that the higher the microbial diversity in the gut, the healthier the gut [51,52].

Although there is a broad consensus on the concept of a core microbiome, gut composition shifts dynamically. For example, the diversity of the gut microbiome in zebrafish and Southern catfish increases with host age [36,53], while the microbial diversity in *Oncorhynchus mykiss* decreases with a reduction in nutrients [54]. Surrounding habitats, such as water and sediment, are the reservoirs for gut microbiota acquisition and enrichment [55,56]. Although the taxonomic composition of the microbiota in terms of species composition differs substantially, the functional composition measured from bacterial RNA data is well conserved [49].

3. Species-Specific, Native Probiotics

Most of the probiotics used in aquaculture do not originate from the aquatic host organism itself but from terrestrial sources or different environments. Some evidence suggests that host-associated, native (autochthonous) probiotics reveal a higher performance than those isolated from other sources (allochthonous). One possible explanation is that autochthonous probiotics are better adapted to their natural habitat, the gut, than allochthonous probiotics. Hence, autochthonous probionts are expected to be readily able to colonize the host's GIT and perform to a better extent [57]. Furthermore, microorganisms seem to obtain the highest physiological activities in their natural habitat [58]. In addition, the survival rate of probiotics is likely higher when they are applied in their natural environment [57]. This suggests that large proportions of the gut microbiota have coevolved with the host and consequently reveal some degree of species-specificity. Indeed, interactions between multicellular organisms and microorganisms may promote beneficial mutations to the bacteria [46].

In the past, most studies regarding the specificity of probiotics have focused on lactic acid bacteria (LAB); host specificity of the adhesion and colonization of microorganisms remains controversial. Fuller was the first author to state that epithelial attachment is host-specific [11], but later studies reported that LAB from one host may indeed adhere to the epithelium of another species [59–63]. In sturgeon, the host specificity of LAB has been reported repeatedly [64,65]. This led to the controversial hypothesis that specificity is most apparent in ancient taxa such as sturgeon, whereas modern teleosts do not exhibit such stringent specificity [19]. Nevertheless, due to their beneficial effects on livestock and humans, several allochthonous LAB strains of terrestrial origin have been used successfully in fish [19]. Such an administration is particularly effective during early development [66]. Nevertheless, disruption of gut integrity occurs for some LAB strains [65]. It seems, therefore, advisable to check probionts for pathomorphological modification of the gut during their evaluation. Several studies suggest that LAB strains are suitable probiotic candidates due to their ability to withstand acidity and bile salts and adhere to the gut, their lactic acid production (which partially inhibit the growth of pathogenic bacteria), and their strengthening of the mucosal barrier. Still, one should keep in mind that other studies could not confirm the beneficial effects (particularly when LAB from terrestrial sources were used), which, in part, may be attributed to the relative aerobic conditions in the fish gut (compared to terrestrial animals) [45]. Here, LAB may be outcompeted by other bacteria.

Mucus is a gel-like layer that functions as a transit tissue and pathogen barrier [67,68]. It consists mainly of water, glycoproteins, lipids, and salts. Among the glycoproteins, mucins are important for the successful adherence of microbes. Not surprisingly, *Lactobacillus* species were reported to adhere to mucin as well as intestinal cells with specific surface-associated proteins, including mucus-binding proteins (MUBs), surface layer proteins (SLPs), surface-layer-associated proteins (SLAPs), and moonlighting proteins [69–71]. As a screening criterion, hydrophobicity is a desirable trait of probiotics that should generally be used in the analysis [72,73].

The isolation of probiotic candidates from fish relies on culture-dependent techniques using selective and nonselective growth media. In practice, one should use several media and differing culture conditions (e.g., nutrient and pH gradients) in order to increase the diversity of isolates [74]. In fish, due to the dominance of aerobe and facultative anaerobe bacteria in the gut (see above), but also because anaerobic bacteria are difficult to handle, aerobic and facultative anaerobic bacteria are the prime targets of screening. Once a probiotic candidate is established, the cultivation protocol can easily be adapted to biotechnological production in a bioreactor. Most investigations on host-associated bacteria have used farmed fish because the isolation of bacteria from wild fish must be performed in the field and sterile work near the capture site is often difficult. Previous findings indicate that the use of multistrain probiotics improves functionality and efficacy. Provided that mutual inhibition can be excluded, the combination of multiple beneficial isolates is recommended [75–77].

4. Modes of Action

Beneficial effects for finfish farmers exerted by probiotic applications encompass nutritional, metabolic, and health effects. This particularly includes increased growth performance and appetite, enhanced food conversion (e.g., by an enzymatic contribution to digestion), improved feed value (macro- and micronutrients made available by the probiotic), inhibition of pathogenic microorganisms (adherence and colonization), stimulation of the immune system, increased stress resistance, and improved general vigor (Figure 1). Furthermore, higher reproductive output, reduced malformations, and higher flesh quality have been reported.

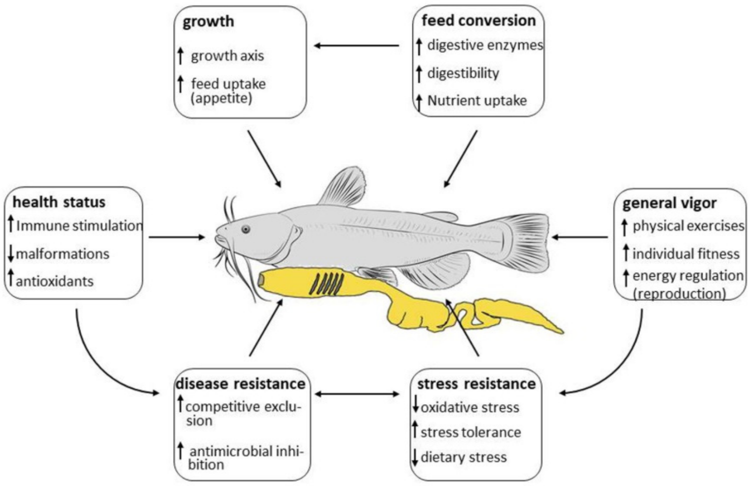

Figure 1. Mode of action of probiotics, including increased growth performance and appetite, enhanced feed conversion (e.g., by secretion of microbial digestive enzymes) or improved feed value (macro- and micronutrients), increased stress tolerance, improved disease resistance due to the inhibition of pathogenic microorganisms (adherence and colonization), improved health status by stimulating the fish's immune system, and enhancement of general vigor.

4.1. Growth

Numerous studies have found that probiotic administration often results in improved growth performance [78–82]. In this regard, probiotics can act either directly by increasing the appetite and growth regulation or indirectly via improved digestibility (see feed conversion). In tilapia, weight can increase by up to 115.3% [78], but growth performance might have been linked to the better feed conversion reported. Nevertheless, probiotics actually stimulate the growth axis, increasing the transcription of insulin-like growth factor 1 (IGF-1) and the growth hormone receptor [81]. Furthermore, metabolism-related key enzymes such as glucokinase, hexokinase, glucose-6-phosphatase, and pyruvate kinase were upregulated in the host. In addition, an extract of probiotic *Rhodobacter sphaeroides* induced growth-associated genes and stimulated muscle growth, in particular [83]. The use of an additive containing *Bacillus subtilis*, *Bifidobacterium bifidum*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus lactis*, *Lactobacillus plantarum,* and *Pediococcus acidilactici* reduced the expression of myostatin, thereby enhancing the growth of white muscle [80]. Still, in a study on *Lactobacillus acidophilus*, the food-intake-stimulating hormone ghrelin was downregulated [84]. In contrast, correlated to increased food intake, Giorgia et al. [85] reported an upregulation of orexigenic genes (neuropeptide Y, agouti-related protein, ghrelin) and a decrease in anorexigenic leptin. In this context, we recommend that potential probiotics for use in practice be routinely tested for palatability [86,87]. If one detects any adverse effects, it is advisable to enhance the taste and, thus, the appetite with stimulating additives. In conclusion, probiotics modulate the regulation of growth and appetite, either directly or indirectly (Figure 1). Additionally, the host-associated microbiota seems to play an important role in the proper development and differentiation of gut components. Here, an optimal microbiota is suggested to positively influence the proliferation of epithelia cells, including the formation of mucosal layers [38,88]. For example, Merrifield et al. [89,90] observed that feed-supplemented *Pediococcus acidilactici* significantly enlarged the absorptive surface of the gut via an increased microvilli length in the proximal gut of rainbow trout, whereas *Bacillus subtilis*, *Bacillus licheniformis,* and *Enterococcus faecium* did not exhibit such an effect.

4.2. Feed Conversion

There are several studies on probiotics that report increased feed conversion, but the actual mechanism is rarely revealed [91,92]. Significantly, germ-free zebrafish were arrested in their differentiation and were subsequently unable to absorb proteins [88].

Only establishing a complex microbiota restored nutrient uptake, suggesting that the gut microbiota contributes substantially to the nutrient uptake and assimilation of the host. Probiotics also convert less degradable compounds into forms that can be easily digested by the host (Figure 2). Here, various microbial enzymes, such as lipases, phytases, amylases, cellulases, trypsin, and other proteases, can be involved [93–97]. In addition, microbes can stimulate the activity and secretion of host enzymes directly [98–100]. In modern aquafeeds, supplemented with high amounts of plant ingredients, specific probiotics may increase the digestion of feed components such as nonstarch hydrocarbons, cellulose, or chitin, which are indigestible for the fish host. In addition, probiotics such as LAB may be sources of vitamins [101–103]. However, it remains controversial if the host actually absorbs these vitamins [104]. Furthermore, bacteria may represent a source of PUFA, but concentrations vary substantially between bacteria species [74]. The *Vibrio* species are especially rich in EPA and DHA [105–109]. Interestingly, high contents of DHA are observed particularly in deep-sea fish and seem to be an evolutionary adaptation towards high pressure and low temperature. Although screening techniques have improved, screening for PUFA-producing bacteria is not commonly addressed [74,110,111]. These examples show that probiotics can increase the nutritional value of the feed by increasing digestibility or providing microbial metabolites such as cofactors, vitamins, or essential fatty acids.

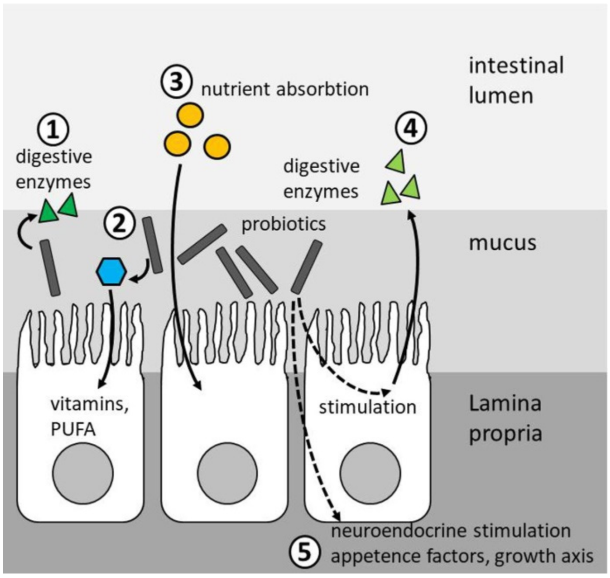

Figure 2. Nutrition- and growth-related effects (modes of action) of probiotics in the gastrointestinal tract (GIT), including direct effects such as (1) secretion of digestive enzymes; (2) absorption of (micro)nutrients such as cofactors, vitamins, and polyunsaturated fatty acids; (3) indirect effects, including elevated nutrient uptake/absorption; (4) stimulation of enzyme secretion, as well as (5) neuroendocrine stimulation of appetence and growth. For further details, refer to the text.

4.3. Stress Resistance

Stress resistance and immune stimulation may translate into improved disease resistance, observed upon pathogen challenge. Therefore, in practice, it is often difficult to differentiate between these modes of action. Here, we will focus on the probiotic effects that modulate the hypothalamus–pituitary–interrenal axis (HPI axis) and regulate the oxidative stress system. In fact, the use of probiotics in counteracting stress is desirable since stress in the rearing facility often translates into an increased risk of disease transmission. In Nile tilapia, administration of *Saccharomyces cerevisiae* and *Bacillus* spp. revealed lower plasma cortisol concentrations upon transportation stress, suggesting better stress resistance after probiotic treatment [112]. Similarly, after an evoked stress, sea bream larvae pretreated with *Lactobacillus plantarum* and *Lactobacillus fructivorans* exhibited lower plasma cortisol levels [113], suggesting higher stress tolerance upon probiotic treatment. Since cortisol has an immunosuppressive effect on antibody production, there is a clear linkage with the health status (Figure 1). In a stress challenge trial, yeast-fed fish exhibited high tolerance to acute heat stress as well as hypoxia exposure [114], but neither cortisol nor components of the oxidative stress defense were determined. Still, typical signs of stress such as the darkening of the skin or behavioral responses such as disoriented swimming, accelerated ventilation, or flaring of the gills indicate an increased stress tolerance after probiotic treatment, but no quantitative data exist. In gnotobiotic European sea bass (*Dicentrarchus labrax*) larvae, *Vibrio lentus* in the diet lowered the glucocorticoid baseline level [34]. Similarly, feeding of LAB *Lactobacillus delbrueckii delbrueckii* resulted in lower cortisol levels [115]. Before and after a hypoxia challenge, decreased glucose and cortisol levels were also reported in Nile tilapia treated with *Aspergillus oryzae* [116]. Controversially, antioxidant enzymes, as well as heat shock protein HSP70, increased upon treatment. In contrast, in zebrafish treated with *Bacillus amyloliquefaciens* R8, mRNA of antioxidant enzymes and HSP70 decreased [117]. Lower oxidative stress levels were also observed in zebrafish after probiotic treatment [118], suggesting increased hepatic stress tolerance.

4.4. Health Status

We distinguish between an immunostimulation of the host's immune system and direct interactions of the probiotic with a respective pathogen after challenge. Below, we refer to the latter as disease resistance. Still, there is a close interlinkage (Figures 1 and 3). For example, probiotic-treated fish revealed an increased expression of innate immune-related genes (IL-1β, IL-6, IL-21, TNF-α, TLR-1, -3, and -4) and, after exposure to pathogens *Aeromonas hydrophila* and *Streptococcus agalactiae*, showed a higher survival rate than control fish, which confirms that the immune stimulation leads to an enhanced immune response towards pathogens [117]. On the other hand, immunostimulation, including lysozyme, serum peroxidase, alternative peroxidase, phagocytosis, and respiratory burst activities, may not result in increased survival upon pathogen challenge [119]. Still, it is widely accepted that the stimulation of innate immunity in terms of either gene or protein synthesis, enzyme activity, or cellular response may benefit the organism during pathogen exposure. Immune stimulation frequently involves immune parameters such as a higher number of leukocytes [120,121], increased phagocytic activity [122–124], respiratory bursts [122,125], immunoglobulin M (IgM) [126,127], ß-defensins [128], proinflammatory cytokines (interleukin IL-8 and IL-1 β) [124,129], and the modulation of immunity-related genes [129,130]. Probiotics can stimulate elements of the nonspecific immune system, such as mononuclear phagocytes (monocytes, macrophages), neutrophils, and natural killer (NK) cells [131]. It seems that different probiotics have different effects on the expression of immune-related genes of a respective fish species [79]. Still, differences could also be attributed to differences in experimental conditions and the strains of fish used.

Among the components of the innate immune response, the mucosal epithelium is one of the oldest and most common. Accordingly, mucin glycoproteins form a physical barrier that covers the epithelium and prevents the adhesion of pathogens. Mucin-producing cells influence the distribution of the microbes by secretion of antimicrobial peptides (AMPs). In striped catfish *Pangasianodon hypophthalmus*, the administration of a *Bacillus* mixture increased AMP levels [132]. Germ-free fish can exhibit both an undeveloped immune system and a downregulated immune response due to undeveloped intestinal vasculature and gut-associated lymphoid tissue (GALT) [133]. However, the immune status is largely restored once the microbiota is re-established [37,88].

Additionally, some microorganisms produce volatile, short-chain fatty acids (SCFAs), mainly acetic, propionic, and butyric acids, which stimulate the immune system of the fish [134,135]. Gut cell proliferation and differentiation, apoptosis, mucin production, and lipid metabolism are largely mediated by SCFAs. Moreover, SCFAs are potent immunostimulators and improve lymphocyte function. Indeed, SCFAs play a key role in the modulation of the immune system in higher vertebrates, including T-cell differentiation, stimulation of heat shock proteins, and evocation of immune-related effects upon binding to G-coupled protein receptors, which are expressed in neutrophils, macrophages, and monocytes [136,137].

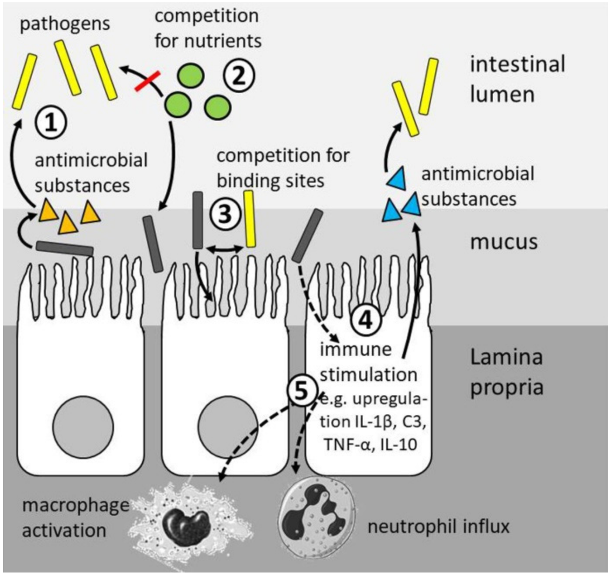

Figure 3. Health- and disease-resistance-related effects (modes of action) of probiotics in the gastrointestinal tract (GIT), including direct effects such as (1) secretion of antimicrobial substances, (2) competition for nutrients, and (3) binding sites, as well as (4) indirect effects such as stimulation of immune parameters and (5) activation of macrophages and the influx of neutrophils. For further details, refer to the text.

4.5. Disease Resistance

In recent decades, efficient vaccines against common viral, bacterial, or fungal diseases infecting commercial livestock have been developed. However, especially in the early life stages, fish vaccination is not possible due to an incomplete immune system [63]. Larvae and fry are most susceptible to pathogens at these stages, and the resulting mortality is highly relevant [138,139]. Here, probiotics may be particularly useful.

Most surveys on probiotic applications in aquaculture consider the ability to inhibit or exclude pathogenic bacteria. The majority of the studies addressing disease resistance use in vitro assays to identify antagonistic effects as one of the most important selection criteria for candidate probiotics. However, it is essential to confirm such effects in an in vivo challenge experiment. Improved resistance towards pathogens, expressed as higher survival rates, occurs in several aquaculture species, including tilapia [124,140], carb [141,142], rainbow trout [143], cod [144], and several other species.

Prophylactic treatment of the host can lead to competitive exclusion of pathogen bacteria. Here, the probiotics compete with the pathogens for nutrients, oxygen, and binding sites to mucosa or epithelial surfaces (Figure 3). Thereby, bacteria are able to prevent potential infections. Competition for binding sites inhibits pathogen adherence and colonization, involving factors such as hydrophobicity, electrostatic interactions, lipoteichoic acids, and passive and steric forces [145]. Indeed, Brunt and Austin [146] demonstrated that the inhibition of pathogenic *Lactobacillus garvieae* and *Streptococcus iniae* by *Bacillus* species was not a result of antibiosis via the production of antimicrobial compounds. Instead, under iron-limited conditions, siderophore-producing probiotics such as *Bacillus* spp. outcompete pathogens by depriving them of available iron ions [147]. Furthermore, several probiotic organisms produce secondary metabolites that may inhibit pathogen growth, e.g., natural antibiotics, hydrogen peroxide, organic acids, bacteriocins or proteases, antibiotics, and lytic enzymes, which can eradicate harmful bacteria directly [14]. For example, *Bacillus velezensis* produced four bacteriocin gene clusters and, additionally, lytic enzymes such as ß-1,3-glucanase [148]. In the genome of *Bacillus subtilis*, 4–5% of the genes are devoted to the production of antibiotics [149].

Undoubtedly, the innate immune system plays a major role in shaping the microbiota and, therefore, keeps the occurrence of pathogenic infections under control [150]. However, adult wild-type zebrafish display a decreased abundance of *Vibrio* spp. compared to zebrafish lacking adaptive immunity (rag1-/-), indicating that the innate immune system alone cannot fully regulate all members of the microbiota in the intestine [150]. It has also been suggested that the adaptive immune system, e.g., via T-cells, is involved in the regulation of the microbial composition.

Since farmers cannot predict the onset of a disease, it is of utmost importance that the probiotic is either administered as prophylactic during a susceptible life stage (e.g., larval stage) or is persistent for a long time. Here, a persistent contribution to the microbiome is highly desirable. In this context, the effects of a probiotic administered after the onset of a disease should be investigated. We would recommend probiotic applications that exert persistent proliferation in the GIT since prolonged administration could have adverse effects (dysbiosis) on the host [97]. In addition, long-term administration of probiotics could become obsolete.

4.6. General Vigor (Fitness)

Undoubtedly, immune stimulation and disease resistance are closely interlinked with the physical performance of an individual. Still, few studies exist that indicate better physical fitness due to probiotic administration [151,152]. In mice and humans, probiotics can enhance exercise performance and reduce fatigue after exercise [153,154]. For example, a swimming exercise endurance test revealed improved muscle strength and endurance, including increased hepatic and muscular glycogen and decreased lactate, after four weeks of probiotic supplementation [153]. At the same time, dramatic changes in the microbial community occurred. Indeed, several studies have reported increased microbial diversity in athletes compared to non-athletes [155,156]. The mechanisms that influence physical performance and potentially support improved general vigor upon probiotic supplementation are still unknown. Additionally, probiotics may increase maximum oxygen uptake, aerobic capacity, and training load [151]. Probiotic supplementation can significantly increase SCFA production to generate more ATP required for exercise, but with regard to the limited number of studies, it is unknown if other pathways might be involved and further studies are needed. Other effects observed after probiotic applications include a reduction of malformations [157,158] and accelerated maturation [159–161].

Interestingly, *Lactobacillus rhamnosus* IMC 501®® accelerates the reproductive performance of female zebrafish (*Danio rerio*), as indicated by the gonadosomatic index and gene expression of reproductive parameters such as aromatase, vitellogenin, and estradiol receptors [162,163]. This may be driven by increased energy availability, supporting the energy demand of developing gonads. Indeed, treatment with *L. rhamnosus* modulates lipid metabolism, decreasing cholesterol and triglyceride content and rising fatty acid levels [164].

5. In Vitro Screening for Candidate Probiotics

To reduce the actual costs, screening commonly involves in vitro assays. In general, these assays should ensure that the probiotic candidates meet the following criteria: (1) harmlessness towards the host, (2) absence of virulent and antibiotic resistance genes, (3) reach target habitat intact (e.g., survival of stomach passage at low pH), (4) acceptance by the host (adherence and host-specificity), and (5) in vivo as opposed to in vitro findings (e.g., pathogen antagonism, dietary enzymes). In humans, regulatory authorities have tried to standardize the in vitro assays by publishing detailed protocols and directives, but tests are performed in a rather arbitrary manner. Here, a standardization of tests will improve future screening efforts.

For easy handling, culturing is carried out in aerobic conditions because target candidates are aerobic and facultative anaerobic bacteria. In addition, 16S-rRNA sequencing is used for taxonomic classification. Moreover, genes involved in the response to low pH, e.g., F1F0-ATPase or heat shock proteins such as DnaK, GroES, and GroEL, are determined. However, to assess pH and bile tolerance in vitro, culturing approaches are easy to implement and are mostly cost-effective. Here, simple approaches with modified PBS have been carried out, but strategies including artificial gastric and pancreatic juices should be preferred [165–167]. An utmost important aspect of candidate screening is the safety of the respective candidate. Here, monitoring the minimum inhibitory concentrations (MICs) for the most relevant antibiotics usually relies on EFSA (European Food Safety Authority) protocols [168]. Additionally, blood agar plates are used to determine hemolytic activity [169]. The screening of pathogenic traits may also assess the ability of a candidate to bind to host cells, such as platelets [170]. The problem with the in vitro safety assays is that virulence may simply not develop under the specific conditions of the assay and, thus, remain undetected. Indeed, interaction with the host in vivo may only trigger virulence. Adherence to the mucus or epithelium is essential to persistently colonize the GIT of the host. Some probiotic strains are equipped with protein surface appendages such as pili or fimbriae, which are identified microscopically or by screening the genome for the respective encoding sequences. The adhesive capacity correlates with the hydrophobicity of the bacterial cell surface, which is determined according to the partition into hydrocarbons [171,172]. However, easy-to-perform surface hydrophobicity is often regarded as rather outdated [173]. Recently, high-throughput adhesion screening methods have utilized immobilized commercially available mucin [174].

As outlined, several bacteria contain high contents of essential fatty acids, cofactors, or vitamins [175,176]. An analysis is usually carried out under standard culture conditions using chromatographic methods, but conditions mimicking the proximal intestine during culturing are preferential. Similarly, candidate screening may target extracellular enzyme activity, allowing high throughput analysis. Most studies involve the monitoring of antagonistic activity towards selected pathogens. This is commonly assessed using simple inhibition assays performed on solid media, e.g., paper-disk diffusion assay or well diffusion assay [74]. In vitro pathogen adhesion assays are used to assess competitive inhibition. Again, conditions should mimic those in the intestine. Nevertheless, in vitro production of antimicrobial compounds may not result in similar observations in vivo since adverse effects may not be triggered. Vice versa, in vitro assays may not detect adverse effects. In addition, the desired antimicrobial effects may not be restricted to pathogens and, consequently, affect the entire microbiota, resulting in adverse effects in vivo.

6. Administration

Currently, in most studies, single-species probiotics are evaluated, but, as outlined above, multistrain probiotics are recommended in many application areas. For spore-forming species such as *Bacillus*, spores should be used as the delivery form because they exhibit improved resistance towards hostile environments (reduced moisture of dry feeds, low pH in the stomach). Probiotics, for which the intestinal tract is the intended site of action, are primarily administered orally via the diet. Here, the enrichment of probiotic cells on or in feeding pellets is the most common practice. In larvae, probiotics are typically added to the rearing water or incorporated into the live feed, for example, *Artemia*, as biological vectors [144]. For feed pellets, an easy-to-perform stepwise top coating technique has been described [74].

Although the pH of the GIT is not as low in fish as in higher vertebrates [103], pH sensitivity is an issue in probiotic administration. There are interspecific variations in pH along the different compartments of the GIT. In general, herbivores tend to have higher pH than omnivorous and carnivores. The channel catfish, as a typical representative of carnivores, reveals a pH between 2–4 in the stomach and 7–9 in the duodenum, a stable value of 8.6 in the upper intestine, and a near-neutral pH in the lower intestine [177].

In larvae, particularly those with prominent metamorphosis, the pH is generally higher. Hoehne-Reitan [178] reported, for example, an alkaline pH throughout the GIT until Day 24 after hatching. Still, after weaning, the pH in the stomach dropped to a minimum of 3.5. Furthermore, several teleosts are agastric (approximately 20% of the species) and do not have acidic digestion. Experimentally, artificial gastric juices stimulate the passage of the stomach [165,166,179,180]. As a rule of thumb, the lower the actual pH, the more sophisticated the strategies of administration required, most of which are adopted from application to higher vertebrates. These include delivery systems such as microencapsulation in cellulose sulfate or calcium alginate [180], legume protein encapsulation [181], hydrogels [182], and coated mucoadhesive films [183] or bioencapsulation in live biological vectors [184]. The probiotic should be retained within the delivery system, and the system should be stable until it is exposed to a specific set of environmental conditions (most importantly pH, bile salts, and enzyme activities). Consequently, the biomaterials used should be stable under acidic conditions in the stomach, and decomposition should only occur after subjecting them to the pH of the small intestine or to pancreatic enzymes.

7. Conclusions

Undoubtedly, in the context of rapidly increasing global aquaculture production, alternative ecofriendly methods for the prevention and therapy of diseases as well as the improvement of growth performance are pressing issues. Here, probiotics offer the tempting opportunity to modulate the GIT microbiome persistently, exerting beneficial effects such as increased growth, feed conversion, health, disease, and stress resistance. Interestingly, probiotics may be capable of degrading compounds that the fish host cannot digest alone. Carbohydrate digestion and detoxification of antinutrients are the focus of aquaculture research. Probiotic-derived chitinase, for example, may allow the use of novel feedstuff such as insect meal or krill. Similarly, the probiotic digestion of secondary plant metabolites with antinutritional effects may improve the rate of fishmeal replacement with a respective plant feed ingredient. Multispecies probiotics exhibit better probiotic effects than single-species applications. The modulation of the gastrointestinal microbiome through dietary administration of probiotics represents a potential strategy to improve microbial metabolite production, stimulate immune signaling, and increase defense mechanisms against pathogens. However, modulation of the microbiome may induce adverse effects and may even bear the risk of paving the road for pathogens. Therefore, we need to deepen our knowledge of microbiome regulation. Additionally, species-specific studies are required before a given probiotic is applied in a novel species. Moreover, the antiviral activity of several *Lactobacillus* strains towards murine norovirus (MNV) has been reported, and it will be interesting to see if microbes can also alter fish-specific viral infectivity [185].

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