Review

Reducing the Use of Antibiotics in European Aquaculture with Vaccines, Functional Feed Additives and Optimization of the Gut Microbiota

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Abstract: This review presents several alternatives to replace antibiotic therapy and make the European aquaculture industry more friendly and environmentally sustainable. The first part of this review highlights the growing importance of the aquaculture industry worldwide for its ability to supply low-cost proteins and lipids. The second part discusses different strategies for these replacements, from recombinant vaccines to diets with low environmental impact and rich in bioactive molecules that can benefit other species. Specifically, the beneficial effects of bioactive compounds present within insect meals are discussed. In addition, particular focus is placed on the importance of adopting sustainable protocols for fish farming, including supplements such as probiotics, prebiotics, and synbiotics capable of modulating the gut microbiota as the second brain. Those feed additives can stimulate European farmed species' immunological systems, growth, and welfare.

Keywords: fish health; environmental sustainability; antibiotic resistance in aquaculture; gut microbiota; feed additives

1. Introduction

In 2016, 37 countries, accounting for more than half of the world's human population, produced more farmed than wild-caught fish [1]. It is now clearly established that aquaculture continues to be the fastest-growing business globally in the food production sector but at a slower pace compared to the past. However, the growth rates by country are profoundly different and reflect country-specific frameworks of environmental regulations, bureaucracy, and licenses given to the sector [2]. In this context, the European Union (EU) accounted only for 3.7% of global production in 2016. However, it represented the largest and most valuable fish and fish products market, increasing from 2016 to 2017 [1]. Despite good overall growing performance in aquaculture production, the EU is still lagging behind growth rates in other regions. To enhance competitiveness, the EU strategic plan (2016) identified guidelines related to improving the efficiency of the production processes, supporting technological innovations, implementing the Marine Strategy Framework Directive (MSFD), and exploiting advantages due to high-quality environmental standards. On the other side, setting strict environmental regulations is a double-edged sword. It represents a

critical trade-off between prompting the growth of the aquaculture business and conserving the natural environment [3].

Among the main environmental concerns arising from aquaculture activities are the impacts of escaped farmed organisms, the spreading of diseases, the considerable nutrient enrichment of the water column, the release of chemicals (i.e., metals, antifoulants, antibiotics, and chemotherapeutics), depletion of wild fish stocks for feeding purposes and habitat alteration [4–6].

The impacts of escaped farmed organisms are one of the leading environmental issues associated with aquaculture activities [6]. This is almost inevitable, resulting from either damage to cages by aquatic predators (i.e., seals and dolphins) or human errors during routine handling [5,6]. Influences of these fish with the local wild stocks can alter the natural genetic structure, resulting in the transfer of genotypes among differentiated populations used in aquaculture [7]. By using a regression model based on a 37-year study regarding the spawning in the wild of both farmed and wild Atlantic salmon (*Salmo salar*), it was shown that the escape of captive animals into the wild could substantially impact recruitment and, more specifically, disrupt the capacity of natural populations to adapt to water temperatures associated with climate variability [8]. These authors concluded that farmed fish are genetically manipulated through selected breeding regimes to meet commercially desirable features, such as high growth rates, disease resistance, altered aggression, and adaptation to high stocking densities, and these factors may have a significant impact on the biodiversity, fitness, and reproductive performance of the wild stocks.

Another problem linked to escaped fish is the input of alien species into ecosystems. As reviewed by Grigorakis and Rigos [6], two polychaetes, 48 algae species, eight crustacean species, and 15 mollusks were introduced through the Mediterranean aquaculture. The impacts of introducing alien farmed animals to the indigenous populations can occur through direct predation or competition, the reduction in environmental well-being, or the indirect reduction of local biodiversity [7].

In addition to the problems linked to the transfer of genotypes among local and farmed populations and the introduction of alien species, the caged open systems of mariculture may promote the spreading of diseases [8]. Sea lice in salmon farms represent the well-known infection case between farmed fish and the wild stock [9].

Furthermore, the release of organic pollutants and eutrophication of sediments, such as organic nitrogen and phosphorus, in the environment represents an additional source of adverse effects of aquaculture practices on the ecosystem [4]. The feeding wastes released in the water column can be derived from multiple sources, such as non-ingested feed, non-digested feed components, and fish excretions [6]. Metals are used to enrich the food for mineral requirements, antifouling products to prevent the development of fouling organisms in the nets, and antibacterial agents to cope with bacterial fish infections or bacterial pathogens for which no effective prevention exists [6–8]. Costello [5] reported that 75% of organic effluents in Norwegian salmon farms are dispersed to near-field sites up to 500 m from the release point. A small proportion of particulate organic effluents (up to 2.7%) are distributed to far-field sites (up to 2 km), suggesting that organic effluents from fish farms may be spread over large areas of the fjord systems. Thus, the spreading potential of organic and chemical wastes over vast areas is noteworthy and can cause a reduction of oxygen, algal bloom, a decrease in water quality, and habitat destruction [9,10]. In aquaculture, antimicrobials are routinely used for treatment therapy, prophylactic reasons, or growth promotion. However, subtherapeutic doses of antibiotics have contributed to promoting antimicrobial resistance with adverse effects on the health of fish, human beings, and the aquatic ecosystem [4,10–12]. Antibiotics and chemotherapeutics have been used to prevent or control bacterial infections in aquaculture for many years. Unfortunately, the use of antibiotics for treatment is not successful and sustainable due to the increase in antibiotic-resistant bacteria, and the negative effect on the environment and human health. These negative effects caused the establishment of strict regulations for the administration of

antimicrobial agents in many European countries [1]. An effective way to prevent diseases in fish is through vaccination. However, aside from the high cost, vaccines act specifically and provide immunity only against particular pathogens [2]. Therefore, the development of vaccines for fish is limited due to the restricted information on fish immunology, the necessity of obtaining approval, the stress associated with handling during injection, and the limited economic feasibility. On the other hand, compared to terrestrial animals, fish require a higher dosage of antigens [3]. Thus, there is a need to look for alternative disease prevention techniques which are environmentally friendly and highly effective. Therefore, over the past decade, research interests in the improvement of fish health and welfare status through the use of bioactive feeds in health, such as prebiotics, probiotics, and, in some cases, the combination of them as a synbiotics, have arisen [4].

Furthermore, there is much discussion about the paradox of fish farming, which should aim to relieve pressure on wild stocks but, instead, negatively affect wild resources since most of the feeds are based on wild-caught raw fish. For example, 4 t of wild-caught anchovies are necessary as feed or fishmeal to produce 1 t of aquaculture-raised salmon [13]. So, indirectly, aquaculture reduces the food available for marine predators [4]. Recently, high demand and high prices for fishmeal and increasing aquacultural production have pushed new research into developing a renewable source of food for aquatic animal feeding. Insects are a nutrient source to digest into absorbable metabolites, which give energy and immune factors. Available documentation of the nutritional composition and value of different insect species considered candidates for use in animal feeds has become substantial [5–7]. Therefore, research on the use of insect meal in aquafeeds has developed rapidly in recent years, leading to an increased number of scientific contributions on this topic [6,7]. The inability of reproduction in captivity for some species, i.e., bluefin tuna, eel, and shellfish, obligates the farmers to capture larvae, juveniles, or adults of wild stocks for rearing [6].

In conclusion, aquaculture is far from being considered a sustainable resource. But in the last years, with the increasing development of the aquaculture industry, significant signs of progress in the sustainability field have been made.

The application of feed additives, probiotics, and prebiotics has the potential to provide the proper solutions to meet current and future market needs and to face challenges related to the production of numerous valuable species for the future of the European aquaculture sector. Future efforts should be made toward understanding the stage-specific nutritional requirements of these species and formulating cost-effective dry feeds to be adopted by the industry.

2. The Microbiota: A Second Brain Affecting Fish Physiology

The microbiota is a complex and interconnected ecosystem arising from the collection of microorganisms, including bacteria, archaea, viruses, fungi, and protozoa found throughout the body, mainly internal and external surfaces, including the gastrointestinal tract, skin, and mouth [14]. Most of these microorganisms are found in the gastrointestinal tract, where they play a crucial role in maintaining host health and preventing the insurgence of diseases by producing microbial metabolites [15–17]. The microbiota composition is usually similar at the phylum level between individuals of the same species. However, diet, stress, genetics, and environmental factors can significantly shape a host's microbial communities [18]. Given the potential of microbial communities to be manipulated by environmental cues, studying the microbiota has gained particular attention in the aquaculture industry to improve fish health. More specifically, the development of disease management strategies has always relied on using antibiotics in the past. Only recently, this unsustainable strategy has been replaced by environmentally integrated approaches to the modulation of fish microbiota [19]. Duan and collaborators analyzed the microbial communities of the white-leg shrimp (*Litopenaeus vannamei*) fed with different concentrations of *Clostridium butyricum*, a bacterium producing butyric acid which provides energy to the host's intestinal epithelial cells. They found that microbiota composition was altered

with decreased pathogens and an increased abundance of beneficial bacteria, which may contribute to the expression of the host's digestive genes and immune-related genes [20]. Schmidt and collaborators have tested whether probiotic administration in the black molly (*Poecilia sphenops*) could reverse antibiotic-induced losses of disease resistance and found that antibiotic treatment significantly increased fish mortality. However, probiotic bacterial species could colonize black molly microbiota without influencing the overall microbiome structure, reversing antibiotics' adverse effects [21]. Given these results, leveraging microbiota manipulations is paramount in advancing the aquaculture industry.

3. The Consequences of Antibiotic Misuse in Aquaculture

Antibiotics have been used traditionally in aquaculture as growth promoters. However, the adverse effects of antibiotic misuse have been highlighted over past years, which resulted in strict limiting regulations. McGinnity et al. [8] reported that between 2000 and 2010, antibiotic consumption increased by 36% worldwide. There are estimations that by 2050, about 10 million people are expected to die due to antibiotic resistance [9]. On the other hand, it has been reported that the prophylactic use of antibiotics reduces the synbionts in aquatic animals, with consequences for host immunity [22]. Even the legally allowed concentrations of antibiotics like sulfamethoxazole and oxytetracycline can have detrimental effects on the health of the gastrointestinal tract if used in the long term, which was confirmed using zebrafish as a model [23]. Furthermore, the irreversible effects caused by antibiotic exposure on the microbiota of aquatic animals were confirmed in mosquitofish (*Gambusia affinis*) [24]. In this section, the various aspects of antibiotic misuse in aquaculture are discussed.

3.1. Regulation of Antibiotic Application in Europe

The increase and dissemination of studies on the effects of inappropriate misuse of antibiotics in aquaculture for human and environmental health have prompted the national and international authorities to regulate the use of these products in farms. At the European level, this has led to the birth of a new discipline in veterinary medicine called pharmacovigilance, which is regulated by the Directive 2004/28/CE. Moreover, it involves the control of maximum residue levels (MRL) of veterinary drugs in food products of animal origin, the assessment of the risks for the environment related to the use of veterinary medicines, and control of the development of drug resistance, with particular concern to antibiotic resistances [25]. Furthermore, the EU banned certain antimicrobials used in food-producing animals as feed supplements [25]. Each member state has individually taken the European regulations and developed measures to respond to the sector's specific needs. This has been accomplished by the institution of national monitoring organizations that, through collaboration with veterinarians, have to adopt strategies that help to prevent and control bacterial infection, therefore limiting the use of antibiotics and/or using rationale criteria to choose therapeutic protocols favoring the risk/benefit/cost ratio.

In this context, scientists are studying and proposing different alternatives to antibiotics. Vaccination is ideal for preventing disease, but commercially available vaccines are still very limited [26]. Other alternatives are using probiotics and microorganisms to avoid bacterial infections in aquatic organisms, improving the balance of digestive tract flora, feed utilization, and promoting the growth of aquatic animals [27].

The use of antibiotics as growth promoters in European aquaculture was banned in 2006, and even the regulated medicinal use has decreased significantly, especially in significant salmon-producing areas such as Norway and Scotland [28]. It is still widespread in many developing countries to use antibiotics prophylactically. Considering that more than 90% of the world's aquaculture production is accounted for from developing countries, the adverse effects due to the misuse of antibiotics are still relevant, as some of those adverse effects discussed here have global relevance.

3.2. Antibiotic Resistance

The accumulation of antibiotics in fish is influenced by various factors, including the fish species, specific substances used, and the distribution patterns within the fish's tissues [29]. Scientific studies have provided evidence that the extensive utilization and release of antibiotics into the environment, including in aquaculture settings, can lead to their accumulation in fish. However, the accumulation behavior of antibiotics in fish can vary depending on their unique combined ionic/hydrophobic properties [30]. For example, the physicochemical characteristics of antibiotics, such as their low fat solubility, can affect the extent of their accumulation. Although there have been investigations into the accumulation of antibiotics in aquaculture organisms, further research is necessary to comprehensively comprehend the potential for accumulation and the distinct distribution patterns of antibiotics based on their individual combined ionic/hydrophobic properties in aquaculture systems. On the other side, antibiotics can easily find their way to sediments [31] and the surrounding waters through uneaten food and feces released from aquaculture farms [32]. Once the antibiotics reach the sediments or surrounding environment, they promote the development of antibiotic-resistant bacteria. Antibiotic resistance can be developed in bacteria through mutation [33] or horizontal transfer of resistant genes through transformation, transduction, and conjugation [34], among which conjugation seems to be the most common [35]. With the farming of shrimps in mangrove areas, the residues of antibiotics like trimethoprim (TMP), sulfamethoxazole (SMX), norfloxacin (NFXC), and oxolinic acid (OXLA), and resistant bacteria were found in farms of Vietnam [36]. Even legally approved antibiotics can lead to the emergence of resistant strains, as observed in Denmark, where sulphonamide and trimethoprim resistant strains of *Aeromonas* spp. were found [37]. Some of the significant issues following the development of antibiotic resistance are as follows:

a. *Decreased effectiveness of the drugs*

In Danish trout farms, resistant bacteria were identified around the farm [21]. Occasionally, antibiotic-resistant species were found in a mussel species due to antibiotic use in a marine Atlantic salmon farm [38]. Moreover, resistant bacteria can also increase antibiotic resistance in several pathogens affecting the fish, which inevitably leads to the decreased effectiveness of the drugs used for specific treatment [37].

b. *Cross-transfer to the terrestrial environment*

Sometimes, the resistant strains were found in areas away from the aquaculture site of the application of antibiotics, as seen in the case of salmon culture in Chile [39]. The resistant strains can be transferred to the terrestrial environment and humans [40]. The quinolone resistance of certain Gram-negative bacteria in humans gives additional evidence for the aquatic-terrestrial transfer of antibiotic resistance between bacteria from both habitats [41]. Clear evidence of the transmission of antibiotic-resistant determinants by fish pathogens like *Aeromonas* spp. to human pathogens like *Escherichia coli* was identified in several areas of Europe [42]. To check the diagram mapping the linkages between reservoirs of antibiotic resistance, see [10].

These cross-transfers can happen when aquaculture is practiced with agriculture, where aquafeed and waste are used as agricultural manure [43]. This high transfer capacity has led to the WHO's hypothesis that by 2050, the death toll due to antibiotic resistance will be greater than 10^6 yearly (World Health Organization 2014). All these pieces of evidence prove the detrimental effects of antibiotic usage in aquaculture on other niches, including marine and terrestrial.

Apart from these environmental issues, aquaculture commercial fishery products also seem to have some residual antibiotics due to excessive usage, which can lead to toxicity, allergies, or even antibiotic resistance to unaware consumers [44]. Even the same can occur to the people who handle the fish in the aquaculture industry through unintentional absorption through the skin or bronchial tracts [45].

3.3. The Fate of Antibiotics

The abuse and high dose of antibiotic usage, low absorption after administration (occurring with food, bath, or injections) [37], solubility, bioactivity, and persistence can cause ecological and health problems in aquatic organisms [46]. Municipal and marine wastewater contains antibiotics due to the problems in removing these substances from water. A wastewater treatment plant cannot remove residual antibiotics in the aquatic environment, which causes health problems for wildlife, exerts selective pressure, alters the composition of sediment microflora, and selects for antibiotic-resistant bacteria [37]. Given the mobile genetic elements in the environment, bacteria can access a gene pool that transfers from one cell to another in a chain process, favoring exponential diffusion in bacterial populations [26].

In addition, residues of antibiotics can be present in the fish after exposure, both in plasma, liver, muscle, and bile. The route of exposure may happen in several ways. Indeed, the distribution of antibiotics in different tissues is mainly influenced by species and the type of drug. Moreover, the accumulation of antibiotics in fish tissue is related to their bioaccumulative potential, which can vary based on the specific substances and fish species. The tissue-specific bioaccumulation of antibiotics in fish is influenced by various factors, including tissue phospholipids and liposome-water [11].

An additional concern is associated with a mixture of antibiotics and how they potentially interact with each other (synergically or antagonistically). Indeed, environmental concentrations of individual compounds may be below the threshold of concern. However, their mixture could elicit significant toxicity to aquatic organisms, and despite the study of mixtures being quite challenging, little information is available [47]. For this reason, many countries have established guidelines for using veterinary antibiotics [48]. The antibiotic residue, as well as the half-life in the farming environment (e.g., sediments), largely depends on the sediment type as well as the type of antibiotic. For more information, refer to [12].

4. Alternative to Antibiotic Treatment

4.1. Recombinant Vaccine

The use of antibiotics and parasiticides in aquaculture has certainly brought good results in the short term. However, in the long-term, it tends to harm the environment and fish. First, because it contributes to bacteria resistance to antibiotics, and second, it reduces the microbial diversity in fish intestines. For these reasons, scientific research is focusing heavily on vaccine development. The vaccine is a "preparation" that can stimulate the immune system to produce a response that confers immunity and protects against a specific disease.

The interest in vaccines for the aquaculture industry has a lengthy background. The first fish vaccine has been available since the 1970s [49]. However, due to several obstacles, such as economic, effective adjuvants, and environmental considerations of the different countries, it was not easy to introduce various vaccines [50]. An effective way to prevent diseases in fish is through vaccination. However, aside from the high cost, vaccines act specifically and provide immunity only against particular pathogens [2]. Therefore, the development of vaccines for fish is limited due to the restricted information on fish immunology, the necessity of obtaining approval, the stress associated with handling during injection, and the limited economic feasibility. In addition, compared to terrestrial animals, fish require a higher dosage of antigens [2,3]. That is why, so far, many vaccines are available in other countries, such as in America and Asia but not in Europe, due to different regulations.

There are different types of vaccines, but recombinant vaccines, also known as new-generation vaccines, are a breakthrough for both the aquaculture industry and scientific research. This is because recombinant DNA technology allows us to combine the genetic material of different organisms artificially. Individual DNA sequences encoding specific antigens are isolated and inserted into a plasmid vector using biomolecular tech-

niques. Plasmid allows correct antigen expression and can be inoculated directly in the host or in prokaryotic/eukaryotic expression systems that produce the antigen that can be used to vaccinate the host after appropriate purification. Given its efficiency against various diseases, the recombinant vaccine application in aquaculture has received increasing attention [13]. Many reports are available regarding effective protection caused by recombinant subunit vaccines, nucleic acid vaccines, recombinant live vector vaccines, and gene deletion/mutation vaccines [13]. However, the economics of using such a kind of vaccine must be considered, which is influenced by cost of production, safety concerns, effectiveness, and ease of application [14].

In recent years, more and more scientists have begun to explore the effects of recombinant vaccines on the gut microbiota. The importance of the composition of intestinal microbiota as one of the main factors contributing to the effectiveness of immunization is known [51]. It has been established that some members of the resident microbiota are intrinsically linked to the production of mucosal immunity [52]. Some studies have indicated that a particular composition of microbial communities is associated with differential responses to the vaccine [53]. Recently, it has been shown how the administration of a recombinant vaccine obtained from engineered *Lactobacillus plantarum* could stimulate immunity through two different pathways, on the one hand, colonizing and strengthening the intestinal mucosa, on the other hand by crossing the intestinal barrier and reaching the other organs [54]. Several studies indicate that recombinant vaccines may influence the composition of gut microbiota. Indeed, an alteration in the diversity of the resident intestinal microbial community immediately after the administration of recombinant vaccines is observed. However, after a few days, it is restored without significant consequences [55].

This identifies not only the function of infection responder by the intestinal microbiota but also demonstrates that recombinant vaccines do not irreversibly affect the composition of the microbiota but can maintain the main intestinal bacterial communities [55]. However, this hypothesis may be true for one vaccine and one species of fish, but to generalize, therefore, needs to be confirmed and merits additional studies. It is worthy to mention that another concern in case of vaccines administration in aquaculture is the development of resistance to the host response to vaccines by specific pathogens [56]. Aquaculture pathogens have various mechanisms by which they can acquire resistance to the host's immune response. These mechanisms include antigenic variation, immune suppression, immune camouflage, and the production of immune evasion proteins [56]. Therefore, to mitigate the possibility of vaccine evasion in aquaculture, it is crucial to routinely evaluate the effectiveness of vaccines and monitor the pathogen populations. This enables the identification of any alterations in the pathogen's behavior and the formulation of strategies to revise or adjust vaccines to preserve their efficacy against evolving aquatic pathogens.

4.2. Insects' Meal and Its Bioactive Compounds

To date, fish meal and fish oil still represent the optimal protein source for aquafeeds [57] due to the high nutritional values matching several cultured fish's dietary requirements. Nevertheless, environmental and economic implications have addressed researchers' efforts to find alternatives and more sustainable protein sources. Recently, high demand and high prices for fishmeal, together with increasing aquacultural production, have pushed new research into developing a renewable source of food for aquaculture feeding. Among several candidates, insects have received significant attention as an ingredient for aquafeeds due to their high amount of protein (60–80%), good aminoacidic profile, and abundance of vitamins and minerals [58].

A recent EU commission regulation (2017/893-24/05/2017) authorized the use of seven insect species (two flies, two mealworms, and three cricket species) in aquafeeds and the potential of insects as a valuable ingredient for aquafeed formulation has been determined for different fish species under the interest of European farmers, including Atlantic salmon [59], European sea bass (*Dicentrarchus labrax*) [60], turbot (*Psetta maxima*) [61], and

rainbow trout (*Oncorhynchus mykiss*) [62,63]. In addition to their nutritional values, insects present bioactive compounds that are supposed to modulate microbiota and positively affect animal health [64], even if the effects are host specific.

A differential shift in the dominant bacteria in the gut of rainbow trout (*O. mykiss*), gilthead seabream (*S. aurata*), and European seabass (*D. labrax*) was observed after replacing fishmeal with yellow mealworm (*Tenebrio molitor*) meal [65]. The significant role of an insect-based diet in modulating and enriching fish microbiota was attributed to the high chitin content [66]. Indeed, the dietary inclusion of chitin in Atlantic cod (*Gadus morhua*) and salmon has been proven to improve the biodiversity of their intestinal microbial community [67].

Chitin is the primary constituent of arthropods' exoskeleton [68] and represents one of the most abundant polysaccharides in nature, second only to cellulose on a world scale. Chitin can be considered an insoluble fiber since most fish cannot digest it and, similarly to vegetable fibers, may have potential prebiotic properties. Among the substances obtained from natural sources, chitin has received little attention in fish cultured in Europe [67,69]. Data reported for chitin are often inconsistent, in particular for salmonids.

In some cases, chitin is suspected to affect fish health adversely (such as turbot), diminishing feed intake and digestibility [61]; even if most studies contradict these findings. However, chitin has been shown to have antimicrobial properties and bacteriostatic effects, particularly on some Gram-negative pathogen bacteria [70]. Recently, the results of an insect-based diet were investigated in rainbow trout (*O. mykiss*) without adverse effects on the immune system [63] and gut microbiota [71]. The partial substitution (25–50%) of fishmeal with defatted insect meal in the trout diet was essential in modulating the intestinal microbial communities, increasing gut microbial richness and diversity [71,72].

In this regard, the gut microbiota of rainbow trout is dominated by Firmicutes, Proteobacteria, and Actinobacteria, regardless of the diet administered [73,74]. These phyla and Bacteroidetes usually represent up to 90% of fish intestinal microflora in different marine and freshwater species, constituting the "core gut microbiota" [75,76]. In trout, the dietary administration of insects leads to a significant increase in gut Firmicutes and Actinobacteria and, in particular, in lactic acid bacteria (LAB) such as Aerococcaceae, Leuconostocaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, and Carnobacteriacee [71,72,74], which are generally considered beneficial microorganisms and used as probiotics for fish and other vertebrates [77–80]. Several studies reported positive effects of LAB on disease resistance, survival, and growth parameters for a wide variety of European fish species [81,82]. The increasing number of LAB could be promoted by chitin which acts as a prebiotic, providing the preferential growing substrate for this family of bacteria [67] that, in turn, promotes the digestibility of indigestible carbohydrates, such as resistant starch and dietary fibers, thus contributing to more efficient food energy utilization [83]. In addition, the increased number of LAB could have an active role in host defense against pathogenic bacteria, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Pseudomonas aeruginosa*. It is known that LAB produces bactericidal compounds such as lactic acid, hydrogen peroxide, and bacteriocins or biosurfactants, which create a biofilm able to prevent pathogens from adhering to the intestinal surface [74,84].

Moreover, in the gut of trout fed an insect-based diet, an enrichment of Clostridiales was observed, an order that contains many butyrate-producing bacteria belonging to the *Clostridium* genus, such as *Clostridium butyricum* [85]. Butyrate is considered the most essential short-chain fatty acid due to its numerous positive and well-documented properties on the health of the intestinal tract and peripheral tissues in vertebrates, including fish [86]. Butyrate, the primary energy source for colonocytes, has anti-inflammatory potential and is crucial in regulating the immune system [87].

In addition to the high content of chitin, insects contain high amounts of lauric acid (C12:0), a medium-chain fatty acid (MCFA) known for its antiviral and antibacterial effects on Gram-positive bacteria [88]. Furthermore, this fatty acid has been demonstrated to positively affect fish gut welfare by mitigating inflammatory conditions [89]. The data

available up to now show in most studies a beneficial role of the bioactive compounds present in insect meal for the cultivated Mediterranean species. Readers with specific interests in economic feasibility and impacts and the environmental footprint of insect meal application in aquaculture see [15,16].

4.3. Probiotics

Probiotics are live microbial feed supplements that beneficially affect the host animal by improving its intestinal microbial balance [90]. Given the adverse effects of antibiotics discussed above over the past decades, there were extensive attempts to find alternatives [91]. On the other hand, recent works highlighted the importance of gut microbiota composition due to its effects on host growth, health, and well-being. Therefore, this section discusses the gut microbiota modulators as an alternative to the prophylactic and therapeutic administration of antibiotics and their effects on growth and welfare.

The Food and Agricultural Organization and World Health Organization (FAO/WHO) defined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [92]. Probiotics are considered alive or dead microorganisms (whole or part of them) that positively affect farmed fish by improving their intestinal balance and thus enhance feed utilization and growth performance, as well as resistance to disease and stress [93,94]. Furthermore, their use in aquaculture as an environmentally friendly alternative to medicines and chemical additives has received significant attention in recent years [95].

Source of probiotics and administration: Probiotics have been obtained from the aquatic environment or aquatic animals [93,96,97]. In particular, fish skin mucus and the gastrointestinal tract can be valuable sources [98]. Probiotics isolated from different natural sources are putative, in contrast to the non-putative ones derived from commercial sources [99]. Bacteria isolated from various sources should possess certain qualities to be chosen as probiotics [99,100]. The determination of the inhibitory activity against target pathogens in vitro, such as mucus adhesion, absence of harmful effects on the host, fermentative action, tolerance to freeze-drying, and viability in feed during the packaging and storing process are some of the criteria with which a potential probiotic is chosen [101]. Good probiotic performance is strictly connected to the choice of the proper administration method and the knowledge of their action modes [102]. Aquatic probiotics can be provided through direct addition to culture water [95], live food [103], intramuscular or intraperitoneal injection, or by addition to an artificial diet or culture water.

Considering liquid-form probiotics, the direct addition to the rearing water is the most applicable for all fish ages, and it represents the best solution for larval rearing, where microorganisms could be an essential cause of epizootic mortality [104]. Since the larval epidermal mucus layer represents the primary interface with the environment, the administration through rearing water can improve and stimulate larval non-specific defense [104,105].

Dry form-probiotics can be provided through direct incorporation in live (such as rotifers, copepods, and artemia) or pellet feed via injection [106]. In this regard, a widely used strategy is microencapsulation, which ensures proper probiotic delivery to the host [81]. Independently of the administration method, probiotics delivery should preferably begin in the early stages of larval development preceding exogenous feeding. Furthermore, the frequency of the addition of probiotics in all aquatic species usually depends on the probiotic species, culture conditions, stage of fish or shrimp development, diet, and selected probiotic concentration [82].

It is worthy to mention that there are some concerns regarding the administration of high levels of probiotics in the aquaculture environment. The concerns are mainly about potential alteration of the natural microbial communities in aquaculture systems and disturbing the existing microbial balance and ecological dynamics within the aquaculture environment [107]. On the other hands, the excessive utilization of probiotics in aquaculture has the potential to cause the proliferation of specific microbial species or the accumulation

of an excessive amount of biomass. As a consequence, this can disrupt the equilibrium of the microbial community, negatively impacting nutrient utilization and waste management processes within the aquaculture system [107]. Given such concerns, it is crucial to (1) select appropriate probiotic strains that are native to the specific aquaculture environment, (2) select the optimum inclusion levels, and (3) perform regular monitoring of the ecosystem to check potential impacts of probiotics.

Effects of probiotics: Probiotics are used in aquaculture to improve the growth performance of the farmed species by increasing appetite and increasing digestive enzyme activities and, thus, the digestive capacity, breaking down indigestible components as well as having critical beneficial effects on intestine morphology [93]. The nutrient absorption capacity of the host increases due to the ability of the gut microbiota to metabolize and convert many essential nutrients to end products [108]. Most often, probiotics are Gram-positive bacteria belonging to the following geni: *Bacillus*, *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, and *Pediococcus*, positively affecting reproductive performance and gamete quality. In particular, *Lactobacillus rhamnosus*, used as a feed additive, had positively affected zebrafish follicle maturation, fecundity, and egg quality [109–112]. Moreover, probiotic *L. rhamnosus* has a significant potential for ovarian physiology and embryo development [109,113]. Numerous studies have addressed the efficacy of probiotics in female fish species. However, very few studies have focused on sperm and semen quality (motility, concentration, quality of seminal plasma, sperm morphology, and volume) [114]. As shown in Gioacchini, Maradonna, Lombardo, Bizzaro, Olivotto, and Carnevali [112], and Carnevali, Maradonna, and Gioacchini [114], probiotic effects on fish growth and reproduction are also mediated by modulation of metabolic neuropeptides at the level of the central nervous system. Lactogen 13 administration (*L. rhamnosus* IMC 501) in tilapia (*Oreochromis niloticus*) modulates the expression of genes involved in appetite regulation, such as neuropeptide y (npy), agouti-related protein (agrp), leptin, and ghrelin [115].

Probiotics are a promising and eco-friendly approach against bacterial infections, the most common cause of disease in aquaculture [116]. In particular, *Bacillus aerius* B81e diet supplementation has shown to increase resistance to the most dangerous bacterial pathogens in aquaculture, *Streptococcus agalactiae* [117] and *Aeromonas hydrophila* [118], in various aquatic species like rainbow trout (*O. mykiss*), Nile tilapia (*O. niloticus*) and white-leg shrimp (*L. vannamei*) [119–121]. Several *Lactobacillus* species showed significant antimicrobial activity against *Aeromonas* sp. and *Vibrio* spp. [122,123] or inhibited the *Listeria innocua* growth [124]. Furthermore, *Lactobacillus* sp. increases resistance to lymphocystis viral disease found in *Paralichthys olivaceus* [125]. A positive effect against WSSV (white-spot syndrome virus) was found in white-leg shrimp (*Litopenaeus vannamei*) after administration of strain *Bacillus megaterium* [126]. Positive results against the IHNV (infectious hematopoietic necrosis virus) are recorded after administration of *Aeromonas* spp., *Corynebacterium* spp., *Pseudomonas* spp., and *Vibrio* spp. [82,127]. Only a few studies have been reported about the antifungal activity of probiotics. The positive effects of *Saprolegnia* spp. are produced by *Aeromonas* strain A199 isolated from the short-finned eel (*Anguilla australis*) culture water, *Pseudomonas* sp. M162, *Pseudomonas* sp. M174, and *Janthinobacterium* sp. M169, a *Lactobacillus* spp., *L. plantarum* FNCC 226 has been reported [128].

Prolonged administrations of probiotics strengthen both innate immunity by the modulation of humoral immune responses, the expression of immune-related genes [129,130], and cellular immunity by increasing numbers of leucocytes, lymphocytes, monocytes, and erythrocytes, neutrophil adherence, migration of neutrophils and plasma bactericidal activity and complement activity [131]. Stress-related diseases could modify the innate immune responses, but probiotics have been reported to improve the stress tolerance of fish to rearing conditions [132]. For example, mixing *Bacillus* spp. can reduce cellular stress in sea bream (*Sparus aurata*) larvae by lowering HSP70 gene expression [133]. Furthermore, *Bacillus* spp. as probiotics are involved in producing antioxidant enzymes like SOD and glutathione [134]. Finally, probiotics can improve water quality by reducing ammonia levels [95] and the degradation of starch and proteins from underutilized

feed [135]. Gram-positive bacteria are especially more efficient in converting organic matter into CO_2, or bacterial biomass [136]. The probiotic bacteria also possess significant algicidal activity [137].

Mode of action: Probiotics colonize sites in the digestive tract of aquatic animals, particularly the gastrointestinal mucosal epithelium [138,139]. Probiotics form a physical barrier in the host intestinal mucosa that prevents pathogenic bacteria's growth on the gut surface with a competitive exclusion [136]. In addition to competition for space, probiotics can reduce the number of nutrients that pathogenic bacteria may use, limiting their maintenance in the host [128]. Furthermore, disruption of the quorum sensing system of pathogens has been proposed as a new anti-infective strategy in aquaculture [140]. Finally, the exclusion of pathogen bacteria can occur by producing compounds with antibacterial action and organic acids that lower the gut's pH, preventing pathogens' growth [141].

Application of probiotics: Probiotics can be applied using single culture or multiple strain combinations. Using *Carnobacterium* sp. reduced disease caused by *Vibrio ordalii*, *Aeromonas salmonicida*, and *Yersinia ruckeri* in Atlantic salmon (*Salmon salar*) and rainbow trout (*O. mykiss*) [142]. A mixture of *Bacillus subtilis* and *Lactobacillus acidophilus* evidenced a higher protection against harmful pathogens in tilapia [143]. Mixed probiotic strains are more efficient than probiotics based on a single strain [144,145].

Probiotics can be used with plant products, and this combination can improve growth performance, hematological parameters, immune response, and disease resistance in both farmed fish and crustaceans [146]. Finally, several studies reported that probiotics could be applied with yeast extract to increase immune response, specific growth rate (SGR), feed conversion ratio (FCR), and survival rate in many farmed species such as Nile tilapia, shrimps, and gilthead seabream [147].

4.4. Prebiotics

Prebiotics are defined as non-digestible fibers that benefit the host by selectively stimulating the growth and/or activity of specific health-promoting bacteria that can improve the host's health [90]. Several types of prebiotic oligosaccharides have been used in aquafeeds. The common prebiotics established in aquafeeds to date include inulin, fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), mannanoligosaccharides (MOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltooligosaccharides (IMO), and GroBiotic-A [70]. These prebiotics are mostly plant-derived, although some commercial prebiotics used in European aquaculture are yeast (Actigen®, Samut Sakhon, Thailand) or dairy/yeast-derived (GroBiotic-A™, International Ingredient Corporation, Fenton, MO, USA). As a result, they often contain glucans, which provide additional immunomodulatory effects. In addition, some commercial prebiotics are enriched with functional additives such as β-glucans to stimulate further the fish immune system (Immunogen®, A-Max, Waltham, MA, USA).

Table 1 shows the studies assessing the effects of prebiotic supplementation on European species aquaculture; after the review by Dimitroglou, Merrifield, Carnevali, Picchietti, Avella, Daniels, Guroy, and Davies [91], a total of ten fish species and one of shellfish were found. The most studied fish species were the common carp, seabass, gilthead seabream, and rainbow trout. Surprisingly, only four studies were found for Atlantic salmon, a highly commercial species. Less studied species include the channel catfish, *Senegalese sole*, hybrid striped bass, the Siberian, and the starry sturgeon. Other species relevant to European aquaculture of which no studies were found in the last years include the Atlantic halibut, the Atlantic cod, European catfish, brown trout, the Artic charr, and the European eel (*Anguilla anguilla*), among others. Regarding shellfish species, only one study has been found in recent years that aimed to assess a synbiotic mixture (this study will be discussed in the synbiotic subsection) [90]. No studies were found for the other important European shellfish species (mussels, oysters, and clams).

Table 1. An overview of prebiotics administration for species of interest in European aquaculture.

Prebiotic	Fish Species	Main Results	Reference
GOS, FOS, INL	Common carp	Improved immune response and antioxidant capacity	[148]
Agaricus bisporus powder	Common carp	Improved growth performance and immune response	[149]
Profeed® (scFOS; Maxflow, Jefo, France)	Common carp	Improved survival rate, digestive enzyme activities and microbiota	[150]
INL	Common carp	Improved survival but no effect on growth and digestive enzyme activities	[151]
FOS	Common carp	No effects on growth performance and hematological parameters but increased leucocytes, respiratory burst activity, cultivable bacteria and LAB	[152]
Immunogen® (MOS + β-Glucan)	Common carp	Improved growth and disease resistance	[153]
MOS, INL	Grass carp	Positive effects on non-specific immunity and growth	[154]
GroBiotic-A™ (dairy/yeast) International Ingredient Corporation, Fenton, MO, USA	Channel catfish	Higher weight gain, feed consumption and lysozyme activity; prebiotics probably induced n6 fatty acid biosynthesis	[155]
Actigen® (ACTIGEN Co., Ltd., Samut Sakhon, Thailand)	Channel catfish	Improved disease resistance, with changes in mannose receptor DEC205 and IL4 signaling	[156]
Bio-MOS® (MOS) Alltech, Nicholasville, KY, USA	Senegalese sole	Increased microvilli density and higher intestinal folds	[91]
Inulin, Bio-MOS® (MOS), TOS, GroBiotic®-A	Hybrid striped bass	Prebiotics correlated more with gut structure than digestive enzyme activity.	[157]
EWOS prebiosal®	Atlantic salmon	The enhanced adhering capacity of the probiotic bacteria *Carnobacterium divergens* to the gut epithelium.	[158]
Bio-MOS® (MOS)	Atlantic salmon	Increased protein composition, hepatic glycogen, intestinal absorptive surface, microvilli density and length, and lower occurrence of sea lice.	[159]
Mix of prebiotics, nucleotides, vitamin C and E	Atlantic salmon	Decreased protein turnover with down-regulation of adaptive and innate immune response genes	[160]
Agri-pro (not specified)	Atlantic salmon	Limited effect on microbiome and LAB	[161]
scFOS, XOS, GOS	Sea bass	scFOS and XOS increased the number of OTUs and species richness; 7 days was sufficient to modulate allochthonous gut microbiota	[162]
scFOS, XOS	Sea bass	Increased nitric oxide and lysozyme production, while reduced Ig, monocytes and il10 gene expression	[163]

Prebiotic	Fish Species	Main Results	Reference
scFOS, XOS	Sea bass	XOS decreased lipogenesis, whereas XOS and scFOS increased glycolytic activity. Prebiotics were not effective in counterbalancing the negative effects of plant-based diets on gut morphology; however, XOS reduced antioxidant enzymatic activity	[164]
Bio-MOS® (MOS)	Sea bass	1% dietary Bio-MOS® improved fish growth, feed efficiency, intestinal microvilli length, and survival	[165]
Actigen® (cMOS)	Sea bass	Improved growth, immune parameters, liver and muscle LC-PUFAs, and β-oxidation	[166]
Bio-MOS® (MOS)	Sea bass	Enhanced posterior gut epithelial defense by increasing membrane polar lipids content in relation to a stimulation of the eicosanoid cascade and GALT	[167]
Bio-MOS ® (MOS)	Sea bass	Enhanced disease resistance and protected gut microbiota	[168]
Bio-MOS ® (MOS)	Sea bass	Improved feed utilization, liver morphology and lipid vacuolization, immune parameters and mucus secretion, Increased disease resistance by enhanced gut mucosal barrier protection	[169]
Profeed® (scFOS)	Gilthead seabream	Limited effects on gut microbiota composition, digestive enzyme activities, gut histomorphology and innate immune parameters	[170]
Profeed® (scFOS)	Gilthead seabream	Limited effects on fish metabolism but improved growth at low temperatures (18 °C) with respect to the control group	[164]
Bio-MOS® (MOS)	Gilthead seabream	Improved growth and digestibility	[171]
Immunogen® (MOS + β-Glucan)	Siberian sturgeon	Improved growth and hematological parameters	[172]
AXOS	Siberian sturgeon	Beneficial shift towards the phylum firmicutes with a subsequent acetate, butyrate and total SCFAs increase, dependent on AXOS degree of polymerization	[173]
AXOS	Siberian sturgeon	Improved growth performance, immune response and gut microbiota composition, especially in AXOS with a high degree of polymerization	[174]
FOS	Starry sturgeon	Improved growth performance, survival, LAB, hematological and immunological parameters	[175]
MOS	Rainbow trout	Modulated amino acid catabolism of gut microbes, which influenced feed utilization	[176]

Table 1. *Cont.*

Prebiotic	Fish Species	Main Results	Reference
Immunogen® (MOS + β-Glucan)	Rainbow trout	Enhanced stress tolerance and survival after pathogen challenge	[177]
Active MOS® (MOS, Orffa)	Rainbow trout	Enhanced stress tolerance and survival after pathogen challenge	[178]
Alphamune® (MOS + β-Glucan) Zoetis, Italy	Rainbow trout	Improved growth but limited effects on ADC and digestive enzyme activity	[179]
INL, FOS	Rainbow trout	Improved growth, body composition, intestinal absorption of calcium, and inhibition of *Vibrio*. spp.	[180]
scFOS	Turbot	No effects on innate immune response or hematological parameters; however, oxidative stress was temperature-dependent	[181]

Optimal prebiotic inclusion levels varied depending on factors such as fish species and prebiotic type, with most tested levels ranging from 0.4% to 1%. Dose length typically lasted eight weeks, although one study found significant effects after seven days of exposure [162]. Increasing numbers of prebiotics have been tested in aquafeeds, but MOS was by far the most used one, followed by scFOS, FOS, XOS, GOS, and inulin. Chitosan is another interesting prebiotic, but no studies have been found in species cultured in Europe for the last few years.

Most studies have found beneficial effects on growth and immune responses, but the exact mechanisms of action remain unknown. In this context, a central premise of the prebiotic concept is the positive modulation of intestinal microbiota; however, only a few studies have analyzed the intestinal microbiota. The most frequently used techniques were the culturable bacteria analysis, including LAB analysis and PCR-DGGE, despite these techniques only providing a very limited knowledge of the microbiota composition. Today, to the authors' knowledge, there is only one study with a detailed characterization of gut microbiota modulation using prebiotics, despite the increasing availability of high-throughput sequencing tools [182].

There have been many studies to understand the effects of prebiotics on various species and parameters relevant to aquaculture. The outcomes ranged from many positive results in several species to few adverse effects and no significant effects in some cases. Atlantic salmon (*S. salar*) is one of the essential aquaculture candidates in Europe, and many positive results were found with prebiotic administration in this species. MOS-fed Atlantic salmon smolts had significantly lower sea lice attachment and infection along with various other health benefits like increased intestine absorptive area and more microvilli density but no effect on growth [159]. Another transcriptomic study showed better growth in Atlantic salmon by reducing energy wastage through prebiotic administration [160]. A commercial prebiotic formulation (EWOS prebiosal®) enhanced the adhering capacity of the probiotic bacteria *Carnobacterium divergens* to the gut epithelium of Atlantic salmon [158].

European seabass (*Dicentrarchus labrax*) also showed positive effects on being treated with various prebiotics. MOS improved gut mucus lysozyme activity and increased phagocytic activity of the head kidney, whereas, in another study, it enhanced the disease resistance against *Vibrio anguillarum* [168,169,183]. It was also discovered that MOS promoted the health of the posterior gut through improving the epithelial defense in addition to better growth rates, and later studies showed that inclusion at the rate of 0.16% MOS in the diet could promote growth, increase LC-PUFA accumulation and β-oxidation in European sea bass [166]. The same was confirmed with MOS-Bio-Mos in seabass, which improved sea bass survival [165]. Also, in seabass, it was found that both XOS and scFOS increased glycolytic activity in the fish meal-fed fishes.

In contrast, dietary XOS improved growth and decreased lipogenesis in fish fed with plant protein diets, proving the potential of XOS to be used in European sea bass culture as a prebiotic [164]. These XOS and scFOS effects were explained later as gut microbial community modulation, which lasted throughout time [162]. On the contrary, XOS supplementation in a plant protein-based seabass diet decreased some immune gene expression in the gut [163].

Another essential aquaculture species, rainbow trout (*Oncorhynchus mykiss*), has been under study several times on the prebiotic effects. Immunogen was found to upregulate various immunological parameters, including immune gene expression, and showed protection and disease resistance against *A. hydrophila* [184,185]. Alphamune, a prebiotic mix of MOS and β-glucans, improved growth and nutrient efficiency in rainbow trout [179]. Another prebiotic blend of MOS and Polyhydroxybutyrate improved the disease resistance in the fingerlings of rainbow trout but without affecting growth [178]. Similarly, MOS, GOS, FOS, and inulin in three different studies with rainbow trout were also found to improve growth, body composition, intestinal microbiota, antioxidant enzyme activity, disease resistance, intestinal absorption of Ca, etc. [148,180]. In contrast, inulin and FOS incorporation reduced crude protein in rainbow trout [180].

Positive effects were seen mainly in carp species too. Immunogen improved resistance to *A. hydrophila* and growth in common carp (*Cyprinus carpio*) fingerlings, similar to the rainbow trout (*O. mykiss*) studies [153,184]. A study using GOS, FOS, and inulin showed that GOS has the best effect on growth and improved immune response in carps [148], and a similar positive outcome was obtained with a diet supplemented with White-Button Mushroom Powder (WBMP) in early stages of carp culture [186]. FOS was found to have improved stress resistance and survival and increased the count of LAB and heterotrophic aerobic bacteria. In another study, scFOS enhanced survival rates, digestive enzyme activities, and LAB counts; in both studies, it did not affect growth or other hematological parameters [150]. MOS and inulin positively impacted non-specific immunity and growth of grass carp (*Ctenopharyngodon idella*) [154]. In contrast, carcass protein content was reduced by inulin in a study on common carp fry [151].

Prebiotic supplementation has had positive outcomes in various sturgeon species, too. FOS at 1% showed better growth and improved immunity and intestinal microbiota in juvenile stellate sturgeon (*Acipenser stellatus*) [175]. Arabinoxylan-oligosaccharides (AXOS) with higher polymerization seem to have improved the health of juvenile Siberian sturgeon (*Acipenser baerii*) [174]. Using 454 pyrosequencing, it was found that AXOS modulates the microbiota of the hindgut in juvenile Siberian sturgeon [173].

Channel catfish (*Ictalurus punctatus*) fingerlings showed improved surface mucosal health on prebiotic treatment [156]. MOS has demonstrated a positive effect in hindering pathogen infection in sole (*Solea senegalensis*) by modulating the morphology of the intestine [91]. In another study using hybrid striped bass (*Morone chrysops* x *M. saxatilis*), four prebiotics, including FOS and Bio-MOS, had altered gastrointestinal structure [157]. Two studies were conducted in gilthead sea bream (*Sparus aurata*) on the effects of short-chain fructooligosaccharides (scFOS) on microbiota and immunity and the combined impact of scFOS and rearing temperature on growth and a similar second study in turbot (*Scophthalmus maximus*), where none of them showed any significant effects [170].

4.5. Synbiotics

Synbiotics are nutritional supplements that combine probiotics and prebiotics in the form of synergism. Synbiotics were proposed to characterize some colonic foods with interesting dietary properties, making these compounds candidates for classification as health-enhancing functional ingredients [187]. In humans, it has been well established that prebiotics, probiotics, and synbiotics modulate health conditions mainly by excluding pathogenic bacteria, regulating signaling pathways, and modulating the intestinal immune system of the host. Research on farmed fish has now started describing some of these mechanisms. The probiotic bacteria can either directly inhibit/compete (competing for

nutrients, iron, and adhesion sites) with the pathogenic bacteria or indirectly influence them via autochthonous bacteria [139]. Further, these beneficial organisms can help produce mucus and antimicrobial peptides (such as defensins and chemokines by the epithelium, which play essential roles in these mechanisms), prevent apoptosis, or improve tight junction function, thereby enhancing epithelial barrier function. The inhibitory molecules of the probiotics (e.g., hydrogen peroxide, bacteriocins, and biosurfactants) can deter the pathogen from establishing on the epithelial cell surface [96].

Production of short-chain fatty acids (SCFAs), namely acetic, propionic, and butyric acids, causes a pH drop in the gut via prebiotic fermentation by probiotics in the intestine. It has been reported that SCFAs interact with immune components (e.g., pathogen pattern recognition receptors (PRRs)) in the intestine and can modulate the immune responses in the gut [17,18].

These alternative disease prevention methods have been used to reduce the presence of opportunistic pathogens and simultaneously stimulate the host immune responses. However, other effects not immune-related have been observed, such as improved growth performance, feed utilization, digestive enzyme activity, glucose and lipid metabolism, antioxidant enzyme activity, gene expression, disease resistance, larval survival, gut morphology, alteration of gut microbiota, mediate stress response and improve nutrition [187].

Synbiotics for European commercial species: Table 2 shows the studies assessing the effects of synbiotics supplementation on European species aquaculture after the review by Dimitroglou et al. [91]. A total of six fish species and one shellfish were found. The most studied fish species was the rainbow trout, followed by the gilthead seabream. Surprisingly, only one study was found for Atlantic salmon and sea bass, highly commercial European species. Other species with available information include the common carp and the Siberian sturgeon. Other fish species relevant for European aquaculture of which no studies were found in the last years include channel catfish, European catfish, Senegalese sole, Atlantic halibut, Atlantic cod, starry sturgeon, brown trout, Arctic char, and European eel, among others. As regards shellfish species, only one study was found [188]. No studies were found for the other crucial European shellfish species (mussels, oysters, and clams).

Table 2. An overview of synbiotics administration for species of interest in European aquaculture.

Synbiotic	Fish Species	Main Results	Reference
GOS + *Pediococcus acidilactici*	Common carp	Improved skin mucus and serum immunity	[189]
COS + *Bacillus coagullans*	Common carp	Synergistic effects on enhancing immunity and disease resistance.	[190]
scFOS + *Pediococcus acidilactici*	Atlantic salmon	Improved intestinal morphology and innate immune system without negatively impacting growth performance.	[75]
MOS + *Pediococcus acidilactici*	Sea bass	No effects on gut histology but increased weight and improved disease resistance (survival).	[191]
INL + *Bacillus subtilis*	Gilt head seabream	Upregulation of gene markers involved in the immune system and reinforcement in the junctions between enterocytes.	[192]
INL + *Bacillus subtilis*	Gilt head seabream	Reduction in microbiota diversity and impaired gut morphology.	[192]
INL + *Bacillus subtilis*	Gilt head seabream	Impaired disease resistance despite increased Immunomodulation.	[193]

Table 2. *Cont.*

Synbiotic	Fish Species	Main Results	Reference
INL + Yeast *Debaromycess hansonii* L2	Gilt head seabream	Modulated microbiota and stimulation of the immune system.	[98]
MOS + *Bacillus* spp.	European lobster	Synbiotics enhanced microbial richness and diversity in addition to improved growth, survival, and disease resistance.	[90]
AXOS + *Lactococcus lactis*	Siberian sturgeon	Synergistic effects on the immune system	[173]
MOS + *Saccharomyces cerevisiae*	Rainbow trout	Dynamic microbiome composition shifted towards Firmicutes and Fusobacteria with a positive correlation with plasma cholesterol levels.	[182]
INL, FOS, XOS, GOS or IMO + *Pediococcus acidilactici*	Rainbow trout	P. acidilactici + GOS was found to be a good synbiotic, which indeed promoted fish growth LAC levels	[194]
MOS + *Saccharomyces cerevisiae*	Rainbow trout	Upregulation of genes involved in membrane transport and epithelial barrier defense	[195]
GOS + *Pediococcus acidilactici*	Rainbow trout	The additive effect of synbiotic treatment increased antioxidant defense and disease resistance	[196]
GOS + *Pediococcus acidilactici*	Rainbow trout	Increased disease resistance, innate immune response, skin mucus parameters, and protein levels in synbiotic treatment	[197]
FOS + *Saprolegnia parasitica*	Rainbow trout	Increased feed conversion efficiency, hematological parameters, and disease resistance in 0.1% synbiotic treatment	[198]
FOS + *Entercoccus faecium*	Rainbow trout	Best growth, fish efficiency, and survival after disease challenge in the 0.1 and 0.15% synbiotic treatment	[199]
MOS + Bacillus spp.	European lobster (*Homarus gammarus*)	improves growth, survival, and microbial parameters	[90]

Optimal synbiotic inclusion levels varied depending on factors such as fish species, prebiotic type, and probiotic type, with most tested levels ranging (in Table 2, percentage of prebiotic and probiotic components in the feed) from 0.2% to 1% for prebiotics plus 0.1% to 0.5% for probiotics. The most common synbiotic mix included MOS, INL or GOS, *Pediococcus acidilactici*, *Bacillus subtilis*, or *S. cerevisiae*. Unlike prebiotics, synbiotics studies generally included microbiota analysis (8 out of 19). However, the most frequently used techniques were culturable bacteria analysis, including LAB analysis and PCR-DGGE, which only provide a very limited knowledge of the microbiota composition. Today, to the author's knowledge, there is only one study with a detailed characterization of the gut microbiota modulation using synbiotics, despite the increasing availability of high-throughput sequencing tools [200] showing that Proteobacteria, Firmicutes, *Actinobacteria, Acidobacteria, Bacteroidetes, and Fusobacteria* were dominant phyla. Feeding with synbiotics decreases Gammaproteobacteria abundance and increases *Firmicutes* and *Fusobacteria*, which are supposed to be beneficial.

Generally, most studies conducted to date have evaluated the effects of synbiotics on growth parameters. A good-described positive action of synbiotics in fish is their effect on growth performance and feed utilization [70]. These effects seem mainly attributed to enhanced nutrient availability due to changes in digestive enzyme activity, vitamin synthesis, or gut morphology [201]. It has been exhibited that administration of dietary *Pediococcus acidilactici* in combination with *Saccharomyces cerevisiae* in Pollack, *Pollachius pollachius* larvae [202], or galactooligosaccharide in rainbow trout, *O. mykiss* [196], common carp, *C. carpio* [189] or fructooligosaccharide in angelfish, *Pterophyllum scalare* [203] remarkably promoted growth performance. Also, the improvement of growth was observed in European sea bass (*Dicentrarchus labrax*), rainbow trout (*O. mykiss*), and European lobster (*Homarus gammarus*) fed a synbiotic diet [90] (Table 2).

Effects of synbiotics on fish gut microbiota and morphology: Modulations of performance, health, and disease resistance in fish when fed bioactive feeds are frequently attributed to changes in gut bacterial communities [204]. Gut microbiota affects the host in several ways, including development, digestion, nutrition, disease resistance, and immunity [205]. Prebiotic fermentation by probiotic or beneficial bacteria in the intestine affected bacterial gut communities. In European fish, gut microbiota was studied in a few species, gilthead sea bream *Sparus aurata* L., rainbow trout *O. mykiss*, and European lobster *H. gammarus.* Cerezuela et al. [192] indicated that microbiota diversity was reduced in gilthead sea bream (*S. aurata* L.) fed inulin + *Bacillus subtilis*. In this case, inulin, a prebiotic, might be favored for some specific bacterial groups, but others cannot hydrolyze the prebiotic.

The short-chain fatty acids (SCFAs) and vitamins produced as end-products of prebiotic fermentation by probiotics are responsible for the beneficial effects of feeding synbiotics. SCFAs, as an energy source and vitamins, are absorbed and metabolized by the enterocyte cells, improving the histoarchitectural structure of the intestine to modulate the physiological activities [206]. Abid, Davies, Waines, Emery, Castex, Gioacchini, Carnevali, Bickerdike, Romero, and Merrifield [75] indicated that incorporating a diet with short-chain fructooligosaccharides (scFOS) and *Pediococcus acidilactici* in the synbiotic form improved the intestinal morphology in Atlantic salmon (*S. salar*). Prebiotics and probiotics were reported to enhance gut morphology in European fish by increasing intestine absorptive surfaces, microvilli density, and villi structure [91]. Improving gut morphology directly affects fish immunological status and, consequently, fish health, as preserving a healthy mucosal epithelium reduces the odds of opportunistic bacterial infections [207].

Dietary synbiotics are generally reported to enhance immune status in fish, which were indicated to present enhanced immune parameters such as white blood cell counts, lysozyme activity, alternative complements pathway hemolytic activity (ACH50), and immunoglobulins [94]. Prebiotics (one of the synbiotic-forming parts) interact with gut-associated lymphoid tissue (GALT) in the gut. GALT in fish is formed by intraepithelial and lamina propria leucocytes, including B and T lymphocytes, macrophages, and eosinophilic and neutrophilic granulocytes [208], which are vital defense cells and stimulated in the supplemented diet with synbiotics.

Regarding the immune responses to dietary administration of synbiotics, they were studied in European commercial fish species, namely common carp, *C. carpio*, European sea bass, *D. labrax*, Atlantic salmon, *S. salar*, gilthead sea bream, *S. aurata* L., rainbow trout, *O. mykiss*, and Siberian sturgeon, *A. baerii* or in shellfish, European lobster, *H. gammarus*. Almost all references reported a positive effect of synbiotics on immune responses and disease resistance, except the results on supplementation of dietary inulin in combination with *Bacillus subtilis* in gilthead sea bream (*S. aurata* L.), which was found to impair disease resistance despite increased immunomodulation [192].

The increased survival of fish fed synbiotics against pathogens was reported (Table 2). A primary defense strategy against pathogen bacteria is the augmentation of the innate immune system, particularly that of the GALT, which synbiotics may enhance. Another possibility is prebiotic binding to gut pathogen bacteria, preventing bacterial adhesion to

the colonic epithelium. The cluster formed by prebiotics and pathogens is then excreted with the feces [90].

Probiotics can stimulate the immune responses of fish through different pathways, including the production of short-chain fatty acids (SCFAs) via the fermentation of prebiotics by probiotics in the intestine. It has been reported that SCFAs interact with immune components (e.g., pathogen pattern recognition receptors (PRRs)) in the intestine and can modulate the immune responses in the gut [209]. Also, probiotic bacteria have cell wall components such as lipopolysaccharides, which can stimulate the immune responses of fish (via microbe-associated molecular patterns (MAMPs)). Also, probiotic bacteria can produce bacteriocins and antimicrobial substances, and probiotics can eliminate pathogens and prevent growth by competitive exclusion (competing for iron, space, and food).

5. Conclusions

In this review, we have reported a series of studies that highlight the beneficial effect of using insect meal, recombinant vaccines, and beneficial bacteria individually or in a mix with natural substances that can act as substrates for bacteria present in the intestine or the use of synbionts in the farming of the most representative species of the European aquaculture industry. The vast majority of studies have highlighted a very positive role of probiotics, prebiotics, and synbiotics, demonstrating how using these natural compounds or bacteria, usually present in the intestine or the surrounding environment, can improve the production of many farmed species.

The clear effect of the administration of probiotics, prebiotics, and synbiotics, as well as novel therapies and alternative diets, may represent the future for the development of sustainable aquaculture, which, thanks to their effectiveness, could promote the reduction or even the elimination of the use of antibiotics in aquaculture, fully meeting the suggestions of FAO and WHO.

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