RESEARCH ARTICLE

Dietary Approaches to Attain Fish Health with Special Reference to their Immune System

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Abstract: Fish despite their low collocation in the vertebrate *phylum* possess a complete immune system. In teleost fish both innate and adaptive immune responses have been described with melanomacrophage centers (MMCs) equivalent to mammalian germinal centers. Primary lymphoid organs are represented by the thymus and kidney, while spleen and mucosa-associated lymphoid tissues act as secondary lymphoid organs. Functions of either innate immune cells (*e.g.,* macrophages and dendritic cells) or adaptive immune cells (T and B lymphocytes) will be described in detail, even including their products, such as cytokines and antibodies. In spite of a robust immune arsenal, fish are very much exposed to infectious agents (marine bacteria, parasites, fungi, and viruses) and, consequentially, mortality is very much enhanced especially in farmed fish. In fact, in aquaculture stressful events (overcrowding), microbial infections very frequently lead to a high rate of mortality. With the aim to reduce mortality of farmed fish through the reinforcement of their immune status the current trend is to administer natural products together with the conventional feed. Then, in the second part of the present review emphasis will be placed on a series of products, such as prebiotics, probiotics and synbiotics, β-glucans, vitamins, fatty acids and polyphenols all used to feed farmed fish. With special reference to polyphenols, results of our group using red grape extracts to feed farmed European sea bass will be illustrated. In particular, determination of cytokine production at intestinal and splenic levels, areas of MMCs and development of hepatopancreas will represent the main biomarkers considered. All together, our own data and those of current literature suggests that natural product administration to farmed fish for their beneficial effects may, in part, solve the problem of fish mortality in aquaculture, enhancing their immune responses.

1. INTRODUCTION

Despite their low position in the vertebrate *phylum*, fish possess quite robust immune machinery, which allows them to survive in a very hostile environment. In analogy to mammals, fish immunity can be divided into two major compartments, namely the innate and the adaptive immune system. In fact, teleost fish possess both primary and secondary lymphoid tissues and, specifically, thymus and head-kidney represent primary organs while the spleen and the mucosa-associated lymphoid tissues (MALTs) are secondary organs. In turn, MALTs can be divided into gut-associated lymphoid tissue, gill-associated lymphoid tissue (GIALT), skin-associated lymphoid tissue and nasopharinx-associated lymphoid tissue [1]. MALTs are covered by a protective layer of mucus containing antibodies which interact with both commensal and invading microorganisms [2-4]. They are not organized lymphoid structures as in the case of mammal lymph nodes but fish myeloid and lymphoid cells constitute a diffuse network, even if an interbranchial lymphoid tissue has been identified in GIALT [5]. As later discussed in this review, melanomacrophage centers (MMCs) are pigmented phagocytes distributed in kidney, spleen and liver of fish [6-8]. MMCs belong to the innate immunity and are considered as precursors of the mammal germinal centers (GCs) [9]. Furthermore, in fish, innate immunity is based on the function of other immune cells, such as granulocytes, macrophages and dendritic cells (DCs), but also non-immune cells, such as intestinal M-like cells, red blood cells and thrombocytes participate to immune defense [10-17].

Fish innate-like lymphocytes are positioned between innate and adaptive immunity, and, in particular, extrathymic γδ T cells have been identified [18-20]. As discussed in the next section of this review, fish intestine represents the major source of innate-like lymphocytes in analogy to mammalian-like lymphocytes [21].

With special reference to fish adaptive immunity, both CD4^{+} and CD8^{+} cells are largely present in MALTs and their phenotypes and functions will be discussed in a specific section of this review [22-24]. B cells are also contained in MALTs and immunoglobulin (Ig)T represents the equivalent of secretory IgA in mammals, thus, protecting mucosal sites from invading pathogens [25]. Despite a robust immune system, fish succumb because of severe infections and, especially, in farmed fish mortality is very high due to invasion by pathogens and stressful environmental conditions [26, 27]. Therefore, preserving fish health in aquaculture and prevention of disease is a major focus of the current research in this field. This achievement may result from the combination of different strategies such as innovative feeds, low fish density and corroboration of the immune response [28]. Particularly, administration to fish of dietary supplements has been increasing in the last few years and, here, this procedure will be elucidated in detail.

On these grounds, aims of the present review are the illustration of piscine innate and adaptive immune responses but also of the role played by natural products in the enhancement of fish immunity against infectious challenges and stressful conditions.

2. INNATE IMMUNITY

2.1. Melanomacrophage Centers (MMCs)

MMCs are nodular accumulations of melanin-macrophages mostly distributed in kidney, spleen and liver of fish. Pigments

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(lipofuscin, melanin and hemosiderin) contained in MMCs can be of both exogenous and endogenous origin and the latter is derived from phagocytosed erythrocytes [29]. This last event suggests that MMCs play a role in iron recycling similarly to hemosiderin-laden splenic macrophages in mammals [30]. Besides erythrocytes, there is evidence that MMCs phagocytose pathogens [31, 32]. In turbot, the evidence that splenic MMCs are in close association with ellipsoids (which are capillaries) may suggest their property to phagocytose blood-borne pathogens [33]. Besides the above mentioned functions, MMCs have been considered as primitive precursors of mammalian GCs, even if such a contention needs further validation. In fact, fish lack GCs and MMCs, which may also be due to the adaptive immune responses [34, 35]. In this framework, fish immunized with bacteria are able to retain antigens in and around splenic MMCs, trapped within immune complexes (ICs) and such retention can be enhanced by injection of preformed ICs [36-38]. More specifically, the above results support a similarity between MMCs and mammalian follicular DCs in the context of GCs, thus, allowing antigen presentation to T helper (h) 2 cells, and ultimately, promoting B-cell antibody formation [39].

Conclusively, as recently reviewed by Steinel and Bolnick [40], one can hypothesize that MMCs have evolved as "garbage dumps" from a common precursor of vertebrates, and, then, after meeting pathogens and antigens have acquired immunological functions, finally, evolving into GCs.

2.2. Macrophages

Fish macrophages are distributed in mucosal and non-mucosal tissues (spleen, kidney and liver) and in analogy to the mammal counterpart can also be divided into subsets. In mammals, one can distinguish M1 (classically activated macrophages) and M2 a, b and c (alternatively activated macrophages) [41]. Functionally, M1 are inflammatory, while M2 and, mostly, M2c are anti-inflammatory cells, also secreting angiogenic mediators for the resolution of inflammation. In particular, M1 cells exert phagocytosis of pathogens, produce free radicals and pro-inflammatory mediators [42-44]. M2 fish macrophages have been analyzed for arginase gene expression and activity, as well as, gene expression and function of interleukin (IL)-10 and transforming growth factor-β. However, the fine regulatory mechanisms involved in the polarization toward M2a-c cells need further investigation [45-47].

Colony stimulating factor (CSF)-1 represents the major growth factor for fish macrophages and in most fish CSF-1.1 and CSF-1.2 molecules have been identified [48]. CSF-1.1 promotes the differentiation toward M1 macrophages in goldfish (*Carassius auratus*) [49]. In support of the above finding, evidence has been provided that a soluble CSF-1 receptor inhibits the pro-inflammatory pathway in goldfish macrophages. Quite interestingly, in some fish species, two distinct *csf1r* genes have been detected, thus, suggesting that these animals have elaborated more complex mechanisms of macrophage differentiation in comparison to mammals [50].

In order to cope with pathogens, fish macrophages bear both extracellular and intracellular pattern recognition receptors (PRRs). At least five types of PRRs have been identified, namely, Toll-like receptors (TLRs), c-type lectins, nucleotide-binding domain-leucine-rich repeat containing receptors (NLRs or NOD-like), retinoic acid inducible gene I-like receptors and absence in melanoma-like receptors [51]. Besides macrophages, also neutrophils, monocytes, DCs, epithelial and endothelial cells possess PRRs. Bony fish display 17 TLRs with TLR**s** 20-23 present only on fish [52, 53]. Of note, not all fish bear TLR-4, a specific receptor for Gram-negative lipopolysaccharides (LPS) and, in particular, zebrafish TLR-4 does not recognize LPS [54]. NLRs have been identified in fish and seem to share conserved structural domains with mammalian NLRs. In this respect, studies have been focused on gene expression and to a lesser extent on signaling following NLR stimulation [55, 56].

Fish M1 macrophages exert a potent anti-microbial activity which encompasses different phases. Following phagocytosis of pathogens, generation of superoxide anions (respiratory burst) and nitric oxide (NO) production are two independent macrophage-mediated anti-microbial functions [57]. Another mechanism related to the microbicidal activity of M1 macrophages is the indoleamine 2,3-dioxygenase (IDO)-mediated degradation of tryptophan [58]. However, IDO can control immune response via production of kinurenins and, in turn, interferon (IFN)-γ-stimulated macrophages produce IDO [59, 60]. Fish M2 macrophages play both anti-inflammatory and pro-healing effects. The fish IL-4/13a and IL-4/13b exert anti-inflammatory effects, dampening the gene expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β and IFN-γ [61, 62]. Quite interestingly, fish M2c macrophages under influence of cortisol up-regulate the IL-10 gene expression and in the gold fish recombinant IL-10 suppresses reactive oxygen species production and inflammatory gene expression [63]. Arginase production is a marker of M2 cell activity in fish [64]. In fact, arginase converts L-arginine to L-ornithine which, in turn, represents a precursor for polyamine and proline constituents of collagen for tissue repair to occur [65]. Fish possess both *arginase-1* and *arginase-2* genes with M1 cells expressing more *inducible nitric oxide synthase* gene (inflammatory profile) and M2 cells expressing more *arginase* genes (anti-inflammatory/tissue repairing profile) [66].

Fish macrophages also serve as antigen presenting cells (APCs) and interact with T lymphocytes via the major histocompatibility complex (MHC)-1 expression, whose extent is different according to species [67]. Of note, some fish species have lost the MHC-II, even if they mount an effective immune response against pathogens [68, 69]. Other fish species have expressed non-classical MHCs whose lineages have separated before the emergence of tetrapods [70]. In this framework, it is important to emphasize the role of fish DCs which act as APCs. DCs express MHC-II and CD83 markers, also exerting potent phagocytic properties and induction of CD4+ T cell production [70-72].

In this context, the immune role exerted by fish erythrocytes is worth mentioning. *In vitro* studies have demonstrated that trout erythrocytes are able to engulf *Candida* (*C.*) *albicans* particles and present them to surrounding head-kidney macrophages [13, 14]. Furthermore, mature fish erythrocytes which have engulfed *C. albicans* have been shown to release in supernatants cytokine-like factors able to potentiate macrophage function, while inhibiting their migration (macrophage inhibition factor-like activity) [15]. Furthermore, evidence has been provided that *C. albicans* phagocyting trout erythrocytes form aggregates with macrophages (the so-called rosettes), thus traveling via circulation to distant organs, and, finally, contributing to the formation of MMCs [16]. Quite interestingly, in infected trout with *C. albicans,* evidence of rosette formation in blood has been provided, and, in addition, fish thrombocytes were present in close association with erythrocytes [17]. These data suggest that fish thrombocytes may also play an immunological role in the clearance of *C. albicans*. Major innate immune cells functions are summarized in Table **1**.

3. FISH LYMPHOCYTES

In a recent review [73], the question has been poised on the equivalence between fish lymphocytes and mammalian innate-like T cells. In mammals and fish, innate-like T cells are mostly located in the intestine as intestinal intraepithelial lymphocytes (IELs) characterized by an archaic T cell receptor (TCR), the so called γδTCR [74, 75]. γ+δ+ cells can develop extrathymically in the absence of antigen encountering and exert an array of functions, such as direct killing of infected cells, tumor immunosurveillance, cytotoxicity and release of both regulatory and pro-inflammatory cytokines [76]. Quite interestingly, subsets of murine γδ cells have been identified which are activated by innate stimuli rather than TCR

Table 1.   Main innate immune cells in fish.

Cell types	Functions
Macrophages Melanomacrophage centers	Accumulation of pigmented macrophages with phagocytic activity [35] and primitive precursors of mammalian germinal centers [34, 35].
M1 and M2 (a, b, c) macrophages	M1 are phagocytic macrophages that produce free radicals and pro-inflammatory cytokines [42-44]. M2 macrophages prevalently exert anti-inflammatory activities [45-47]. In general terms, macrophages act as antigen presenting cells to T lymphocytes via MHC-I.

ligation and known as IL-17 producing (γδT-17) and IFN-γ producing (γδT-IFN-γ) cells [77].

Innate-like B cells in the mammals are the B1-B cells also subdivided into B1a and B1b-B cells [78]. B1 cells secrete natural polyreactive antibodies in a T-independent fashion, being phagocytic, microbicidal and involved in autoimmune processes [79, 80].

In teleost fish, T cells are largely present in gills and intestine and express the TCRαβ on their surface in the presence of either CD4 coreceptor or CD8 coreceptor. Then, CD4$^+$ Th cells, CD8$^+$ T cytotoxic cells, T regulatory (Treg) cells and Th17 cells have been identified [81-83].

Fish intestine is the major site of immune responses as described below. In the intestine, IEL *in vivo* undergo recombinant-activating gene (RAG)-mediated somatic rearrangement of a given V/C combination in the CDR3 junction length of TCRβ-chain/TCRγ-chain [84, 85]. Conversely, gill T cells *in vitro* proliferate when stimulated by lectins but do not express RAG [86]. In the sea bass intestine, evidence has been provided that T cells express RAG-1, TCRα, TCRγ, CD8α and CD4, thus, suggesting that gut is the main source of T cells in adult fish [87]. Conversely, gills can be considered as a source of T cells committed as effector/helper cells [86]. In this context, a special issue is undoubtedly represented by fish immune tolerance. Sugimoto and associates [88] have clearly demonstrated that zebrafish lacking a functional FoxP3 ortholog exhibit a reduced survival with the development of a pro-inflammatory profile. In fact, in analogy to mammals, Treg cells are controlled by a master regulatory gene encoding FoxP3. The same group has also demonstrated that zebrafish Treg cells mediate organ-specific regeneration, while in the absence of FoxP3 deficient Treg cells infiltrate damaged organs (*e.g.,* spinal cord, heart and retina) but do not express regenerative factors [89]. Furthermore, the intrabranchial lymphoid tissue in gills promotes immune tolerance in view of its high expression of FoxP3 [90]. This homeostatic mechanism seems to be very effective in the gills where continuous exposure to a variety of antigens may lead to exaggerated inflammatory immune responses. Main functions of T lymphocytes are expressed in **Table 2**.

Table 2.   Subsets of fish T lymphocytes.

Cell types	Functions
γ$^+$δ$^+$ T cells	Direct killing of infected cells, immunosurveillance, cytotoxicity and release of both regulatory and pro-inflammatory cytokines [76].
α$^+$β$^+$ T cells (CD4$^+$ Th cells, CD8$^+$ T cytotoxic cells, Treg cells and Th17 cells)	Gill and intestinal T cells are the major sites of these lymphocytes and their anti-inflammatory activity is prevalent [89, 90].

The origin of fish B cells is still uncertain. In zebrafish, the pancreas has been indicated as the primary source of B cells [91]. In sea bass, the presence of IgM producing cells in kidney may suggest that this organ is the primary site of B cell production [92]. Fish B cells express *μ, θ, δ* genes which lead to three different Ig classes, namely, IgM, IgT and IgD, as recently reviewed by Flajink [93]. IgM are tetrameric and their concentration is very abundant in biological fluids [94]. IgT (for immunoglobulin teleost) is a mucosal antibody isotype produced as a monomer [95]. However, in trout mucus a non-covalent polymeric IgM association has been reported [96]. Finally, IgD is expressed in monomeric form and in analogy to humans it can be involved in inflammatory responses binding to a still unknown receptor on the surface of basophils [97]. In **Table 3**, fish antibody classes and functions are outlined.

Table 3.   Antibody classes in fish.

Class	Functions
IgM	They are tetrameric and very abundant in biologic fluids of non-immunized fish [93].
IgT	This is a mucosal antibody equivalent to mammalian IgA and produced as a monomer [95].
IgD	They are monomeric, being involved in inflammatory activities via binding to basophils [97, 98].

Conclusively, according to Scapigliati and associates [73] fish B cells have certain properties, such as elevated concentration of IgM in non-immunized fish, low increase of IgM affinity following secondary immunization, possible expression of TLRs and pathogen phagocytosis, kidney B1-B cells similar to the splenic murine counterpart and proliferating B cells in peritoneum. Quite interestingly, the first teleost CD5 molecule has recently been identified [98]. This finding represents an additional evidence of phenotypical and functional similarities between fish IgM$^+$ B cells and mammalian B1 cells.

There are experimental evidences in favor of immunological memory in fish which are mostly based on adoptive transfer of peripheral leukocytes from vaccinated donors to naïve histocompatible recipients [99]. Cell transfer experiments afford long term protection in recipients but if the mechanisms triggered by these cells may be considered "memory" needs further clarification.

As indicated in the previous paragraphs, fish lymphocytes are able to secrete an array of cytokines. In this regard, the best studied fish T lymphocyte cytokine is IFN-γ as a product of Th1 cells [100]. Two different types of IFN-γ have been identified in fish, namely IFN-γ and IFN-γ rel [101]. A functional dichotomy has been observed in these two types of fish IFN-γ, mostly, in terms of their effects on macrophages and monocytes, as extensively reported by Grayfer and associates [102, 103]. It would be very interesting to decipher whether there are differences between these two

IFNs in exerting antimicrobial activities against various fish pathogens.

At the end of this section, one has to emphasize that teleost fish possess a commensal microbiota able to shape the immune response, as recently reviewed [104]. Studies on the interaction between fish microbiota and immune system will contribute to solve problems in aquaculture system, preventing development of infections.

4. NATURAL PRODUCTS AS MODULATORS OF FISH IMMUNE RESPONSE

Fish disease (viral, bacterial, fungal and parasitic infections) have been increasing, thus, causing noticeable economical losses in the aquaculture industry [105-107]. Particularly, juvenile marine fish undergo elevated mortality rate during the larvae culture phase due to chronic stress and bacterial infections [108, 109]. In aquacultures, high population density and manipulation seem to favor growth of opportunistic bacteria with reduction of beneficial microorganisms, thus, leading to mortality [110, 111]. In order to overcome the problem of fish mortality three main strategies have been adopted, namely: **i.** the maintenance of microbiota equilibrium in aquaculture; **ii.** the improvement of water quality and, finally, **iii.** the enhancement of the immune response by larvae [112]. With regard to immune enhancement in teleost fish, immune stimulation strategy is based on the application of compounds which potentiate the function of phagocytic cells, thus, increasing their bactericidal and fungicidal functions [113]. The observed high mortality in larvae may depend on their immature adaptive immune system, and, therefore, immune stimulation may be very effective on the**ir** natural arm of immunity [114]. In synthesis, larval stage is a very critical phase in aquaculture where application of immunostimulant strategies appears to be more effective. On these grounds, in the next paragraphs several compounds of natural origin will be illustrated for their capacity to regulate/potentiate fish immune response in aquaculture.

4.1. Prebiotics, Probiotics, Synbiotics

According to Bindels and associates [115], "prebiotic concept typically refers to non digestible food ingredients or substances that pass undigested through the upper part of the gastrointestinal tract and stimulate the growth and/or activity of health-promoting bacteria that colonize the large bowel." Major prebiotics are fructose oligosaccharides (FOS), galactose oligosaccharides (GOS), xylose oligosaccharides (XOS), inulin, polydextrose, dietary fibers and mannan-oligosaccharides [116]. Prebiotics administered to fish as food or feed ingredients beneficially impact on the fish intestinal immune system [117]. Common carp fed FOS, GOS and inulin underwent a significant increase in skin mucus lysozyme activity, especially in GOS-fed group [118]. Administration of GOS to Caspian white fish increased immunoglobulin levels [119]. In zebrafish, administration of sodium propionate increased appetite and mucus gene expression [120]. In a series of reports, evidence has been provided that administration of prebiotics to fish has led to increased anti-inflammatory cytokine expression and regulation of appetite gene expression also via production of short chain fatty acids (SCFAs) [121-126]. Other lines of evidence have documented that prebiotics are able to increase intestinal digestion and absorption in juvenile golden pompano, augmenting villus length, villus width and their numbers as well muscle thickness at all gut segments [127]. In zebrafish, administration of chitosan silver nanocomposites reduced the number of Proteobacteria (a *phylum* of Gram-negative bacteria enriched in pathogens) [128]. Conclusively, as recently reviewed [129], prebiotics in addition to a direct effect on the fish gut immune system are digested by intestinal microbiota for the production of SCFAs which, in turn, promote the killing of pathogens.

Probiotics are defined as "microorganisms that when consumed (as in food or a dietary supplement) maintain or restore beneficial bacteria to digestive tract" [130]. There are a few evidences on the beneficial effects of probiotic supplementation to fish. Bioflocs are rich in various probiotics and bioactive compounds and their effects on juvenile sea cucumber *Apostichopus japonicus* have been analyzed [131]. From an immunological point of view, biofloc increased phagocytosis and respiratory burst in comparison to untreated fish, also upregulating immune-related genes such as heat shock proteins [90, 70, 105], rel, nitric oxidase and lysozyme. In treated fish challenged with *Vibrio splendidus,* mortality significantly decreased in comparison to controls. *Lactobacillus* (*L.*) *delbrueckii* administration to *Cyprinus* carpio huanghe var has recently been investigated to evaluate resistance against *Aeromonas (A.) hydrophila* [132]. Results have demonstrated that *L. delbrueckii* supplementation upregulated lysozyme, acid phosphatase and myeloperoxidase activities and antioxidant enzymes [superoxide dismutase (SOD), catalase, gluthatione peroxidase)], while downregulating mRNA levels of IL-1β, IL-8, TNF-α and NF-κB p65. In view of these effects growth performance and survival rate improved. In another study, two recombinant *L. casei* (surface-displayed or secretory) expressing the OmpAI of *A. veronii* were administered to *Cyprinus carpio* challenged with *A. veronii* [133]. In comparison to untreated fish after *A. veronii* infection, fish fed with recombinant *L. casei* exhibited higher titers of serum and skin mucus antibodies, lysozymes and gene expression of IL-1β, IFN-γ, IL-10 and TNF-α. Immune modifications positively correlated with higher survival in treated fish in comparison to controls.

In view of the beneficial effects exerted by pre- and probiotics when independently administered to fish, their association, the so-called synbiotics, has also been experimented in fish. As extensively reviewed by Huynh and associates [134], synbiotics administration to fish increased nutritional absorptive capacities, on the one hand, and, increased natural and adaptive immune responses on the other hand. Besides that, synbiotics were able to modify intestinal microbiota in *Apostichopus japonicus* during vibrio infection, increasing proportions of *Bacillus* and *Lactococcus* in comparison to controls [135].

4.2. β-Glucans

β-glucans are natural polysaccharides largely produced by bacteria, fungi and yeast [136]. β-glucans act through a specific receptor dectin-1 (not yet well defined in fish) and TLR-2 on leukocytes [137]. Activation of dectin-1 triggers intracellular signaling, *e.g.,* Syk/NF-κB pathways, thus, leading to increased expression of immune genes and release of pro-inflammatory cytokines (IL-1, IL-8, IL-12, TNF-α). β-glucans are profusely used in aquaculture for their beneficial effects on the immune system and increased resistance to stress. In cod larvae, β-glucans have been administered via encapsulation by live food [138]. Mostly, β-glucans extracted from diatoms were very effective in survival and growth. In a recent study [139], *Sterigmatomyces halophilus* β-glucans, when administered to pacific red snapper, increased phagocytic activity, respiratory burst, NO production, SOD and catalase activities and immune genes for IL-1β, IL-10 and IL-17. This led to an increased resistance against *A. hydrophila*. Similar protective effects were observed in *Oreochromis niloticus* challenged with *Staphylococcus iniae* [140]. Increase in anti-oxidant, anti-inflammatory, anti-stress and immune-related genes contributed to the protective effect against bacterial infections. Furthermore, in an *in vitro* model, fungal β-glucans increased in head-kidney leukocytes from pacific red snapper NO production, myeloperoxidase, SOD and catalase activities as well as induction of pro-inflammatory cytokines [141].

4.3. Lipids

There is a large body of evidence that polyunsaturated fatty acids (PUFAs), when ingested with food or as supplements exert

beneficial effects to the host, even including the switch-on of an intestinal immune tolerogenic pathway and the improvement of gut microbiota [142, 143]. In farmed fish, a few experimentations with PUFAs have been conducted in order to promote growth of healthy fish despite pollution, overcrowding and risk of infections. In a recent report [144], rainbow trout juveniles were administered with four PUFAs, namely, α-linoleic (ALA), linoleic acid (LA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). All four PUFAs were combined with an environmentally-realistic concentration of cadmium. Some fish were challenged with *A. salmonicida* at the end of cadmium conditioning period. Despite the fact that cadmium even at low doses could down-regulate the fish immune response, PUFA administration improved growth (DHA), protected against oxidative stress (ALA and EPA) and enhanced innate immunity. Hepatic steatosis frequently occurs in farmed fish, ultimately leading to severe inflammation. In large yellow croaker, administration of omega-3 PUFAs mitigated liver steatosis-induced inflammation [145]. The protective mechanism exerted by omega-3 seems to rely on the sirtuin-1-mediated nuclear translocation of NF-κB p65 subunit in hepatocytes.

Arachidonic acid supplemented to rabbit fish *Siganus rivulatus* during an outbreak of *S. iniae* prolonged survival in comparison to controls, while increasing lysozyme, complement and total serum Ig levels [146]. Conclusively, this regimen could improve growth survival and immune functions.

α-lipoic acid (α-LA) when supplemented to grass carp has been demonstrated to prevent omega-3-induced lipid peroxidation [147]. In fact, in grass carp omega-3 dietary regimen could increase production of malondialdehyde, thus, inducing lipid peroxidation in liver and muscle. This α-LA-mediated effect was based on the increased expression of NF-E2-related nuclear factor 2 and decrease of kelch-like-ECH-associated protein 1 mRNA levels.

Sodium butyrate (SB) administration to young grass carp enhanced intestinal immune function associated with NF-κB and p38 MAPK signaling [148]. In general, SB ameliorated growth performance, intestinal growth and function, increasing the number of Lactobacilli in the gut microbiota. In particular, either natural immunity (increase in C3 and C4, IgM and antimicrobial peptides) or adaptive immunity (increase in anti-inflammatory cytokines) were modulated by SB.

4.4. Vitamins

Vitamins are essential micronutrients endowed with multiple immune functions in animals and humans. In an *in vitro* study, using the rainbow trout monocyte-macrophage cell line RT811 and head-kidney leukocytes vitamin C increased production of phagocytic cell number [149]. Furthermore, vitamin C also enhanced the transcription of several pro-inflammatory and antimicrobial genes triggered by *Escherichia coli*. Of note, evidence has been provided that vitamin C attenuates the toxic effects of 17α-methyltestosterone, reducing up-regulation of pro-inflammatory cytokines, chemokines, TLR-7, IgM and apoptosis [150]. Finally, as recently reviewed by Dawood and Koshio [151] vitamin C is essential for growth health and stress resistance in fish.

In European sea bass administered with vitamin D3 an increase in phagocytic capacities was observed [152]. In head-kidney and gut, increase in fbl and rbl transcripts and *hep* gene, respectively, were detected.

Vitamin E effects on fish have also been investigated. In turbot fed high-fat diet vitamin E administration prevented induction of hepatic oxidative stress [153]. Furthermore, increase in non-specific immunity and modulation of inflammatory genes was documented. In *Tilapia nilotica*, vitamin E supplementation attenuated the ZnONPs-mediated oxidative stress [154]. In particular, ZnONPs administration in the absence of vitamin E increased alanine transaminase, aspartate transaminase and alkaline phosphatase ac-

tivities and generated oxidative stress inhibiting SOD and catalase activities. In Chinese mitten crab fed the diet with fish oil and vitamin E improved growth and anti-oxidant functions along with high resistance to *A. hydrophila* were observed [155]. In *Anguilla japonica,* dietary vitamin E led to higher hemoglobin concentration and white blood cell numbers also increasing lysozyme and SOD activity [156].

4.5. Polyphenols

Polyphenols are natural products largely present in the vegetal kingdom and, especially, in vegetables, fruits, cereals and beverages such as wine, tea, chocolate and juices [157]. Structurally, polyphenols can be divided into flavonoids and non-flavonoids (*e.g.,* resveratrol) compounds [158]. Numerous reports have firmly confirmed the anti-oxidant and anti-inflammatory abilities of polyphenols. In the last decade, our own group has documented the efficacy of polyphenols extracted from red wine or fermented grape marc to exert anti-inflammatory activities, promoting a tolerogenic pathway either *in vitro* or *in vivo* in experimental models and humans [155-168]. Also in farmed fish polyphenols have been used as supplement to conventional feed. In gold fish, subjected to a hypercholesterolemic diet, administration of polyphenols derived from waste water from an olive mill reduced steatotic damage [169]. In farmed turbot, resveratrol administration prevented the outcome of Scutociliatosis, exerting potent anti-protozoal activity against the Hystiophagous ciliate *Philasterides dicentrarchi* [170]. In this framework, in juvenile GIFT tilapia administration of resveratrol at higher doses (0.3 g/kg) induced hepatic damage and intestinal deformation in comparison to the lower doses (0.1 g/kg) [171]. In zebrafish, caffeic acid and hydroxytyrosol prevented the obesogenic effect generated by the peroxisome proliferator-activated receptor γ agonist, rosiglitazone [172]. In the same test system, both compounds prevented lipid accumulation in primary-cultured rainbow trout adipocytes with hydroxytyrosol suppressing the mRNA levels of the *fasn* gene in the adipose tissue of this fish. In another report, farmed European sea bass juveniles have been administered with polyphenols extracted from red grape (Canosina Nero di Troia, *Vitis vinifera*) at two different concentrations (100 and 200 mg/kg) [173]. Fish were evaluated for gut and splenic cytokine release and MMCs areas and distribution. A decrease in intestinal IL-1β and IL-6 release with an increase in MMCs areas was observed. Conclusively, following administration of polyphenols, reduction of pro-inflammatory cytokines and increase in protective natural immunity and adaptive immune response (splenic IFN-γ) led to a reduced fish mortality in comparison to controls. Using the same dietary regimen, polyphenols led to an expansion of farmed sea bass hepatopancreas, which may have contributed to reduced mortality [174]. In Table **4*** major interventions with natural products in farmed fish are summarized.

CONCLUSION AND FUTURE PERSPECTIVES

As extensively discussed in the previous sections of this review, fish mortality in aquaculture is still high due to stressful conditions and mostly infectious agents. As recently reviewed by Mweemba Munang'Andu and associates [175], genomic studies have been applied to farmed fish for discovering new pathogens, genes linked to disease resistance and innovative approaches to control and prevent piscine disease. For instance, hepcidin-like antimicrobial peptides (Th1-5, Th2-2, and Th2-3) have been identified in tilapia, using phage hybridization [176]. Hepcidin has been shown to exhibit antimicrobial activities against a wide range of extracellular and intracellular bacteria and viruses in many fish either *in vitro* or *in vivo* [177-180]. Recombinant IFNs and, especially, IFN-α2 have been shown to be protective against viruses in rainbow trout, even if immunity had a short duration [181]. IFN encapsulated in nanoparticles when administered to fish increased relative percent survival against nervous necrosis virus infection [182]. However, in Atlantic salmon infectious pancreatic necrosis virus could replicate

Table 4. **Dietary products administered to farmed fish.**

Products	Main functions
Prebiotics (FOS, GOS, XOS), inulin, polydextrose, dietary fibers and mannan oligosaccharides	Increase in immune performance [117-120]. Digestion by fish intestinal microbiota and production of short chain fatty acids [129].
Probiotics	Increase in innate immunity, anti-inflammatory activities, enhanced resistance against microorganisms [131-133].
Synbiotics (pre+ probiotics)	Increase in natural and adaptive immunity [134].
β-glucans	Activation of the receptor dectin-1 and increased expression of immune genes and release of pro-inflammatory cytokines [137-140].
Lipids: 1. polyunsaturated fatty acids	Enhancement of innate immunity, protection against oxidative stress, mitigation of liver steatosis [144, 145].
2. α-lipoic acid	Prevention of omega-3 induced lipid peroxidation [147].
3. Sodium butyrate	Increase in intestinal immune functions [148].
Vitamins (C, D, E)	Vitamin C increases production of reactive oxygen species and phagocytic number [149]. Vitamin D increases phagocytic capacities [152]. Vitamin E prevents hepatic oxidative stress, increases non-specific immunity and modulates inflammatory genes [153, 154].
Polyphenols	Prevention of steatotic damage and obesogenic effect mediated by rosiglitazone, reduction of intestinal pro-inflammatory cytokines, increase in splenic IFN-γ release and expansion of hepatopancreas [169, 172, 173].

*Further details have been added in the text

at higher rate despite the presence of type I IFN [183]. In synthesis, according to Ooi and associates and Xu and associates [182, 184], type I IFNs are able to induce the expression of antiviral genes, also activating signaling pathways, which, in turn, generate other antiviral genes in fish. Our group has recently contributed to the issue of better health of farmed fish demonstrating that conventional feeds enriched in polyphenols (extracted from seeds of red grapes) improve the gut immune response also decreasing mortality in sea bass (*Dicentrarchus Labrax L.*) [173]. Furthermore, in these fish the same dietary regimen increased the size and morphology of hepatopancreas, thus, further contributing to fish health [174]. Conclusively, all the above cited data are representative of the current studies aimed at preventing and controlling farmed fish disease.

LIST OF ABBREVIATIONS

α-LA	=	α-lipoic acid
ALA	=	α-linoleic
APCs	=	Antigen presenting cells
CSF	=	Colony stimulating factor
DCs	=	Dendritic cells
DHA	=	Docosahexaenoic acid
EPA	=	Eicosapentaenoic acid
FOS	=	Fructose oligosaccharides
GCs	=	Germinal centers
GIALT	=	Gill-associated lymphoid tissue
GOS	=	Galactose oligosaccharides
ICs	=	Immune complexes
IDO	=	Indoleamine 2,3-dioxygenase
IFN	=	Interferon
Ig	=	Immunoglobulin
IELs	=	Intraepithelial lymphocytes
IL	=	Interleukin
LA	=	Linoleic acid
LPS	=	Lipopolysaccharides
MALTs	=	Mucosa-associated lymphoid tissue
MHC	=	Major histocompatibility complex
MMCs	=	Melanomacrophage centers
NLRs	=	Nucleotide-binding domain-leucine-rich repeat containing receptors
NO	=	Nitric oxide
PRRs	=	Pattern recognition receptors
PUFAs	=	Polyunsaturated fatty acids
RAG	=	Recombinant-activating gene
SB	=	Sodium butyrate
SCFAs	=	Short fatty acids
SOD	=	Superoxide dismutase
TCR	=	T cell receptor
Th	=	T helper
TLRs	=	Toll-like receptors
TNF	=	Tumor necrosis factor
Treg	=	T regulatory
XOS	=	Xylose oligosaccharides

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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