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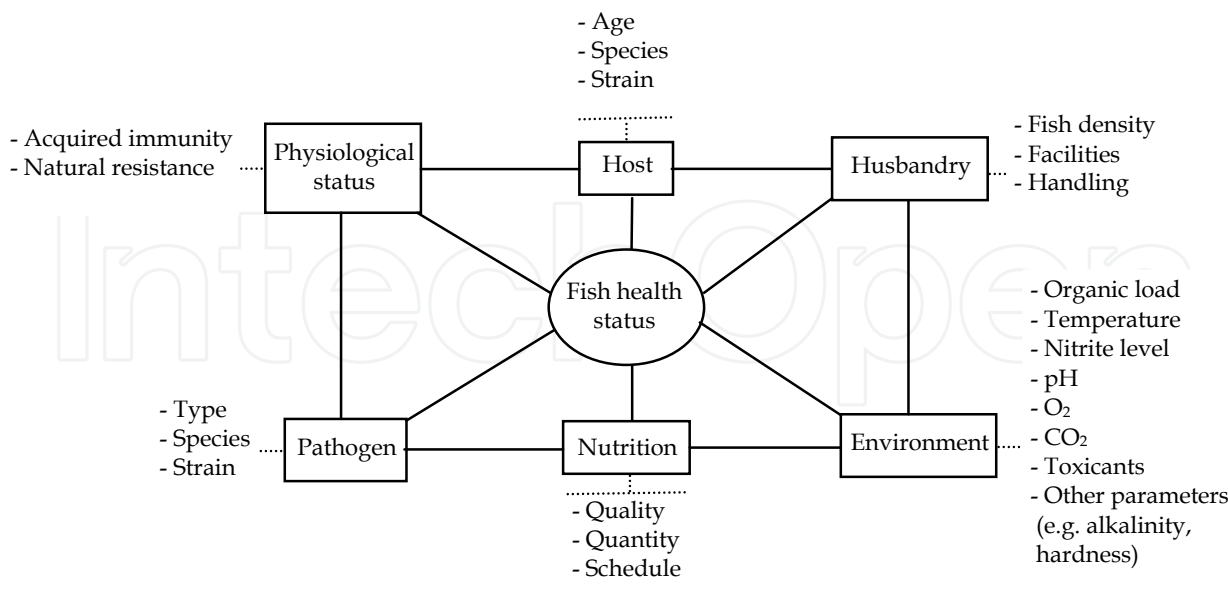
The Immune System Drugs in Fish: Immune Function, Immunoassay, Drugs

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1. Introduction

Fish is a heterogeneous group of different organisms which include the agnathans (hagfishes and lampreys), condryctians (sharks and rays) and teleosts (bony fish). Like in all vertebrates, fish have cellular and humoral immune responses, and central organs whose the main function is involved in immune defence. Fish and mammals show some similarities and some differences regarding immune function (Cabezas, 2006; Nelson, 1994; Tort et al., 2003; Zapata et al., 1996). The fish defence system is basically similar to that described in mammals. For cellular defence systems in fish, teleosts have phagocytic cells similar to macrophages, neutrophils, and natural killer (NK) cells, as well as T and B lymphocytes. Teleosts also have various humoral defence components such as complement (classical and alternative pathways), lysozyme, natural hemolysin, transferrin and C-reactive protein (CRP). The existence of cytokines (such as interferon, interleukin 2 (IL-2), macrophage activating factors (MAF)) has also been reported (Secombes et al., 1996, Sakai, 1999). On the contrary, the morphology of the immune system is quite different between fish and mammals. Most obvious is the fact that fish lack bone marrow and lymph nodes. Instead, the head kidney serves as a major lymphoid organ, in addition to the thymus and spleen (Press & Evensen, 1999). Gut associated lymphoid tissues are also known lymphoid organs, and have been shown to function in eliciting immune responses in carp (Joosten et al., 1996). Some teleosts, such as plaice, have been shown to possess a lymphatic system that is differentiated from the blood vascular system, though the existence of such a system has been challenged in other species (Hølvold, 2007).

Health of fish depends on the interrelationship of some major components of the fish and the environment in which they live (Figure 1). Tolerance of these various factors is dependent on the host and in many case the husbandry practices. The environment may be the most critical component of the fish health matrix because environmental quality influences the fish's physiological well-being, species cultured, feeding regimes, rate of growth, and ability to maintain natural and acquired resistance and immunity. Overall physiological status of the fish host is determined by the husbandry practice, environmental quality, the fish's nutritional well-being and the pathogen, all of which influence the natural resistance and acquired immunity of the host. It is common knowledge that fish stressed by one of these factors are more susceptible to infection (Magnadóttir, 2010; Plumb & Hanson, 2011).



(modified from Magnadóttir, 2006 and Plumb & Hanson, 2011).

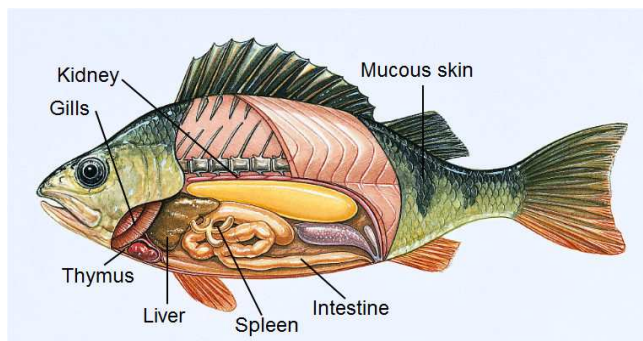
Fig. 1. The relationship of various factors in fish health status.

In addition, in the Food and Drug Administration (FDA) and the European Union (EU) member states, although a limited number of antimicrobial agents are licensed for use in fin fish culture, various drugs such as chemotherapeutics have been used to an increasing levels treat bacterial infections in cultured fish in the last decades years. However, the incidence of drug-resistant (including multiple and cross-resistance) bacteria has become a major problem in fish culture and public health (Alderman & Hasting, 1998; Aoki, 1992; Horsberg, 2003). Vaccination is a useful prophylaxis for infectious diseases of fish and is already commercially available for bacterial infections such as vibriosis, enteric red mouth disease (ERD) and furunculosis and some viral infection such as infectious pancreatic necrosis (IPN). Vaccination may be the most effective method of controlling fish disease. Furthermore, the development of vaccines against intracellular pathogens such as *Renibacterium salmoninarum* has not so far been successful. Therefore, the immediate control of all fish diseases using only vaccines is impossible. Immunostimulants such as synthetic chemicals, bacterial derivatives, polysaccharides or animal and plant extracts increase resistance to infectious disease, not by enhancing specific immune responses, but by enhancing non-specific immune defence mechanisms. Although, there is no memory component and the response is likely to be of short duration. Use of these immunostimulants is an effective means of increasing the immunocompetency and disease resistance of fish. Research into fish immunostimulants is developing and many agents are currently in use in the aquaculture industry (Klesius et al., 2001; Sakai, 1999; Subasinghe, 2009). Besides, the additions of various food additives like vitamins, carotenoids, probiotics, prebiotics, synbiotics and herbal remedies to the fish feed have been tested in fish. Overall the effects have been beneficial such as reducing stress response, increasing the activity of innate parameters and improving disease resistance (Austin & Brunt, 2009; Hoffmann, 2009; Magnadóttir, 2010; Nayak, 2010).

2. Immune system components

2.1 Tissues and cells

Types of immune organs vary between different types of fish. In the jawless fish (hagfishes and lampreys), true lymphoid organs are absent. Instead, these fish rely on region of lymphoid tissue within other organs to produce their immune cells (Zapata et al., 1996). However, genetic differences may be small and some molecular and cellular agents similar, the anatomical and functional organisation such as the structure and form of the immune system (Press & Evensen, 1999; Randeli et al., 2008). The immune system of fish has cellular and humoral immune responses, and organs whose main function is involved in immune defence (Jimeno, 2008). Most of the generative and secondary lymphoid organs present in mammals are also found in fish, except for the lymphatic nodules and the bone marrow (Alvarez-Pellitero, 2008; Jimeno, 2008; Press & Evensen, 1999; Zapata et al., 1996). Instead, the anterior part of kidney usually called head kidney, aglomerular, assumes hemopoietic functions (Jimeno, 2008; Meseguer et al., 1995; Tort et al., 2003), and unlike higher vertebrates is the principal immune organ responsible for phagocytosis (Dannevig et al., 1994; Galindo-Villegas & Hosokowa, 2004), antigen processing activity and formation of IgM and immune memory through melanomacrophagic centres (Tort et al., 2003). The most important immunocompetent organs and tissue of fish include the *kidney* (anterior/or head and posterior/or caudal), *thymus*, *spleen*, *liver*, and *mucosa-associated lymphoid tissues* (Figure 2) (Press & Evensen, 1999; Shoemaker et al., 2001). In fish, myelopoiesis generally occurs in the head kidney and/or spleen, whereas thymus, kidney and spleen are the major lymphoid organs (Zapata et al., 2006). Next to the thymus as the primary T cell organ head kidney is considered the primary B cell organ. Also, head kidney and spleen present macrophage aggregates, also known as melano-macrophage centres (Alvarez-Pellitero, 2008).



(modified from <http://www.dkimages.com/discover/previews/1171/10686362.JPG>).

Fig. 2. Immune structures in teleost fish.

The kidney often referred to as the head kidney tissue is important in hematopoiesis and immunity in fish. And it is predominantly a lympho-myeloid compartment (Press & Evensen, 1999). Early in development, the entire kidney is involved in production of immune cells and the early immune response. As the fish mature, blood flow through the kidney is slow, and exposure to antigens occurs. There appears to be a concentration of melanomacrophage centers are aggregates of reticular cells, macrophages, lymphocytes and plasma cells; they may be involved in antigen trapping and may play a role in immunologic memory (Galindo-Villegas & Hosokowa, 2004; Press et al., 1996; Secombes et al., 1982). The head kidney or anterior kidney (pronephros), the active immune part, is formed with two

Y-arms, which penetrate underneath the gills. In addition, this structure of the kidney has a unique feature, and it is a well innervated organ, and the kidney is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones. Thus, the kidney is a valuable organ with key regulatory functions and the central organ for immune-endocrine interactions and even neuroimmuno-endocrine connections (Press & Evensen, 1999; Tort et al., 2003).

The thymus is a paired bilateral organ situated beneath the pharyngeal epithelium in the dorso-lateral region of the gill chamber. But it seems that the size of the thymus varies with seasonal changes and hormonal cycles (Galindo-Villegas & Hosokowa, 2004; Meseguer et al., 1995; Press & Evensen, 1999; Zapata et al., 1996). The thymus appears to have no executive function. It is regarded, as a primary lymphoid organ where the pool of virgin lymphocytes in the circulation and other lymphoid organs. However, much of the data supporting this is indirect evidence obtained either by immunizing with T-dependent antigens (Ellsaesser et al., 1988) or by using monoclonal antibodies as cell surface markers (Passer et al., 1996) and functional *in vitro* assay. In addition, trout-labeled blood lymphocytes migrate through the thymus before reaching the spleen and kidney (Tatner & Findlay, 1991). It suggest that teleost thymus, despite its striking morphology, has the same function as in higher vertebrates, that is, it is the main source of immunocomponent T cells (Zapata et al., 1996), and research shows that the thymus is responsible for the development of T-lymphocytes, as in other jawed vertebrates (Alvarez-Pellitero, 2008; Galindo-Villegas & Hosokowa, 2004). In general, the available data support a correlation between the histological maturation of the teleost thymus, appearance of the lymphocytes in peripheral lymphoid organs, and development of the cell-mediated immune response (Zapata et al., 1996).

The spleen is the major peripheral and a secondary lymphoid organ in fish which contains fewer haemopoietic and lymphoid cells than the kidney, being composed mainly of blood held in sinuses, and it is believed to be involved in immune reactivity and blood cell formation (Galindo-Villegas & Hosokowa, 2004; Manning, 1994; Zapata et al., 1996). Most fish spleen is not distinctly organized into red and white pulp, as in mammals, but white and red pulp is identifiable. It contains different sized lymphocytes, numerous developing and mature plasma cells, and macrophages in a supporting network of fibroblastic reticular cells. Lymphocyte and macrophage are present in the spleen of fish, contained in specialized capillary walls, termed ellipsoids. In addition, ellipsoids appear to have a specialised function for plasma filtration and particularly immune complex. Most macrophage is arranged in malanomacrophage centers, and it is defined that they are primarily responsible for the breakdown of erythrocytes. These centers may retain antigens as immune complexes for long periods. Although the lymphoid tissue is poorly developed in the teleost spleen, after antigenic stimulation, increased amount of lymphoid tissue does appear, and indirectly suggesting the presence of T-like and B-like cells in this group fish (Espenes et al., 1995; Galindo-Villegas & Hosokowa, 2004; Zapata et al., 1996). The spleen of teleosts has also been implicated in the clearance of blood-borne antigens and immune complexes in splenic ellipsoids and also has a role in the antigen presentation and the initiation of the adaptive immune response (Alvarez-Pellitero, 2008; Chaves-Pozo et al., 2005; Whyte, 2007).

The liver is included under this chapter, because in mammals, it is responsible for production of components of the complement cascade and acute phase proteins (such as CRP), which are important in the natural resistance of the animal, defined that the liver of

fish plays a similar role (Fletcher, 1981). On the contrary, research to support this claim is lacking (Galindo-Villegas & Hosokowa, 2004; Shoemaker et al., 2001).

The mucosa-associated lymphoid tissues in fish are distributed around the intestine referred to as the gut, skin and gills, thus complementing the physical and chemical protection provided by the structure (Jimeno, 2008; Press & Evensen, 1999; Tort et al., 2003). Teleost lack organized mucosa-associated lymphoid tissues such as Peyer's patches of mammals, though there is evidence that skin, gills and intestine contains populations of leucocytes (Jimeno, 2008; Press & Evensen, 1999) and innate and adaptive immunity act in case of attack of microorganisms (Ellis, 2001; Schluter et al., 1999). This equipment is completed with immunocompetent cells such as leucocytes and intraepithelial plasmatic cells (Dorin et al., 1994; Moore et al., 1998; Tort et al., 2003). Recently, several additional defences have been discovered in fish mucous membranes (Bols et al., 2001), such as the production of nitric oxide by the gill as well as antibacterial peptides and proteins by skin (Campos-Perez et al., 2000; Galindo-Villegas & Hosokowa, 2004; Ebran et al., 1999; Tort et al., 2003). Not only the mucous membranes of these tissues are an important physical barrier in fish, but also contain several components with a role in the host-parasite interaction, and release antimicrobial agents or proteins. Besides that among the epidermal secretions, complement, lysozyme, lectins (or pentraxins), alkaline phosphatase and esterase, trypsin (or trypsin-like), natural antibodies or immunoglobulins are often found, although their amount and activity depend on the species, and hemolysine are among the substances present with biostatic or biocidal activities (Alexander & Ingram, 1992; Alvarez-Pellitero, 2008; Aranishi & Mano, 2000; Arason, 1996; Balfry & Higgs, 2001; Ellis, 1999; Galindo-Villegas & Hosokowa, 2004; Jones, 2001; Fast et al., 2002; Magnadóttir, 2006; Palaksha et al., 2008; Shoemaker et al., 2001; Tort et al., 2003). Most research on the presence of immunoglobulin or antibody in the mucus suggests that mucus immunoglobulin is not a result of the transduction of immunoglobulin from the serum (Shoemaker et al., 2001). Mucous or goblet cells secrete mucus, which has at least three different types of defensive roles: (1) Mucus interrupts establishment of microbes by being continually sloughed off. (2) If establishment is accomplished, mucus acts as a barrier to be crossed. (3) The mucus on skin, and presumably the other surfaces, contains a variety of humoral factors with antimicrobial properties (Galindo-Villegas & Hosokowa, 2004; Tort et al., 2003).

All multicellular organisms possess a selection of cells and molecules that interact in order to ensure production from pathogens (Abbas & Lichtmann, 2006). This collection of highly specialised components makes up the immune system, and poses a physiological defence against microbe invasion (Jimeno, 2008). Fish immune cells show the same main features as those of other vertebrates, and lymphoid and myeloid cell families have been defined. Key cell types involved in non-specific cellular defence responses of teleost fish include the phagocytic cells monocytes/macrophages, non-specific cytotoxic cells (or NK cells), thrombocytes, granulocytes (or neutrophils) and lymphocytes (Table 1) (Buonocore & Scapigliati, 2009; Hamerman et al., 2005; Hølvold, 2007; Magnadóttir, 2006; Jimeno, 2008; Shoemaker et al., 2001).

Epithelial and antigen presenting cell also participate in the innate defence in fish, and some teleost have been reported to have both acidophilic and basophilic granulocytes in the peripheral blood in addition to the neutrophils. Furthermore, recently it has been observed that basophilic granular cells (acidophilic/eosinophilic granule cells or mast cells) of fish Perciformes order, the largest and most evolutionarily advanced order of teleosts, are endowed with histamine (Garcia-Ayala & Chaves-Pozo, 2009; Jimeno, 2008; Magnadóttir,

2006; Murelo et al., 2007; Whyte, 2007). Mononuclear cells in fish include the macrophages (and/or tissue macrophages) and monocytes. These cells are probably the single most important cell in the immune response in fish. Not only are they important in the production of cytokines, but they also are the primary cells involved in phagocytosis and the killing of pathogens upon first recognition and subsequent infection (Buonocore & Scapigliati, 2009; Cabezas, 2006; Clem et al., 1985; Garcia-Ayala & Chaves-Pozo, 2009; Secombes et al., 2001; Shoemaker et al., 2001). Macrophages also play major roles as being the primary antigen-presenting cell in teleost, thus linking the non-specific and acquired immune response (Balfry & Higgs, 2001; Galindo-Villegas & Hosokowa, 2004; Jimeno, 2008; Shoemaker et al., 2001; Vallejo et al., 1992). Thrombocytes are thought to be a nucleated version of the mammalian platelet. These cells are involved in blood clotting and have recently been thought to have phagocytic properties (Balfry & Higgs, 2001; Secombes, 1996).

Cellular components	Functional characteristics and mode of action
Monocytes/Macrophages	Phagocytosis, and phagocyte activation, cytokine production, intracellular killing, antigen processing and presentation, Secretion of growth factors and enzymes to remodel injured tissue, T-lymphocyte stimulation.
Granulocytes (or Neutrophils)	Phagocytosis, secretion and phagocyte activation, cytokine production, extracellular killing, inflammation.
Non-specific cytotoxic cells (or natural killer cells)	Recognition and target cell lysis, induce apoptosis of infected cells, Synthesize and secrete interferon-gamma (IFN- γ).

(modified from Hølvold, 2007; Shoemaker et al., 2001).

Table 1. Non-specific immune cells in fish and their functional characteristics and mode of action.

Fish possess polymorph nuclear cells, or granulocytes (especially neutrophils, and eosinophils, and basophils), that contain granules, the contents of which are released upon stimulation (Balfry & Higgs, 2001). These cells are highly mobile cell, phagocytic, produce reactive oxygen species, traveling via the blood and lymphatic systems to sites of infection and injure, thereby playing a vital role in the inflammatory response. Also, neutrophils are the primary cells involved in the initial stages of inflammation in fish, between 12 to 24 hours, and the function of the granulocytes may be cytokine production to recruit immune cells to the area of damage or infection (Galindo-Villegas & Hosokowa, 2004; Manning, 1994; Shoemaker et al., 2001). However, eosinophilic granular cells found in the stratum granuloma of the gut, gills and skin, and surrounding major blood vessels, are not considered to be eosinophils but rather mast cells (Vallejo & Ellis, 1989; Reite, 1998; Galindo-Villegas & Hosokowa, 2004). Cells mediating the lytic cycle to occur and destroy tumour target cells lines following receptor binding in fish have been denominated non-specific cytotoxic cells (Galindo-Villegas & Hosokowa, 2004), and are similar to (or closely related in function) the mammalian NK cells (Shoemaker et al., 2001). These cells capable of be important in protozoan parasites (Evans & Gratzek, 1989; Evans & Jaso-Friedman, 1992), and viral immunity of fish (Hogan et al., 1996), and are found in the blood, lymphoid tissue, and gut of fish (Balfry & Higgs, 2001). Lymphocytes are the cells responsible for the specificity of the specific immune response. The two different classes of lymphocytes (T and B) are the acknowledged cellular pillars of adaptive immunity, and can be distinguished by their cell surface markers and subsequent function (Balfry & Higgs, 2001; Garcia-Ayala &

Chaves-Pozo, 2009; Pancer & Cooper, 2006). T lymphocytes recognize antigen that is presented by antigen-presenting cells such as macrophages, and are primarily responsible for cell-mediated immunity. These cells are also important sources of cytokines, which are particularly important in the inflammatory response (Balfry & Higgs, 2001). On the other hand, B lymphocytes are responsible for humoral immunity, and recognize antigen and produce specific antibodies to that antigen. T and B cells can be worked together and with other types of cells to mediate effective adaptive immunity (Garcia-Ayala & Chaves-Pozo, 2009; Jimeno, 2008; Miller et al., 1998; Pancer & Cooper, 2006). Interestingly, B cells from rainbow trout have high phagocytic capacity, suggesting a transitional period in B lymphocyte evolution during which a cell type important in innate immunity and phagocytosis evolved into a highly specialized component of the adaptive arm of the immune response in higher vertebrates (Jimeno, 2008; Li et al., 2006).

2.2 Humoral molecules

The classification of humoral parameters is commonly based on their pattern recognition specificities or effector function. Most non-specific humoral molecules involved in the natural resistance of fish are presented with composition and mode of action in Table 2 (Magnadóttir, 2006; Shoemaker et al., 2001). These components act in several ways to kill and/or prevent the growth and spread of pathogens. Other acts as agglutinins (aggregate cells) or precipitins (aggregate molecules). There are also opsonins that bind with the pathogen and, in doing so, facilitates its uptake and removal by phagocytic cells. In addition, some of these substances have important role in the inflammatory immune response, such as opsonins, anaphylatoxins, neutrophil, and macrophage chemo-attractants. Briefly, these factors involve various lytic substances/or hydrolase enzymes (lysozyme, cathepsin L and B, chitinase, chitinase, trypsin-like), agglutinins /or precipitins (CRP, serum amyloid P (SAP), lectins, α - and natural precipitins, natural antibodies, natural hemagglutinins), enzyme inhibitors (α_2 -macroglobulin, serine-/cysteine-/and metal-proteinase inhibitors) and pathogen growth inhibitors (interferon (IFN), myxovirus (Mx)-protein, transferrin, ceruloplasmin, metallothionein). Antimicrobial peptides such as cathelicidins (CATH-1, -2), defensins (DB-1, -2, -3), hepsidins (hepsidinLEAP-1, -2), piscidins (e.g. pleurocidin, epinecidin-1, dicentracin), ribosomal proteins, histone derivatives (e.g. parasin, histon H2B, SAMP H1, oncorhynchins, hipposin), which widespread in nature as defence mechanism in plant and animals are also substances that have been identified in the tissue such as mucus, liver, skin and gills of some teleost species, including halibut and flounder (Alvarez-Pellitero, 2008; Aoki et al., 2008; Aranishi & Mano, 2000; Balfry & Higgs, 2001; Buonocore & Scapigliati, 2009; Cole et al., 1997; Ellis, 1999; Ellis, 2001; Galindo-Villegas & Hosokawa, 2004; Hølvold, 2007; Lemaître et al., 1996; Magnadóttir, 2006; Rodriguez-Tovar et al., 2011; Shoemaker et al., 2001; Smith & Fernandes, 2009; Smith et al., 2000; Tort et al., 2003; Whyte, 2007; Yano, 1996).

In addition, in teleost fish, evaluating the complement system as a humoral component is an essential part of the innate immune systems, and can be activated through the two /or three pathways of complement; (1) the classical pathway such as specific immunoglobulin or IgM is triggered by binding of antibody to the cell surface but can also be activated by acute phase proteins such as ligand-bound CRP or directly by viruses, bacteria and virus-infected cells, (2) the alternative pathway such as bacteria cell wall and viral components or surface molecules of parasites is independent of antibody and activated directly by foreign

microorganisms, (3) the lectin pathway is elicited by binding of a protein complex consisting mannose-binding lectins to mannans on bacterial cell surfaces. All three pathways converge to the lytic pathway, leading to opsonisation or direct killing of the microorganism (Aoki et al., 2008; Balfry & Higgs, 2001; Ellis, 1999; Ellis, 2001; Galindo-Villegas & Hosokowa, 2004; Holand & Lambris, 2002; Nakao et al., 2003; Randelli et al., 2008; Shoemaker et al., 2001; Tort et al., 2003; Whyte, 2007; Yano, 1996).

Humoral components	Composition	Mode of action
Antibacterial peptides (e.g. histone H2B, cecropin P1, pleurocidin, parasin, hipposin, SAMP H1)	Protein	Constitutive and inducible innate defence mechanism, active against bacteria, defence before development of the specific immune response in the larval fish
Antiproteases (e.g. α_1 -anti-protease, α_2 -anti-plasmin, α_2 -macroglobulin)	----	Restricts the ability of bacteria to invade and growth <i>in vivo</i> , active against bacteria
Ceruloplasmin	Protein	Copper binding
Complement system (e.g. C3, C4, C5, C7, C8, C9 and their isoforms, B- and D-factors)	Protein	Promote binding of microbes to phagocytes, promote inflammation at the of complement activation, cause osmotic lysis or apoptotic death
Interferons (IFNs) /Myxovirus (Mx)-proteins (e.g. IFN- $\alpha\beta$, IFN- γ)	Glycoprotein /or Protein	Aid in resistance to viral infection, inhibit virus replication, inducible IFN-stimulated genes
Lectins (e.g. legume and cereal lectins, mannose-binding lectin, C-type lectins, intelectin, cod, ladder lectin)	Glycoprotein and/or specific sugar binding protein	Induce precipitation and agglutination reactions, recognition, promote binding of different carbohydrates in the presence of Ca ⁺² ions, active complement system, opsonin activity and phagocytosis
Lytic enzymes (e.g. lysozyme, chitinase, chitobiase)	Catalytic proteins lysozyme, complement components	Change the surface charge of microbes to facilitate phagocytosis, haemolytic and antibacterial and/or antivirucidal, antiparasitical effects, opsonic activity, inactivation of bacterial endotoxin(s)
Natural antibodies	----	Recognition and removal of senescent and apoptotic cells and other self-antigens, control and coordinate the innate and acquired immune response, activity against haptenated proteins
Pentaxins (e.g. C-reactive protein, serum amyloid P)	Protein	Opsonisation or activation of complement, promote binding of polysaccharide structures in the presence of Ca ⁺² ions, induce cytokine release, coast microbes for phagocytosis by macrophage
Proteases (e.g. cathepsine L and B, trypsin-like),	----	Defence against bacteria, activity against <i>Vibrio anguillarum</i>
Transferrin/Lactoferrin	Glycoprotein	Iron binding, acts as growth inhibitors of bacteria, activates macrophage

(modified from Hølvold, 2007; Shoemaker et al., 2001).

Table 2. Non-specific humoral molecules and their composition and mode of action in fish.

2.3 Cytokines and chemokines

The initiation, maintenance, and amplification of the immune response are regulated by soluble mediators named cytokines. Cytokines are the soluble messengers of the immune system and have the capacity to regulate many different cells in an autocrine, paracrine, and endocrine fashion, and can also be immune effectors (King et al., 2001). In the last few years, much interest has been generated in the study of fish cytokines and chemokines and significant progress, and has been made in isolating these molecules from fish. In recent years, various cytokines have been described in fish, but the major drawback in identifying fish cytokines is the low sequence identity compared to their mammalian counterparts. The low sequence identities also limit the detection of proteins of fish cytokines by using the antibodies of human cytokines (Plouffe et al., 2006). Most of these have been identified in biological assays on the basis of their functional similarity to mammalian cytokine activities. Some have also been detected through their cross-reactivity with mammalian cytokines (Manning & Nakanishi, 1996).

The predominant pro-inflammatory cytokines are interleukins (ILs) (especially IL-1 β and IL-6) and tumour necrosis factor- α (TNF- α) (Balfry & Higgs, 2001; Bird et al., 2005; Corripio-Miyar et al., 2006; Garcia-Ayala & Chaves-Pozo, 2009; Hølvold, 2007; Jimeno, 2008; King et al., 2001; Magnadóttir, 2010; Randelli et al., 2008; Savan et al., 2005; Tort et al., 2003). These cytokines have a number of systemic effects, including body temperature elevation neutrophil mobilization, and stimulation of acute phase protein production in the liver (Balfry & Higgs, 2001; King et al., 2001; Randelli et al., 2008). Additional several cytokine /or cytokine homologues found in fish include IL-2, IL-4, IL-10, IL-11, IL-12, IL-15, IL-18, IL21, IL22, IL-26 and IFN- γ , (Balfry & Higgs, 2001; Bei et al., 2006; Bird et al., 2004; Buonocore & Scapigliati, 2009; Corripio-Miyar et al., 2006; Garcia-Ayala & Chaves-Pozo, 2009; Hølvold, 2007; Igawa et al., 2006; Inoue et al., 2005; Jimeno, 2008; King et al., 2001; Li et al., 2007; Magnadóttir, 2010; Randelli et al., 2008; Tort et al., 2003; Wang et al., 2005; Whyte, 2007; Yoshiura et al., 2003; Zou et al., 2004), and others cytokines in some fish species include transforming growth factor- β family such as TGF- β_1 , - β_2 , - β_3 , - β_A , and - β_B , macrophage-migration inhibition factor (MIF), macrophage-colony stimulating (M-CSF or CSF-1; such as CSF-1R or sCSF-1R), chemotactic factor (CF) and platelet activating factor (PAF). However, no antibody markers are at present available for fish TGF- β , M-CFS and PAF (Belosevic et al., 2006; Garcia-Ayala & Chaves-Pozo, 2009; Klesius et al., 2010; Manning & Nakanishi, 1996; Randelli et al., 2008; Tafalla et al., 2003). On the other hand, orthologous cytokines in teleost fish have been classed as Class I, Class II, chemokines, TNF superfamily and IL-1 family (Table 3) (Alvarez-Pellitero, 2008; Aoki et al., 2008; Lutfalla et al., 2003).

IL-1 β has been identified in 13 different species of teleost, and is produced by macrophage and also by a variety of other cells such as neutrophilic granulocytes. These ones are play a role in immune regulation through stimulation of T cells which is analogous to mammalian IL-1 β . In addition, it is an important mediator of inflammation in response to infection and it has been reported in the trout to directly affect hypothalamic-pituitary-interrenal axis function, stimulating cortisol secretion. Another potentially important cytokines, TNF- α has been cloned in various fish. Besides, TNF-like protein activity has been shown to induce apoptosis, and to enhance neutrophil migration and macrophage respiratory burst activity. The number of studies in fish have provided indirect evidence suggesting that TNF- α is an important macrophage activating factor (MAF) produced by leukocytes. In some fish species, homologous MAF containing supernatants have been shown to induce a typical

activated-macrophage response, evidence by increases in phagocytosis and nitric oxide production (Balfry & Higgs, 2001; Garcia-Ayala & Chaves-Pozo, 2009; Holland et al., 2002; Hølvold, 2007; Tort et al., 2003; Whyte, 2007). In addition, TNF- α has been shown increase chemotaxis of rainbow trout anterior kidney leukocytes and induces the expression of a number of genes in the immune response including IL-1 β , IL-8 and cyclooxygenase-2 (COX-2) (Zou et al., 2003). Other vital cytokines, IFNs are secreted proteins, are also pH-resistant cytokines which are produced by many cell types in response to a viral infection (within 2 days in rainbow trout injected viral haemorrhagic septicemia virus), and occurs in very young fish. In isolated Atlantic salmon macrophage stimulated with polyinosinic polycytidylic acid (poly I:C), peak IFN production occurred within 24 h and peak Mx protein production after 48 hours (Ellis, 2001; Nygaard et al., 2000). Therefore, IFN-mediated antiviral defence mechanisms are able to response during the early stages of a viral infection, which is mediated by the innate non-specific IFN responses while long-term protection is mediated by the specific immune response (Galindo-Villegas & Hosokowa, 2004; Ellis, 2001).

Class	Function /or Structure	Members
Cytokine class I	Involved in expansion and differentiation of cells. Have a 4- α helix bundle structure	IL-a and -b, IL-1 1-a and -b*, epo, GCSF-a and -b*, leptin, PRL, GH, M17*, M17 homologue (MSH)*
Cytokine class I	Involved in minimizing damage to host after insult. Contain more than 4- α helices.	IFN- α 1, IFN- α 2, IFN- γ , IL-10, IL-20, IL-24
Chemokines	Regulate cell migration under both inflammatory and homeostasis. Small proteins with 4 conserved Cys residues.	CXC (CXCL8-like, CXC-10, -12, -13, -14), CC (CCL19/21/25, CCL20, CCL27/28, CCL17/22, MIP, MCP)
TNF super family	Involved in inflammation and lymphoid organ development. Compact trimmers as membrane bound or soluble proteins.	Lymphotoxin- β , lymphotixin- β , TNF- α
IL-1 family	Involved in pro-inflammatory responses. Fold rich in β -strands.	IL-1 α , IL-1 β , IL-18

*: Only found in fish. (modified from Aoki et al., 2008).

Table 3. Cytokines of teleost fish, and their function/or structure and members.

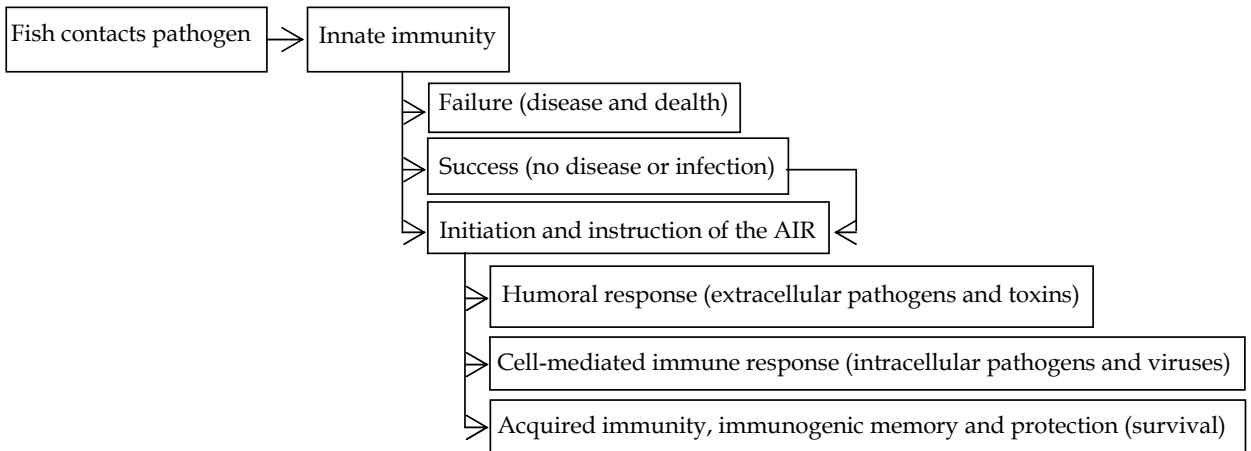
Chemokines are known as second-order /or chemotactic cytokines, are a superfamily of small secreted cytokines that direct migration of immune cells to sites of infection, produced by different cell types that have, among other function, chemoattractant properties stimulating the recruitment activation and adhesion of cells to sites of infection injury (Alvarez-Pellitero, 2008; Aoki et al., 2008; Ellis, 2001; Hølvold, 2007). Different chemokines have been characterized in some fish species such as rainbow trout, carp, catfish, flounder and Atlantic halibut, including members of the first two conserved cysteines in their sequence: CXC, CC, C and CX₃C class /or family. Although, the CC chemokines represent the largest subfamily of chemokines, IL-8 was the first known chemokines, and other chemokines such as CXCL8 (or IL-8), γ IP-10, CK-1 and CK-2 belongs to the subfamily. Chemokines play a key role in the movement if immune effector cells to sites of infection and it is becoming increasingly clear that their function is also necessary to translate an

innate immune response into an acquired adaptive immune (Alvarez-Pellitero, 2008; Aoki et al., 2008; Hølvold, 2007; Peatman & Liu, 2007; Whyte, 2007).

3. Fish immune system description

In this section, since complexity and due this component of the immune system including innate (non-specific) and acquired (specific / or adaptive) immune systems in fish is out of the scopes of this chapter, will not be described in detail, but will be briefly mentioned here-in. Hereof, components of these systems and its mode of action were given in detail at Section 2.

The classical division of the immune system is into the innate and the adaptive systems. Despite the fact that dividing immune system into the innate and the acquired immunity is a common practice, recent studies in both fish and mammalian immunology demonstrate that these are combined systems rather than independent systems. Thus, the innate immune response is also important in activating the acquired immune response (Figure 3) (Fearon & Locksley, 1996; Jimeno, 2008; Medzhitov, 2007; Shoemaker et al., 2001).



AIR: Acquired immune response. (modified from Shoemaker et al., 2001).

Fig. 3. Schematic representation of the response of a fish following an encounter with a pathogen.

3.1 Innate (non-specific) immune system

The innate immune system is of prime importance in the immune defence of fish. It is commonly divided into 3 compartments: (1) physiochemical barriers and/or the epithelial and/or mucosal barrier such as scales, epithelial surface (on gills, skin and gut) with secreted mucus, (2) the humoral parameters such as cell secretions of complement, CRP, IFN, lysozyme, transferrin, lectins, antimicrobial peptides, and (3) the cellular components such as non-specific cytotoxic cells (or NK cells), monocytes/macrophages, thrombocytes, granulocytes (or neutrophils), lymphocytes (see Section 2) (Buonocore & Scapigliati, 2009; Jansson, 2002; Magnadóttir, 2010; Rodriguez-Tovar et al., 2011). The general term for these innate parameters is pattern recognition proteins or receptors. These parameters recognize pathogen associated molecular patterns (PAMPs) associated with microbes and also inherited danger signals from malignant tissue or apoptotic cells. Typical PAMP are polysaccharides and glycoproteins like bacterial lipopolysaccharide, fragellins, teichoic acid and

peptidoglycans, bacterial CpG and virus associated double-stranded RNA (Alvarez-Pellitero, 2008; Cabezas, 2006; Ellis, 2001; Hølvold, 2007; Jimeno, 2008; Magnadóttir, 2010; Medzhitov & Janeway, 2002; Whyte, 2007). However, under normal conditions the fish maintains a healthy state by defending itself against the potential invaders by a complex system of innate defence mechanisms. These mechanisms are both constitutive and responsive and provide protection by preventing the attachment, invasion or multiplication of microbes on or in the tissue. Immune systems effecting drugs such as immunostimulants, probiotics, prebiotics and synbiotics should act through the enhancement of the innate immune response (Austin & Brunt, 2009; Galindo-Villegas & Hosokowa, 2004; Hoffmann, 2009; Magnadóttir, 2006; Nayak, 2010).

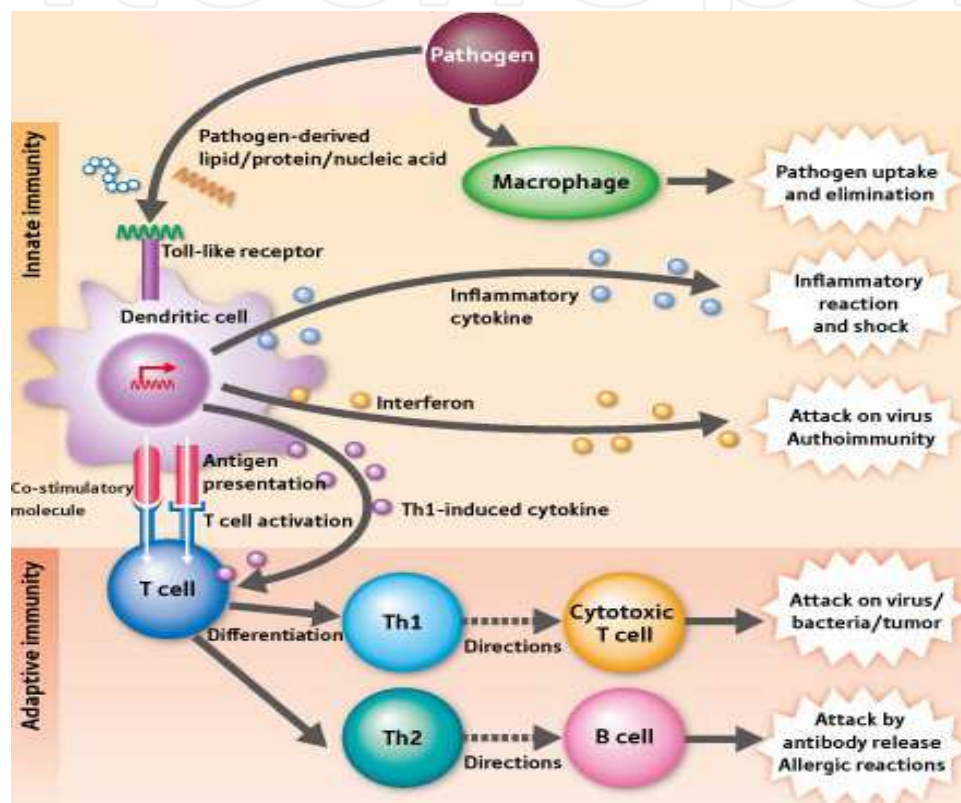
The production or expression of both humoral and cellular innate parameters is commonly amplified or up-regulated during immune response, but there is believed to be no memory. This mean that a second encounter with the same pathogen will not result in enhance response as is seen in acquired immune response (Magnadóttir, 2010).

3.2 Acquired (specific) immune system

If a pathogen evades or overwhelms the innate defence mechanism of the host, causing the foreign antigen to persist beyond the first several days of infection, an acquired immune system components is initiated. In addition, the antigen-specific lymphocytes of acquired immune response are capable of swift clonal expansion and of a more rapid and effective immune response on subsequent exposures to the pathogen (King et al., 2001). However, activation of the acquired immune system is relatively slow, requiring specific receptor selection, cellular proliferation and protein synthesis but it is long lasting (Magnadóttir, 2010).

In contrast to the innate immune systems components, the acquired immune system produces effector cells (T- and B-lymphocytes) and molecules (immunoglobulins (Igs)/or specific antibodies), which are highly specific to the antigen of the invading microbe. The B-cells, similar to the B1-subset of mammalian B-cells, are involved in the humoral response while the T-cells are responsible for the cell-mediated response (Galindo-Villegas & Hosokowa, 2004; Jansson, 2002; King et al., 2001; Magnadóttir, 2010). Furthermore, the other key elements in the evolution of the acquired immune system are the appearance of the thymus, the recombination activation gene (RAG; especially RAG 1 and 2 genes) enzymes, which through gene rearrangement generate the great diversity of the Ig superfamily (T- and B-cell receptors) and major histocompatibility complex (MHC). On the other hand, the key humoral parameter of the acquired system is the Igs (antibodies), expressed either as B-lymphocytes receptor or secreted in plasma. The trigger for activation of the acquired immune system, the activation and proliferation of lymphocytes, take place in organized lymphoid tissue. Following activation by a specific antigen, either in soluble form or in association with the MHC marker on antigen presenting cells, the B-cells proliferate and differentiate into long lasting memory cells and plasma cells, which secrete the specific antibody. Also, T-cells, using a specific receptor, recognise pathogen only in association with the MHC marker on antigen presenting cells (Alvarez-Pellitero, 2008; Buonocore & Scapigliati, 2009; Galindo-Villegas & Hosokowa, 2004; Jansson, 2002; King et al., 2001; Magnadóttir, 2010; Rodriguez-Tovar et al., 2011). Effectively only one functional Ig class, a tetrameric IgM, is demonstrated in teleost fish, and these molecule is also made up of eight heavy (μ)-/and light (λ)-

chains. This is in contrast to the pentameric Ig classes and sub-classes mammals on the basis of heavy chain molecular weight and on their surface and secrete-antibodies only of the Ig class. Other Ig-like molecules have been described in some fish species, which may increase the diversity of the B-cell recognition capacity (Lorenzen, 1993; Magnadóttir, 2010; Randelli et al., 2008; Shoemaker et al., 2001; Wilson et al., 1997). Resistance to and recovery from first infection are a results of complex interactions between innate and acquired defence mechanism (Lorenzen, 1993). A summary of innate and acquired immune systems in fish is shown in Figure 4 (Jimeno, 2008).



Th1: T-helper 1, Th2: T-helper 2. (Jimeno, 2008).

Fig. 4. Cross-talk between innate and acquired immune systems.

Briefly, the immune reaction in fish is influenced by endogen rhythms and environmental parameters, of which temperature is by far the most important. Another important factor is nutrition, which may be subject to enormous variation within and between wild populations (Lorenzen, 1993). The immunosuppressive effects of population and stress resulting in higher disease susceptibility are well known. Choosing a universal trait or an innate component that could act as a biomarker for adverse conditions in aquaculture is however problematic. This is because of the variable effects on innate and acquired parameters depending on the type and duration of adverse conditions and on the fish species (Magnadóttir, 2006; Ortuño et al., 2001). The innate and acquired immune systems are given activity/or factor, cells involved and cellular markers in Table 4 (Jansson, 2002; Randelli et al., 2008).

Activity/Factor	Cell involved	cDNA sequence coding for	Cellular marker
<i>Innate immunity</i>			
Phagocytosis	Mononuclear phagocytes B-cells	-	mAb to MΦ, and IgM, neutrophils, pAb to granulocytes, granulin
ROS species	Mononuclear phagocytes	iNOS	NBT, no antibodies
Complement, APR	Hepatocytes	C3, C4, C5, C7, C8, CRP, SAP	pAb to C3
Antibacterial	Various types	Families of peptides	None
Antiviral	Leucocytes, fibroblasts	IFN-1, IFN, Mx-protein	None
Enzymes	Various types	Lysozyme, caspases, proteases	None
Inflammation, cytokines, monokines	Leucocytes	TNF-α, COX-2, PLA2, TLRs, ILs (1, 6, 12, 14, 16, 17, 18, 20, 21, 22), >16 chemokines	pAb for IL-1, pAb and mAb for TNF-α
Non-specific killing	Leucocytes	NCCRP-1	mAb to 5C6
<i>Acquired immunity</i>			
Memory, specific antibody,	B-cells	IgM, IgD, IgT, RAGs	mAb to IgM, B-cells
Memory, cellular recognition,	T-cells	TcR-α, -, β -γ, -δ, CD3, RAGs	DLT15, WCL38
Specific killing	T-cells	CD8-α, CD8-β, MHC _I	None
Helper activity	T-cells	CD4, MHC _{II}	None
	Th1 /or Th2	IFN-γ, IL-2 /or IL-4, IL-10	None
	Leucocytes	ILs (7, 15, 21, 22, 26), LtB	None

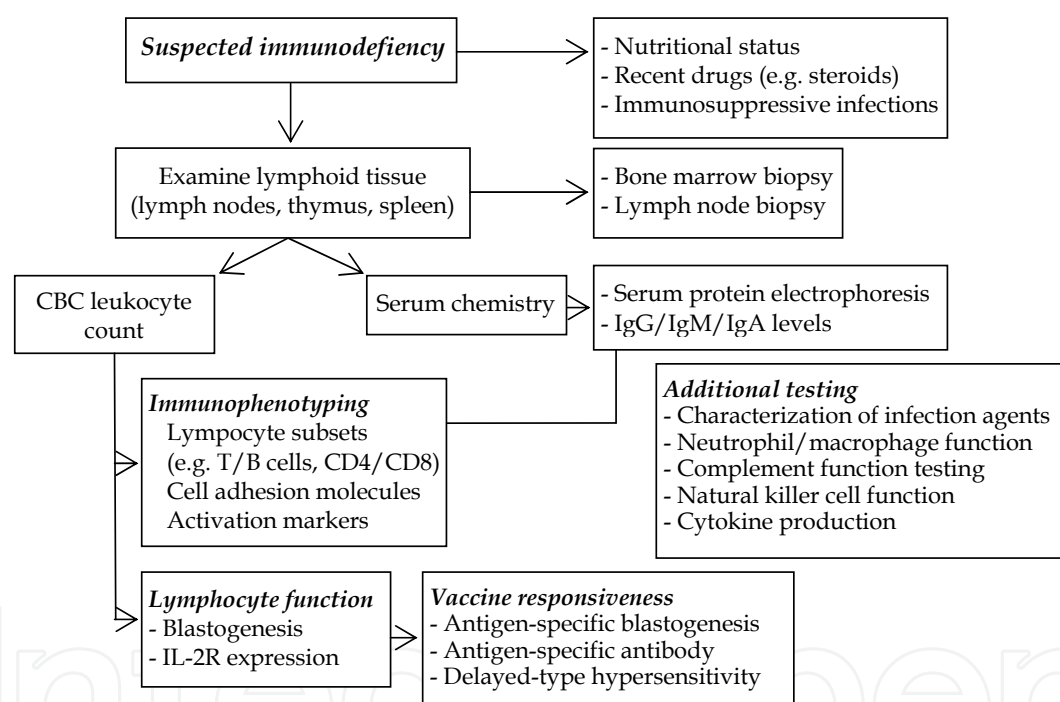
APR: Acute phase response, CD: Cell-differentiation cluster; COX-2: Cyclooxygenase 2, CRP: C-reactive protein, iNOS: Inducible nitric oxide synthase, IFN: Interferon, Ig: Immunoglobulin, IL: Interleukin, LtB: Lymphotoxin B, MΦ: Macrophage, MHC: Major histocompatibility complex, NCCRP-1: Non-specific cytotoxic cells receptor protein-1, NBT: Nitroblue tetrazolium, PLA2: Phospholypase A2, RAGs: Recombinase-activating genes, ROS: Reactive oxygen species, SAP: Serum amyloid P, TcR: T-cell receptor, Th1: T-helper 1, Th2: T-helper 2, TLRs: Toll-like receptors, TNF: Tumor necrosis factor, mAb: Monoclonal antibodies, pAb: Polyclonal antibodies. (modified from Randelli et al., 2008).

Table 4. The innate and acquired immune systems activity and/or factors and cellular markers.

4. Immunoassay

Diagnostics is the determination of the cause of a disease or clinical pathology. The techniques used range from gross observation to highly technical biomolecular-based tools. Pathogen screening is another health management technique, which focuses on detection of pathogens in sub-clinical, or apparently healthy, hosts. Schematic representation of the diagnosis using a stepwise clinical approach is presented in Figure 5 (King et al., 2001; Subasinghe, 2009).

In recent years, fish immunological research has been mainly focused on two aspects: (1) Firstly, comparative and development studies have contributed to a better understanding of the characterize, the structural and functional evolution of the immune system mechanisms and pathways from invertebrate, through fish to mammals, (2) The second aspect, and one that has received the major funding, is the requirement of the fish farming industries, and also has understated how the fish immune system responds the foreign agents. The word-wide growth in aquaculture in the past 2-3 decades has demanded the development of a comprehensive knowledge of the immune system of the commercially important fish species, and also has understated how the fish immune system responds the foreign agents. The purpose has been twofold: to secure to optimum activity of the natural immune defence of the fish through cultural conditions and the choice of fish stock (or by breeding to produce stock of fish with superior disease resistance), and also to develop and improve prophylactic measure such as vaccination, immunostimulants and probiotics (Alvarez-Pellitero, 2008; Galindo-Villegas & Hosokowa, 2004; Ellis, 2001; Magnadóttir, 2010).



CBC: Complete blood count, Ig: Immunoglobulin, IL: Interleukin. (modified from King et al., 2001).

Fig. 5. Schematic representation clinical evaluation of the immune system.

A variety of technologies have already made an impact in reducing disease risk and many novel methods will contribute in the future (Adams & Thompson, 2006; Adams & Thompson, 2008). Improved nutrition, use of probiotics, improved disease resistance, quality control of water, seed and feed, use of immunostimulants, rapid detection of pathogens and the use of affordable vaccines have all assisted in health control in aquaculture. The success of vaccination in reducing the risk of furunculosis in salmon is an excellent example of technology having made a significant impact. This is turn led to a reduction of the use of antibiotics that has been sustained, and productivity has increased as a result of vaccination (Gudding et al., 1999; Adams et al., 2008).

Many of the assays for detecting the changes in the protective mechanism of the fish due to immunomodulations are divided from those used in fish disease diagnostics and immunization programs. Although, most used tests in the last decades, most used assays for fish immunomodulation diagnosis are given as list in Table 5 (Adams & Thompson, 2008; Anderson, 1996; Brown-Treves, 2000, Jeney & Anderson, 1993; King et al., 2001; Lorenzen, 1993; Roque et al., 2009; Plumb & Hanson, 2011; Subasinghe, 2009). A large number of methods have been developed for immunodiagnostics and these are used routinely in many laboratories for the detection of fish and shellfish pathogens. These tools include both immunoassay and DNA-based diagnostic methods such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), polymerase chain reaction (PCR), quantitative (or real-time)-PCR (QPCR), reverse transcriptase-PCR (RT-PCR), fluorescent antibody assays (FAT), indirect-IFAT, quantitative-FAT (QFAT), immunohistochemistry (IHC), *in situ* hybridization (ISH) and blot (dot-blot/dip-stick/western blot) amplification techniques (Adams et al., 2008; King et al., 2001; Plumb & Hanson, 2011; Roque et al., 2009; Subasinghe, 2009). However, with the development of Rapid Kits (immunochromatography/lateral flow) which are simple to use, sensitive and inexpensive (Adams & Thompson, 2008).

Hematological/physiological assays-blood samples <i>Hematocrit</i> : Percent of red blood cell pack <i>Leukocrit</i> : Percent of white blood cell pack <i>Cell counts and differentials</i> : Numbers of cells and types <i>Lysozyme levels</i> : Enzyme level in blood <i>Serum immunoglobulin level</i> : Specific and nonspecific antibody <i>Serum protein level</i> : Total protein in serum	Specific immune response assays <i>Scale rejection</i> : Transplantation indicator <i>Delayed hypersensitivity</i> : Allergenic reactions <i>Trypan blue</i> : Killer cell activity <i>Chromium release</i> : Killer cell activity <i>Melanomacrophage centers</i> : Antigen processing cells, Antigen accumulation: Concentration in spleen or kidney areas, Cell aggregates: Increase in numbers of melanomacrophage cells <i>Passive hemolytic plaque assay (Jerne assay)</i> : Antibody- producing cells <i>Assays measuring serum antibody levels</i>
Innate defensive mechanism or acquired immune response assays (These assays can be used for either response) <i>Phagocytosis</i> : Percents and indexes; engulfment by phagocytic cells/or phagocytic activity: By incubating blood with a killed bacterial culture <i>Bactericidal activity</i> : By incubating macrophages with a live bacterial culture <i>Rosette-forming cells</i> : Adherence of particles around lymphocytes <i>Glass or plastic adherence</i> : Stickiness of phagocytic cells <i>Pinocytosis</i> : Engulfment of fluids by phagocytic cells <i>Neutrophil activation</i> : Myelo-peroxidase production and NBT dye reduction by oxidative burst e.g. oxidative radicals, Chemiluminescence: light detection from oxidative burst <i>Blastogenesis</i> : Mitosis of lymphocytes cells;	Immunoelectrophoresis/or immunoassay and DNA-based diagnosis <i>CF</i> : Complement fixation <i>DBH or WB</i> : Dot blot hybridization or Western blot <i>ELISA</i> : Enzyme-linked immunosorbent assay <i>FAT</i> : Fluorescent antibody assays (or technique) <i>IFAT</i> : Indirect-FAT <i>QFAT</i> : Quantitative-FAT <i>ISH</i> : In situ hybridization <i>LAMP</i> : Loop-mediated isothermal amplification <i>PCR</i> : Polymerase chain reaction <i>QPCR</i> : Quantitative (or real-time)-PCR <i>rcb-PCR</i> : Reserve cross blot-PCR <i>RT-PCR</i> : Reverse transcriptase-PCR <i>RIA</i> : Radioimmunoassay <i>SNT or VNT</i> : Serum- /or virus- neutralization test <i>Lateral-flow immunoassays</i> Multiplex assays (e.g. <i>Protein array system, Micro-arrays</i>)
Agglutination / or Hemagglutination <i>Preciptinin (Ouchterlony gel)</i> : Measures soluble antigens in gels <i>Immunoelectrophoresis</i> : For defining blood or antigenic components	

(modified from Anderson, 1996).

Table 5. Hematological, innate and acquired immune response assays.

These molecular-based techniques (immunoassay and nucleic acid assay) provide quick results, adaptable to field situation, with high sensitivity and specificity, at relatively low cost, and can be easily applied to a large number of samples, and are also particularly valuable for infections which are difficult to detect such as sub-clinical infections using standard histology and tissue-culture procedures such as histopathology, bacteriology, virology, parasitology and mycology. They can be used for non-lethal sampling, and are valuable for monitoring challenge experiments under controlled laboratory conditions. Further development of this technology is likely to enhance more rapid detection and diagnosis of disease, which is crucial for early and effective control emergent disease situations (Adams & Thompson, 2008; Subasinghe, 2009). Although, modern immunoassays are very sensitive, sometimes their result may not be easy to analyse. This is partly because the blood chemistry and/or immune parameters of fish is highly depended on environmental conditions, nutrition, and other factors such as degree of antigen purity, genetic make-up, maternal effects, age and sexual maturation. There are also differences in sensitivities and specificities for each method and in the type of samples that can be used such as formalin fixed, fresh, tissue, blood, water. Further limitations of some immunoassays are that they can be lengthy assay to perform, required cell culture expertise, specific reagent and equipment, and requiring up to 7 to 14 days before they can be evaluated. In addition, non-specific reactions in immunoassays may vary by an order of magnitude between fish caught at the same time and place, and may eventually obscure specific antibody activity (Table 6) (Adams et al., 2008; Adams & Thompson, 2008; King et al., 2001; Lorenzen, 1993; Magnadóttir, 2010; Vatsos et al., 2003).

Any antibody-based test is only as good as the antibody used in it, and a standard protocol and reliable source of standard specific antibody is crucial. Antibody probes can be produced in a number of ways, including polyclonal antibodies (prepared in animal species, and can also be very useful tools for the detection of pathogens), monoclonal antibodies (prepared using hybridoma technology), phage display antibodies or antibody fragments. However, serum contains many different types of antibodies and mixed populations of antibodies can create problems in some immunological techniques (Adams, 2004; Adams & Thompson, 2006; Adams et al., 2008), some of which are now commercially available. Although some antibody-based methods can be very sensitive and carrier status can be detected, such technology can be limited in sensitivity when environmental samples are used, such as water samples, and molecular methods are ideal in this situation. (Adams et al., 2008; Zhang et al., 2004; Zhang et al., 2006)

Molecular technologies are also widely used for the detection of fish pathogens (Adams & Thompson, 2006; Cunningham, 2004; Wilson & Carson, 2003). They have been successfully utilized for the detection and identification of low levels of aquatic pathogens. In addition, molecular methods can be used for the identification of pathogens to species level and in epidemiology for the identification of individual strains and differentiating closely related strains. The DNA-based methods such as PCR are extremely sensitive. However, false positive and false negative results can cause problems due to contamination or inhibition. Real-time PCR (closed tube to reduce contamination) and Nucleic Acid Sequence Based Amplification (NASBA) are alternatives that reduce this risk and offer high sample throughput. Some of the most common PCR-based technologies used for the detection of pathogens are nested PCR, random amplification of polymorphic-DNA (RAPD), reverse transcriptase-PCR (RT-PCR), reverse cross blot-PCR (rcb-PCR) and RT-PCR enzyme hybridisation assay. *In situ* hybridisation is also widely used in the detection of shrimp

Method	Advantage	Disadvantage
<i>Conventional methods</i>		
Culture	Useful because the pathogen is isolated and the etiological agent can be confirmed	Labour intensive, can be expensive, not always possible to confirm identity of etiological agent
Histopathology	Useful for assisting in the diagnosis of disease, particularly where the causative agents of new diseases have not yet been identified	Labour intensive; skilled personnel required, not always possible to identify agent
Microscopy	It is an important tool in many of the methods shown in this Table. Many different types of microscopes are now available	Can be labour intensive; skilled personnel required; can be expensive if using confocal microscope or TEM. Not always possible to identify agent
Biochemical analysis	Useful for identifying bacteria with characteristic biochemical profiles; commercial kits available for this purpose	Can be labour intensive; skilled personnel required. Not always possible to identify agent
<i>Molecular methods</i>		
PCR	Very sensitive, can be automated to analyse large sample numbers	Only detects presence of DNA of pathogen, not the whole organism. False positive and negative results can occur
Nested-PCR	Extremely sensitive method, more sensitive and specific than one-round PCR	Takes longer than the one-round PCR. False positive and negative results can occur
RT-PCR	Can detect live pathogens (e.g. detects RNA)	Care needed to ensure RNA is not degraded
Random amplified polymorphic DNA	Useful method for determining the identity of microorganisms at a strain level, assessing the genetic relationship of samples or analysing mixed pathogen populations in samples	Can be labour intensive. Skilled personnel required
Reverse cross blot-PCR	Useful for distinguishing closely related species	Expensive. Labour intensive. Skilled personnel required
RT-PCR enzyme hybridisation assay	Can detect live pathogens. Large sample numbers can be analysed	Labour intensive. Skilled personnel required
<i>In situ</i> hybridisation	Detects DNA or RNA of pathogen, therefore there is no need for antibodies to detect protein	Labour intensive. Skilled personnel required. Expensive, sometimes difficult to see pathology in tissue sections after procedure
LAMP	Fast, with results obtained in a couple of hours. Suitable for field application. Does not require skilled operator. Results easy to interpret. Sensitive	Complex to set up initially
Quantitative-PCR	Allows quantification of DNA that can be related to pathogen level in infected tissue. Extremely sensitive	Labour intensive. Requires specialised equipment. Skilled personnel required. Expensive
<i>Immunological methods</i>		
Agglutination	Simple method, no requirement for specialised equipment	Not very sensitive in comparison to other immunological methods
ELISA-detection of pathogen	Versatile method that can be used to identify pathogens or antibodies depending on how assay is set up. Microassay- therefore small amounts of reagent needed. Quantitative; can be automated to analyse large sample numbers. Sensitive	Standardised reagents and specialised equipment needed. Need careful selection of controls and a skilled operator

Immuno-histochemistry	An extension of histopathology–the pathology can be observed around the infected tissue as the slide is counterstained. Can be amplified to increase sensitivity	Need formalin-fixed, wax embedded tissue sections, therefore procedure is labour intensive. Need a skilled operator to analyse results
Western blot	Particularly useful for serology to identify pathogen-specific proteins	Standardised reagents and specialised equipment needed. Need careful selection of controls and a skilled operator
Dot blot	Versatile method which can be used to identify pathogens or antibodies depending on how assay is set up. Microassay–therefore only small amounts of reagent needed. Protein not denatured in process unlike Western blotting	Standardised reagents need to be available to perform analysis. Need a skilled operator
FAT/IFAT	Fast method if performed directly on infected tissue smears, takes longer if fixed tissue sections are used (e.g need to process infected tissue). Sensitive. Useful for detection of viruses	Need a skilled operator to analyse results, auto-fluorescence on tissue sections can interfere with interpretation of results. Requires specialised equipment
Serology-ELISA detection of fish antibodies	Non-destructive sampling method, uses ELISA format therefore can screen large numbers of samples	Indirectly detects the presence of the pathogen. Most suitable for viral infections as antibodies against Gram-negative bacteria may cross-react in assay. In order to perform the assay a specific anti-fish species antibody is required. Needs careful interpretation
Rapid kits	Fast (results obtained in minutes), inexpensive, suitable for field application. Easy to interpret results. Sensitive	Designed to be used with fresh tissue. Using frozen or fixed tissue may affect sensitivity of results
Multiplex methods		
Protein array system (Luminex)	Versatile method that can be used to identify pathogens or antibodies depending on how assay is set up. Can detect proteins or DNA. Microassay–therefore only small amounts of reagent needed. Quantitative. Can measure several pathogens or analytes simultaneously. Sensitive	Labour intensive. Needs a skilled operator. Expensive. Standardised reagents need to be available to perform analysis. Requires specialised equipment
Multiplex-PCR assays	Can detect more than one pathogen with the assay. Sensitive	Difficult to standardise. Expensive
Micro-arrays	Can detect more than one pathogen with the assay. Allows up and down regulation of genes to be examined. Very sensitive	Needs a skilled operator, very expensive, labour intensive, designated software needed to analyse results. Requires specialised equipment

ELISA: Enzyme-linked immunosorbent assay, FAT: Fluorescent antibody assays (or technique), IFAT: Indirect FAT, LAMP: Loop-mediated isothermal amplification, PCR: Polymerase chain reaction, RT-PCR: Reverse transcriptase-PCR, TEM: Transmission electron microscopy (modified from Adams & Thompson, 2008).

Table 6. Used methods, advantages and disadvantages to diagnose fish disease.

viruses and confirmation of mollusc parasites. Colony hybridisation has also been used successfully for the rapid identification of *Vibrio anguillarum* in fish (Powell & Loutit, 2004), and has the advantage of detecting both pathogenic and environmental strains (Adams et al., 2008).

Serology is an alternative approach to pathogen detection, and can also be applied to the detection of pathogen-specific antibodies in fish. The ELISA is well suited to large scale screening and this can be performed in any species of fish when an anti-fish species antibody is available (Adams et al., 2008). A number of new technologies are being developed for the rapid detection of pathogens and monitoring host responses. These include immunochromatography, such as lateral flow technology, and multiplex testing using the Bio-Plex Protein Array System or microarray technologies (Adams and Thompson, 2006). Lateral Flow is simple methodology enabling accurate (high sensitivity, specificity), simple, easy to use (2 steps, no instrument required) testing that is also economic (time/labor saving). The Protein Array System (Luminex) theoretically offers simultaneous quantitative analysis of up to 100 different biomolecules from a single drop of sample in an integrated, 96-well formatted system, mainly focusing on the detection of cytokines. Therefore, it can be used in molecular and immunodiagnostics to detect pathogens directly from tissue samples or culture, or it can be used in serology to measure fish antibodies (Adams et al., 2008; Adams & Thompson, 2008; Dupont, 2005; Giavedoni, 2005).

5. Immunosuppression

Aquatic environment of fish is in close contact with numerous pollutants. Aquatic pollutants such as heavy metals, aromatic hydrocarbons, pesticides and mycotoxins modulate the immune system of fish, thus increasing the host susceptibility to infectious pathogens. Pollutants in the water which may be particulate or soluble can also be natural source such as metals showing the seasonal increase in lakes as well as drugs used in the prevention or treatment of disease such as cortico-steroid hormones, used drugs in terrestrial animal health in aquaculture such as florfenicol, oxolinic acid, and oxytetracycline (Table 7). Immunosuppressive effects of these compounds may occur at high concentrations or long-term exposures (Anderson, 1996; Bols et al., 2001; Brown-Treves, 2000; Duffy et al., 2002; El-Gohary et al., 2005; Enis-Yonar et al., 2011; Kusher & Crim, 1991; Lumlertdacha & Lovell, 1995; Lundén & Bylund, 2002; Lundén et al., 1998; Lundén et al., 1999; Manning, 2001; Manning, 2010).

Substances	Parameters	Fish species
<i>Metals and organometallies</i>		
Aluminum	Reduced chemiluminescence	Rainbow trout
Arsenic	Phagocytosis elevated or lowered	Rainbow trout
Cadmium	Elevated serum antibody	Rainbow trout
	Chemiluminescence reduced	Rainbow trout
	Lymphocyte number and mitogenic response reduced	Goldfish
	Antibody-binding lymphocyte reduced	Bluegill
Chromium	Serum antibody reduced	Brown trout, carp
Copper	Chemiluminescence reduced	Rainbow trout
	Susceptibility to IHNV increased	Rainbow trout
	Leukocyte respiratory burst activity inhihed	Rainbow trout
	Serum antibody reduced	Brown trout
	Antibody-producing cells reduced	Rainbow trout
	Susceptibility to <i>Vibrio anguillarum</i> increased	Eel

Lead	Serum antibody reduced	Brown trout
Mercury	Lymphocyte numbers reduced	Barb
Nikel	Serum antibody reduced	Brown trout
Zinc	Serum antibody reduced Phagocytosis decreased	Brown trout Rainbow trout
<i>Aromatic hydrocarbon</i>		
Benzidine	Non-specific agglutination rise	Estuarine fish
PAHs	Macrophage activity reduced Melanomacrophage numbers reduced	Spot, Hogchoker Flounder
PCBs Benzo[a]pyrene PCB 126 Aroclor 1254 Aroclor 1232 Aroclor 254/1260	Phagocytic capacity reduced Antibody-producing cells reduced Non-specific cytotoxic cell activity reduced Antibody-producing cells reduced Susceptibility to disease increased Susceptibility to disease increased	Rainbow trout Medaka Catfish Coho salmon Channel catfish Rainbow trout
Phenols Hydroquinone	Antibody- producing cells reduced Non-specific cytotoxic cell activity reduced	Rainbow trout Carp
TCDD	Mitogenic response partially suppressed Susceptibility to IHNV	Rainbow trout
<i>Pesticides</i>		
Bayluscide	Serum African antibody reduced	Catfish
Dichlorvos	Lysozyme activity reduced	Carp
DDT	Antibody-producing cell, serum antibody reduced	Goldfish
Endrin	Phagocytic, antibody-producing cell activities reduced	Rainbow trout
Malathion	Lymphocyte number reduced	Channel catfish
Metrifonate	Phagocytic, neutrophilic and lysozyme activity reduced , antibody-producing cell reduced,	Cichlid fish
Methyl bromide	Thymic necrosis	Medaka
Tributyltin	Chemiluminescence reduced	Oyster, Hogchoker
Trichlorophon	Phagocytic, neutrophilic, lysozyme activity reduced	Carp
<i>Mycotoxins</i>		
Aflatoxin-B ₁	B-cell memory loss, neutrophilic activity reduced	Rainbow trout
Fumonisin-B ₁	Antibody-producing cells reduced	Catfish
<i>Antibiotics</i>		
Florfenicol	Chemiluminescence reduced Phagocytic cells counts reduced after 5-6 weeks	Rainbow trout
Oxolinic acid	Antibody-producing cells reduced	Rainbow trout
Oxytetracycline	Mitogenic response reduced, Antibody-producing cells reduced, phagocytic activity reduced	Carp Rainbow trout
<i>Other compounds</i>		
Cortisol/ Kenalog-40	Antibody-producing cells reduced	Rainbow trout
Hydrocortisone	Phagocytic activity reduced	Striped bass

DDT: Dichloro-diphenyl-trichloroethane, PAHs: Polynuclear aromatic hydrocarbons, PCBs: Polychlorinated biphenyls, TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin. IHNV: Infectious hematopoietic necrosis virus. (modified from Anderson, 1996).

Table 7. Nonspecific defense mechanisms and specific immune response assays /or parameters in fish effected by presence of some immunosuppressive compounds.

6. Immunomodulation

Immunomodulators present in the diets stimulate the innate immune systems, while antigenic substance such as bacterins and vaccines initiate the more prolonged process of antibody production and acquired immune systems. Prophylactic and therapeutics administration of immunomodulators will need to be adapted to each cultured fish species in anticipation of recognize pathogens, under known environmental conditions (Gannam & Schrock, 2001). Prophylactic and therapeutic compounds and/or drugs against infections are rarely successful or limited effects; currently there are no approved some drugs for the control and treatment fish disease in the aquaculture industry. For example, several substances, such as fumagilin and albendazole have been used in fish with potential value in controlling microsporidian infections. However, other drugs, like sulphaquinoxaline, amprolium and metronidazole have been ineffective to control the disease (Berker & Speare, 2007; Dykova, 2006; Rodriguez-Tovar et al., 2011). Most of similar drugs have ambiguous result and it is has been reported that high concentrations and prolonged treatment of infections with some drugs might cause side-effects. More promising results have been achieved by using immune-prophylactic control components such as probiotics (e.g. basillus P64, yeasts and lactic acid bacteria), prebiotics (e.g. fructo- galacto-, transgalacto-oligosaccharide), vaccination (e.g. *Vibrio spp.*, *Yersinia ruckerii*) and immunostimulants (e.g. β -glucan, chitosan and levamisole) (Austin & Brunt, 2009; Hoffmann, 2009; Magnadóttir, 2010; Nayak, 2010; Rodriguez-Tovar et al., 2011). On the other hand, in recent years, organically produced aquatic products are increasingly available to consumers and, in particular, sea bass and sea bream from certificated fish farms (Perdikaris & Paschos, 2010). The initial legislative framework for organic aquaculture in the European Union (EU) was the Directives (EC) 834/07 and (EC) 889/08 (EU, 2007; 2008).

7. Immunostimulants

Various chemotherapeutic compounds have been extensively used to treat bacterial infections in cultured for about the last 20-30 years. However, the incidence of drug-resistant bacteria has become a major problem in fish culture (see Chapter 11: Section 5.2). Although, vaccination is a useful prophylaxis for infectious disease, and is also already commercially available for bacterial infections such as vibriosis, redmouth disease and for viral infections such as infectious pancreatic necrosis, the development of vaccines against intracellular pathogens such as *Renibacterium salmoninarum* has not so far been unmitigated successful. Therefore, the immediate control of all fish disease using only vaccines is impossible. Even thought, use of immunostimulants, in addition to chemotherapeutic drugs and vaccines, has been widely accepted by the aquaculture industry, many question about the efficacy of immunostimulants from users still continue such as whether this components can protect against infections disease (Table 8). Also, the biological activities of the immunostimulants may be so multiple and potent that some of them may be more harmful than beneficial (Dalmo, 2002; Sakai, 1999).

By definition, an immunostimulant is a naturally occurring compound that molecules that modulates the immune system by increase the host's resistance against disease that in most circumstances are caused by pathogens (Bricknell & Dalmo, 2005). However, synthetic chemicals such as isoprinosine, bestatin, levamisole, muramyl dipeptide and FK-565 well-known as lactoyl tetrapeptide are known to possess immunostimulatory properties. It is

important to note the use of the term “modulate”, as a substance with the potential immunostimulatory properties may lead to a down regulation of the immune response if administered in excess amounts or long-term usage. Hence, administration of an immunestimulant prior to an infection may elevate the defence barriers of the animal and thus provide protection against an otherwise severe or lethal infection. Also, immunostimulants enhance individual components of innate immune response, but this does not always translate into increased survival. An important point to have in mind is that not by enhancing acquired immune response. Therefore, there is no memory component and the response is likely to be of short duration (Gannam & Schrock, 2001; Hølvold, 2007; Maqsood et al., 2011; Raa, 2000; Sakai, 1999).

	Chemotherapeutics	Vaccines	Immunostimulants
When	Therapeutically	Prophylactically	Prophylactically
Efficacy	Excellent	Excellent	Good
Spectrum of activity	Middle	Limited	Wide
Duration	Short	Long	Short

Table 8. A comparison of characteristics of chemotherapeutics, vaccines and immunostimulants (Sakai, 1999).

A division of immunostimulants depended on which effects they include such as anti-bacterial, -viral, -fungal and -parasitic effects may be helpful but hard to accomplish. Some immunostimulants may induce both antibacterial and antiparasitic effects, whereas other may help the organism to fight virus and fungus. Generally, immunostimulants used in fish and shrimp in many countries can be divided into two main groups as biological substances and synthetic chemicals depending on their sources (Table 9) (Anas et al., 2005; Brown-Treves, 2000; Dügenci et al., 2003; Galindo-Villegas & Hosokowa, 2004; Gannam & Schrock, 2001; Gildberg et al., 1996; Glina et al., 2009; Jiye et al., 2009; Lauridsen & Buchmann, 2010; Maqsood et al., 2011; Noga, 2010; Paulsen et al., 2003; Perera & Pathiratne, 2008; Petersen et al., 2004; Raa, 2000; Sakai, 1999). But, some immunostimulants may be included in different subgroups by some researchers, such as schizophyllan and scleroglucan. These substances may be included in bacterial derivatives-subgroups as various β -glucan products from *Schizophyllum commune* and *S. glucanicum*, respectively, or may be included in polysaccharides-subgroups as polysaccharides containing sugars.

7.1 Dose, timing, administration-route and -period of immunostimulants

The effect of timing the administration on immunostimulant function is a very important issue. Usually, the most effective timing of antibiotics is upon the occurrence of disease, and they cannot often be used prophylactically due to risk of fostering the development of drug-resistant bacteria. Researchers proposed that immunostimulants may improve health and performance of fish and shrimp in aquaculture, if used prior to: (1) before the outbreak of disease to reduce disease-related losses, (2) situations known to result in stress and impaired general performance of animals such as handling, change of temperature and environment, weaning of larvae to artificial feeds, (3) expected increased exposure to pathogenic micro-organisms and parasites such as spring and autumn blooms in the marine environment, high stocking density, (4) developmental phases when animals are particularly susceptible to infectious agents such as the larvae phase of shrimp and marine fish, smoltification in

salmon, sexual maturation (Raa, 2000; Sakai, 1999). In addition, the effects of immunostimulants may also be different dependent on the administration route, the dose used, the duration of the treatment and growth period. Immunostimulants does not show a linear dose/effect relationship; instead they most often show a distinct maximum at a certain intermediate concentration and even a complete absence of effect or toxicity, at high concentration. The explanations for these phenomena are still speculative and include competition for receptors (analogous to substrate inhibition of enzyme), over stimulation resulting in exhaustion and fatigue of the immune system (Bright-Singh & Philip, 2002).

Groups	Substances	Compounds
Biological substances	Animal compounds	EF-203 (Chicken egg), Ete (Tunicate, <i>Ecteinascida turbinata</i>), Hde (Abalone, <i>Haliotis discus hannai</i>), cod milt, firefly squid and acid-peptide fractions (fish protein hydrolysate)
	Plant extracts	Glycyrrhizin (Licorice, saponin in <i>Glycyrrhiza glabra</i>), quil-A saponin, ergosan (<i>Laminaria digitata</i>), C-UP III (a Chinese herb mix), laminaran (Seaweed), spirulina (<i>Spirulina plantensis</i>) <i>Quillaja saponica</i> (Soap tree), leaf extract (<i>Ocimum sanctum</i>), scutellaria extract (<i>Scutellaria baicalensis</i>), astragalus extract (<i>Astragalus membranaceus</i>), ganoderma extract (<i>Ganoderma lucidum</i>), lonicera extract (<i>Lonicera japonica</i>), phyllanthus extract (<i>Phyllanthus emblica</i>), azadirachta extract (<i>Azadirachta indica</i>), solanum extract (<i>Solanum trilobatum</i>), mistletoe (<i>Viscum album</i>), nettle (<i>Urtica dioica</i>), ginger (<i>Zingiber officinale</i>) and chevimmun (<i>Echinacea angustifolia</i> - <i>Baptista tinctoria</i> - <i>Eupatorium perfoliatum</i>)
	Bacterial and yeast derivatives	β -glucans (from bacteria and mycelial fungi; MacroGard, VitaStim, SSG, Eco-Activa, Betafectin, Vetregard, Dinamune, Aquatim, AquaStim, Curdlan, Krestin), ascogen (Aquagen), peptidoglycan (<i>Brevibacterium lactofermentum</i> ; <i>Vibrio</i> sp.), pDNA (<i>Escherichia coli</i>), lipopolysaccharide, fragellins (recombinant- <i>Borrelia</i>), <i>Vibrio anguillarum</i> cells, <i>Clostridium butyricum</i> cells, <i>Achromobacter stenohalis</i> cells and streptococcal components (<i>Bordetella pertuosis</i> , <i>Brucella abortus</i> , <i>Bacillus subtilis</i> , <i>Klebsiella pneumonia</i>)
	Cytokines	Interferon, interleukin-2, tumor necrosis factor
	Hormones	Growth hormone, prolactin, melanin stimulating hormone, β -endorphin and melanin concentrating hormone
	Nutritional factors	Vitamin-A, -C, -E, carbohydrate (Acemannan), soybean protein, trace elements (zinc, iron, copper, selenium) and nucleotides
	Polysaccharides	Chitin, chitosan, lentinan, schizophyllan, sclerotium, scleroglucan, protein-bound polysaccharide (PS-K), oligosaccharide and polyglucose
	Others	Lactoferrin
Synthetic chemicals		Avridine, bestatin, DW-2929, ISK, KLP-602, FK-156 (lactoyl tetrapeptide), FK-565, fluoro-quindone, Freund's complete adjuvants, imiquimod, isoprinosine, levamisole, muramyl dipeptide and sodium alginate

(modified from Galindo-Villegas & Hosokowa, 2004; Sakai, 1999).

Table 9. Groups, substances and examples of immunostimulants evaluated in many countries that have been tried to increase disease protection in fish species and/or shrimps.

It is reported that oral administration of an immunostimulant such as lipopolysaccharide is increased larval growth. This may be important in the intensive production of fish larvae and juveniles. In spite of advantages and limitations, the basic methodologies adopted are injection, immersion and oral (Table 10). Injection and immersion methods are suitable only for intensive aquaculture and both require the fish to be handled or at least confined in a small space during the procedures (Dalmo, 2002; Guttvik et al. 2002; Raa, 2000). By injections of immunostimulants enhances the function of leucocytes and protection against pathogens. However, this method is labour intensive, relatively time-consuming and becomes impractical when fish weight less than 15 gram. By immersion, efficacies had been confirmed by several researchers (Anderson et al., 1995; Baba et al., 1993; Jeney & Anderson, 1993; Perera & Pathiratne, 2008), although, since dilution, exposure time and levels efficacy are not well defined, caution must be taken in account by applying this methods. Oral administration is only method economically suited to extensive aquaculture, is non-stressful and allows mass administration regardless of fish size, but of course may be administration only in artificial diet (Galindo-Villegas & Hosokowa, 2004; Noga, 2010).

Route	Dose	Exposure time	Advantages	Limitations
Injection	Variable	1 or 2 doses	Allows use of adjuvants, Most potent immunization route, most cost effective method for large fish	Only for intensive aquaculture, fish must be >10~15 g, stressful (anesthesia, handling), labour hard
Immersion	2-10 mg/L	10 min to hours	Allows mass immuno-stimulation of small (<5 g) fish, most cost effective method for small fish,	Only for intensive aquaculture, dip rise handling stress, potency not as high as injection route
Oral	0.01–4%	Some days or longer	Only not-stressful method, Allows mass immuno-stimulation of fish any size, no extra labour cost	Poor potency, requires large amounts of immunostimulation to achieve protection, suitable only for fish fed artificial diet

(Galindo-Villegas & Hosokowa, 2004).

Table 10. Administration methods, advantages and limitations of immunostimulants in aquaculture.

The effects of immunostimulants were dose and/or application time, route, and period related. For example, low-dose glucan content being beneficial whereas high-dose glucan content had limited effects (Ai et al., 2007). Peptidoglycan is not influence the high-dose (0.1%) in shrimp diets, and not effect after 60 days of oral administration in rainbow trout growth. On the other hand, Ete exerted a protective effect in eels injected intra-peritoneal 2 days after challenge with *A. hydrophila*. However, the protection was not seen when Ete was administered intra-peritoneal 2 days before or concurrently with the bacteria. The adjuvant effects of glucan against *A. salmonicida* vaccine oral delivery (7 days administration) and immersion (15 min). No adjuvant effects were seen with the immersion treatment, although the fish administered glucan orally showed enhanced vaccine effects (Sakai, 1999). The

number of NBT-positive cell in catfish increased following oral administration of glucan and oligosaccharide over 30 days, but not over 45 days (Yoshida et al., 1995). The effects of long-term oral administration of immunostimulants are still unclear. However, the dilution, the effective administration period and the levels of efficacy require more complete investigation for each immunostimulants.

7.2 In vivo and in vitro effects of immunostimulants

The benefit of immunostimulants is considerable. They have the potential to elevate the innate defence mechanisms of fish prior to exposure to a pathogen, or improve survival following exposure to a specific pathogen when treated with an immunostimulant. There are two main procedures for evaluating the efficacy of an immunostimulant; (1) *in vivo* such as protection test against fish pathogen, (2) *in vitro* such as the measurements of the efficiency of cellular and humoral immune mechanism (Bricknell & Dalmo, 2005; Maqsood et al., 2011). *In vivo* evaluation should be based at least on the following parameters: phagocytosis, antibody production, free radical production, lysozyme activity, natural cytotoxic activity, complement activity, mitogen activity, macrophage activating factor (MAF), nitroblue tetrazolium reaction (NBT), etc. The evaluation of an immunostimulant by the *in vitro* methods which test the effects of that substance on the immune system is to be preferred in preliminary studies (Table 11 and Figure 6) (Aly & Mohamed, 2010; Anas et al., 2005; Barman et al., 2011; Brown-Treves, 2000; Dügenci et al., 2003; Galina et al., 2009; Galindo-Villegas & Hosokowa, 2004, Gannam & Schrock, 2001; Magnadóttir, 2010; Maqsood et al., 2011; Noga, 2010; Paulsen et al., 2003; Peddie et al., 2002; Raa, 2000; Sakai, 1999; Yin et al., 2009). Nevertheless, if possible *in vitro* test should be performed together wit *in vivo* experiments in order to elucidate the basic mechanisms responsible for the protection (Bricknell & Dalmo, 2005; Brown, 2006; Brown-Treves, 2000; Dalmo, 2002; Sakai, 1999). Used aquaculture potential immunostimulants with *in vivo* and/or *in vitro* effects and administration route are given at Table 12, as well as with doses at Table 13.

In vivo effects	In vitro effects
Increased survival after challenges with bacteria, antiparasitic effects including reduced settlement of sea lice, improved resistance to viral infection and increased interferon levels	Increased macrophage activity including: - Phagocytosis, free radical production, enzyme activity, migration activity, production of cytokines, nitric oxide production, bacterial killing, antibody production, respiratory burst, MAF, NBT
Growth enhancement	Increased cytotoxicity
Increased antibody production following vaccination	Increased lysozyme activity
Increased lysozyme levels	Increased cytokine induction Increased oxygen radical induction Increased cell proliferation

MAF: Macrophage activating factor, NBT: Nitroblue tetrazolium reaction. (modified from Bricknell & Dalmo, 2005).

Table 11. The *in vivo* and *in vitro* responses seen in fish treated with immunostimulants.

A number of studies have been reported that potential immunostimulants may be showed at *in vivo* and/or *in vitro* effects in fish species in different countries and various periods (Aly & Mohamed, 2010; Anas et al., 2005; Dugenci et al., 2003; Galindo-Villegas et al., 1996; Galindo-

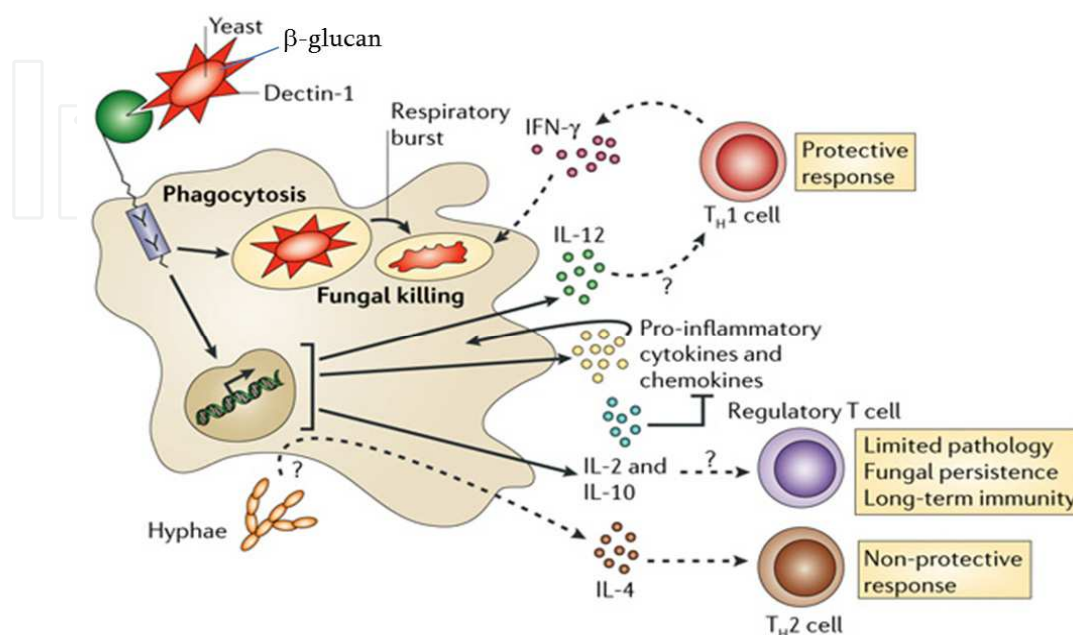
Villegas et al., 2006; Gildberg et al., 1996; Ispir & Dorucu, 2005; Kunttu et al., 2009; Lauridsen & Buchmann, 2010; Ortuño et al., 2002; Peddie et al., 2002; Perera & Pathiratne, 2008; Sakai et al., 1995; Seker et al., 2011; Soltani et al., 2010; Yin et al., 2009; Zhao et al., 2010).

Immunostimulant	Species	Route	In vivo or in vitro effects	Resistance
Synthetic chemicals				
FK-565	Trout	ip	phagocytosis ↑	<i>A. salmonicida</i> ↑
		in vitro	antibody ↑	-
Freund's adjuvants	Trout	ip	-	<i>V. anguillarum</i> ↑, <i>Y. ruckeri</i> ↑
	Yellowtail	ip	-	<i>P. piscicida</i> →
Levamisole	Carp	ip/oral	phagocytosis ↑ / NBT →	-
		im	phagocytosis ↑, CL ↑	<i>A. hydrophila</i> ↑
	Trout	in vitro	phagocytosis ↑, NBT →	-
		ip	phagocytosis ↑, CL ↑, complement ↑	<i>V. anguillarum</i> ↑
		im	-	<i>A. salmonicida</i> ↑
Muramyl dipeptide	Trout	ip	phagocytosis ↑, CL ↑	<i>V. anguillarum</i> ↑
Bacterial and yeast derivatives				
<i>Achromobacter stenohalis</i> cells	Char	ip	CL ↑, complement ↑	<i>A. salmonicida</i> ↑
<i>Clostridium butyricum</i> cells	Trout	oral	phagocytosis ↑, NBT ↑	<i>V. anguillarum</i> ↑
Glucan	Trout	im / ip	phagocytosis ↑ / NBT ↑	-
Lipopolysaccharide	Plaice	ip	macrophage migration ↑	-
	Red sea bream		phagocytosis ↑	
	Goldfish	in vitro	macrophage activating factor ↑	
	Salmon		phagocytosis ↑, NBT ↑	
	Catfish		IL-1 ↑	
Peptidoglucon	Trout	oral	-	<i>V. anguillarum</i> ↑
	Shrimp		-	YHB ↑
	Yellow tail		phagocytosis ↑	<i>E. seriolicida</i> ↑
	J. flounder		lysozyme ↑, phagocytosis ↑	<i>E. tarta</i> ↑
Vibrio bacteria	Trout	im	-	<i>A. salmonicida</i> ↑, <i>E. seriolicida</i> ↑
	Prawn/shrimp	ip,im,oral	-	<i>Vibrio sp.</i> ↑
VitaStim	Coho	ip, oral	-	<i>A. salmonicida</i> ↑
	Chinook	oral / im	-	<i>A. salmonicida</i> ↑/→
	Catfish	oral	antibody ↑	<i>E. ictaluri</i> →
Yeast glucan	Salmon	oral	-	<i>V. anguillarum</i> ↑, <i>A. salmonicida</i> ↑
		ip	complement ↑, lysozyme ↑, antibody ↑, NBT ↑, killing →	<i>A. salmonicida</i> →, <i>Y. ruckeri</i> ↑
	Catfish	ip	phagocytosis ↑, NBT ↑, antibody ↑, killing ↑	<i>E. ictaluri</i> ↑
		oral	CL ↑, migration ↑	<i>E. ictaluri</i> →
	Shrimp	im,in vitro	phenoloxidase ↑, lysozyme →, NBT ↑, CL ↑	-
	Trout	ip	lysozyme ↑, killing ↑, O ₂ ⁻ ↑, NBT →	<i>V. anguillarum</i> →
	Turbot	oral	lysozyme ↑, complement →, CL ↑	<i>V. anguillarum</i> ↑
Yeast glucan + Vit-C	Trout	oral	lysozyme →, complement ↑, CL ↑	-

<i>Animal and plant extracts</i>				
Acid-peptide fraction	Salmon	<i>in vitro</i>	NBT ↑	-
EF-203	Trout	oral	phagocytosis ↑, NBT ↑, antibody →	<i>R. salmoninarum</i> ↑
			phagocytosis ↑, CL ↑	<i>Streptococcus</i> sp. ↑
Ete (Tunicate)	Eel	ip	phagocytosis ↑	<i>A. hydrophila</i> ↑
	Catfish	ip	phagocytosis ↑, antibody →	<i>E. ictaluri</i> ↓
Firefly squid	Trout	ip	NBT mitogen Con A, LPS killing	-
Glycyrrhizin	Yellowtail	oral	complement ↑	<i>E. seriolicida</i> ↑
	Trout	<i>in vitro</i>	mitogen (Con-A, LPS) ↑ macrophage activating factor ↑, O ₂ ⁻ ↑,	-
Hde (Abalone)	Trout	ip	phagocytosis ↑, CL↑, NK ↑	<i>V. anguillarum</i> ↑
Quil-A saponin	Yellowtail	oral	leucocyte migration ↑	-
	Trout	im	serum bactericidal activity ↑	-
<i>Polysaccharides</i>				
Chitin	Trout	ip	phagocytosis ↑, lysozyme →	<i>V. anguillarum</i> ↑
Chitosan	Trout	oral/ip,im	phagocytosis ↑, NBT ↑/NBT ↑, killing ↑	<i>A. salmonicida</i> ↑/ -
Lentinan + Schizophyllan	Carp	ip	phagocytosis ↑	<i>E. tarda</i> ↑
Oligosaccharide	Catfish	oral	NBT ↑	-
Polyglucose	Salmon	<i>in vitro</i>	NBT ↑, pinocytosis ↑, acid phosphatase ↑	-
PS-K	Tilapia	oral	phagocytosis ↑	<i>E. tarta</i> ↑
Schizophyllan	Prawn	oral	phagocytosis ↑	<i>Vibrio</i> sp. ↑
Schizophyllan + Scleroglucan	Yellow tail	ip	complement ↑	<i>E. seriolicida</i> ↑
			phagocytic index ↑, lysozyme ↑	<i>P. piscicida</i> →
Scleroglucan	Carp	ip	-	<i>A. hydrophila</i> ↑
<i>Hormones, Cytokines and Others</i>				
Growth hormone	Trout	ip	phagocytosis ↑, mitogen ↑, CL↑, NK ↑	<i>V. anguillarum</i> ↑
Interferon	Flatfish	<i>in vitro</i>	-	HRV ↑
Lactoferrin	Trout	oral	phagocytosis ↑	<i>V. anguillarum</i> ↑
			CL ↑	<i>Streptococcus</i> sp. →
		<i>in vitro</i>	CL ↑, NBT ↑	-
	Red sea bream	oral	lectin ↑, lysozyme →	<i>Cryptocaryon</i>
Prolactin	Trout	<i>in vitro</i>	NBT ↑	-

↑: Increase, ↓: Decrease, →: No change, Brook: Brook trout, Chinook: Chinook salmon, CL: Chemiluminescent response, Coho: Coho salmon, Con-A: Concanavalin-A, IL-1: Interleukin-1 production, im: immersion, ip: Intraperitoneal injection, J. flounder: Japanese flounder, Killing: Bactericidal activity of macrophage, LPS: Lipopolysaccharide, NBT: Nitroblue tetrazolium reaction, NK: Natural killer cell, O₂⁻: Production of superoxide anion, Prawn: Kuruma prawn, PS-K: Protein-bound polysaccharide, Salmon: Atlantic salmon, Shrimp: Black tiger shrimp, Trout: Rainbow trout, Turbot: *Scophthalmus maximus*, Vit-C: Vitamin-C, *A. hydrophila*: *Aeromonas hydrophila*, *A. salmonicida*: *Aeromonas salmonicida*, *E. ictaluri*: *Edwardsiella ictaluri*, *E. seriolicida*: *Enterococcus seriolicida*, *E. tarda*: *Edwardsiella tarda*, HRV: *Hirame rhabdo virus*, *P. piscicida*: *Pasteurella piscicida*, *R. salmoninarum*: *Renibacterium salmoninarum*, *V. anguillarum*: *Vibrio anguillarum*, *Y. ruckeri*: *Yersinia ruckeri*, YHB: Yellow-head baculo virus. (modified from Gannam & Schrock, 2001; Sakai, 1999).

Table 12. Potential immunostimulants, *in vivo* and/or *in vitro* effects of this immunostimulants with administration route.



Recognition of β -glucans on fungal particles induces several dectin-1-mediated cellular responses, which might contribute to anti-fungal immunity *in vivo*. These include fungal uptake and killing and the production of pro-inflammatory cytokines and chemokines, such as tumour-necrosis factor (TNF) and CXC-chemokine ligand 2, in collaboration with the Toll-like receptors (TLRs), which is likely to lead to cellular recruitment and activation. Dectin-1-mediated recognition also stimulates the production of interleukin-12 (IL-12), which might result in a protective T-helper 1 (TH1)-cell response and the production of interferon- γ (IFN- γ), thereby activating the fungicidal activities of phagocytes. In dendritic cells, β -glucan recognition by dectin-1 can also induce the production of IL-10 and IL-2, which could potentially contribute to the development of regulatory T cells, thereby limiting inflammatory pathology and promoting fungal persistence and long-term immunity, as proposed previously. IL-10 would also inhibit the production of pro-inflammatory cytokines and chemokines. Fungi might also mask their β -glucan, by conversion from yeast to hyphal forms. This could result in the induction of non-protective TH2-cell immune responses, mediated by IL-4; this could be the result of preventing recognition by dectin-1 although the pathways leading to this response are unknown. Although dectin-1 is described here as having a central role in the generation of protective immune responses, it should be noted that many other opsonic and non-opsonic receptors such as the mannose receptor, complement receptor 3, dendritic-cell-specific ICAM3-grabbing non-integrin and TLRs also contribute to this process. (modified from Brown, 2006).

Fig. 6. Recognition of β -glucans on fungal particles induces several dectin-1-mediated cellular responses, which might contribute to anti-fungal immunity *in vivo*.

Immunostimulant	Species	Dose - Route	<i>In vivo or in vitro</i> effects
β-glucan	Atlantic salmon	15 mg/kg, inj	ROS ↑, lysozyme ↑
		150 mg/kg, oral	acid phosphatase ↑
		1 ml/fish, inj	lysozyme ↑, complement ↑, antibody ↑
	Coho salmon	5 and 15 mg/kg, inj	→
	Japanese flounder	3 g/kg, oral	NBT ↑
	Sea bass	2% wet bw, oral	humoral activation ↑
	Dab	0.5 µg/kg, iv	ROS ↑
	Dentex	0.5%, oral	→
	Turbot	0.5 – 500 µg/kg, iv	ROS ↑
		2 g/kg	leukocyte number ↑
	Yellow tail	2 – 10 mg/ml, inj	phagocytic activity ↑
	Rainbow trout	88 and 350 µg/g, oral	lysozyme ↑, ROS ↑, complement bacteriolytic activity ↑
β-glucan + LPS	Atlantic salmon	1 – 250 + 10 µg/ml	lysozyme ↑
β-glucan + Mannose	Japanese flounder	1%, oral	NBT ↑, lysozyme ↑
	Pink snapper	0.1 – 1% w/w, oral	ROS ↑, macrophage activation ↑
β-glucan + FKC + Quillaja saponica	Pink snapper	122 + 34 + 5 mg/kg	agglutination titers ↑
CFA	Sockeye salmon	5 mg/kg, inj	antibody ↑
Chitosan	<i>M. rosenbergi</i> larva	0.25 – 1% v/v, 150 ml <i>in vitro</i>	Antibacterial activity ↑
Chitin	Gilthead sea bream	0.1 ml/fish, inj	→
		1 mg/fih, inj	humoral and cellular activation ↑
		25 – 100 mg/kg	NCCs ↑, ROS ↑, phagocytic activity ↑
EF-203	Rainbow trout	30 µg/kg, oral	NBT ↑, phagocytic activity ↑
Ergosan	Rainbow trout	1 mg/fish	complement ↑
Fungi	Sockeye salmon	10 g/kg, oral	→
Glycyrrhizin	Yellow tail	0 – 50 mg/kg, oral	complement ↑
Laminaran	Blue gourami	20 mg/kg, inj	CL ↑
Levamisole	Atlantic salmon	2.5 mg/L, bath	ROS ↑, phagocytic activity ↑, lysozyme ↑
	Coho salmon	5 mg/kg, inj	→
	Sockeye salmon	125 – 500 µg/ml, oral	phagocytosis ↑, complement ↑, ROS ↑, lymphokine ↑
		0.5 – 500 µg/ml, iv	ROS ↑
		75 – 300 mg/kg, oral	NCCs ↑
	Japanese flounder	125 – 500 mg/kg, oral	phagocytic activity ↑, NBT ↑, lysozyme ↑
	Rainbow trout	10 and 50 µg/ml, <i>in vitro</i>	phagocytic activity ↑, NBT ↑
		5 mg/kg, inj	NBT ↑, lysozyme ↑, phagocytic activity ↑, killing ↑
LPS	Red sea bream	1 ml/fish, inj	phagocytic activity ↑

MCFA	Coho salmon	5 mg/kg, inj	→
Microsporidian	Flounder	106 spores, inj	antibody ↑
Myxosporean	Sea bass	multiple, iv	ROS ↑
Peptidoglycan	Japanese flounder	1.5 – 4.5 g/kg, oral	phagocytosis ↑, complement ↑, MAF ↑, ROS ↑
	Yellow tail	0.2 mg/kg, oral	phagocytic activity ↑
Quillaja	Yellow tail	0.5 – 50 mg/kg	chemotaxis ↑
Yeast	Gilhead sea bream	1 – 10 g/kg	cellular response ↑
	Sea cucumber	5%, oral	phagocytic activity ↑, phagocytic index ↑, lysozyme ↑
Wy-18, 251	Coho salmon	10 mg/kg, inj	→

↑: Increase, ↓: Decrease, →: No change, µg: Microgram, inj: Injection, iv: Intra venous, bw: Body weight, CFA: Complete Freund’s adjuvant, CL: Chemiluminescent response, FKC: Formalin-killed *Edwardsiella tarda* cells, L: Litre, LPS: Lipopolysaccharide, Killing: Bactericidal activity of neutrophil and monocytes, MAF: Macrophage activating factor, M. rosenbergi: Macrobrachium rosenbergi, MCFA: Medium-chain fatty acid(s), NBT: Nitroblue tetrazolium reaction, NCCs: Non-specific cytotoxic cells, ROS: Reactive oxygen species, Turbot: *Scophthalmus maximus*, Wy-18, 251: an analog of levamisole at structure 3-(p-chlorophenyl) thiazolol [3,2-a] benzimidazole-2-acetic acid. (modified from Galindo-Villegas & Hosokowa 2004).

Table 13. Doses and effects of immunostimulants as nutritional factors and nucleotides in some fish species.

Improvements in the health status of fish can certainly be achieved by balancing the diets with regard to nutritional factors (see Table 9), in particular lipids such as fatty acids, essential oils, and anti-oxidative vitamins or minerals such as vitamin-C, -E and selenium, and also minerals iron and fluoride, but this is primarily a result of an input of substrates and cofactors in a complex metabolic system. These compounds were identified as micronutrients that could affect disease resistance. This is unlike immunostimulants, which interact directly with the cells of the immune system and make them more active, because they enhance in immune system by providing substrate and co-factors necessary for the immune system to work properly. Nevertheless, some nutritional factors are so intimately interwoven with the biochemical processes of the immune system that significant health benefits can be obtained by adjusting the concentration of such factors beyond the concentration range sufficient to avoid deficiency symptoms or below a certain concentration range (Balfry & Higgs, 2001; Gannam & Schrock, 2001; Lim et al., 2001a, 2001b; Raa, 2000; Soltani et al., 2010). The modulatory effects of dietary nutritional factors on macrophage-, haemolytic-, lysozyme- and complement activation, lymphocyte proliferation, macrophage phagocytic response as well as oxidative burst, pinocytosis and bactericidal activity have been reported in aquaculture (Balfry & Higgs, 2001; Galindo-Villegas & Hosokowa, 2004; Gannam & Schrock, 2001, Sakai et al., 1999).

Other a type of nutritional factors as immunostimulants in aquaculture, nucleotides have essential physiological and biochemical functions including encoding and deciphering genetic information, mediating energy metabolism and cell signaling as well as serving as components of co-enzymes, allosteric effectors and cellular agonists. Also, these compounds have traditionally been considered to be non-essential nutrients. Nucleotides consist of a purine or a pyrimidine base, a ribose or 2'-deoxyribose sugar and one or more phosphate groups. The term nucleotide in this context refers not only to a specific form of the compounds but also to all forms that contain purine or pyrimidine bases.

Immunostimulant	Species	Dose - Period	In vivo or in vitro effects / Resistance
Nutritional factors			
Vitamin-C	Catfish	150 mg/kg, 14 w	<i>E. tarta</i> ↑
		1000 mg/kg, 7 w	neutrophil → (phagocytosis)
		3000 mg/kg	complement ↑, phagocytic index ↑ antibody → macrophage killing → <i>E. tarta</i> ↑
		4000 mg/kg, 9 w	complement →, antibody →, <i>E. tarta</i> ↑
	Red sea bream	1000 mg/kg	complement →
		10 000 mg/kg	phagocytic activity ↑
	Atlantic salmon	2750 mg/kg, 26 w	complement ↑, NBT →, phagocytosis →, MAF ↑, <i>A. salmonicida</i> ↑
		2980 mg/kg	antibody ↑
		3170 mg/kg, 16 w	NBT ↑, killing ↑, migration ↑, antibody ↑, <i>A. salmonicida</i> ↑
		4000 mg/kg, 52 d	<i>V. salmonicida</i> ↑
		5000 mg/kg	antibody →
	Coho salmon	3000 mg/kg	phagocytosis ↑, ROS ↑, complement ↑
	J. flounder	6100 mg/kg	NBT ↑
	Trout	244 mg/kg, 16 w	proliferation ↑, NBT →, MAF ↑
		550 mg/kg, 10 d	IHNV ↑
		2000 mg/kg, 4 w/12 w	<i>I. mutifiliis</i> ↑ / <i>V. anguillarum</i> ↑
		2000 mg/kg, 127 d	phagocytic index ↑, lysozyme ↑→
	Turbot	300 - 200 mg/kg	phagocytic activity ↑, lysozyme ↑
	Sockey salmon	> requirement	→
	Yellow tail	122 - 6100 mg/kg	phagocytic activity ↑, lysozyme ↑
Vit-C + Vit-E	Gilhead sea bream	2900 + 1200 mg/kg	lysozyme ↑, NCCs ↑
		Many concentration, <i>in vitro</i>	migration ↑, phagocytic activity ↑, ROS (mix) ↑
Vit-C + Yeast glucan	Trout	oral	lysozyme →, complement ↑, CL ↑
Vitamin-E	Catfish	2500 mg/kg, 180 d	phagocytic index ↑, antibody →
	Chinook	300 mg/kg and > requirement	→
	J. flounder	600 mg/kg	phagocytic activity ↑, lysozyme ↑
	Atlantic salmon	low levels	IHNV ↓
		> requirement	→
		800 mg/kg, 20 w	NBT →, complement ↑, lysozyme ↓, <i>A. salmonicida</i> ↑
	Trout	500 mg/kg, 12 w	phagocytosis ↑
		low levels	antibody ↓
	Turbot	500 mg/kg	phagocytic activity ↑
Vit-E + Selenium	Catfish	240 + 0.8 mg/kg, 120 d	NBT ↑
Vitamin-A (retinol)	Atlantic salmon	oral	anti-protease activity ↑, migration ↑
	Gilhead sea bream	50 -300 mg/kg	ROS ↑
α-tocopherol	Gilhead sea bream	600 - 1800 mg/kg	complement ↑
α-tocopherol acetate	Yellow tail	119 - 5950 mg/kg	phagocytic activity ↑, lysozyme ↑

Arginine	J. flounder	150 mg/kg	NBT ↑, lysozyme ↑	
Ascorbate 2-monophospate	Atlantic salmon	20 – 1000 mg/kg	→	
Ascorbil 2-sulfate	Atlantic salmon	4770 mg/kg	→	
		82, 44, 3170 mg/kg, 23 w	antibody ↑	
		1000 mg/kg	ROS ↑, lymphocyte number ↑	
		2750 mg/kg	complement ↑	
		4000 mg/kg	lysozyme ↑	
Axtahantin	J. flounder	100 mg/kg	chemotaxis ↑, NBT ↑	
Essential oil	Common carp	30, 60, 120 ppm diet, 1% bw, 8 d	antibody ↑, bactericidal activity ↑	
Protein hydrosilate	Atlantic salmon	1 – 25 <i>in vitro</i>	ROS ↑	
Soybean protein	Trout	oral	phagocytosis ↑, NBT ↑, killing ↑	
Nucleotides				
Ascogen P ¹	Hybrid striped bass	5 g/kg, fixed ration approaching satiation daily	neutrophil oxidative radical ↑, survival after challenge with <i>Streptococcus iniae</i> ↑	
Ascogen S ²	Hybrid tilapia	2 and 5 g/kg, 16 w	growth ↑, survival ↑	
		5 g/kg, 120 d	antibody after vaccination ↑ lymphocyte mitogenic response ↑	
	Trout	0.62, 2.5 and 5 g/kg, diet at 1% bw/d, 37 d	growth ↑	
Optimun ²	Trout	2 g/kg, containing 0.03% nucleotide	2% bw/d, 3 w	survival after challenge with, <i>V. anguillarum</i> ↑
			1% bw/d, 2 w	survival after challenge with infectious salmon anaemia virus ↑
	Coho salmon		2% bw/d, 3 w	survival after challenge with <i>Piscirickettsia salmonis</i> ↑
			2% bw/d, 3 w	sea lice infection ↓
	Atlantic salmon		1.5% bw/d, 3 w ^a and 5 w ^b	antibody ↑, mortality ↓
			1.5% bw/d, 8 w	plasma chloride ↓, growth ↑
			10 w	intestinal fold ↑
	Turbot		to hand sanitation daily	Altered immunogene expression in various tissues
Ribonuclease-digested yeast RNA ³	Common carp	15 mg/fish, by intubation, 3 d	phagocytosis ↑, complement ↑, lysozyme ↑, respiratory burst ↑, <i>A. hydrophila</i> infection ↓	

1: Canadian Biosystem Inc. Calgary-Canada, 2: Chemoforma Augst-Swithzerland, 3: Amano Siyaku Co-op Tokyo, a: Before vaccination, b: Post-vaccination, bw: Body weight, d: Day, w: Week, ↑: Increase, ↓: Decrease, →: No change, Chinook: Chinook salmon, CL: Chemiluminescent response, J. flounder: Japanese flounder, Killing: Bactericidal activity of macrophage, NCCs: Non-specific cytotoxic cells, NBT: Nitroblue tetrazolium reaction, NT: Nucleotide, ROS: Reactive oxygen species, Trout: Rainbow trout, Turbot: *Scophthalmus maximus*, Vit-C: Vitamin-C, Vit-E: Vitamin-E, *A. hydrophila*: *Aeromonas hydrophila*, *A. salmonicida*: *Aeromonas salmonicida*, *E. tarda*: *Edwardsiella tarda*, *V. anguillarum*: *Vibrio anguillarum*. (modified from Galindo-Villegas & Hosokowa, 2004; Li & Gatlin, 2006; Sakai et al., 1999).

Table 14. Doses and effects of immunostimulants as nutritional factors and nucleotides in fish species.

In recent years, world-wide heightened attention on nucleotide supplementation for fishes was aroused by the reports of some researches, indicating that dietary supplementation of nucleotides enhanced resistance of salmonids to viral, bacterial and parasitic infections as well as improved efficacy of vaccination and osmoregulation capacity (Burrells et al., 2001a, 2001b; Grimble & Westwood, 2000; Li & Gatlin, 2006). The modulatory effects of dietary nucleotides on lymphocyte maturation, activation and proliferation, macrophage phagocytosis, immunoglobulin responses as well as genetic expression of certain cytokines have been reported in humans and animals including some fish species such as hybrid tilapia, rainbow trout, Coho salmon, Atlantic salmon and common carp (Gil, 2002; Li & Gatlin, 2006). To date, research pertaining to nucleotide nutrition in fishes has shown rather consistent and encouraging beneficial results in fish health management, although most of the suggested explanations remain hypothetical and systematic research on fishes is far from complete. Because increasing concerns of antibiotic use have resulted in a ban on sub-therapeutic antibiotic usage in some countries, research on immune nutrition for aquatic animals is becoming increasingly important. Also, research on nucleotide nutrition in fish is needed to provide insights concerning interactions between nutrition and physiological responses as well as provide practical solutions to reduce basic risks from infectious diseases for the aquaculture industry (Burrells et al., 2001a, 2001b; Li & Gatlin, 2006). In aquaculture, used immunostimulants as nutritional factors and nucleotides with dose, administration route and effects are given at Table 14 (Galindo-Villegas & Hosokawa, 2004; Li & Gatlin, 2006; Sakai et al., 1999).

7.3 Risks and benefits using immunostimulants

Immunostimulants are more widely applied both within the aquaculture sector and in traditional animal husbandry. There are many examples of successful use of immunostimulants to improve fish welfare, and also *in vivo* or *in vitro* effects of immune system (see Table 12, Table 13 and Table 14). One of the earliest applications of immunostimulants in fish was the use of glucans in salmon diets. These diets were considered to be effective in managing disease outbreaks after stressful events such as grading and there was believed to be some benefit in reducing sea lice settlement; allowing the stock to go longer between anti-sea lice treatments. Certainly, the use of in-diet immunomodulators has become widely accepted in aquaculture with commercially available diets supplemented with nucleotides which have been demonstrated to reduce sea lice settlement and provide better protection against *A. salmonicida* and *V. anguillarum* infection (Bricknell & Dalmo, 2005; Burrells et al., 2001a; 2001b). Immunostimulants can provide particular benefits when used in order to: (1) reduce mortality due to opportunistic pathogens, (2) prevent virus disease such as Vitamin-C on infectious hematopoietic necrosis (IHN) virus and yeast glucan on yellow-head baculovirus, (3) enhance disease resistance of farmed fish and shrimp, (4) reduce mortality of juvenile fish especially in fry and larval fish, (5) enhance the efficacy of antimicrobial as adjuvant substances, if used in combination with curative antimicrobial drugs at an early phase of disease development, or prior to anticipated disease outbreak, (6) enhance the resistance to parasites or microsporidias, such as Vitamin-C on *Ichthyophthirius multifiliis*, lactoferrin on *Cryptocaryon irritans*, or glucans and chitin on *Loma salmonea*, (7) enhance the efficacy of vaccines, (8) improve fish welfare against stress (e.g grading, sea transfer, vaccination and environmental change), such as glucans may be helped reduce the negative effects of stress on the innate immune response,

soybean lecithin may be provided higher tolerance for increased water temperature, and vitamin-E may be protected the complement system against stress-related reduction of activity, (9) promoting a greater and more effective sustained immune response to those infectious agents producing subclinical disease without risks of toxicity, carcinogenicity or tissue residues, (10) maintaining immune surveillance at heightened level to ensure early recognition and elimination of neoplastic changes in tissues, and (11) selectively stimulating the relevant components of the immune system or non-specific immune mechanism that preferentially confer protection against micro-organisms, such as via interferon release, especially for those infectious agents for which no vaccines currently exists (Ai et al., 2007; Bricknell & Dalmo, 2005; Cerezuela et al., 2009; Gannam & Schrock, 2001; Maqsood et al., 2011; Raa, 2000; Rodriguez-Tovar et al., 2011; Yin et al., 2009).

Naturally, there is a risk that use of immunostimulants in aquaculture may cause unforeseen problems. Continual feeding of immunostimulants has generally been abandoned, in adult fish, in favor of pulse feeding. There are two possible outcomes of continuous feeding of an immunostimulant; (1) although, it is a very rare occurrence, the immunostimulant up-regulates the immune system to heightened levels and this is maintained until the immunostimulant is withdrawn, (2) the most obvious contra-indication as it would be in larval fish, continual exposure to an immunostimulant can induce tolerance. This is caused by the immune system of the host becoming de-sensitized to the immunostimulant and the immunostimulant response is lost, or in extreme circumstances the continued expose to an immunostimulant causes the immune response to become suppressed, giving a lower level of innate defences whilst exposure to that particular immunostimulant is maintained (Bricknell & Dalmo, 2005; Sakai et al., 1999). Besides, no research has yet been performed concerning the influence of immunostimulants at some stage such as maturation and spawning of fish. The immune systems become suppressed by sex hormones, testosterone and estradiol-17 β , at these stages. Although the use of immunostimulants could cause recovery of the immune systems suppressed by sex hormones, they may disturb sexual maturation and other essential functions associated with spawning, or may include sterility through polyploidy (Cuesta et al., 2007; Magnadóttir, 2010; Piferrer et al., 2009). On the other hand, the mere deleterious side-effects of immunostimulants have not been completely investigated.

8. Conclusions

Important progress has been made in recent years in our knowledge of the immunological control of fish diseases which has benefitted the growing aquaculture industry worldwide and also provided better understanding of some basic immunological phenomena. There are mainly three methods for control of fish disease: vaccination, chemotherapeutics and immunostimulants. In addition, researches in recent years about probiotics, prebiotics, and synbiotics also exhibited positive health effects in fish species. Immunostimulants and vaccines are used together to prevent infectious diseases. Immunostimulants may be used for treatment of some infectious diseases; they may not as effective as many chemotherapeutics. Antibiotic-resistant bacteria threaten treatment of fish disease using chemotherapeutics. Immunostimulants may compensate these limitations of chemotherapeutics. Immunostimulants are thought to be safer than chemotherapeutics and their range of efficacy is wider than vaccination. The combination of vaccination and immunostimulant administration may also increase the potency of vaccines. In addition,

continued pressure on the use of antimicrobials associated with food residue and environmental issue will encourage the use of immunostimulants. However, cautions have to be taken regarding issues such as tolerance, non-wanted side effects such as immunosuppression using too high doses of immunostimulants or non-desirable effects caused by a prolonged use of such compounds. Actual knowledge of potential immunostimulants is still obscure in several aspects, especially in those related to pathways and mechanisms in which such substances can reach their specific cell targets.

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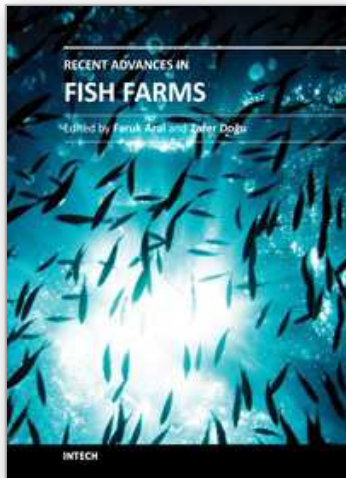
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The world keeps changing. There are always risks associated with change. To make careful risk assessment it is always needed to re-evaluate the information according to new findings in research. Scientific knowledge is essential in determining the strategy for fish farming. This information should be updated and brought into line with the required conditions of the farm. Therefore, books are one of the indispensable tools for following the results in research and sources to draw information from. The chapters in this book include photos and figures based on scientific literature. Each section is labeled with references for readers to understand, figures, tables and text. Another advantage of the book is the "systematic writing" style of each chapter. There are several existing scientific volumes that focus specially on fish farms. The book consists of twelve distinct chapters. A wide variety of scientists, researchers and other will benefit from this book.

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