

Immunostimulants for Aquaculture Health Management

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Abstract

Aquaculture is gaining momentum in several parts of the world in recent years. Intensification has become a common practice in both finfish and shellfish culture to optimize the returns. High stocking densities, artificial feeding and fertilization have become common husbandry practices in both carp and shrimp culture systems. Due to intensification of culture practices, diseases of microbial etiology of economical significance has surfaced in rearing and grow out ponds and are major threat to the sustainability of the aquaculture industry. Synthetic chemicals and antibiotics have been used to prevent or treat fish and shrimp and have achieved at least partial success. Vaccination against specific pathogens has been developed recently with some success depending on the particular disease. An alternative approach has been the application of various compounds to boost or stimulate the innate immune system of farmed fish and shrimp. These compounds, termed immunostimulants is considered an attractive and promising agent for the prevention of diseases in fish and shellfish. In recent years, the established beneficial effects of immunostimulants in many livings systems promote their application for disease management in aquaculture practices.

Keywords: Immunostimulants; Immune system; Aquaculture management

Introduction

During the last two decades, the problems of diseases have emerged as the major constraints in aquaculture industry. The increased disease occurrences have resulted in the transfer of pathogenic organisms among countries. Due to this, the shrimp industry of India as well as other Southeast Asian countries has suffered significant economic losses. As there is no effective remedies against these viral diseases, immunostimulants can become powerful tools to control fish and shrimp diseases [1].

An immunostimulant is defined as a chemical, drug, stressor or action that enhances the innate or non-specific immune response by interacting directly with cells of the system activating them. Immunostimulants can be grouped under chemical agents, bacterial preparations, polysaccharides, animal or plant extracts, nutritional factors and cytokines [2]. List of pathogen successfully controlled by using immunostimulants exposure in fish/shrimp like bacteria such as *Aeromonas hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *E. ictaluri*, *Vibrio anguillarum*, *V. vulnificus*, *V. salmonicida*, *Yersinia ruckeri*, *Streptococcus* spp.; virus such as infectious hematopoietic necrosis, yellow head virus, viral hemorrhagic septicemia and parasite *Ichthyophthirius multifiliis*.

Immunostimulants are dietary additives that enhance the innate (non-specific) defense mechanisms and increase resistance to specific pathogens [2]. There is no memory component developed and duration of the immune response is very short. Immunostimulants are chemical substances which activate leukocytes [3]. Adjuvant (FCA) is one of the first immunostimulants used in animals to elevate the specific immune response, and it has also been successfully used in conjunction with injection of fish bacterins [4]. So far glucans, which are polymer of glucose found in the cell walls of plants, fungi and bacteria appear to be most promising of all examined in fish and shrimp and oral application found to be the route of choice [5]. Use of these different types of immunostimulants is an effective means to increase the immune competency and disease resistance of fish and shellfish. Research on

immune stimulants in aquaculture is under progress and many agents are currently in use in aquaculture industry.

Concept of Immunostimulants

Aquaculture has been grown rapidly for food production in the last few decades. Several commercial fish species have been cultured intensively in narrow or enclosed spaces such as ponds, cages or tanks under overcrowding or high density leading to adversely affect the health of cultured fish with a potentially stressful environment and infectious diseases [2]. The infectious disease-outbreaks have emerged as constraints for the development of aquaculture. The occurrences have spread through the uncontrolled movement of live aquatic animals resulting in the transfer of pathogenic organisms among the countries (Table 3) [1]. Antibiotics and chemotherapeutics have been used to prevent or control bacterial infections in aquaculture for about 20 years [2]. Unfortunately, the use of antibiotics for treatment is not successful and sustainable due to increase in antibiotic-resistant bacteria, negative effect on the indigenous microflora of juveniles or adult fish [6], and the accumulation of antibiotic residues in fish tissue and environment causing human and animal health issues. Vaccination is an effective prophylactic treatment for infectious diseases in fish culture, but it may be very expensive and stressful to fish. A single vaccine is effective against only one specific type of pathogens, but limits the effectiveness for wide range of pathogens due to the complex antigenic structure [7].

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Name	Company	Type	Mode of action	Instruction for use	Specific supporting literature	Recommended level of inclusion (kg/ton)
Immestim	Immudyne, USA	B (1,6) branched B (1,3) glucan from yeast	Activates macrophages	4 days-yes 3 days-no	General on similar products	0.5-12.5
Macroguard	Biotech-Mackzymal, Norway	B (1,6) branched B (1,3) glucan from yeast	Activates macrophages	-With vaccines in injection - in feed <6-8 weeks cont.	Yes, on salmonids and tiger shrimp	1.0
Vitastim	Taitoand Company, Japan	B (1,6) branched B (1,3) glucan from fungi	Activates macrophages	As feed ingredient	Yes, on shrimp and fish	1.0 50 mg/kg body weight
Aqua-Mune	Park Tonks	B (1,6) branched B (1,3) glucan from baker's yeast	Activates macrophages	As feed ingredient	No	1.0
Penstim	AURUM Aquaculture Ltd, USA	Beta-glucan	Activates macrophages	Immersion for larvae and feed ingredient for larger animals	-	2.0 to 5.0 in feed
Laminarn	Pronova, Noway	B (1,6) branched B (1,3) glucan from brown algal laminariae	Activates macrophages	As feed ingredient	Yes	-
Calcium spirulan	Kelly Moorhead, Hawaii	Sulfated polysaccharide from spirulina	Inhibit viral envelop replication, inhibits virus penetration in host cell	Not known	Yes, on mammals	Not known
SP 604	Alltech Inc, USA	Premix of mannan (Biomos), Cr and Se yeast, probiotics	Multiple: activates macrophages, source of trace minerals			
Agrimis	Agrimerica, USA Santel, France	Mannan based oligosaccharide from	Good substrate for lactic bacteria, occupies binding sites of pathogens in gut, stimulates immune response	As feed ingredient	Yes, on mammals	1.0
Elorisan	BUGICO, Switzerland	Organic silicon	Lower lipid permeability, protection of nervous tissues	Intraperitoneal injection	Yes	0.1 ml of 1% solution
DS 1999	International Aquaculture Biotechnologies Ltd	Bacterin	Activates macrophage	Add directly in culture medium (larval culture)	Yes, field trails	0.5 in larval diets
Levamisole	Janssen Pharmaceutica, Belgium	Tetrahydro-6- phenylimidazolthiazole hydrochloride	Activates macrophages	As feed ingredient in bath	Yes, on mammal and fish	5-10mg/kg body weight
Lysozyme Hydrochloride	Belovo, Belgium	Lysozyme from hen's eggs	Kills/lyses bacterias	As feed ingredient	Yes, mammals (Cheese)	-
Lactoferrin	DMV international, the Netherlands	Lactoferrin from bovine milk	Binds Iron, makes it unavailable to pathogens	As feed ingredient	Yes, on mammals	-
Selenium yeast	Alko, Finland	Selenium Yeast	Acts a yeast (activates macrophages) and source of selenium	As feed ingredient	-	1.0
Polypeptides fish hydrolysates	Tepual, Chile Sopropeche, France	Short peptides	Activates macrophages	As feed ingredient	-	-
Blood plasma	Harimex, The Netherlands	Blood serum (immunoglobulins, glycoproteins)	Activates macrophages, Binds on gut bacterial receptors	As feed ingredient	Yes, on mammals	50-100
Fish oils	Fish oil producer	Omega-3 fatty acids	Lower prostaglandin E-2 production (Immunodepressor), increase membrane fluidity	As feed ingredient	Yes, on mammals	10-100

Status of commercial immunostimulants usage in different countries

(Source: Devresse et al. 1997. Nutrition and Health. Aquaculture Division, INVE scientific department, Belgium)

Table 3: Some commercial immunostimulants currently available on the market in different countries.

Therefore, the needs to look for alternative techniques with eco-friendly disease-prevention have been taken into account.

The alternative technique to prevent the diseases has been proposed that the strengthening of fish immune systems through the application of immunostimulants is one of the most promising methods Immunomodulation by contrast, is a consequence of a change in the number or function of the cells involved in the immune response. The most proven effect of immunostimulants is to facilitate the function of phagocytic cells and increase their bactericidal and fungicidal activities [2]. Immunostimulants can promote recovery from immunosuppressive states caused by any form of stress.

Fish will either survive if they successfully fight against the infection of pathogens, or die if they may not be successful in preventing the spread of the infection. The outcomes of survival or death are largely depended on the efficacy of the immune system to combat the initial infection or the spread of the pathogens. The immune system of fish can be grouped into acquired immunity (specific) and innate immunity (non-specific). Both use physical, cellular and humoral mechanisms to protect against infectious pathogens. The specific immune systems recognize specific antigen on a pathogen, and provide a protection against that specific pathogen. Non-specific immune systems provide a

set of protective mechanisms that are inherently available for immediate protection against a wide variety of pathogens [8].

An immunostimulant is a chemical, drug, stressor, or action that elevates the non-specific defense mechanism or the specific immune response [4]. Immunostimulants are practiced in aquaculture as a means to overcome the immunosuppressive effects due to stressors [9,10] or might be used as a prophylactic treatment for expected seasonal outbreaks of known endemic diseases [11] or a suppressive treatment for latent or sub-lethal pathogens. Immunostimulants can also promote recovery from immunosuppression states caused by stress [2]. Various types of immunostimulants evaluated in fish and shrimp are summarized in Table 1.

Vaccines vis-a-vis Immunostimulants

Vaccination is an important tool in preventing infectious disease in humans and animals and both passive and active vaccinations are extensively employed in fish. It is a term that should strictly be applied only when the purpose is long lasting protection through immunological memory. A vaccine targets the specific immune response. It requires primary challenge with antigen and is dependent upon the clonally derived lymphocytes subsets to be implemented [12]. However, most commercial vaccine usually enhances resistance to only one or two specific pathogens and confers only a temporary resistance to disease. Immunostimulants by contrast, can boost immunity to a wide variety of pathogens, thus are nonspecific. Immunostimulations can be achieved in a more general sense by, for instance, targeting complement activation, phagocytosis and cytokines secretion, without necessary or purposefully requiring a specific response to a defined antigen. Examples include zymosan, glucans and lipopolysaccharides and these are best called as true immunostimulants. Comparisons of characteristics of vaccines vis-a-vis immunostimulants are given in Table 2.

Application of Immunostimulants used in Fish and Shrimp

Synthetic chemicals

Levamisole is an antihelminthic chemical compounds used to treat

Synthetic chemicals	Levamisole, FK-565 (Lactoyltetrapeptide from <i>Streptomyces olvaceogriseus</i>), Quaternary ammonium compounds (QAC).
Biological substances	
1) Bacterial derivatives	MDP (Muramyl dipeptide from <i>Mycobacterium species</i>), Lipopolysaccharide (LPS), Freund's complete adjuvant (FCA), EF203 (fermented egg product), Peptidoglycan (from <i>Brevibacterium lactofermentum</i> and <i>Vibrio sp.</i>), <i>Clostridium butyricum</i> cells, <i>Achromobacter stenohaliscells</i> , <i>Vibrio anguillarum</i> cells (<i>Vibrio</i> vaccine)
2) Yeast derivatives	β -1, 3 glucan, β -1, 6 glucan
3) Nutritional factors	Vitamin C and E, n-3 fatty acid
4) Hormones	Growth hormone, Prolactin, tri-iodothyronine
4) Cytokines	Interferon, Interleukin
5) Polysaccharides	Chitosan, Chitin, Lentinan, Schizophyllan, Oligo-saccharide
6) Animal and plant extracts	Ete (tunicate), Hde (Abalone), Glycyrrhizin, Firefly squid, Quillaja, Saponin (Soap tree)
7) Others	Lactoferrin, Soyabean protein, Quil A, Spirulina, <i>Achyranthes aspera</i> (Herb), <i>Mucorcircinelloides</i> (Fungi)

Table 1: Immunostimulants evaluated in fish and shrimp.

the nematodes infection in human and animals as well (Table 1). It can stimulate immune response *in vitro*. Levamisole enhanced phagocytic activity, the NBT reaction and increase antibody producing cells oral administration of levamisole increased the number of leucocytes, lysozyme activities in serum and the stimulated NBT reduction and phagocytic index of phagocytic cells [13]. However, no differences were found in the levels of hematocrit, leucocrit or immunoglobulin using levamisole in rainbow trout [14]. Findlay et al. [15] have recommended application of levamisole as immunostimulant in fish. It has been observed that rainbow trout exposed to a bath treatment containing 5, 10, 25 μ g/ml levamisole for 2 hrs period showed resistant to *Y. ruckeri* [16].

Biological substances

Bacterial derivatives: MDP (*Muramyl dipeptide*): MDP (*N*-acetyl-muramyl-L-alanyl-D-isoglutamine), obtained from *Mycobacterium*. Kodama et al. [17] revealed that intra peritoneal injection of rainbow trout with MDP-Lys increased the phagocytic activities, respiratory burst and migration activities of kidney leucocytes as well as resistance of the fish to *A. salmonicida*.

LPS (lipopolysaccharide): LPS is a cell wall component of Gram-negative bacteria. It was reported that LPS effective in preventing *A. hydrophilla* disease and stimulating innate immune response of rainbow trout [18]. Salati et al. [19] reported that LPS can stimulate phagocytosis and the production of superoxide anions in Atlantic salmon macrophages and LPS can stimulate B-cell proliferation and enhance macrophage phagocytic activity in red sea bream *Pagrus major*. Also, LPS stimulates the production of macrophage activating factor in goldfish lymphocytes [20]. These substances are very potent even in very low doses and may occur as contaminants in bacterin preparations and used in fish immunizing programmes. LPS stimulate hemocytes proliferation; enhance phagocytic activity as well as the microbicidal activity of shrimp [21].

FCA (Freund's complete adjuvant): FCA is a mineral oil adjuvant containing killed *Mycobacterium butyricum*, increase the immune response in fish. FCA can increase respiratory burst, phagocytic and NK cell activity of leucocytes in rainbow trout protect against *V. anguillarum* infection [22]. In contrast, yellowtail injected with FCA did not show increased resistance to *P. piscicida* infection, although the adjuvant effect of FCA on a *P. piscicida* vaccine was observed in fish [23].

Vibrio bacterin: *Vibrio anguillarum* bacterin (inactivated whole cell vaccine) is the most successful vaccine for salmonid fish, administered through injection, oral and immersion methods [2].

Sl. No.	Vaccine	Immunostimulants
1.	Prophylactic for long duration with only one or two treatments.	Prophylactic for short duration, require more treatments.
2.	Efficacy of vaccine is excellent	Efficacy of immunostimulants is good
3.	Limited spectrum of activity.	Wide spectrum of activity
4.	No toxic side effects	No toxic side effects
5.	No accumulation of toxic residues	No accumulation of toxic residues
6.	No environmental impact	No environmental impact
7.	Enhance specific and nonspecific immune response	Mainly enhance nonspecific immune system of larvae before specific immune system matures.
8.	Difficult to vaccinate larvae of fish and shrimp	Easy to vaccinate larvae of fish and shrimp
9.	Costly	Cost effective

Table 2: A comparison of characteristics of vaccines and immunostimulants.

Immunostimulation of *V. anguillarum* bacterin was seen in fish and shellfish. In black tiger shrimp the migration of hemocytes treated with vibrio bacterin can be increased [24]. Further, Norqvist et al. [25] also reported that vaccination of rainbow trout with attenuated *V. anguillarum* stimulates protection against *A. salmonicida* challenge.

Clostridium butyricum cells: *C. butyricum* bacterin can enhance the resistance to vibriosis in rainbow trout by oral administration by leucocyte activation, including phagocytosis and increased superoxide anion production [26]. Young et al. [27] revealed that *C. butyricum* shows immunostimulatory effects like stimulation of macrophages and NK cells and improves further protection against *Candida* infection.

Achromobacter stenohalis cells: *A. stenohalis* is a gram-negative aerobic organism which has been isolated from sea water. Inactivated *A. stenohalis* can enhance immune responses of kidney cells, complement activity and increase protection against *A. salmonicida* challenge. The LPS of this bacterium activates mouse macrophages and B-lymphocytes [28].

EF203: EF203 is a fermented product of chicken eggs and oral administration of it to rainbow trout stimulates the activity of leucocytes such as phagocytosis and chemiluminescence and increases protection against *Streptococcus* infection [29].

Yeast derivatives: Glucans, long chain polysaccharide extracted from yeast, are good stimulators of non-specific defence mechanism in animals including fish and shellfish like phagocytic activity and protection against bacterial pathogens. Several types of glucan has been investigated in fish such as yeast glucan, peptide-glucan β -1,3, glucan (VST). Yeast glucan (β 1-3- and β 1-6-linked glucan) and β -1,3glucan (VST) is derived from cell walls of baker's yeast like *Saccharomyces cerevisiae* and *Schizophyllum commune*, respectively [30]. β -glucans comprised diverse group of polysaccharides of D-glucose monomers linked with β -glycosidic bonds. Cellular and non-cellular defense mechanisms are increased in activity after treatment with β -glucan like lysozyme activity, phagocyte activity, complement activity and bactericidal activity of macrophages. A number of reports reveal that dietary β -glucan administration increases resistance to infection like Selvaraj et al. [31] reported that highest antibody titre against *A. hydrophila* injected with β -glucan (100-1000 μ g glucans/fish). In addition, Robertsen et al. [32] recorded that intraperitoneal injection of β -glucan prepared from cell walls of *Saccharomyces cerevisiae* injected to Atlantic salmon showed increased resistance to *V. anguillarum*, *V. salmonicida* and *Y. ruckeri*.

Polysaccharides

Chitin and Chitosan: Chitin is a polysaccharide which constitutes the principal component of exoskeletons of crustacean and insect and cell walls of few fungi [2]. It can stimulate macrophage activity and give resistance from certain bacteria. Kawakami et al. [23] Chitosan, de-N-acetylated chitin can increase protection against *A. salmonicida* infection when injected with or immersed in chitosan solution in brook trout, *Salvelinus fontinalis* [33].

Lentinan, Schizophyllan and Oligosaccharide: Lentinan, Schizophyllan and Oligosaccharide can increase cellular and non-cellular defense mechanisms like lysozyme activity, phagocyte activity and complement activity in fish.

Animal and plant extracts

Ete (Tunicate) and Hde (Abalone): An extract from the marine tunicate, *Ecteinascida turbinata* (Ete) and a glucoprotein fraction of

water extract (Hde) from abalone, *Haliotis discus hannai*. It can enhance the killing of tumor cells *in vitro* and inhibits tumor growth *in vivo*. *Ete* (Tunicate) can enhance the phagocytosis and increased survival of Eel when injected against *A. hydrophila* [34]. In addition, when rainbow trout injected with Hde against *V. anguillarum* infection showed increased survival along with enhanced phagocytic activities [35].

Firefly squid: Firefly squid, *Watasenia scintillans*, can stimulate the immune system of rainbow trout such as the production of superoxide anion, potential killing activities by macrophages and the lymphoblastic transformation of lymphocytes *in vitro*.

Chinese medicinal herbs (*Astragalus membranaceus* and *Lonicera japonica*) extracts can be used as immunostimulants to enhance immune response and disease resistance of cultured fish species [7]. The herbal immunostimulants such as *Embllica officinalis*, *Cynodon dactylon* and *Adathoda vasica* improved the immune system and reduced microbial infection in the goldfish *Carassius auratus* [36] and similar work was also carried out on another ornamental fish *Poecilia sphenops* using herbal immunostimulants. Nile tilapia shows enhanced phagocytic activity after treated with *Astragalus* extract for one week [7,37]. Dugenci et al. [38] documented that ginger extract to be very effective in enhanced phagocytic and extracellular burst activity of white blood cells in rainbow trout.

Nutritional factors

Vitamin C: Vitamin C is involved in several physiological functions including growth, development, reproduction, wound healing, response to stressors and possibly lipid metabolism through its action on carnitine synthesis while administering in feed. Vitamin C (Ascorbic acid) is a co-factor in many biological processes including collagen synthesis and cellular functions related to neuromodulation, hormone and immune systems. It has been observed by Tewary and Patra [39] that higher levels of dietary vitamin C significantly increased the protection against *A. hydrophila*.

Vitamin E: Vitamin E can enhance specific and cell-mediated immunity against infection in Japanese Flounder *Paralichthys olivaceus* [40] and macrophage phagocytosis in fish such as channel catfish *Ictalurus punctatus* [41] and turbot *Scophthalmus maximus* [42]. Vitamin E deficiencies in trout result in reduced protection against *Y. ruckeri* [43].

Hormones

Growth hormone (GH): GH directly affects immune competent cells like macrophages, lymphocytes and NK cells. In fish, exogenous growth hormone (GH) has mitogenic activity on lymphocytes and activates NK cells and production of superoxide anions of leucocytes.

Prolactin: Prolactin also directly affects immune competent cells like macrophages, lymphocytes and NK cells. It can enhance the production of superoxide anions of leucocytes. Sakai et al. [44] documented that prolactin helps in increased level of production of superoxide anion in rainbow trout by leucocytes.

Lactoferrin, consist of a single peptide chain with molecular wt. ~ 87,000 Da and posses 2 Fe-binding sites per molecule, most popular physiological fluids of mammals [2].

Cytokines: Cytokines are polypeptides or glyco-proteins which act as modulators in the immune System. Cytokines may be useful as powerful immunostimulants if their structures can be identified and recombinant molecules prepared.

Algal derivatives

Laminaran is a β (1, 6)-branched β (1, 3)-D-glucan, a major component in sub-littoral brown algae, e.g. Phaeophyceae. Almost all B-(1, 3) D-glucan display poor water solubility which makes them less easy to handle than aqueous soluble laminaria. Laminaran obtained from *Laminaria hyperborea* has immune modulatory effect on immune system as well.

Immunological system in fish

The immune system is the system which continuously fights against the pathogen and give proper protection to our body. The two types of immune system are innate immune system or non specific immune system and acquired immune system or adaptive immune system. The essence of the immunological system of the vertebrates is to react and protect against the infections. Proper work of the immunological system of a fish involves different cells and organs. There are different factors that affect the immunological response of the fish. Inherent factors like health and age, extrinsic like temperature or changes in abiotic parameters them all together affect the health condition, and thus the response. Those changes cause in some cases stress, which if achieves high level generates an immunological system collapse [45].

Cells involving on the immune system are leucocytes or white cells. Those can be found in the blood stream or on tissues. Lymphoid tissues on fishes are thymus, spleen, anterior kidney and lymphoid tissues associated to mucus and intestine [46]. The classification of leucocytes as in the vertebrates has been done following morphological criteria whereby various groups can be distinguished such as lymphocytes, granulocytes and macrophages [47]. A short explanation of each group is done below in order to know the main characteristic and functions.

Lymphocytes

High differentiated cells with capability to respond on stimuli. The most common are mature lymphocytes with an irregular surface or border [46]. Previous studies have defined lymphocytes as a high metabolically potential due to its high number of organelles in the cytoplasm as golgi apparatus, mitochondria, ribosome, and endoplasmic reticule. They are found in all over the body circulating on the blood stream and gathered on lymphoid organs and the quantity is very variable. The main function is to produce antibodies, immunological memory, and regulatory factors as lymphokines in response of the humoral and cell specific immune [48]. Lymphocytes B are bone marrow derived while T is thymus derived. T cells are responsible for cell mediated immunity as well as providing assist to B lymphocytes; those last are responsible to produce antibodies against antigen [49].

Granulocytes

Occurrence and functions varies within species of fish, the origin is focus on the kidney tissues. In teleost, there are describe three based on morphology [46,50] including Neutrophil, eosinophil and basophil, the first being the most common. Granulocytes react responding in the presence of foreign material going into the body but without recognizing specific antigens. This kind of defending is called in non-specific defense mechanism, explained little forward. When the invasion is occurred those cells migrate and destroy the estrange particles by phagocytosis or just by killing by a cytotoxic response.

Macrophages

Based on Literature many test has been done in several species of fish. After several tests it seems that macrophages can be use to

evaluate the health of the fish as a kind of indicators. Those cells play an important role on killing pathogens as immune response. As exposed in the work of Fernández et al. [46], based also in many previous studies, macrophages are the main phagocyte cells on fishes. The pathogens are killed by two ways, releasing toxic substances or by ingestion, known as phagocytosis. It involves producing ROS-reactive oxygen species or microbiocidal oxygen radicals. This generating activity is known as respiratory burst, and is not only produce by phagocytosis. Lymphokines can regulate macrophage functions like MAF, macrophage activating factor [51]. The immunological system of fish can be divided in two branches depending on the functionality such as natural or non-specific and acquired or specific. The non-specific immune system is considered to be the most important ones for immunisation.

Immunostimulation of non-specific defense mechanisms

Most immune-stimulatory compounds examined in fish and shellfish have been shown to have immune enhancing potential through heightening of nonspecific immune responses of the organisms. The nonspecific immune responses in contrast to the specific response does not require prior exposure to an antigen and consists of barriers such as skin, scales, lytic enzymes and phagocytic cells. The nonspecific immune response is also considered to be the first line of defence against invading pathogenic microorganisms and is the sole immunological mechanism by which invertebrates protect themselves from disease. In contrast to specific immunity, which only recognizes a particular antigen or pathogen, each component of the nonspecific response can recognize a broad array of foreign agents. It has been hypothesized that fish and shellfish are more reliant on nonspecific immune response. For these reasons, a large portion of the research on immunostimulation has focused on up-regulating the nonspecific immune response of the organisms. It is well established that mononuclear phagocytes or macrophages plays a central role in the cellular part of the nonspecific defence mechanism of fish [52]. Haemocytes and the prophenoloxidase (proPO) system are the primary defence mechanisms of shrimp. Both semi-granular and granular cells carry out the functions of the proPO system [53]. Phenoloxidase is the terminal enzyme in the proPO activation system and is activated by lipopolysaccharides or peptidoglycans from bacteria and β -1, 3 glucan from fungi through the pattern recognition molecules [54]. Phenoloxidase activity has been detected in many species of penaeid shrimp such as Sao Paulo shrimp *Farfantepenaeus paulensis*, yellow leg shrimp *Fafantepenaeus californiensis*, tiger shrimp *Penaeus monodon*, blue shrimp *Litopenaeus stylirostris* and white shrimp *L. vannamei* [55-59]. The activation state of these cells and enzyme systems are often used as measures of non-specific immunostimulation. Other measures employed for this purpose include cell migration, phagocytosis and bactericidal activity as well as changes in numbers of leucocytes and the activation potential of cells upon stimulation, as measured by oxidative radicals and enzymes [52]. The nonspecific immune responses such as phagocytosis and the production of oxidative radicals are quickly activated by the immunostimulants and help to protect the host against a broad spectrum of pathogens [4].

Method of administration

Immunostimulants potentiate the immunity of the host itself, enabling it to defend more strongly against pathogens. Several immunostimulants also stimulate the natural killer cells, complement, and lysozyme and antibody response of fish [53]. There are mainly 3 ways to deliver immunostimulants including injection, immersion and oral uptake. Injection of immunostimulants can produce strong

non-specific response but its costly affairs with lots of time and labour intensive as well, applicable only for large size of fish more than 10-15g in body weight in intensive aquaculture system. It has been reported that injection has wide protection against a range of pathogens like intra-peritoneal injection with glucan injected to channel catfish shows increased in phagocytic activity reducing fish mortality challenge with *Edwardsiella ictaluri* [54]. For small fish vaccination is impractical. Immersion produces less non-specific immune response, but more cost effective than injection, increase more stress to fish while handling, applicable in intensive culture system. Immersion method is very effective during acclimation of juveniles to ponds in field condition. Using immersion of levamisole showed increase in circulating leukocytes, phagocytic rate and increase protection against *P. damsela* sub sp. *Piscicida* in European Seabass [55].

Oral ingestion produces good non specific immune response and can be the most cost effective method with economically viable. It is mostly suited for extensive aquaculture system. Immunostimulants powders are mixed with feed using a fish oil coating. Now a day, bioencapsulation method is also followed to immunize the fish larvae during their early larval stages with live fed organisms.

Timing of administration

It is necessary to apply immunostimulants at the right time. Anderson [4] proposed that the application of immunostimulants should be implemented before the outbreak of disease to reduce disease-related losses. Effective dosage and exposure time will be further more complicated based on different culture systems with feeding regime [56]. In Atlantic salmon injection with high dose of glucans @ 100 mg/kg led to absence of protection for 1 week, but maximum benefits only occurs after 3-4 weeks. Also, at low dose of injection @ 2-10 mg/kg, give protection only 1 week [32]. Similarly, it has been noticed that increase in the number of NBT positive cells in African Catfish fed with glucan or oligosaccharide over 30 days, but not over 45 days [57].

Mode of action

The mode of action of immunostimulants is to activate the immune systems of organisms, to enhance the immunity level against invading pathogens. The approach is very diverse in nature or may be poorly understood and also depends on the type of immunostimulants, dose, route of administration, time and length of exposure.

Following are some of the mechanism of actions:

- Stimulators of T-lymphocytes- Levamisole, Freund's Complete Adjuvant (FCA), Glucans, Muramyl dipeptide, FK-565 (Lactoyl tetrapeptide from *Streptomyces olivaceogriseus*).
- Stimulates of B-cells- Bacterial endotoxions, Lipopolysaccharides.
- Macrophage activator- Glucans, Chitin and Chitosan
- Inflammatory agents including chemotoxins
- Cell membrane modifiers- Detergents and Sodium dodecyl sulphate, Quaternary ammonium compounds (QAC), Saponins
- Nutritional factors- Vitamin C and E, n-3 fatty acids
- Cytokines- Leukotriene, Interferon
- Heavy metals- Cadmium
- Animal and fish extracts- Mitogens

In general immunostimulants enhance the phagocytosis and bacterial killing ability of macrophage, complements, lymphocytes and nonspecific cytotoxic cells, resulting in resistance and protection to various diseases and invading microorganisms.

Detection of immunostimulation

An increase in any characteristics such as phagocytosis, production of superoxide anions etc in treated fish and shellfish over controls is evidence of immunostimulation. Following are some of the methods of detection of immunostimulation.

1. Haematocrit and leucocyte count: Leucocytes mediate nonspecific immunity. So raised leucocytes count with an essentially unchanged haematocrit is an indication of immunostimulation.
2. Phagocytic activity: Phagocytosis is a common reaction of cellular defence and generally recognized as a central and important way to eliminate microorganisms or foreign particles. Phagocytosis can be assayed by incubating blood with a killed bacterial culture and examining stained smears for phagocytes containing bacteria [58].

The phagocytic activity are defined as phagocytic ratio (PR) and phagocytic index and are expressed as

$$PR = \frac{\text{Number of phagocytic cells with engulfed bacteria}}{\text{Number of phagocytes}} \times 100$$

$$PI = \frac{\text{Number of engulfed bacteria}}{\text{Phagocytic cells}}$$

Bactericidal activity: Bactericidal activity can be assayed by incubating macrophages with a live bacterial culture and then washing off the supernatant liquid, lysing the macrophages and examine the numbers of live bacteria [59].

3. Oxidative radical production: A major way in, which neutrophil granulocytes contribute to nonspecific immunity, is by the production of oxidative radicals. Nitro-blue tetrazolium (NBT) reacts with oxidative radicals producing a dark blue color and is used to identify neutrophils actively producing them.
4. Myelo-peroxidase production: Activated neutrophils also produce myelo-peroxidase. The level of activation can be determined by incubating blood smears in an indicator reagent and examining cells under the microscope for degree of staining.
5. Immunoglobulin concentration: Some serum immunoglobulins are humoral antibodies and therefore heighten specific immunity, many others regulate nonspecific immunity.
6. *In vitro* measurement: Jeney and Anderson [10] have described an *in vitro* method for screening substances for immunostimulation. In essence finally divided pieces of rainbow trout spleen are maintained in a tissue culture medium with a test substance and after 4 days cell suspension are prepared. For neutrophils the cell suspensions are treated with NBT and examined by spectrophotometry; for phagocytes aliquots of the cell suspension are shaken for 15 min. with a suspension of fixed sheep erythrocytes and then smears are made for microscopy.

7. *In vivo* measurement: In fish, specific immunity develops slowly and thus it is possible to assess immunostimulation by a challenge test with virulent bacteria, which rapidly kills large number of fish at a time. Any delay or reduction in mortality in treated fish compared to untreated group may be attributed nonspecific immunity systems.

Attributes of immunostimulants

- Safe for the environment and human health, biocompatible and biodegradable
- Promote a healthy body status by triggering the immune system of the host
- Nontoxic to finfish and shellfish with no known side effects
- Enhance disease resistance against broad spectrum of pathogens
- Reduce mortality due to opportunistic pathogens
- Prevent viral diseases
- Enhance the efficacy of antimicrobial substances
- Enhance the efficacy of vaccines and antibiotics
- Cheap, ecofriendly and easily available.

Efficacy and limitation of immunostimulants

The use of immunostimulants can protect fish from several infectious diseases and decrease mortality rates by increasing fish resistance against infectious bacteria such as *Vibrio anguillarum*, *V. salmonicida*, *Aeromonas salmonicida* and *Streptococcus* sp., viral infections such as IHN (Infectious Hematopoietic Necrosis) and yellow-head (YHV) disease and parasitic infections such as white spot disease and sea lice; and immunostimulants do not increase resistance against *Renibacterium salmoninarum*, *Pseudomonas piscicida* or *Edwardsiella ictaluri* infections due to their resistances to phagocytosis and abilities to survive within macrophages [60]. Use of immunostimulants in cultured fish result in macrophage activation, increased phagocytosis by neutrophils and monocytes, increased lymphocyte numbers, increased serum immunoglobulins, and increased lysozyme [2]. Different immunostimulants have effectiveness for different life stages based on solubility and give a different degree of protection against the pathogens [56]. An algal extract, laminaran, more soluble than the fungal and yeast glucans, has also proven to activate macrophages [61] and to increase respiratory burst activity in anterior kidney leucocytes of Atlantic salmon [62]. The laminaran has been promoted for its superior solubility compared with other β -1, 3-glucans, considering as candidate substances for diet application [56]. Absorption of laminaran from water by yolk-sac larvae of Atlantic halibut, *Hippoglossus hippoglossus*, through the skin and intestinal epithelium [63] suggests that it may have the potential to enhance immunity in early life stages before the development of acquired immunity.

However, effect of immune stimulants on nonspecific immune mechanism is normally of short duration [64]. It has been shown in salmon gave maximum leucocyte responses just 2 days after injection with M-glucan [59], and that yeast β -glucan gave an increase in respiratory burst activity 4-7 days after treatment [65]. The protection in trout using glucans and chitosan was greatly reduced after 14 days of immune stimulation [33]. The use of several immunostimulants for prolonged periods does not appear to provide additional advantages with respect to a single dose [64]. Tseng et al. [66] observed that after 29 days, the protection induced in blue gourami by injection of

20 mg/kg laminaran for 22 days, was not significantly different from the effect of a single injection of 20 mg/kg laminaran. But, [6] Misra et al. (2006) reported that most of the immune parameters such as leucocyte count, phagocytic ratio, phagocytic index, lysozyme activity, complement activity, serum bactericidal activity were significantly enhanced on 42 days after three i.p. injection of 10 mg of β -glucan/kg body weight of *Labeo rohita* fingerlings, which would lead to long-term protection in fishes. The effects of immunostimulants are not directly dose dependent, and high dose or overdosage may not enhance and may inhibit the immune responses [64], reported that the increase in respiratory burst activity of glucans-treated macrophages was maximal at glucan concentrations of 0.1-1 μ g/ml, whereas at 10 μ g/ml no effect was seen and at 50 μ g/ml glucan was inhibitory [2]. Vitamin E is immunostimulatory in concentration of 50-300 IE/kg feed, but very high vitamin E levels (>1000-5000 IE/kg diet) fed for a prolonged time have an immunosuppressive effect [64]. Galeotti et al. [56] concluded that genetics of the species, life history stage, and the culture environment all interact with the type and dosage of immunostimulants to contribute to the efficacy of the immunostimulatory substance.

Immunostimulants in aquaculture health management

Immunostimulants have been extensively studied in fish and shellfish both at whole animal and on a cellular level. It has been used as prophylactics to control infectious disease of animals and also playing the role of alarm molecules that activate the immune system [67]. Fish and shrimp depends more heavily on nonspecific defence mechanisms than mammals and therefore immunostimulants play a vital role in health management strategies of aquatic organisms. There are at least 20 different compounds, including levamisole, lipopolysaccharides, glucan, vitamin C and E etc. that are used as immunostimulants, adjuvants and vaccine carries in fish [4]. Among these compounds, glucan is one of the most promising stimulants for nonspecific defence mechanism and also the most studied immunostimulant in aquatic species. Glucan has been reported to enhance resistance against bacterial pathogens such as *Vibrio anguillarum*, *Aeromonas salmonicida*, *A. hydrophila* and *Yersinia ruckeri* in several species in fish such as the carp *Cyprinus carpio*, Atlantic salmon *Salmo salar*, rainbow trout *Onchorhynchus mykiss*, yellow tail *Seriola quinqueradiata* and African catfish *Clarias gariepinus* [10,68].

Applications of β -1,3 glucan enhances the nonspecific cellular defence mechanisms of animals by increasing the number of phagocytes and the bacterial killing activity of macrophages in rainbow trout, Atlantic salmon, catfish, and carp [69] and through production of superoxide anions by macrophages [70]. In an Indian major carps, *Labeo rohita*, yeast glucan have been observed to enhance the phagocytic activity of leucocytes and stimulate generation of reactive oxygen species (ROS) in phagocytes [71].

In recent years, nucleotides and their metabolites have received heightened attention as potential immunomodulators [72]. They play key roles in numerous essential physiologic functions, including encoding genetic information, mediating energy metabolism and signal transduction [73]. The beneficial influences of oral administration of nucleotides on immune functions, vaccine efficiency or disease resistance has also been demonstrated in fish such as Atlantic salmon, coho salmon, rainbow trout, common carp, hybrid tilapia and hybrid striped bass [74,75]. Burrells et al. [76] reported beneficial effects of dietary nucleotides when challenging salmonids with infectious salmon anaemia virus, *Vibrio anguillarum*, *Piscirickettsia salmonis* and sea lice. They hypothesized that dietary nucleotides are capable of enhancing the potential of the immune system in general to mount

greater and more rapid specific responses, as compared to the primarily nonspecific capacity of phagocytes induced by glucan. Choudhury et al. [77] reported that dietary yeast ribonucleic acid at 0.4% enhances phagocyte respiratory burst and protection of *Labeo rohita* juveniles by *Aeromonas hydrophila*. In addition, dietary nucleotides have also been observed to modulate gene expression [78], a phenomenon also confirmed in fish by Low et al. [79]. However, the way in which dietary nucleotides modulate gene expression during development of adaptive immunity is still not clear.

The immunostimulatory potential of levamisole in fish is of considerable interest in the USA and elsewhere, because it has approval by the U.S Food and Drug Administration for treatment of helminthes infections in ruminants. Levamisole a synthetic phenylimidazolthiazole has also been found to be possible modulator of the immune responses of carp and rainbow trout [13,80]. After treatment with levamisole both fish species showed enhanced nonspecific immune activity and resistance to an experimental challenge with pathogenic bacteria [58]. The increased protection may be correlated with increased phagocytosis, cytokine expression by macrophages, lymphocyte proliferation following exposure to mitogens and antibody response.

The immunostimulants properties of whole microorganisms, chitin particles, lactoferrin, sodium alginates, vitamin C and E, dietary carbohydrates also received considerable attention in fish and shellfish health management strategies. Little information exists regarding the *in vitro* effect of chitin on fish immune system. Some studies do exist on the *in vivo* administration of chitin and it has been found that injection or dietary administration of chitin may enhance the innate immune system of several fish species [81]. Esteban [82] reported that dietary administration of lactoferrin, a glycoprotein enhances the nonspecific immune responses of gilthead seabream *Sparus auratus*. Sodium alginate extracted from brown algae has been reported to enhance the resistance of common carp *Cyprinus carpio* against *Edwardsiella tarda* infection and increase the nonspecific defence system of *C. carpio* [83]. Bagni et al. [84] reported that alginic acid (Ergosan) and yeast β -glucan (Macrogard) activate innate immune response in sea bass (*Dicentrarchus labrax*), particularly under conditions of immunodepression related to environmental stress.

In recent years, whole microorganisms have been tested for their possible immunostimulant properties in fish. The oral administration or injection of yeasts *Saccharomyces cerevisiae* or *Candida utilis* and fungus *Mucor circinelloides* have been shown to increase both humoral and cellular immune responses and to increase or confer resistance against pathogenic bacteria in channel catfish, rainbow trout and gilthead sea bream [85,86]. Rao and Chakrabarti [87] reported that *Achyranthes aspera*, a herb that stimulates both specific and nonspecific immunity in Indian major carp, *Catla catla*.

Dietary supplementation of certain vitamins may be effective means of increasing immunocompetency and disease resistance of fish [88]. Elevated doses of vitamin C have been shown to enhance immune responses such as macrophage activities, cell proliferation, natural killer cell activity, complement and lysozyme levels in fish [89]. A positive effect of vitamin C together with vitamin E on the immune response of fish has also been found [90,91]. Feeding high levels of ascorbic acid has been reported to enhance protection against bacterial infections viz. *Edwardsiella tarda*, *E. ictaluri*, *V. anguillarum*, *A. salmonicida* and against parasitic infection (*Ichthyophthirius multifiliis*). Sahoo and Mukherjee [92] reported that high level of dietary vitamin C has been used to counteract immunosuppression caused by aflatoxin B1 contaminated feed in immunocompromised rohu (*Labeo rohita*).

Recently, Kumar et al. [93] reported that non-gelatinized carbohydrates (46%) along with supplementation with 50 mg kg⁻¹ amylase stimulated the immune system in *L. rohita* juveniles. Therefore, immunostimulants have an immense importance in disease management in aquaculture.

Studies on the effect of immunostimulants on shrimp and prawn are still at an infant stage. β -glucan have received attention in recent years for their ability to increase disease resistance in shrimp and prawn because the limitation of the specific immune response of these animals and the nature of the disease agents make the development of effective vaccine impractical. β -glucan have been successfully used to increase the resistance of *Penaeus japonicus* against vibriosis [94], further studies using *Penaeus monodon* showed protection against vibriosis, white spot syndrome virus and *Vibrio damsela* and *V. harveyi* [95] and also enhancement of survival and immunity during brood stock rearing [96]. All these effects were caused by direct impact on haemocytes via stimulation of phagocytosis, cell adhesion and superoxide anion [97]. Surprisingly the glucan-induced resistance is maternally transmitted [98]. Surprising data has been obtained in the fresh water crayfish *Pacifastatus lenieusculus*. The injection of glucan caused a short-term severe loss of haemocytes, followed by a rapid recovery due to accelerated release of cells from the haematopoietic organs.

Macrobrachium rosenbergii post larvae showed enhanced growth and resistance to *V. alginolyticus* by dietary administration of β -glucan. Cheng et al. [99] reported that dietary administration of sodium alginate enhances the immune ability of white shrimp *L. vannamei* and increase its resistance against *V. alginolyticus* infection. Vitamin C has also plays a key role in animal health as an antioxidant by inactivating damaging free radicals producing through normal cellular activity and diverse stressors. Lightner et al. [100] reported that inadequate dietary levels of vitamin C in juvenile shrimp may result in black dead syndrome, reduced growth rates, poor feed conversion ratios, decreased resistance to stress and reduced capability to heal wounds. According to Merchie et al. [101], vitamin C plays a role as immunostimulants, as evidenced by the ability of *P. monodon* postlarvae and juveniles to avoid baculovirus and to resist disease caused by *V. harveyi* and saline shock. However, the mode of action of vitamin C as an immunostimulant is not clear, although its antioxidant role and in consequence cell protection could be a mechanism to preserve haemocytes, improving the general immunological system of shrimp. Therefore results suggest that although the shrimp immune system is nonspecific, it would be possible to enhance disease resistance against pathogens in shrimp by careful and regular use of immunostimulants.

Conclusion

Immunostimulants appear to be most promising and useful tools for prophylactic treatment of farmed fish and shrimp. It is safer than chemotherapeutics and their range of efficacy is wider than vaccination. However, these compounds will not replace vaccines proper nutrition or good management techniques. The strength of these compounds appear to lie in their ability to enhance larval culture before the specific immune system matures and the animals can be vaccinated and able to improve nonspecific immune function against a broad spectrum of pathogens.

Thus, application of immunostimulants to aquatic health management has immense potential, but in order to capitalize on this issue a lot of scientific research is necessary to understand the mode of action. Many of the *in vitro* tests are expensive to conduct, limiting the ability to rapidly screen potential immunostimulant regimens for efficacy. Additional research is needed to define the specific dosage

rates and efficacy of various compounds for a variety of aquatic species and their pathogens and to decrease costs of the immunostimulants. It is expected that during coming years immunostimulants will find more application to make aquaculture sustainable. Therefore, immunostimulants may be an effective tool for controlling infectious diseases in aquaculture.

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