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Review

Emerging role of immunostimulants in combating the disease outbreak in aquaculture

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Abstract

The contribution of aquaculture to fish production is steadily increasing. Among various kinds of cultivated aquatic organisms, many marine and freshwater finfish and shellfish species constitute an important industry with their production increasing every year. Development of the aquaculture industry and increasing demand for fish production further provocate intensive fish culture, where the fish is at high risk of infectious disease caused by bacteria, virus, parasites etc. Use of expensive chemotherapeutants and antibiotics for controlling disease have widely been criticized for their negative impacts like residual accumulation in the tissue, development of the drug resistance and immunosuppression, thus resulting in reduced consumer preference for food fish treated with antibiotics. Hence, instead of chemotherapeutic agents, increasing attention is being paid to the use of immunostimulants for disease control measures in aquaculture. Immunostimulants comprise a group of biological and synthetic compounds that enhance the non-specific cellular and humoral defense mechanism in animals. These substances such as levamisole and glucan, peptidoglycon, chitin, chitosan, yeast and vitamin combinations as well as various products derived from plants and animals are effective in prevention of diseases. An application of immunostimulants for the prevention of fish diseases are considered as an attractive and promising area. They mainly promotes the function of phagocytes and increase their microbicidal power. However, a controversy exists over the use of these substances, as in a few cases they have failed to render enhanced protection or increase in immunity. This review article mainly considers the recent developments in this area to control fish diseases and enhance the growth of fish in the culture setups, and describing all different types and sources of immunostimulants, objective and evaluation of the mode of work.

Keywords: Immunostimulants, Aquaculture, Immune response, Fish disease, Growth

Introduction

Aquaculture represents one of the fast growing food producing sectors of the world and aims to increase productivity per unit space. Among various kinds of cultivated organisms, many marine and freshwater finfish and

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shellfish species constitute an important industry with their production increasing every year. Recently, due to intensive farming practices, infectious diseases pose a major problem in aquaculture industry, causing heavy loss to farmers. In order to address this problem, several studies have been conducted on the modulation of fish immune system in order to prevent the outbreak as reviewed recently by Sakai (1999). Disease outbreaks are increasingly being recognized as a potential constraint on aquaculture production and trade, and cause massive financial loss either through mortality or reduced meat quality, resulting in reduced profit margins (Smith et al. 2003).

The economic loss due to the diseases outbreak in the aquaculture sector can be considerable. For example, economic loss attributed due to the diseases in the Asian region countries was estimated to be at least US$ 1400 million in 1990 (ADB/NACA 1991). The best way to overcome the disease problems in a system is through effective management practices, i.e. management of stock, soil, water, nutrition and environment. As proper management is not always delivered, pathogens become established in animals and produce disease. The use of chemicals to control bacterial or parasitic populations brings lots of disadvantages viz. bioaccumulation, human carryover, pollution, etc. Similarly, the widespread use of antibiotics leads to development of antibiotic resistant bacteria, immunosuppression and destabilization of helpful bacterial populations.

The application of antibiotics and other chemicals to pond culture is also quite expensive and undesireable because of its risk of environment and culture contamination as well as imparing the growth of fish. The use of antimicrobial drugs in Norway, a major fish producer dropped from approximately 50 metric tonnes per year in 1987 to 746.5 kg in 1997, measured as active components, and this was mainly due to mass vaccination and selection programmes for important diseases (Verschuere. et al. 2000). Use of expensive chemotherapeutants and antibiotics for controlling disease have widely been criticized for their negative impacts like accumulation in the tissue as residues, development of the drug resistance, immunosuppression and reduced consumer preference for food fish treated with antibiotics (Anderson 1992). Therefore, instead of antibiotics and chemotherapeutic agents, increasing attention is being paid to the use of immunostimulants for disease control measures in aquaculture.

The immunostimulants mainly facilitate the function of phagocytic cells, increase their bactericidal activities, and stimulate the natural killer cells, complement system, lysozyme activity, and antibody responses in fish and shellfish which confer enhanced protection from infectious diseases (Harikrishnan et al. 2011a). Considering the recent successes of these alternative approaches, the Food and Agriculture organization of the United Nations (FAO) defined the development of affordable yet efficient vaccines, the use of immunostimulants and non-specific immune enhancers, and the use of probiotics and bioaugmentation methods for the improvement of aquatic environmental quality as major areas for further research in disease control in Aquaculture (Subasinghe 1997).

Currently, increased consumer demand for perfection in fish and shellfish farms has put new dimensions to the quality, safety, elimination of concomitant pollutants, antibiotics, and carcinogens during the production process. In this context, plants or their byproducts are preferred since they contain several phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectine, and polypeptide compounds, many of which have been shown to be very effective alternatives to antibiotics, chemicals, vaccines, and other synthetic compounds. In aquaculture, the herbal medicines are also known to exhibit anti-microbial activity, facilitate growth, and maturation of cultured species; besides, under intensive farming, the anti-stress characteristics of herbs will be of immense use without posing any environmental hazard. Administration of herbal extracts or their products at various concentrations through oral (diet) or injection route enhance the innate and adaptive immune response of different freshwater and marine fish and shellfish against bacterial, viral, and parasitic diseases (Harikrishnan et al. 2011a).

Despite the partially successful preventive measures including sanitary prophylaxis, disinfection, antibiotics, vaccines, and chemotherapy for the last 20 years, the estimated annual economic loss due to diseases in aquaculture is more than US$ 400 million in China (1993), US$ 17.6 million in India (1994), and over US$ 500 million in Thailand (1996) (Harikrishnan et al. 2011a). Therefore, the research conducted during the last two decades has emphasized on the use of compounds that could enhance immunity or disease resistance in fish. Many of these compounds are commercially available, some are still in the developmental stage and others are on the sidelines awaiting for major investment. This is the right time for appraisal of the current state of the art of these substances for disease resistance in fish.

This review aims to provide an overview of the work done on the use of different immunostimulants in preventing the outbreak of diseases in aquaculture and enhancing the immune status of fish by various mechanisms.

Immunostimulants and immunomodulators

Immunostimulants and immunomodulators comprise a group of biological and synthetic compounds that enhance the non-specific cellular and humoral defense mechanism in mammals. These substances such as levamisole and glucan, peptidoglycon, chitin, chitosan, yeast and vitamin combinations as well as various products derived from plants and animals are effective in prevention of disease (Baulny et al. 1996; Verlhac et al. 1998; Mulero et al. 1998; Esteban et al. 2001). By definition, immunostimulant is a chemical, drug, stressor or action that enhances the innate or non-specific immune response by interacting directly with cells of the system, thus activating them. In practice, immunostimulants are the promising dietary supplement to potentially aid in disease control of several organism including fishes and increase disease resistance by causing up-regulation of host defense mechanism against opportunistic pathogen microorganisms in the environment. Immunostimulants also have ability to increase resistance to viral, bacterial and fungal infection (Anderson 1992).

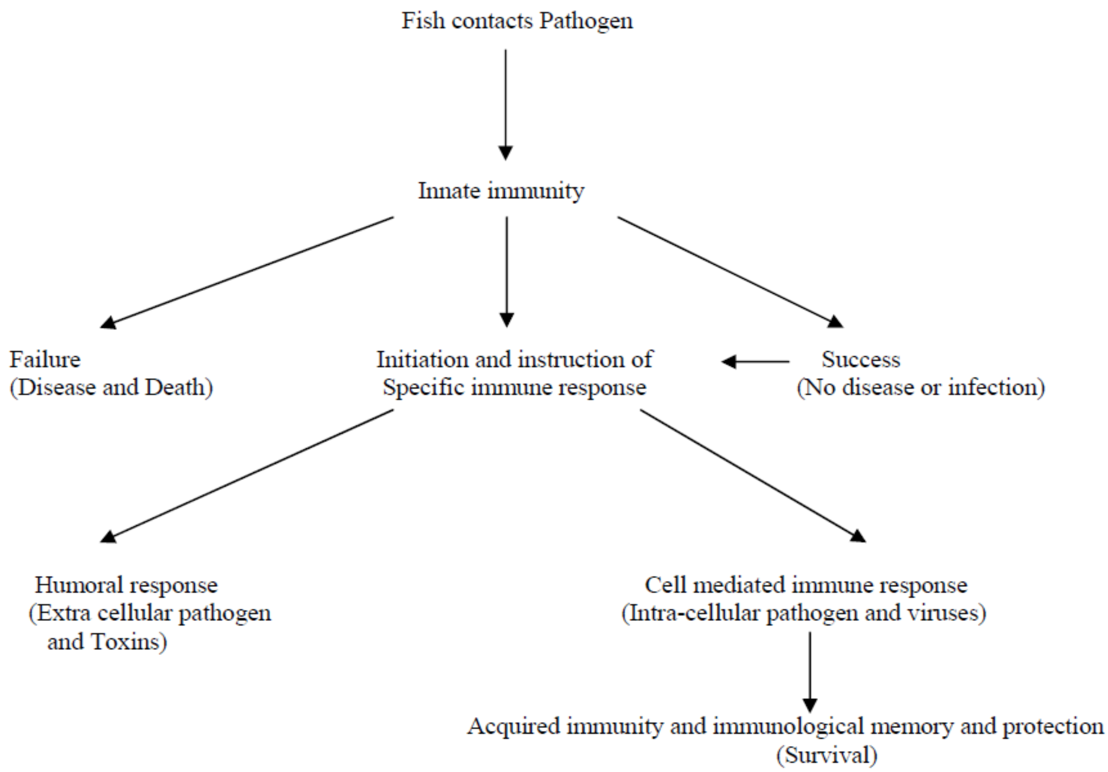

Fig. 1. Schematic representation of response of a fish following an encounter with a pathogen (Shoemaker et al. 2001)

Immunostimulants enhance the humoral and cellular response in both specific and non-specific ways (Figure 1). These agents are widely used for impaired immune function and to stabilize the improved immune status. The use of immunostimulants for the prevention of disease in fishes is considered as an attractive and promising area in the field of aquaculture (Anderson 1992; Secombes 1994). Immunostimulants are valuable for the prevention and control of fish diseases in aquaculture as they represent an alternative and supplementary treatment to vaccination. The immunostimulants also have additional effects such as growth enhancement and increase in the survival rates of the fishes under stress (Heo et al. 2004).

Immunostimulants may be given either alone to activate non-specific defence mechanisms or along with the antigen to heighten the specific immune response. Research on immunostimulants is being intensified particularly in the areas of Acquired Immuno Deficiency Syndrome (AIDS) and cancer research. Its application is rampant in animal science to protect the animals at early stages as well as from immunosuppressive diseases and sometimes in vaccine failure cases. Immunostimulants are sometimes classified more generally as biological response modifiers. However, these substances are more often cytokines or soluble chemicals that allow communication between different cell populations, i.e. they are secreted by one cell population to signal to another one to activate specific processes. In terms of the immune response, a biological response modifier is a chemical that may cause immunostimulation or immunosuppression (Anderson 1992).

Many substances from different sources (bacterial components, chemical agents, animal or plant extracts) have been studied as prospective immunostimulants for fish (Sakai 1999) and some have been reported to confer a degree of protection against several disease causing pathogens such as *Aeromonas hydrophila, Vibrio anguillarum,* and *Aeromonas salmonicida,* which are commonly found in farmed fish (Maqsood et al. 2009, 2010; Kajita et al. 1990; Raa et al. 1992; Baulny et al. 1996; Mulero et al. 1998).

The interest in using immunostimulants is increasing because of the frequent outbreaks of bacterial, viral or parasitic diseases that are limiting factors in culture at many fish farms, hatcheries, and aquaculture stations. Moreover, a serious problem is that only few approved chemotherapeutic agents are available for use in food fish because of growing concern for consumer liability and for accumulation of substance in the environment. Concerns are arising regarding the increase of antibiotic-resistant strain of bacteria in the surrounding areas of aquatic farms, as antibiotics (terramycine, sulfadimethoxine, or mitoprin) are extensively used in fisheries. Indeed, while these antibiotics are often effective in treatment or control of some disease agents, additional methods are needed to control these fish diseases. Furthermore, the greater reliability on non-specific immunity to protect the fish from infections, the short-lived nature of fish, environmental fluctuations, temperature dependent antibody synthesis, and no or relatively few side-effects and residual problems, easy method of exposure, and being economically effective, are the advantages of immunostimulants. Presently, research on immunostimulants is more focused on the routes of administration other than through the diet; however, the information presented here is to indicate the potential of immunostimulants as dietary supplements and the need for further research in the area of their oral administration. Many facts also need to be understood regarding the dose, route, time, frequency, and length of administration of immunostimulants to various fish species at various stages of growth. This is because of the variable behaviour of each of the substances in individuals at various growth stages. Clearly, even for the commercial immunostimulants available today, there are a number of important issues that need to be addressed before confidence can be placed in the benefit of their prolonged use. Again, a deep understanding of the interaction of immunostimulant with various components of the host's immune system is needed, and the mechanisms underlying their immunostimulatory effect require further clarification.

The selection of suitable immunostimulants with a capacity to enhance protection in fish presents a difficult task and, in a strict sense, no such major effort or research funds have been diverted for appropriate screening programmes using model species and test systems. However, immunostimulants receiving major attention and claiming success in rendering enhanced protection in fish under experimental or practical conditions comprise the six major types, independent of their mode of action: (i) synthetic chemicals (levamisole, FK-565 and muramyl dipeptide (MDP)); (ii) microbial derivatives (b-glucans, peptidoglycans, lipopolysaccharides (LPSs), bacterins, Freund's complete adjuvant (FCA) and EF203); (iii) nutritional factors viz. vitamins C and E; (iv) animal and plant extracts; (v) polysaccharides (chitosan, chitin, lentinan, schizophyllan and oligosaccharides); and (vi) hormones (lactoferrin, growth hormone, thyroid hormone and prolactin), cytokines and others (double-stranded RNA (dsRNA), CpG oligodeoxynucleotides (ODNs), recombinant interleukin-1b (IL-1b) and recombinant proteins).

Most commonly used immunostimulants in aquaculture

Glucans

Glucans are the most popular immunostimulants used in aquaculture. They are derived from yeast cell wall and from certain higher plants. They have excellent immunostimulatory properties and work well when injected or fed to fish. Yano et al. (1991) showed that ß-1,6, branched ß-1,3 Glucans were effective in stimulating the non-specific immune response in carp. Jeney and Anderson (1993b) showed that the use of Glucans increased activity in non-specific defense mechanism and in protection against *Yesinia ruckeri*. Glucan treatment of Atlantic salmon (*Salmo salar*) induced protection against *Vibrio salmonicidia.* Samuel et al. (1996) concluded that a single intra-peritoneal injection of 20 mg of laminarian (ß (1,3)-D-Glucan) is sufficient to protect the fish effectively from a virulent strain of *Aeromonas hydrophila*, as laminarian can significantly enhance phagocytic activity in the host. Healthy fish fed with Beta-1, 3 glucan showed the highest antibody response against *E. tarda*. The antibody titre was significantly increased by feeding the immunostimulant to the aflatoxin-treated fish. The lowest mortality rate (5%) was obtained in the glucan-fed healthy vaccinated fish while control healthy vaccinated fish had a 30% mortality rate (Sahoo and Mukherjee 2002). The authors reported that the immune parameters including WBC and RBC counts, serum protein, albumin, globulin, phagocytic ratio, phagocytic index, and serum bactericidal activity rose to their highest level on 42nd day after feeding with diet containing 250 mg of Beta-glucan/kg diet. This dose also resulted in the highest protection in the challenge study. Doses of 250 and 500 mg of Beta-glucan per kg diet resulted in better Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR) (Misra et al. 2006). Selvaraj et al. (2005) studied the use of Beta-glucan as an immunostimulant in carp against the infection of *A. hydrophila* and concluded that when a concentration above 500 µg glucan per fish was administered, the survival was 100% relative to the uninfected control. The total leukocyte count as well as neutrophil and monocyte populations increased after administration of the glucan. This study showed that glucan can be effective in inducing non-specific cellular defense mechanism in carp.

Sea bass (*Dicentrarchus labrax*) were fed a diet supplemented with 2% ß-1,3/ß-1,6-glucan over a 2-week period every 3 months (Bagni et al. 2000). The plasma complement activity was elevated in immunostimulated fish compared with controls. In another trial, alternative pathway of complement activation and lysozyme activity were both significantly enhanced day 15 (just after the first out of four 2-week feeding cycles) of feeding fish with ß-glucan (Macro-Gard) and alginic acid (Ergosan). The immunostimulants did not confer any immune modulation measured as elevated lysozyme levels, increased complement activity, and modulated T and B cell numbers in peripheral blood after the fish were fed for 35 weeks (Bagni et al. 2005).

The dietary effect of ß-1,3-glucan on innate immune responses of large yellow croaker (*Pseudosciaena crocea*) was studied (Ai et al. 2007). The diet was supplemented with 0.09% and 0.18% ß-1,3-glucan and the feeding trial lasted for 8 weeks. Low concentration of glucan (0.09%) significantly enhanced the respiratory burst and phagocytic activity in head kidney macrophages while the high concentration (0.18%) did not. The serum lysozyme activity in fish fed diets with both ß-glucan concentrations were significantly higher than the control, and the 0.18% ß-glucan diet significantly stimulated the lysozyme activity compared to 0.09%. No significant differences were seen in alternative complement pathway activity with any of the ß-glucan concentrations (Ai et al. 2007).

Dietary effect of ß- glucan in fish

Carp (*Cyprinus carpio*) were given ß-glucan by the oral route before vaccination with *Aeromonas hydrophila*. The antibody titre was slightly increased. Orally administered glucan did not result in changes of the alternative complement pathway (Selveraj et al. 2005). ß-Glucan was administered to *Labeo rohita* fingerlings through different dietary doses for 56 days. Leucocyte counts and both cellular and humoral immune parameters were evaluated at 2 weeks intervals (Misra et al. 2006).

Administration of the highest dose (500 mg ß-glucan per kg diet) resulted in a reduced white blood count compared to fish fed 250 and 100 mg. Maximum superoxide anion production and phagocytic activity in head kidney macrophages were found in fish fed 500 mg and 250 mg ß-glucan per kg diet (for 42 days), respectively. Haemolytic complement activity was significantly higher in fish stimulated with ß-glucan except the 500 mg per kg dose. The lysozyme and bactericidal activity were highest at a dose of 250 mg ß-glucan per kg dry diet fed for 42 days. It is known that ß-glucans and lipopolysaccharide (LPS) may activate the alternative pathway of complement. Indeed, an intraperitoneal injection of lipopolysaccharide (e.g. 5 mg LPS/kg body weight) has shown to be exhaustive of the haemolytic activity of complement in the spotted wolf fish (own observation). On the other hand, whether activation of haemolytic complement activity following oral administration of immunostimulants is beneficial in terms of disease resistance remains to be addressed. A commercial ß-glucan (EcoActiva) was administered as a feed supplement to the snapper, *Pagrus auratus* (Cook 2003).

The immunostimulant increased macrophage oxygen radical production during the wintertime, but not during the summer time. In contrast to macrophage activity, EcoActiva did not potentiate neither the classical nor the alternative complement activity. The results of this study suggested that it may be favourable to include ß-glucan in the feed to snapper during the wintertime both to increase resistance against diseases and to increase growth rates. Immunomodulation by a yeast ß-1,3/ß-1,6-glucan in rainbow trout was evaluated with macrophage activities, lysozyme and complement activation. In addition, antibody responses were analysed after vaccination with formalin killed *Yersinia ruckeri* (Verlhac et al. 1998).

Immune response in fish after injection of ß-glucan

Carp (*Cyprinus carpio*) injected intraperitoneally with ß-glucan (100, 500 and 1000 mg) had a significant increase in total leucocyte counts and an increased proportion of neutrophils and monocytes on day 7 (Selvaraj et al. 2005). Classical and alternative complement activation were unaffected by glucan injection. Anterior kidney macrophages from both control and glucan-injected fish were cultured and assayed for superoxide anion production, bacterial killing of *Aermonas hydrophila* and expression of IL-1ß mRNA. Superoxide anion production was significantly increased in macrophages from glucan injected fish at all doses. In addition, *A. hydrophila* was more efficiently killed by macrophages from the glucan stimulated fish. Also, expression of IL-1ß mRNA was higher and dose-dependent in glucan-treated fish.

A combination of ß-glucan and lipopolysaccharide injected intraperitoneally to carp (100 mg ß-glucan and 10 mg LPS) showed a significant increase in total blood leucocyte counts and an increase in the proportion of neutrophils and monocytes (Selvaraj et al. 2006). Superoxide anion production by macrophages was also elevated. Classical and alternative complement pathways were unaffected by the glucan. Also, expression of IL-1ß mRNA was not elevated in the stimulated fish.

Effect of ß-glucan on immunocompromised fish

The dietary effect of ß-1,3-glucan on immune responses in healthy and aflatoxin-induced immunocompromised rohu (*Labeo rohita* Hamilton) was investigated. A single injection of aflatoxin reduced the non-specific immunity as measured by neutrophil phagocytosis, serum bactericidal activity and specific immunity against *Edwardsiella tarda* (Sahoo and Mukherjee 1999, 2002). Feeding of glucan to aflatoxin-immunocompromised fish for 7 days significantly raised non-specific immunity as measured by serum bactericidal activity, phagocytic activity and antibody titres. Cyclophosphamide, a multifunctional alkylating agent as well as a cytotoxic drug, a well-known immunosuppressant, was used to induce an immunocompromised state in Asian catfish (*Clarias batrachus*) (Kumari and Sahoo 2006a, b).

The cyclophosphamide treated fish showed lowered level of respiratory burst, myeloperoxidase and phagocytic activities in blood phagocytes, and a decreased haemagglutination activity. ß-Glucan delivered as feed supplement significantly enhanced these immune parameters. Taken together, the use of, e.g., ß-glucan may have advantages during immunosuppressive states such as during physiological and environmental stress. The feed manufacturers are also advising to use feed with immunostimulants during such circumstances.

Immunostimulation in shrimp by ß-glucan

ß -Glucan has been used to enhance resistance in crustacean against both viral and bacterial infections. The white spot syndrome (WSS) is the major disease of shrimp in India, Southeast Asia and the Southern and Central America. The WSSV causes high mortality in many cultured shrimp species including black tiger shrimp (*Penaeus monodon*) and kuruma shrimp (*Penaeus japonicus*).

Administration of ß-1,3-glucan has been shown to improve the survival of black tiger shrimp when challenged with WSSV (Chang et al. 1999, 2000, 2003). Post larval and juvenile shrimps were fed diets containing 2 g/kg for 10-20 days. Following challenge with WSSV, mortality was significantly lower in the glucan-fed shrimps. None of the un-treated shrimps survived longer than 4 days. By contrast, some of the glucan-treated larvae (12%) and some of the juveniles (20%) were still alive at day 6 and were reared for another 120 days (Chang et al. 1999). *P. monodon* were also fed diets with different levels of ß-1,3-glucan from *Schizophyllum commune* and then challenged with injection of WSSV (Chang et al. 2003). The survival rate of shrimps fed the diet containing 10 g/kg was significantly higher on day 9 than the other groups (0, 1, 2 and 20 g/kg). Haemocyte phagocytic capability, phenoloxidase activity, superoxide anion and superoxide dismutase activity were significantly enhanced after oral ß-1,3-glucan administration (Chang et al. 2003).

Sritunyalucksana et al. (1999) investigated both in vivo and in vitro stimulation of the black tiger prawn by peptidoglycan, LPS and the ß-1,3-glucan laminarin. In this study, laminarin failed to activate the prophenoloxidase, agglutinin and antibacterial activity. Explanation of this lack of effect is uncertain because ß-1,3-glucans have been reported to be a specific activator of prophenoloxidase in several investigations (Sritunyalucksana et al. 1999). Several Glucan products such as vitastim, macrogard, are marketed commercially and are used in supplementing fish feeds. Beta-glucan has been proved to be more effective than all immunostimulants used in aquaculture.

Levamisole

Levamisole, a synthetic phenylimidazolthiazole has been extensively used in both humans and veterinary medicine as an anti-helminthic agent (Janssen 1976). The ability of this agent to enhance the response of mammalian T-lymphocytes and macrophages of both healthy (Renoux 1980) and immunocompromised individuals is well documented (Amery 1978; Morimoto et al. 1979; Ogunbiyi et al. 1988). In fish, levamisole has been used in a few studies with the aim of enhancing the non-specific immune response (Siwicki 1987, 1989; Kajita et al. 1990; Baba et al. 1993) or as adjuvant with a vaccine (Anderson and Jeney 1992; Jeney and Anderson 1993a). The results obtained through these studies point out the potential use of levamisole in fish as an immunostimulant, although special attention must be paid to the doses administered and the timing, as the effect of levamisole is closely dose and time dependent. High dose of levamisole may suppress the immune response and much lower doses may not be effective at all (Anderson et al. 1989; Siwicki et al. 1990). The duration of any increased immune response in fish after the administration of levamisole as well as the duration of this induced protection remains to be determined. The immunostimulatory potential of levamisole in fish is of considerable interest in the present scenario of aquaculture in combating the bacterial and parasitic disease of fish, as the U.S Food and Drug Administration (FDA) has approved it for treatment of helminth infections in ruminants. Levamisole, which is a synthetic phenylimidathiazole, has been shown to have the ability to up-regulate non-specific immune response in carps, rainbow trout and gilthead sea bream (Stickney 2000). Activities of this agent are: a) Enhancement of cell mediated cytotoxicity, lymphokine production and suppression of cell function, and b) Stimulation of phagocytic activity of macrophages and neutrophils.

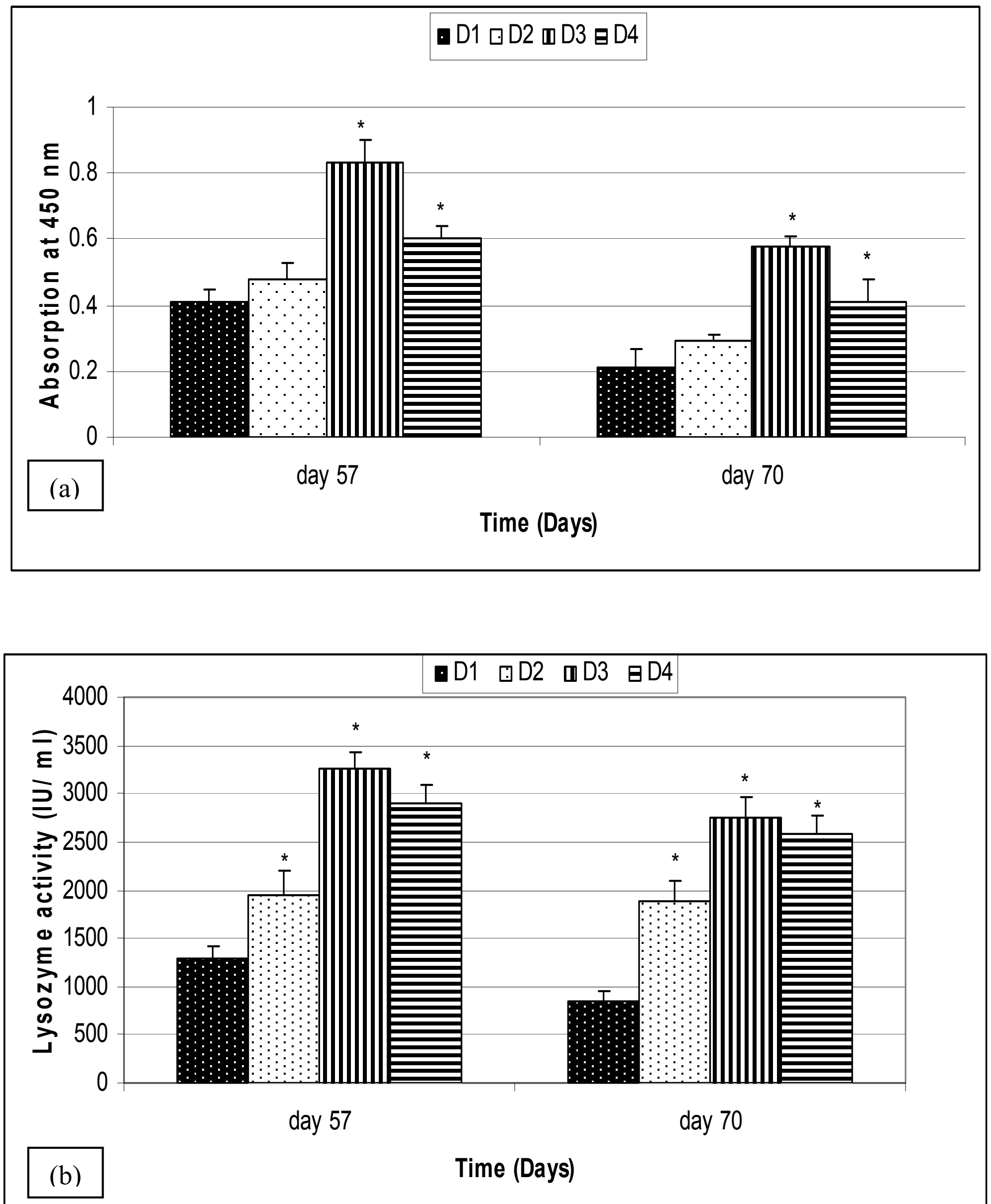

Fig. 2. NBT reduction (a) and Lysozyme activity (b) of common carp fed levamisole (100, 250 and 500 mg/kg diet) and a non-supplemented control diet for 70 days and weighed every 15 days. Asterisks denote statistically significant differences ($P < 0.05$) between control and levamisole treated groups. Data represented as mean ± SE. D1: Control, D2: Levamisole (100 mg/kg diet), D3: Levamisole (200 mg/kg diet), D4: Levamisole (500 mg/kg diet). Source: Maqsood et al. (2009).

Efficacy and immunoreversal effects of four well-known dietary immunomodulators viz., lactoferrin, ß-1,3 glucan, levamisole and vitamin C on the innate immune response of healthy and cyclophosphamide (CYP) induced immunocompromised Asian catfish, *Clarias batrachus* was studied. All the immunostimulants delivered as feed supplements significantly enhanced most of the non-specific immune parameters in both healthy and immunosuppressed subgroups compared to their respective controls (Kumari and Sahoo 2006a). Levamisole induced significantly increase in blood phagocytic activities in both healthy and immunocompromised fish as observed from raised respiratory burst and myeloperoxidase activity as compared to control (Kumari and Sahoo 2006a). Feeding of vitamin C significantly enhanced phagocytic activity and myeloperoxidase content. On the other hand, all parameters were positively influenced by lactoferrin and glucan feeding in both groups compared to their respective controls (Kumari and Sahoo 2006a).

Recently, Maqsood et al. (2009) demonstrated the immunostimulatory effect of levamisole in the common carp against the challenge of *A. hydrophila* by means of enhanced neutrophil and lysozyme activities (Figure 2 a, b). A dose of 250 mg and 500 mg levamisole/kg diet was optimum to stimulate the immune function of common carp and confer a high degree of protection against the invading bacterial pathogen. Same doses of levamisole were equally effective in stimulating the growth and increasing the survival rates of common carp.

Chitin

Chitin is an insoluble, linear Beta-1,4-linked polymer of N-acetyl-D-glucosamine, one of the most abundant polysaccharide in nature and a common constituent of insect and crustacean exoskeleton and fungal cell wall. Chitin is commercially manufactured from the shrimp and crab shells. Outstanding features of chitin are the material, mechanical and chemical properties which offers wide applications in chemistry, medicine and agriculture. Besides many applications in medicine and agriculture, chitin has a major role in aquaculture. There has been a growing interest in chitin because the studies carried out on mammals have shown that it modulates the immune system and also stimulates the main cellular innate immune function (Suzuki et al. 1984; Peluso et al. 1994; Shibata et al. 1997 a,b). Chitin is a non-toxic biodegradable and biocompatible substance. For this reason, chitin and its derivatives have been used in medical practice (Shibata et al. 1997a). Increased protection against *Aeromonas salmonicida* has been observed in brook trout when injected with chitin. Injection of abalone extract and chitin increase phagocytic response and natural killer cell activity in fish (Stickney 2000). Further, chitin is reported to provoke the immunostimulation in a very short time, which renders it as interesting candidate for incorporation in fish diet formulation (Esteban et al. 2000a).

Esteban et al. (2000a) suggested that the administration of a chitin diet at 25 or 50 g/kg enhances immune activity in seabream via non-specific modulation of haemolytic complement activity, leukocyte respiratory burst activity and cytotoxicity. The assayed immune parameters remained unaffected when chitin was intravenously administered. However, the fish that had been intraperitoneally injected showed increased humoral and cellular immune responses. Respiratory burst and phagocytic activities peaked at 3 and 5 days post-injection, respectively, while cytotoxic activities had increased by 3 days post-injection and remained high until 10 days post-injection (Esteban et al. 2000b). Thus, the immunomodulatory effect of chitin has been well proved in different fishes under the culture setups. Choudhury et al. (2005) reported that the dietary yeast RNA and chitin supplementation reduces mortality due to *Aeromonas hydrophila* and enhanced phagocyte respiratory burst, and protection of *Labeo rohita* juveniles to challenge by *A. hydrophila.* Gopalakannan and Venkatesan (2006) reported that WBC count was significantly higher in chitin fed groups as compared to control. The relative percentage survival were higher in chitosan fed fishes (80%) followed by levamisole (66.7%) and chitin (40%). Thus the results indicate that dietary intake of chitin, chitosan and levamisole will enhance the innate immune system and survivability of common carp against *Aeromonas hydrophila* infection in ponds.

Chitosan

Chitosan is a linear homopolymer of ß-(1,4)-2-amino-deoxy-D-glucose and is prepared by the alkaline deacetylation of chitin obtained from crab shell. Administration of chitosan to brook trout (*Salvelinus fontinalis*) by injection and immersion resulted in high levels of protection, which occurred 1, 2, and 3 days afterwards, but protection was greatly reduced by day 14. Injection of chitosan was also more effective than simple immersion (Anderson and Siwicki 1994). Chitosan is used as an immunostimulant in aquaculture to protect salmonids and carps against bacterial diseases (Anderson and Siwicki 1994; Siwicki et al. 1994).

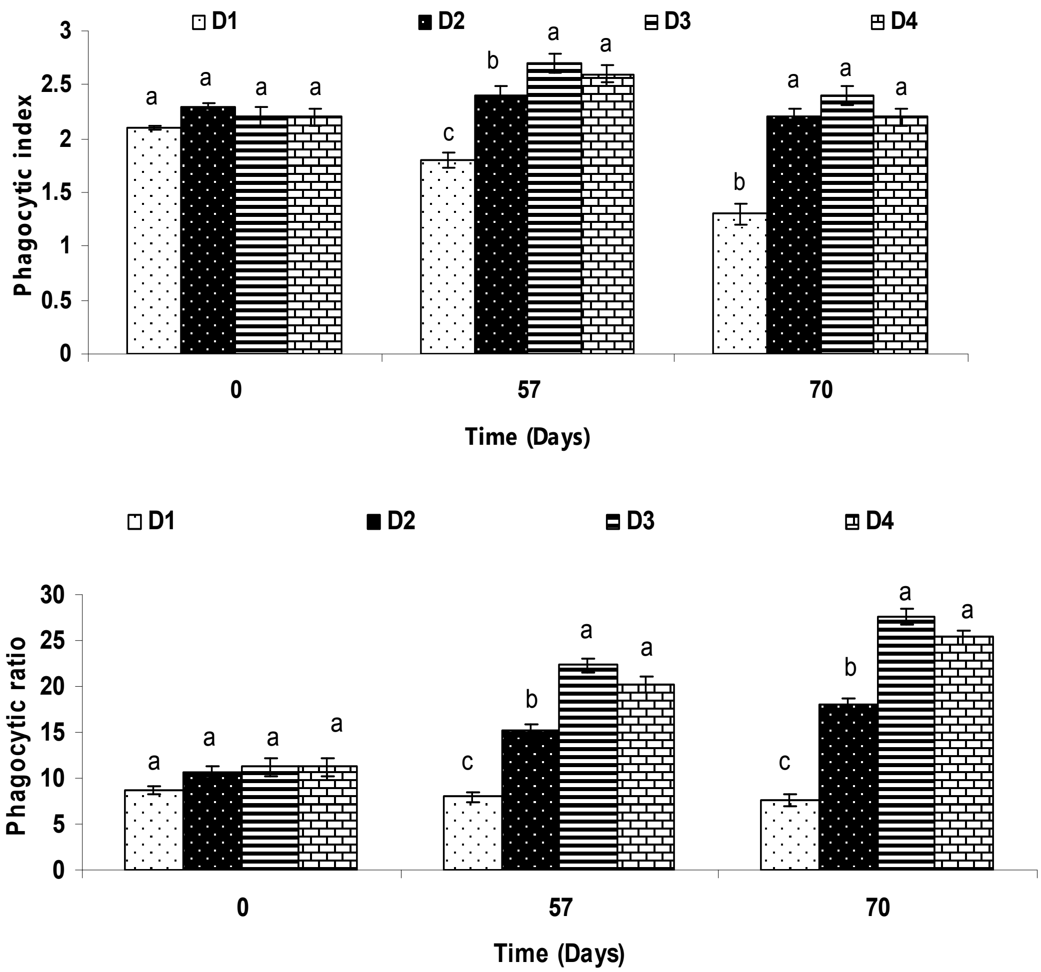

Fig. 3. Change in phagocytic index (a) and phagocytic ratio (b) observed in challenged *Cyprinus carpio* fed chitosan supplemented and control diet. Bars represent the standard deviation (n=3). Same small letters on the bar indicate no significant difference between the different treatment and the control group on the particular sampling day ($P < 0.05$). D1: Control group, D2: Chitosan (1%) fed group, D3: Chitosan (2%) fed group, D4: Chitosan (5%) fed group. Source: Maqsood et al. (2010).

Sahoo and Mukherjee (1999) studied the influence of the chitosan on immune response of healthy and cortisol-treated rohu, *Labeo rohita*, and concluded that the healthy and cortisol-treated fish after chitosan treatment had significantly higher responses in almost all assays of non-specific immunity like total serum protein, serum albumin, serum globulin, albumin: globulin ratio, packed cell volume, phagocytic index, and erythrocyte fragility index compared with healthy controls and cortisol-treated fish without chitosan treatment.

Chitosan is a deacetylated product of chitin. In aquaculture, chitosan has been used as an immunostimulant for protection against bacterial disease in fish, for controlled release of vaccines, and as a diet supplement (Bullock et al. 2000). Gopalakannan and Venkatesan (2006) studied the immunomodulatory effect of dietary intake of chitosan on immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds. They concluded that WBC count, lysozyme activity and neutrophil activity were significantly stimulated in all chitosan fed groups as compared to control. The relative percentage survival (RSP) were higher in chitosan fed fishes (80%) followed by levamisole (66.7%) and chitin (40%).

Recently, Maqsood et al. (2010) reported that the phagocytic index, phagocytic ratio (Figure 3 a and b) and serum bactericidal activity (Figure 4) were increased in the chitosan fed (2 and 5%) common carp, compared to the control. When the fish in all treatments were intra-peritoneally challenged with *Aeromonas hydrophila*, the relative percentage survival (RPS) (82.78%) was higher in chitosan (2%) group (Table 1), when compared to other treatments (Maqsood et al. 2010).

In-vitro intraperitoneal injection and dietary immunostimulatory effects of chitosan and its derivatives with different molecular weight, chitosan, polyglucosamine and N-acetyl-chitooligosaccharides were studied on the grouper, *Epinephelus malabaricus*. Respiratory burst activity was generally decreased with increasing dosage of chitosan products. N-acetyl-chitooligosaccharides were significantly more potent in enhancing respiratory burst activity than the other two chitosans. Feeding the grouper with N-acetyl-chitooligosaccharides at 1g/ 100g diet seems to lower the immunity of the fish (Yu-Li Chen, 2000). Thus, the incorporation of chitosan at a level of 2% in the diet of fish enhances the non-specific immunity and growth of fish under stress conditions, and reduces fish mortality (Maqsood et al. 2010).

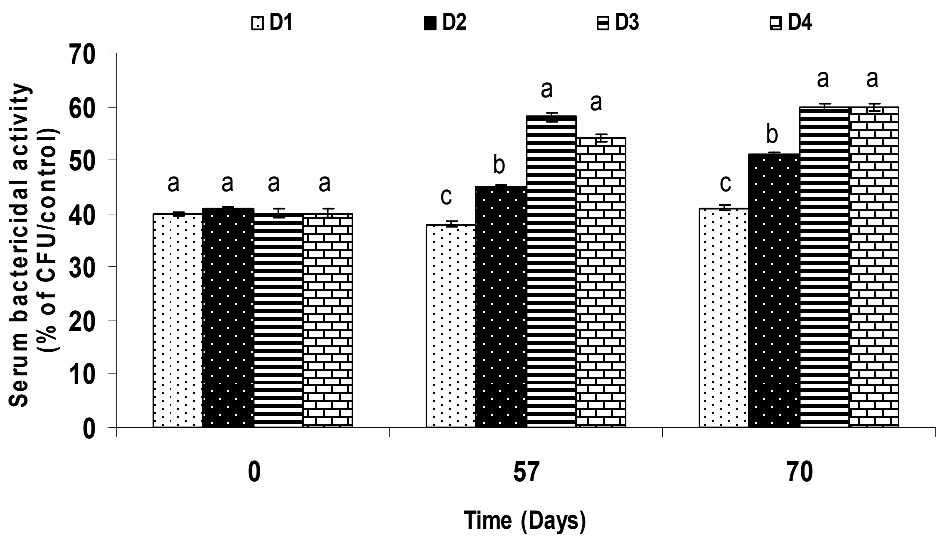

Fig. 4. Change in serum bactericidal activity observed in challenged *Cyprinus carpio* fed chitosan supplemented and control diet. Bars represent the standard deviation (n=3). D1: Control group, D2: Chitosan (1%) fed group, D3: Chitosan (2%) fed group, D4: Chitosan (5%) fed group. Source: Maqsood et al. (2010).

Use of herbal and medicinal plants as immunostimulants in fish

A large number of plants have been used in traditional medicine for the treatment and control of many diseases (Duke 1987). Natural plant products have been reported as antistress, growth promotion, appetite stimulation, tonic and immunostimulation, and to have aphrodisiac and antimicrobial properties in finfish and shrimp larviculture due to the presence of active principle components such as alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids, and essential oils (Citarasu et al. 1998, 1999, 2001, 2002; Sivaram et al. 2004). Herbs, rich sources of immune-enhancing substances, are used in many countries to promote health, increase the body's natural resistance to infection and in prevention and treatment of various diseases (Devasagayam and Sainis 2002). Herbal products are cheaper source for therapeutics, have greater accuracy than chemotherapeutic agents, and offer a viable solution for all problems which aquaculture faces today. Several antimicrobial, anti-stress, immunostimulant and growth-promoting plant products significantly influence the fish or shrimp larviculture (Citarasu et al. 1998, 2002, 2003a, 2003b). Plant phenolics, polysaccharides, proteoglycans, and flavonoids play a major role in preventing or controlling infectious microbes (Citarasu 2010). Herbal compounds have the ability to inhibit the generation of oxygen anions and scavenge free radicals.

A huge list of Indian medicinal plants with Immunomodulatory activities (through stimulated phagocytosis and chemotaxis, increasing production of free radicals and cytokines) in humans and animals has recently been reviewed (Devasagayam and Sainis 2002). Limited reports are available on the use of these substances in fish health management. To cite a few, the inter-peritoneal injection of a water extract of *Ocimum sanctum* leaves showed enhanced antibody titre and increased number of activated neutrophils in *Oreochromis mossambicus* (Venkatalakshmi and Michael 2001).

Table 1. Relative Percentage Survival (RPS) (%) of challenged *cyprinus carpio* fed chitosan supplemented diet and the control diet. Source: Maqsood et al. (2010)

Treatments	Survival (%)	Mortality (%)	RPS (%)
Control D1	26.5	73.5	-
D2	62.2	37.8	48.5
D3	87.35	12.65	82.78
D4	85.00	15.00	79.59

Feeding a diet containing seed of *Achyranthes aspera* (0.5%) to juveniles of *Catla catla* raised immune status as measured through serum anti-trypsin activity, immunoglobulin level, haemagglutination titre as well as RNA/DNA ratio of spleen and kidney (Rao and Chakrabarti 2005). Feeding of a traditional Chinese medicine derived from *Astragalus* root and *Chinese angelica* root to large yellow croaker (*Pseudosciaena crocea*) for 30 days enhanced lysozyme and complement activities and NBT-positive cells as well as survival rate (93.3%) compared with cumulative mortality of 75% in control fish infected with *Vibrio alginolyticus* (Jian and Wu, 2003). A study on rainbow trout with feeding of 1% aqueous extract powder of ginger roots for 3 weeks showed enhanced phagocytosis, respiratory burst activity, and total protein level (Dugenci et al. 2003).

Picrorhiza kurroa has been effectively used as an anti-stress compound for shrimps (Citarasu et al. 1998) and *Ocimum sanctum* positively influenced the immunostimulatory effects, enhanced the antibody response and disease resistance in *Oreochromis mossambicus* against *Aeromonas hydrophila* infection (Logambal et al. 2000). *Curcuma longa* and *Allium sativum* enriched diet also increased the serum bactericidal activity and phagocytosis in rohu *Labeo rohita* (Sahu et al. 2007). Superoxide anion production, serum lysozyme level, and serum bactericidal level were enhanced in fingerling, rohu *L. rohita* fed with diet enriched with *Achyranthus* at different doses (Rao et al. 2006). Application of immunostimulants such as plant extracts significantly enhances the phagocytic activity in various fish (Gopalakannan and Arul 2006; Venkatalakshmi and Michael 2001; Sharifpour 1997; Logambal et al. 2000; Logambal and Michael 2001; Chakrabarti and Rao 2006; Rao and Chakrabarti 2005; Kim et al. 1999).

Recently, a growing interest has emerged in using herbs in animal feeds by both researchers and feed companies. Herbs have been used in various countries to control shrimp and fish diseases. The non-specific immune functions such as bacteriolytic activity and leukocyte function were improved by mixtures of chosen Chinese herbs incorporated in shrimp and fish diet (Chansue et al. 2000). Immanuel et al. (2004) reported that shrimp *Penaeus indicus* juveniles fed with seaweed extracts were protected from *Vibrio parahaemolyticus*. Butanolic extract of *Withania somnifera* through Artemia enriched diet successfully controlled *V. parahaemolyticus* and *V. damsela* infection in shrimp (Praseetha 2005). Ethanol extract with polyvinylpyrolidone (PVP) from *Clinacanthus nutans* enriched diet protect shrimp from yellow head virus (YHV) infection (Direkbusarakom et al. 1996). *Salmonid rhabdovirus* and viral haemorrhagic septicaemia virus (VHSV) could be successfully controlled by using compounds, such as oleuropein (Ole) obtained from *Olea europaea* leaf (Micol et al. 2005).

Rainbow trout fed with ginger (*Zingiber officinale*) extract had significantly higher extracellular activity of phagocytic cells in blood (Dugenci et al. 2003). On the other hand, Scutellaria containing diet significantly inhibited extracellular superoxide anion production in the same fish (Yin et al. 2006). Production of extracellular superoxide anion was increased in trout fed with nettle and mistletoe extracts (Dugenci et al. 2003). *Stella aquatica*, *Impatiens biflora, Oenothera biennis, Artemisia vulgaris,* and *Lonicera japonica* exhibited potentional anti-bacterial and anti-viral properties in fish (Shangliang et al. 1990). Abutbul et al. (2004) used *R. officinalis* containing diet to treat *Streptococcus* infection in tilapia (*Oreochromis sp.*). *Ocimum sanctum* leaf extract contains water soluble phenolic compounds, and various other constituents such as eugenol, methyl eugenol, and caryophyllene that might act as a potential immunostimulant (Chopra et al. 1956). Similar immunostimulatory effect has been observed in *O. mossambicus* administered with azadirachtin, a triterpenoid derived from the seed kernel of *A. indica* (Logambal and Michael 1997). The use of natural products including plant extracts in the treatment of Epizootic Ulcerative Syndrome (EUS) (Campbell et al. 1998), lymphocystis disease virus (LDV) (Harikrishnan et al. 2010a), and some parasitic diseases like myxobolasis, trichodinosis, gyrodactylosis, argulosis, scuticocliates, etc., in farmed tropical freshwater fish has been reported (Harikrishnan et al. 2010 b,c). Medicinal plants contain a number of bioactive compounds e.g., glycyrrhizin (GL) and its aglycon glycyrrhetic acid (GA), liquiritin (LQ), liquiritin apioside (LA), isoliquiritin (IL) and glabridin (GLAB), as well as several active components such as polysaccharides, alkaloids and/or flavonoids (Cinatl et al. 2003).

The herbal active compounds may inhibit or block the transcription of the virus to reduce its replication in the host cells and enhance the innate immunity (Citarasu 2010). Glycyrrhizin is a glycosylated saponin, containing one molecule of glycyrretinic acid, which has anti-inflammatory and anti-tumor activities, mediated by its immunomodulatory activities (Zhang et al. 1990). Yellowtail treated orally with glycyrrhizin showed increased protection against *Edwardsiella seriola* infection, although the lysozyme activity of blood and phagocytic activities of macrophages were not enhanced (Edahiro et al. 1991). Kim et al. (1998) reported that *in vitro* treatment with glycyrrhizin enhanced the respiratory burst activity of macrophages and the proliferative responses of lymphocytes from rainbow trout. A number of plant active compound substances such as saponin (Ninomiya et al. 1995), glycyrrhizin (Jang et al. 1995), aloe (Kim et al. 1999), and azadirachtin (Logambal and Michael 2001; Harikrishnan et al. 2009a, 2009b) have been reported to enhance the innate immunity in fishes. Oral administration of Quil A saponin increased leucocyte migration in yellowtail (Ninomiya et al. 1995).

Spiralina, acid peptide fractions from fish protein hydrolysate, were also reported in tilapia, *O. niloticus* (Park and Jeong 1996), channel catfish (Duncan and Klesius 1996), and Atlantic salmon (Gildberg et al. 1996). Diet with glycyrrhizin enhanced the complement activity and increased the resistance against experimental infection with *Edwardsiella seriola* in yellowtail *Seriola quinqueradiata* (Edahiro et al. 1990). Oral administration of *Quillaja saponin* enhanced the immunity of yellowtail (Ninomiya et al. 1995) and *Astragalus* polysaccharides (APS) component obtained from *Astragalus* membranaceus root can profoundly affect the immune system and therefore have potential as immunomodulators. Quil-A, a fraction from *Quillaja saponaria*, enhanced serum bactericidal activity (Grayson et al. 1987). Feeding with *Catharanthus roseus* plant extract enhanced the immune response of *L. rohita* (Nguyen et al. 2002). Therefore, active compounds and chemical constituents of medicinal plants show positive effects on fish and shellfish in innate and adaptive immune system. Different herbal active compounds and products of herbals such as anthraquinone, essential oil, herbal epimedii, immunplus, polysaccharides, saponin, and azadirachtin act as immunostimulants and have been used individually and combined to control fish and shellfish diseases.

The herbal immunostimulants from *Emblica officinalis*, *Cynodon dactylon*, and *Adathoda vasica* have improved the immune system and reduced microbial infection in goldfish *Carassius auratus* (Minomol 2005). Methanolic extracts of *Ocimum sanctum, Withania somnifera*, and *Myristica fragrans* significantly improved the immune parameters such as phagocytic activity, serum bactericidal activity, albumin– globulin (A/G) ratio, and leukocrit in *Epinephelus tauvina* against *Vibrio harveyi* (Sivaram et al. 2004). *Cynodon dactylon, Aegle marmelos, Tinospora cordifolia, P. kurooa, and Eclipta alba* mixed methanolic extracts containing diet protected against white spot syndrome virus (WSSV) infection and better performance of hematological, biochemical, and immunological parameters of shrimps (Citarasu et al. 2006). Methanolic herbal extracts of *Solanum trilobatum, Andrographis paniculata,* and *Psoralea corylifolia* enriched Artemia reduced the vibrio load and inoculation with *P. aeruginosa*, *S. aureus,* and *S. typhi* (Citarasu 2000; Citarasu et al. 2003 a, b).

Feeding tilapia with two Chinese medicinal herbs (*Astragalus membranaceus* and *Lonicera japonica*) alone or in combination significantly enhanced phagocytic and respiratory burst activity of blood phagocytic cells (Ardo et al. 2008). They had a moderate effect on the plasma lysozyme level and no effect on plasma total protein and total immunoglobulin level. Both herbs reduced the mortality following *Aeromonas hydrophila* infection. The lowest mortality was observed in the group fed with the combination of both herbs and boron (Ardo et al. 2008). Combination of the herbs and boron could even more increase the survival rate of infected fish. There is a growing interest in using medicinal herbs as immunostimulants in aquaculture. In common carp (*Cyprinus carpio*) and large yellow croaker (*Pseudosciena crocea*) respiratory burst activity of phagocytic cells and plasma lysozyme activity were significantly increased after feeding with a ration containing a mixture of *Astragalus membranaceus* and *Angelica sinensis* (Jian and Wu 2003, 2004). Similar results have been reported about experiments on various fish species, e.g. rainbow trout (*Oncorhynchus mykiss*), Indian major carp (*Catla catla*) and Mozambican tilapia (*Oreochromis mossambicus*) (Dügenci et al. 2003; Dey and Chandra 1995; Logambal and Michael 2000). Thus, *Astragalus* and *Lonicera* extracts and boron supplementation added to fish feed can act as immunostimulants and enhance the immune response and disease resistance of cultured fish. Monkey head mushroom, *Hericium erinaceum*, enriched diet fed to olive flounder *Paralichthys olivaceus* infected with *Philasterides dicentrarchi* afforded protection by enhancing the innate immune response (Harikrishnan et al. 2011b).

In the infected fish, the scuticocidal activity, respiratory burst activity, and phagocytic activity did not significantly change at any time when fed with 0.01% *H. erinaceum.* However, on being fed with 0.1% and 1.0% enriched diets, the chosen immune parameters significantly increased from week 1 to week 4 when fish were challenged with parasites. The lysozyme activity significantly increased from weeks 1 to 4 in the infected group than in the control. The cumulative mortality was 90% in control group, whereas, it was 45% and 30% when fed with 0.1% and 1.0% enriched diets, respectively (Harikrishnan et al. 2011b). Thus, feeding with 0.1% and 1.0% enriched diet enhances the immune parameters and affords protection from *P. dicentrarchi* infection in olive flounder.

Rainbow trout fed with diets containing aqueous extracts of mistletoe (*Viscum album*), nettle (*Urtica dioica*), and ginger (*Zingiber officinale*) showed an enhanced extracellular respiratory burst activity ($P < 0.001$) compared to the control group (Dügenci et al. 2003). Especially, the rainbow trout fed with a diet containing 1% aqueous extract of powdered ginger roots for three weeks exhibited a significant non-specific immune response. Phagocytosis and extracellular burst activity of blood leukocytes were significantly higher in this group than those in the control group. All plant extracts added to fish diet increased the total protein level in plasma except 0.1% ginger (Dügenci et al. 2003). The highest level of plasma proteins was observed in the group fed with 1% ginger extract containing feed (Dügenci et al. 2003). Thus, the administration of herbal extracts or their products at various concentrations through oral (diet) or injection routes enhance the innate and adaptive immune response of different freshwater and marine fish and shellfish against bacterial, viral, and parasitic diseases.

Fish treated with or exposed to various immunostimulants have revealed enhanced activities of both humoral and cellular components, although the influencing components varies based upon the nature of substance, fish species and routes of exposure. The specific immunity through augmentation of antibody production is influenced by exposure of fish to immunostimulants.

Conclusions and future trends

The review presents the benefits of use of immunostimulants in aquaculture. Although immunostimulants have wide advantages over vaccines or chemotherapeutics, they are not as effective as many chemotherapeutics, particularly at the time of outbreaks. Ultimately, it is likely that no single compound or method will provide a complete solution to the disease problem in aquaculture system. Hence, a multidimensional comprehensive approach would be of much advantage under practical conditions of disease outbreaks. These include: use of immunostimulants, manipulation of the rearing environment, and use of probiotics and antimicrobial peptides or proteins. For a disease situation that is inevitable in any culture operation, the combination of vaccination and immunostimulation may be of much help. It is also not surprising that many uncertainties remain regarding the use of immunostimulants in aquaculture. The complex mechanism of non-specific stimulation, and interacting with the specific immune system to render enhanced protection need to be further explored. Their efficacies, particularly to combat parasitic or viral disease conditions of fish, are worth studying. A comprehensive research effort should be diverted to study their efficacy on large-scale field applications. The research efforts should also be directed to characterize the receptors on the target cells for recognition of immunostimulatory substances.

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