3. Use of immunostimulants in shrimp culture: An update

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Abstract. Different approaches are used to prevent and control diseases in aquaculture. Immunostimulation is one method that is gaining popularity and is considered a promising development in aquaculture. Immunostimulants were found to be effective in enhancing parameters of non-specific immunity and resistance to diseases of fish and crustaceans. However, some issues raised on the use of immunostimulants pertains to the short-term nature of immune indices used during efficacy evaluation, possible detrimental effects during long-term administration, or self-damage due to unregulated production of immune effectors. Further testing in large-scale production units has been recommended. This chapter presents the various types and sources of immunostimulants commonly used in aquaculture and in shrimp culture in particular. The effects of each immunostimulant vary depending on its source, dose, route of administration, length of exposure, and the species to which it is administered.

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Introduction

Shrimp farming has been practiced in many countries for decades. However, the increasing global demand for shrimp has led to the intensification of shrimp culture that is one of the major contributory factors in the emergence of infectious diseases. The causative agents of infectious diseases in shrimp are mainly viruses and bacteria. *Vibrio* spp. contribute to a number of epizootic diseases causing serious problems in shrimp culture and are responsible for shrimp mass mortalities (Nash et al. 1992). Vaccination in fish and immunostimulation both in shrimp and fish for disease management are being increasingly appreciated in the aquaculture industry. To fight against diseases, fish larvae and aquatic invertebrates rely on their innate immune system (Kurtz & Franz 2003; Little & Kraaijeveld 2004) consisting of cellular and humoral components that cooperate to recognize and eliminate foreign microorganisms and pathogens (Bachère 2003).

Approaches employed to prevent/control fish diseases include the use of vaccines, chemotherapeutics, and immunostimulants. Immunostimulants and vaccines are used as prophylactic interventions. They are not recommended for use when disease is already present in the host. Chemotherapeutants, on the other hand, are effective for treatment of diseases that has already occurred, but they are often subject to abuse and leave residues that affect product quality, among other issues. Vaccination is the most reliable method, however, there are no effective vaccines against most viral diseases. In crustaceans, vaccination is the subject of ongoing investigations but an efficacious product is not expected to be available for commercial application in the immediate future. Immunostimulants may compensate for the limitations of chemotherapeutants and vaccines. They have modulatory effects on the immunocompetence and disease resistance of fish and crustaceans. When combined with vaccination, immunostimulation may also increase the potency of vaccines. Thus, the use of immunostimulants for health management is a promising new development in shrimp aquaculture (Jadhav et al. 2006; Barman et al. 2013).

The shrimp immune system

The immune system of shrimp is relatively primitive/less well-developed when compared to that of fish. Invertebrates such as shrimp lack an antibody-based vertebrate-type adaptive immune system. Thus, non-specific...
immune responses are considered to play crucial roles in the shrimp defence against pathogens. The induction of innate or non-specific immunity is therefore a potentially better tool to combat pathogens present in the culture system compared to vaccination, for example. Phagocytosis, encapsulation, nodule formation, cytotoxicity, lectins, antimicrobial proteins or peptides, and prophenoloxidase (proPO), have been confirmed to perform essential roles in the immune defence of shrimps (Soderhall & Cerenius 1992; Iwanaga & Lee 2005; Amar & Almendras 2010). In recent years, many studies conducted in crustaceans and insects provide evidence supporting the existence of specificity and memory mediated by highly variable receptors in the immune system in these taxonomic groups (Rowley & Powell, 2007). However, this phenomenon was not found to be universal in the crustacean species studied and appears to be pathogen- and host-specific. Moreover, it is not clear at the moment how this specific immune response can provide practical benefits for health improvement in shrimp aquaculture.

What are immunostimulants?

Immunostimulants are substances that activate the immune system of animals to make them more resistant to microbial infections (Raa 1996). The definition has been expanded somewhat to include live organisms or their products that have an impact on the immune system. The use of immunostimulants does not generate a specific response to a certain antigen, but causes an overall response that hastens recognition and elimination of a broad range of infectious agents and foreign substances (Campos et al 1993; Secombes 1994; Sordello et al 1997). Two categories of conserved molecular patterns are recognized by the innate immunity through the various pattern recognition receptors (PRRs): (1) non-self or pathogen-associated molecules; and (2) molecules generated as a result of damage to the host's own tissues signalling danger to the immune system (Matzinger 1998; Medzhitov & Janeway 2002). Pattern recognition is the first step in innate immunity. Immunostimulants, like the presence of infection, are sensed by the various PRRs. So far, 11 types of pattern recognition receptors have been identified in shrimp namely, β-1,3-glucanase-related proteins, β-1,3-glucan-binding proteins, c-type lectins, scavenger receptors, galectins, fibrinogen-related proteins, thioester-containing Down syndrome cell adhesion molecules, serine protease homologs, trans-activation response RNA-binding protein, and Toll-like receptors. Aside from pattern recognition, these PRRs have different binding specificities and effector functions (Wang & Wang 2013). Immunostimulation can strengthen the immune system of farmed aquatic animals and increase their resistance to pathogens during exposure to stress,
such as handling, crowding, sampling, transport, vaccination, during reproduction, and also during the larval stages when high levels of mortality occur. Some commercially available immunostimulants are shown in Table 1.

Sources of immunostimulants

Bacterial preparations

*Vibrio* - is a curved rod-shaped gram-negative bacteria. *Vibrio anguillarum* is a very efficient vaccine for salmonid fish (Johnson et al 1982; Norqvist et al 1989; Sakai et al 1995). The immunostimulant effects of inactivated *Vibrio* cells have been documented in shrimp (Itami et al 1989, 1991, 1992; Horne et al 1995; Teunissen et al 1998; Pereira et al 2009; Powell et al 2011; Lin et al 2013). The authors reported that injection or immersion of shrimp in *Vibrio* bacterin resulted in reduced mortality suggesting immunostimulation by the ”vaccine” as invertebrates do not have an efficient specific immune response. The immunostimulation is mediated by the PRRs that recognize and bind stimulatory components in bacteria. *Vibrio* bacterin may therefore act as an immunostimulant since non-specific immune cells such as phagocytic hemocytes are activated (Sakai 1999). A *Vibrio harveyi* bacterin was also able to protect *P. monodon* against WSSV infection (George et al 2006).

Lipopolysaccharide (LPS) - also known as lipoglycans or endotoxins are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond. They are the major component of the outer membrane of gram-negative bacteria, are responsible for the structural integrity of the bacteria, and stimulate strong immune responses in recipient hosts. The lipid component in association with the main polysaccharide is responsible for the biological activities of LPS (Rietschel et al 1993). The immunostimulant effects of LPS have been demonstrated in fish (Salati et al 1987; Neumann et al 1995; Nya et al 2010) and shrimp (Vargas-Albores et al 1998; Newman 2000; Felix 2005). LPS, at low doses, improves disease resistance and acts as a prophylactic agent (Noworthy 1983). Rungrassame et al., (2013) found that shrimp fed LPS-containing diet exhibited significantly higher survival rates when exposed to *V. harveyi* than those fed the normal diet. Some crucial immune-related transcripts such as anti-lipopolysaccharide factor 3 (ALF3), C-lectin and mucin-like peritrophin were also induced in shrimp digestive tracts by the LPS supplement. These findings led the authors to conclude that LPS supplement is a promising candidate to increase disease resistance in black tiger shrimp farming.
Live bacteria: probiotics as immunostimulants – A number of studies revealed that the supplementation of probiotic bacteria and commercial probiotics in feed or any sort of inclusion can boost the cellular and humoral components of the innate immune system in several species of fish and shellfish including salmonids and shrimps (Gullian et al 2004; Panigrahi et al 2004, 2005, 2007; Song et al 2006; Balcazar et al 2006, 2007a, 2007b; Rodriguez et al 2007; Pais et al 2008; Goncalves et al 2011; Biswas et al 2013; Cerezuela et al 2013; Meena et al 2013; Perez-Sanchez et al 2013; De et al 2014). Immunostimulation by *Bacillus* S11 bacteria increased phagocytic activity in *Penaeus monodon* (Rengpipat et al 2000), whereas the administration of *Lactobacillus plantarum* stimulated phenoloxidase and superoxide dismutase activities leading to enhanced clearance efficiency of *Vibrio alginolyticus* in *Litopenaeus vannamei* (Chiu et al 2007).

Complex carbohydrates

Glucans - are natural biomolecules with immunomodulatory activity. In vertebrates, glucans modulate the immune response through the macrophage and dendritic immune cells. The amount or dose of glucans does not determine its effectiveness as sources, processing, size and uniformity of glucan particles are the actual determinants of its efficacy (www.betaglucan.com). Enhancement of immune responses and protection against infectious agents by treatment with beta-glucans were observed in fish (Robertsen et al 1990; Raa et al 1992; Engstad & Robertsen 1993; Sahoo & Mukherjee 2002; Selvaraj et al 2005; Rodriguez et al 2009; Skov et al 2012; Meena et al 2013; Vetvicka et al 2013) as well as in shrimp (Song & Hsieh 1994; Sung et al 1994; Chang et al 2000, 2003; Wang et al 2008). In shrimp aquaculture, the most widely used immunostimulants are glucan-based (Nahavandi et al 2010).

Prebiotics as immunostimulants – prebiotics are indigestible fibers that increase beneficial gut commensal bacteria resulting in improvements of the host’s health (Song et al 2014). Prebiotics such as fructooligosaccharide (FOS), mannan oligosaccharide (MOS), inulin, or β-glucan are called immunosaccharides because they directly enhance innate immune responses including phagocytic activation, neutrophil activation, stimulation of the alternative complement system, and increased lysozyme activity (Song et al 2014).
**Nutritional factors**

**Vitamins** - dietary supplementation of vitamins such as A, C, and E is effective in increasing the immunocompetence and disease resistance of fish (Waagbo et al 1992; Mulero et al 1998; Verlhac et al 1998; Ortuno et al 1999, 2000; Cuesta et al 2001; Sahoo & Mukherjee 2002; Tewary & Patra 2008). Vitamins A, C, and E in combination with dietary carotenoids were found to enhance the complement, lysozyme, and phagocytic activities in fish (Amar et al 2001). Improved immune function and resistance to pathogens were also demonstrated in several species of penaeid shrimp that received vitamins A, C, and E supplements (Merchie et al 1998; Lee & Shiau 2002, 2003, 2004; Nahavandi et al 2010; Sivakumar & Felix 2011).

**Carotenoids** - the role of carotenoids in the immune response of animals has been established (Chew 1993). Carotenoids improve lymphocyte blastogenesis, lymphocyte cytotoxicity activity and stimulate the production of certain cytokines. Carotenoids also stimulate the phagocytic and bacterial killing ability of neutrophils and macrophages. In fish, dietary carotenoids have been reported to heighten immune responses and increase disease resistance (Christiansen et al 1995; Tachibana et al 1997; Amar et al 2000, 2001, 2004, 2012). Similarly in shrimp, supplementation of dietary carotenoids resulted in increased resistance to stress, salinity shock, and increased the antioxidant response before and after viral infection (Merchie et al 1998; Nahavandi et al 2010; Pacheco et al 2011).

**Trace elements** - deficiencies in trace elements with antioxidant function (cofactors of numerous enzymes) such as zinc (Zn), copper (Cu), manganese (Mn) and selenium (Se) have been reported to depress immunity in animals. Deficiency in Zn, Mn and Cu were found to reduce the natural killer-like activity of leukocytes and antibody production in fish (Kiron et al 1993; Paripatananont and Lovell 1995a; Inoue et al 1998). Dietary Zn and Se protected fish against *Edwardsiella ictaluri* infection (Paripatananont and Lovell 1995b; Wang et al 1997). Enhanced lysozyme activity and total immunoglobulin level, and improved antibody production and survival were exhibited by rainbow trout supplemented with amino acid-chelated trace elements (Apines-Amar et al 2004). Likewise, supplementation of Cu in tiger shrimp resulted in improved growth, total hemocyte count, and superoxide anion production (Lee & Shiau 2002). A similar improvement of immune responses was observed in white shrimp upon dietary supplementation with Zn (Lin et al 2013).
Animal sources/extracts

Chitin/Chitosan - chitin is one of the most abundant polysaccharides in nature, and a common constituent of insects, crustacean exoskeletons, and fungal cell walls (Esteban et al 2000). Chitosan is the product of alkaline deacetylation of chitin obtained from crustacean shells. Injection, immersion, and oral administration of chitin and chitosan have been reported to protect fish and shrimp against bacterial infection (Sakai et al 1992; Siwicki et al 1994; Anderson et al 1995; Wang and Chen 2005; Huxley et al 2010, 2011).

Plant sources/extracts

Seaweeds - plants including seaweeds are rich sources of safe and inexpensive chemical compounds. Products derived from plants exhibit various activities and are utilized as anti-stress agents, growth promoters, appetizers, tonics, immunostimulants and antimicrobials (Citarasu et al 2002; Immanuel et al 2004). Red seaweeds contain biologically active sulphated, galactose-based polysaccharides, and show antibacterial and antiviral activities (Renn 1997; Rajasulochana et al 2009; Wongprasert et al 2013). Rudtanatip et al (2014) reported that sulfated galactans from Gracilaria fisheri elicits anti-WSSV activity by binding to viral proteins which are essential structures for viral attachment to host cells. Fucoidan, a sulfated polysaccharide from brown algae (e.g., Fucus, Laminaria) activated the immune system and increased the resistance to viral infection in penaeid shrimp (Nahavandi et al 2010). An extract from Gracilaria tenuistipitata exhibited protection in shrimp against WSSV as evidenced by the higher survival rate and heightened immune responses (Lin et al 2011). Likewise, white shrimp Litopenaeus vannamei immersed in seawater containing brown seaweed Sargassum hemiphyllum var. chinense powder or its extract showed improved immunity and resistance against Vibrio alginolyticus and WSSV infections (Huynh et al 2011).

Herbs – the benefits of herbs such as onion, ginger, and garlic on human health are well-recognized. Enhanced immune responses and resistance to diseases were recently demonstrated in fish and shrimp supplemented with either onion, ginger, or garlic (Dugenci et al 2003; Nya & Austin 2009, 2011; Chang et al 2012; Apines-Amar et al 2012, 2013; Aruvasu et al 2013; Haghghi & Rohani 2013; Talpur et al 2013; Kanani et al 2014). Elevated expression of the immune-related genes Penaeidin, Crustin, Lysozyme, Toll-receptor, and tumour necrosis factor was detected in kuruma shrimp upon stimulation with garlic (allicin) extract (Tanekhy and Fall 2015). The methanolic extracts of different herbal medicinal plants like Cyanodon
dactylon, Aegle marmelos, Tinospora cordifolia, Picrorhiza kurrooa and Eclipta alba were found to be effective against white spot syndrome virus (WSSV) infection in shrimp (Citarasu et al 2006).

Hormones/cytokines

Growth hormone (GH) – is a peptide hormone (also known as somatotropin or somatropin) that stimulates growth and cell reproduction in humans and other animals. Growth hormone has numerous beneficial effects such as promotion of lipolysis, gluconeogenesis, increase in protein synthesis and stimulation of immune responses among others. The influence of GH on the immune responses of fish have been studied (Harris & Bird 2000; Yada et al 2002, 2004, 2007; Peterson et al 2007). A recombinant bovine growth hormone was found to enhance growth and immunity in shrimp larvae (Kang et al 2005).

Lactoferrin (LF) – formerly known as lactotransferrin (LTF), is a glycoprotein and a component of the innate immune system. Lactoferrin influences the growth and proliferation of various pathogens such as bacteria, viruses, protozoa, or fungi (Kirkpatrick et al 1971). LF affects mainly the respiratory burst and natural cytotoxic activities in fish (Esteban et al 2005). The antimicrobial activities of lactoferrin have been reviewed by Adlerova et al (2008). In crustaceans, dietary administration of bovine lactoferrin enhanced immune indices and resistance against Aeromonas hydrophila challenge in Macrobrachium rosenbergii (Chand et al 2006).

Synthetic sources

Levamisole - known under the tradename Ergamisol, was originally synthesized as an anti-helminthic and has been widely used as an immunomodulator belonging to imidazothiazole derivatives. Levamisole has been used in humans to treat parasitic worm infections, and has been studied in combination with other forms of chemotherapy for colon cancer, melanoma, and head and neck cancer. Oral administration of levamisole resulted in enhanced immunity and improved resistance to diseases in fish (Siwicki 1987, 1989; Siwicki et al 1990; Kajita et al 1990; Sivakumar & Felix 2011). Oral administration of levamisole likewise resulted in increased protection against bacterial infection in tiger shrimp (Huxley et al 2010, 2011).

CpG Oligodeoxynucleotide (CpG ODN) – are short single-stranded synthetic DNA molecules containing cytosine triphosphate deoxynucleotide
"C" followed by a guanine triphosphate deoxynucleotide "G" with phosphodiester "p" linking the two nucleotides. CpG motifs are considered pathogen-associated molecular patterns (PAMPs). CpG motifs are more prevalent in bacterial DNA, and when unmethylated, function as immunostimulants (Krieg et al 1995; Weiner et al 1997). In contrast, the DNA of vertebrates are deficient in CpG motifs and highly methylated. Recently, nucleotides have received much attention as potential immunomudulators. Li & Gatlin (2006) has reviewed nucleotide nutrition in fish. The beneficial influences of oral administration of nucleotides on immune functions, vaccine efficiency or disease resistance have been demonstrated in fish (Ramadan et al 1994; Burrells et al 2001; Sakai et al 2001; Li & Gatlin 2006). It must be pointed out that the nucleotides in the diets of farmed aquatic animals are added to provide the supply of nucleotides (as nutrient) necessary to enhance growth and survival especially under conditions of high requirement or stressful events, whereas CpG is a PAMP and is a non-nutrient nucleotide. Nevertheless, even nucleotides without CpG motifs can be sensed as non-self molecules, such as in cases of autoimmune reactions. Increased immune indices have been reported in shrimp injected with ODNs with or without CpG motifs (Amar & Faisan 2012).

Table 1. Some currently available commercial immunostimulants.
Effects of immunostimulants

Upregulated immune indices such as total hemocyte count, respiratory burst, phenoloxidase activity, phagocytic activity, agglutination titer, lysozyme and SOD activities, etc. have been reported in many immunostimulation studies (Table 2). Growth-promoting activity was also found with some immunostimulants (Song & Hsieh 1994). Growth enhancement could result from improved disease resistance due to immunostimulant supplementation. Shrimp fed with peptidoglycan-supplemented feed also showed better growth and feed conversion rates than those fed a normal diet (Boonyaratpalin et al., 1995). Sung et al (1994) demonstrated that black tiger shrimp grew faster with glucan immersion which could be attributed to the higher activity of glucan delivered by immersion compared to oral administration.

Table 2. Various types of immunostimulants studied in shrimp.

<table>
<thead>
<tr>
<th>Immunostimulants</th>
<th>Effects on host</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>↑ growth, survival, THC, superoxide ion, PO activity</td>
<td>Lee &amp; Shiau 2002</td>
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<td></td>
<td>↑ proPO activity</td>
<td>Sivakumar &amp; Felix 2011</td>
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<tr>
<td>Vitamin C + Cu</td>
<td>↑ growth, respiratory burst, prevented tissue Cu accumulation</td>
<td>Lee &amp; Shiau 2003</td>
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<tr>
<td>Vitamin E</td>
<td>↑ growth, THC, SOD activity ↑ TBA values</td>
<td>Lee &amp; Shiau 2004</td>
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<tr>
<td>Copper (Cu)</td>
<td>↑ growth, THC, superoxide production</td>
<td>Lee &amp; Shiau 2002</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>↑ growth, survival, THC, PO activity, PA, alkaline phosphate and SOD activities</td>
<td>Lin et al 2013</td>
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<tr>
<td>Chitin</td>
<td>↑ proPO activity</td>
<td>Sivakumar &amp; Felix 2011</td>
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<tr>
<td></td>
<td>↑ THC, respiratory burst, PO activity, PA disease resistance</td>
<td>Wang &amp; Chen 2005</td>
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<tr>
<td>Chitosan</td>
<td>↑ THC, respiratory burst, PO activity, PA disease resistance</td>
<td>Wang &amp; Chen 2005</td>
</tr>
<tr>
<td>Thraustochytrid-derived meal</td>
<td>↑ THC, PO activity SOD activity, bactericidal activity, and disease resistance</td>
<td>Nonwachai et al 2010</td>
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<tr>
<td>Levamisole</td>
<td>↑ proPO activity</td>
<td>Sivakumar &amp; Felix 2011</td>
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<tr>
<td><strong>Oligodeoxynucleotide (ODN)</strong></td>
<td>↑ THC, Total protein, hemocyte lysate, agglutination titer, &amp; disease resistance</td>
<td>Amar &amp; Faisal 2012</td>
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<tr>
<td>Sodium alginate</td>
<td>↑ PO activity, respiratory burst, ↑ resistance to <em>V. alginolyticus</em> infection</td>
<td>Cheng et al 2004, 2005</td>
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<tr>
<td></td>
<td>↓ GPx activity</td>
<td>Cheng et al 2005</td>
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<td></td>
<td>↑ SOD activity, expressions of betaGBP, PE, cyt-SOD, PR-5, and SWDP, ↓ respiratory burst</td>
<td>Liu et al 2006</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>↑ PO &amp; SOD activities, bacterial clearance</td>
<td>Chiu et al 2007</td>
</tr>
<tr>
<td>β-1,3-glucan (<em>Schizophyllum commune</em>)</td>
<td>↑ PO activity, superoxide anion, SOD activity &amp; survival against WSSV</td>
<td>Chang et al 2003</td>
</tr>
<tr>
<td></td>
<td>↑ haemocyte phagocytosis, superoxide anion production, &amp; survival</td>
<td>Chang et al 2000</td>
</tr>
<tr>
<td>β-glucan (<em>MacroGard</em>)</td>
<td>↑ mRNA expression of lysozyme &amp; SOD</td>
<td>Wang et al 2008</td>
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<td></td>
<td>↑ growth and disease resistance</td>
<td>Song &amp; Hsieh, 1994</td>
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<tr>
<td>Bacterin+Glucan</td>
<td>↑ survival against white spot syndrome virus, vibriocidal activity</td>
<td>Devaraja et al 1998</td>
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Table 2. Continued

<table>
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<tr>
<th>Compound</th>
<th>Effect</th>
<th>Reference</th>
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<tr>
<td>Bacterial LPS</td>
<td>$\uparrow$ immune gene expression, $\uparrow$ disease resistance</td>
<td>Rungrassamee et al 2013</td>
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<td></td>
<td>$\uparrow$ protection against WSSV</td>
<td>Newman 2000</td>
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<td></td>
<td>$\uparrow$ protection against <em>V. parahaemolyticus</em></td>
<td>Felix 2005</td>
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<td></td>
<td>$\uparrow$ proPO</td>
<td>Vargas-Albores et al 1998</td>
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<td></td>
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<td>Felix 2005</td>
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<tr>
<td>Bacterial Genomic DNA Extract</td>
<td>$\uparrow$ THC, PTP and HLAT</td>
<td>Amar &amp; Faisan 2012</td>
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<td>Peptidoglycan</td>
<td>$\uparrow$ survival against yellow-head baculovirus infection</td>
<td>Boonyaratpalin et al 1995</td>
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<td>MOS</td>
<td>$\uparrow$ growth, PO and SOD activities, resistance to NH$_3$ stress</td>
<td>Zang et al 2012</td>
</tr>
<tr>
<td>Peptidoglycan+MOS</td>
<td>$\uparrow$ growth, THC, respiratory burst activity, protection against WSSV infection,</td>
<td>Apines-Amar et al 2014</td>
</tr>
<tr>
<td>scFOS</td>
<td>$\uparrow$ THC, respiratory burst</td>
<td>Li et al 2007</td>
</tr>
<tr>
<td>Zingerone</td>
<td>$\uparrow$ growth, immunity, and protection against <em>V. aiginolyticus</em></td>
<td>Chang et al 2012</td>
</tr>
<tr>
<td><em>Gracilaria tenuistipitata</em></td>
<td>$\uparrow$ HC, GC, THC, PO &amp; respiratory burst activities, SOD &amp; lysozyme activities, &amp; survival</td>
<td>Lin et al 2011</td>
</tr>
<tr>
<td></td>
<td>$\uparrow$ HC, GC, THC, PO and respiratory burst activities, SOD, GPx &amp; lysozyme activities, &amp; survival</td>
<td>Sirirustanananun et al 2011</td>
</tr>
<tr>
<td><em>Gracilaria fisheri</em></td>
<td>$\uparrow$ immune stimulatory and anti-WSSV activities</td>
<td>Wongprasert et al 2013</td>
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<td></td>
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<td>Rudtanatip et al 2014</td>
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Methods and strategies in using immunostimulants

Immunostimulants can be administered through injection, immersion, or oral administration. Injection and immersion require handling of fish/shrimp or confining them in a small area during application. However, these methods are laborious, time-consuming, and stressful. Injection method is the most cost-effective method for large fish (>10g). Immersion on the other hand, is not as effective as injection but allows mass immunostimulation and is the most cost-effective method for smaller fish (<5g). Oral immunostimulation is a non-stressful method that can be used with any size of fish but requires a large amount of immunostimulant to provide protection. Furthermore, this method is applicable only for fish fed artificial diet.
The outcome of using an immunostimulant is usually determined by the strategy by which it is applied (Bricknell & Dalmo 2005). Continuous use of an immunostimulant may up-regulate the immune system and maintain this status until the immunostimulant is withdrawn, or it may cause adverse effects such as tolerance or immunosuppression. On the other hand, pulse administration (administering immunostimulant-supplemented and non-supplemented diets alternately) oscillate the immune response from a resting level to a heightened response then back to resting, and has been shown to be a better strategy of immunostimulant application.

**Some issues in the use of immunostimulants in shrimp**

Lack of correlation of *in vitro* and *in vivo* studies. Some immunostimulants may enhance the non specific immune response *in vitro* but this does not always result in improved health or increased survival. Some immunostimulants do not show a linear dose/effect relationship; they could be effective at a certain optimum concentration but have no effect or exhibit toxicity at higher concentrations. Consequently, doses determined *in vitro* cannot be directly extrapolated to large-scale production systems. Hence, these immunostimulants may end up being fed at high doses or for long durations resulting in chronic overstimulation and exhaustion of the immune system.

There is lack of unequivocal evidence on the efficacy of some products. Most studies on immune stimulation by microbial polysaccharides claim beneficial effects by improving growth and resistance to pathogen challenge, and/or stimulation of immune responses such as prophenoloxidase, antibacterial, antioxidant, and agglutination activity, and reactive oxygen production. However, these effects are short term, and there are very few studies conducted in large-scale production units on a longer duration. With particular immunostimulants, some studies reported beneficial effects, whereas no positive effects were found by others.

Some immune responses are detrimental. Essentially, the haemocytes perform inflammatory-type reactions such as phagocytosis, haemocyte clumping, production of reactive oxygen metabolites, and the release of microbicidal proteins. Under normal conditions, the inactive form of effector molecules are stored in the hemocytes but are released into the hemolymph through exocytosis and activated upon stimulation by non-self molecules. In the open circulatory system of crustaceans, immune reactions must be localized to avoid self-damage. Some of the responses and reactions that are potentially self-harming include degranulation of hemocytes, cell clumping and hemacytopenia, cytotoxicity at higher doses, depletion of immune
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Effectors, immune exhaustion by repeated immunostimulation, and high energetic cost of regenerating immune components (Smith 2008). With these possible adverse consequences, a rigorous product testing on a large scale over a duration spanning the whole crop cycle is essential. The capacity of the products to upregulate immunity genes should also be considered with the objective of correlating in vitro and in vivo results. Lastly, stimulation that elicits alternative molecules that have no self-harming potential but have direct effect on the pathogens (such as antimicrobial peptides) should be pursued.

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