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REVIEW ARTICLE

Application of immunostimulants in aquaculture: current knowledge and future perspectives

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Abstract

Similar to other industries, aquaculture constantly requires new techniques to increase production yields. Modern technologies and different scientific fields, such as biotechnology and microbiology, provide important tools that could lead to a higher quality and a greater quantity of products. New feeding practices in farming typically play an important role in aquaculture, and the addition of various additives to a balanced feed formula to achieve better growth is a common practice of many fish and shrimp feed manufacturers and farmers. As 'bio-friendly agents', immunostimulants, such as biological factors, probiotics and vitamins, can be introduced into the culture environment to control and kill pathogenic bacteria, as well as to promote growth of the cultured organisms. In addition, immunostimulants are non-pathogenic and non-toxic and do not produce undesirable side effects when administered to aquatic organisms. In this review, we summarize previous studies performed with both traditional immunostimulants and the most promising new generation of immunostimulants, such as polysaccharides, nutrients, oligosaccharides, herbs, microorganisms, prebiotics and different biological factors. This review primarily focuses on their protective efficacies and on what is known concerning their effects on the immune systems of aquatic organisms when delivered *in vivo*.

Keywords: immunostimulant, aquaculture, pathogen, immunity

Introduction

The global aquatic environment has been severely damaged by widespread industrial development. Multiple problems have arisen, especially the deterioration of water quality, and numerous aquatic diseases have become a prominent threat to the healthy development of aquaculture. Antibiotic use is gradually increasing in aquaculture due to the urgent needs of the industry. The use of antibiotics has resulted in several drug residues accumulating within aquatic products, thus raising food safety concerns. In addition, several pathogenic microorganisms have also developed drug resistance. However, many of the current economically disastrous diseases are due to intracellular pathogens, and the production of effective vaccines against these pathogens has not been an easy task. As the demand for an increase in food quality continues and the side effects of antibiotics become an increasing threat to humans, antibiotic use must be eliminated or reduced and replaced with newly developed products. These new products could enhance environmental protection by acting in a manner more conducive to the health of humans and animals (Quesada, Paschoal & Reyes 2013; Arias-Andrés, Mena & Pinnock 2014; Siriyappagouder, Shankar, Naveen, Patil & Byadgi 2014). Vaccines are the most effective method for the prevention and cure of aquatic animal diseases and have been used against several bacterial diseases, such as vibriosis and furunculosis, as well as viral infections. However, vaccinations have not

succeeded in controlling intracellular pathogens. Furthermore, vaccines only control a specific disease, without affecting other diseases. Therefore, it is not possible to control all aquatic animal diseases exclusively through vaccinations. The production of non-toxic, non-polluting and efficient biological agents for use in place of antibiotics has become a global goal in aquaculture research and development.

Immunostimulants are of great interest in health care and have become one of the most active areas of applied medical research. Immunostimulants, which can promote and induce a strong defence response in the host, include polysaccharides, hormones, vitamins, different components of bacteria, biologically active materials, Chinese herbs and synthetic drugs (Mohapatra, Chakraborty, Kumar, DeBoeck & Mohanta 2013; Buchmann 2014; Kasahara & Sutoh 2014; Song, Beck, Kim, Park, Kim, Kim & Ringø 2014; Shrestha, St Clair & O' Neill 2015; Masahiro 1999). Immunostimulants are critical in eliciting immune responses capable of providing complete protection against certain pathogens.

In aquaculture, immunostimulants activate the immune system of aquatic animals and enhance their capacity for disease resistance. Cellular and humoral immunity constitute the specific immune systems of fish. In fish, cellular immunity includes phagocytic cells, neutrophils, natural killer cells and lymphocytes, whereas humoral immunity consists of lysozyme, haemolysin, immunoglobulins and complement molecules. In addition, the existence of cytokines (interferon, interleukin 2, macrophage-activating factors) has also been reported in fish (Sakai 1999; Balcázar, de Blas, Ruizzarzuela, Cunningham, Vendrell & Múzquiz 2006; Aly, Mohamed & John 2008; Aly, Abd-El-Rahman, John & Mohamed 2008; Vasta, Nita-Lazar, Giomarelli, Ahmed, Du, Cammarata, Parrinello, Bianchet & Amzel 2011; Boehm 2012). The non-specific immune system found in crustaceans differs from that in fish. Specifically, the mechanisms of immunostimulation and opsonization in the immune systems of crustaceans are different. According to previous reports, in crustaceans, immunostimulants can increase the phagocytosis of pathogens by activating phagocytic cells in the hemolymph, increase the antibacterial and antiseptic properties of hemolymph, activate the prophenoloxidase system and mediate signal recognition and phagocytosis (Baldmar 2003; Bondad-Reantaso, Subasinghe, Arthur, Ogawa, Chinabut, Adlard, Tan & Shariff 2005; Castex, Lemaire, Wabete & Chim 2010). In fish, immunostimulants enhance the phagocytic capacity of neutrophils and lymphocytes, stimulate the secretion of cytokines from lymphocytes, coordinate cellular and humoral immunity and evoke antibody and complement responses (Jang, Marsden, Kim, Choi & Secombes 1995; Ortuño, Esteban & Meseguer 2000; Qin 2000; Sahoo & Mukherjee 2001; Wang, Yan, Su, Zhou & Shao 2001). Growth rates, survival rates and disease resistance significantly improve after the addition of immunostimulants in aquaculture systems. These effects may be related to the different structure and function of different immunostimulants. The expression of immunostimulant genes *in vivo* in breeding species is expected to improve disease resistance, inhibit the spread of disease and cultivate disease-resistant varieties for use in aquaculture.

New immunostimulants could provide a safer alternative to antibiotic use. However, many aspects of the immune systems of aquatic organism are still unknown, and we are far from understanding which immune mechanisms convey protection against many resident pathogens. As more sources of immunostimulants for marine organisms become available, immunostimulants will likely be industrialized using genetic engineering, ultimately replacing antibiotics as feed additives. This technology also has the potential to improve the safety and reliability of aquaculture products.

The classification of immunostimulants

The use of immunostimulants has only recently became available in aquaculture, but their development is occurring rapidly, with an increase in the types available and the scope of their application. The main immunostimulants applied in aquaculture are as follows: polysaccharides, nutrients, oligosaccharides, herbs, antibacterial peptides and microorganisms.

Effect of polysaccharides on aquatic animals

Polysaccharides are important biological molecules and are present in plants, animals and microbes. Polysaccharides have been considered a broad-

spectrum immunostimulant since the 1960s. In general, applied research on polysaccharides in aquaculture can be divided into research on three delivery types: added to the animal's pond, injected into the organism or used as a feed additive for animals. These delivery mechanisms are used to explore their effects on immune defence. Polysaccharides are widely used in aquatic animal breeding facilities as feed additives because this delivery method is simple and suitable for large-scale application.

β-Glucans

Glucans mainly exists in the cell walls of bacteria and yeast and possess a helical or spiral backbone due to specific intramolecular hydrogen bonding. Glucans are recognized by the immune system of aquatic animals as a foreign molecular pattern. The application of glucans has been extensively studied in aquatic animals, and findings indicate that β-glucans promote growth in certain types of aquatic animals. López, Cuzon, Gaxiola, Taboada, Valenzuela, Pascual, Sánchez and Rosas (2003) demonstrated that β-glucans increase the growth rate of *Litopenaeus vannamei* juveniles when added to feed. Additionally, Ai, Mai, Zhang, Tan, Zhang, Xu and Li (2007) demonstrated that a basal diet supplemented with 0.09% β-glucans significantly enhances the growth of large yellow croakers. The mechanism of how β-glucans promote the growth of aquatic animals is not clear; however, there are two separate hypotheses concerning their function in aquaculture. López *et al.* (2003) hypothesized that *L. vannamei* produces glucanase in its digestive gland. Glucanase decomposes β-glucans to generate energy, which can subsequently be used for protein synthesis, thus promoting the growth of *L. vannamei*. Dalmo and Bogwald (2008) argued that β-glucans improve intestinal immune responses, increasing disease resistance in aquatic animals and promoting their growth. Many studies indicate that β-glucans promote the growth of aquatic animals in relation to the amount included in the feed, the duration of feeding, culture temperature and species being raised.

β-Glucans activate phagocytic cells in fish, improving phagocytosis and the ability of the cells to kill pathogenic organisms (Cook, Hayball, Hutchinson, Nowak & Hayball 2003; Ai *et al.* 2007). They also increase lysozyme and complement activity (Ai *et al.* 2007) in the serum of fish and enhance the resistance of fish to pathogens in the water (Sahoo & Muldaerjee 2002; Kwak, Park, Koo, Cho, Buchholz & Goetz 2003; Selvaraj & Sekar 2005; Sealey, Barrows, Hang, Johansen, Overturk, Lapatra & Harcy 2008). Dalmo and Bogwald (2008) demonstrated that β-glucans improve haemocyte phagocytosis, phenoloxidase activity and respiratory burst activity, as well as enhance the resistance of shrimp to white spot virus (WSSV) (Chang, Su, Chen & Liao 2003; Wang, Chang & Chen 2008). The binding of β-glucan with its receptor is the first step in the enhancement of animal immunity (Brown & Gordon 2003). β-Glucans are recognized by cell surface receptors in vertebrates and are identified by several proteins in the hemolymph of invertebrates. β-Glucans enhance phagocytic activity in the immune defence in mammals through a series of reactions with macrophage surface receptors or the activation of the complement pathway (Misra, Das, Mukherjee & Pattnaik 2006). Studies demonstrate that the surface of the macrophages and neutrophils in several fish also contains receptors for β-glucan. Furthermore, many β-glucan binding proteins (BGBPs) have been identified in crustaceans (VargaS-Albores & Yepiz-Plascencia 2000; Du, Zhao & Wang 2007; Jayaraj, Thiagarajan, Arumugam & Mullainadhan 2008; Lin, Vaseeharan & Chen 2008; Liu, Li, Dong & Xiang 2009; Liu, Song & Chen 2009; Meena, Das, Kumar, Mandal, Prusty, Singh, Akhtar, Behera, Kumar, Pal & Mukherjee 2013).

Glucans are believed to modulate innate immunity by binding to specific receptors on monocyte/macrophages, neutrophils and natural killer (NK) cells (Muller, Raptis, Rice, Kalbfleisch, Stout, Ensley, Browder & Williams 2000). The role and potential influence of β-glucans on immune-related gene and protein expression in different fish species have been reported by many authors. Orally administered β-1, 3-glucan in tilapia (*Oreochromis niloticus*) for 5 days has been shown to stimulate the production of cytokines, including tumour necrosis factor-α (TNF-α), IL (interleukin)-1β, IL-10 and IL-12, in fish plasma (Chansue, Endo, Kono & Sakai 2000). β-Glucans administered for 45 min through a bath treatment to rainbow trout fry at two different doses (0.1 and 1.0 M), with four treatments separated by 1-week intervals, resulted in enhanced gene expression of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 and of the anti-inflammatory cytokines IL-10 and

TGF-β after the first bath. However, no significant change in the transcripts of the pro-inflammatory cytokine IL-17A, relative to its expression in the control, was observed. Gene expression levels in β-glucan-treated fish after the fourth bathing showed no significant differences relative to the controls (Zhang, Swain, Bøgwald, Dalmo & Kumari 2009). Grass carp treated with β-glucans for 15 days prior to injections of grass carp haemorrhage virus (GCHV) showed increased MX gene expression during the early stages (12 and 36 h) of GCHV infection and had a significantly improved survival rate (60%). Increased superoxide dismutase (SOD) and catalase (CAT) activities in erythrocytes and MX gene expression were observed relative to values in the group not pretreated with β-glucans, indicating that β-glucans enhance antiviral responses (Kim, Ke & Zhang 2009). Feeding Pacific white shrimp with different dosages (0, 0.2, 1.0%) of β-glucans from *Schizophyllum commune* for 1 week showed up- and down-regulations of genes within 24 h. Penaeidin 3 (Litvan PEN3) was down-regulated (0–24 h), β-glucan binding protein–high density lipoprotein (BGBP-HDL) and lipopolysaccharide/β-glucan binding protein (LGBP) showed a delayed up-regulation (3–7 days), while haemocyanin, crustin, prophenoloxidase (proPO) and transglutaminase (TGase) showed no response. A diet with 2 g β-glucan per kg feed was sufficient for causing these changes in gene expression (Wang *et al.* 2008). Current research investigating the mechanism by which β-glucans improve the immunity of aquatic animals remains preliminary, and further research is needed.

Other polysaccharides

Peptidoglycans are composed of polymers that contain a chitosan chain, peptide bridge and peptide subunits within the cell wall of bacteria. The composition of peptidoglycans is specific to the cell walls of different prokaryotes, and the activity of peptidoglycans increases after hydrolysis (Itami, Asano, Tokushige, Kubono, Nakagawa, Takeno, Nishimura, Maeda, Kondo & Takahashi 1998). Research demonstrates that peptidoglycans promote growth and enhance the resistance to pathogens and the immunity of aquatic animals (Zhou, Song, Huang & Wang 2006). Peptidoglycans are important immunostimulants that regulate the immune system of aquatic animals.

Chitosan is a de-acetyl chitin, which is a type of alkaline polysaccharide found in the shells of aquatic animals such as shrimp, crab and shellfish. Chitosan is a natural polymer material that is edible, bio-compatible, bio-degradable, non-toxic and safe. The application and study of chitosan has progressed and is currently entering the practical or commercialize stage. The main functions of chitosan in aquaculture are to promote the growth of aquatic organisms (Gopalakannan & Arul 2006; Kumar, Sahu, Saharan, Reddy & Kumar 2006), improve the immunity of aquatic animals (Wang & Chen 2005; Li, Wen & Gatlin 2009), inhibit the growth of aquatic pathogens (Wang & Chen 2005; Hua, Zhou, Zhang & Li 2007), purify the water used in aquaculture (Wang & Chen 2005) and enhance the disease resistance of aquatic animals (Wang & Chen 2005; Gopalakannan & Arul 2006; Kumar *et al.* 2006; Hua *et al.* 2007; Li *et al.* 2009). Chitosan can chelate metal ions through the action of amino acid groups and hydroxyl molecules and can reduce the concentration of heavy metal ions in water, thus protecting aquatic animals (Wu, Fang, Wang & Zheng 2005). The quality of water within an aquaculture system is an important issue, and the appropriate concentration of chitosan increases the transparency of water due to its adsorption and flocculation capacities (Chen, Zhou, Zhou & Chen 2007; Zhang & Wu 2009). *Rehmannia glutinosa* root products enhance the growth and immune parameters of *Cyprinus carpio* (Wang, Meng, Lu, Wu, Luo, Yan, Li, Kong & Nie 2015; Wang, Chan, Rêgo, Chong, Ai, Falcão, Rádis-Baptista & Lee 2015). The seeds of *Achyranthes aspera* enhance immune responses in *Catla catla* (Chakrabarti, Srivastava, Verma & Sharma 2014). The root of *Withania somnifera* improves the disease resistance of *Labeo rohita* against *Aeromonas hydrophila* (Sharma, Deo, Riteshkumar, Chanu & Das 2010). Recently, we found that *Psidium guajava* leaves used as a feed additive increase the growth performance, disease resistance and cytokine gene expression of *L. rohita* (Giri, Sen, Chi, Kim, Yun, Park & Sukumaran 2015a,b,c). In *C. carpio* and *Larimichthys crocea*, the respiratory burst activity (RBA) of phagocytic cells has been shown to be significantly enhanced after feeding with a mixture of two herbal extracts (Jian & Wu 2003). Grass carp fed with polysaccharides from *F. carcica* showed remarkably higher resistance against *Flavobacterium columnare* (Yang, Guo, Ye,

Zhang & Wang 2015). Additionally, Giri *et al.* (2015a,b,c)investigated the effects of dietary supplementation using a polysaccharide extracted from *Chlorophytum borivilianum* roots on the growth performance, immune parameters and expression of several immune-related genes in *L. rohita*. The results of this study show that CBP (*C. borivilianum* polysaccharide) administration at 0.4% for 4 weeks can effectively improve immune responses and disease resistance in *L. rohita*.

With the in-depth study of immunostimulants, polysaccharides are becoming more widely used in aquaculture. Polysaccharides promote the growth, improve the immunity and enhance the disease resistance of aquatic animals. However, special attention should be given regarding the negative impact of the dose, time and pattern of polysaccharides use (Wang & Chen 2005; Wu *et al.* 2005; Ma, Chao & Wu 2006; Chen *et al.* 2007; Hua *et al.* 2007; Li *et al.* 2009; Zhang & Wu 2009). Ongoing research regarding the mechanisms of action of this group of immunostimulants within aquatic animals will provide more scientific evidence for increased standardization and effective application of polysaccharides in aquaculture.

Herbs

Chinese herbs have been used by people as traditional medicines and immune boosters for thousands of years in China. Recently, there has been a growing interest in the immune stimulating functions of several herbs in aquaculture. Non-specific immune properties, such as bacteriolytic activity and leucocyte function, can be improved by mixtures of Chinese herbs in aquaculture. The following characteristics and functions of Chinese herbs, in comparison with those of other immunostimulants, will be discussed. First, Chinese herbs are natural and contain many different types of active components. The immunostimulating activities of many of these components have been most widely studied in mouse, chicken and human cell lines. After a long screening process in humans and other animals, some of the essential compounds in Chinese herbs have been identified. These compounds can be used for a long time without resulting in drug resistance or the accumulation of residues from the medication, and they are beneficial and harmless to humans and other animals (Dügenci, Arda & Candan 2003; Jian & Wu 2004; Magnadóttir 2006; Divyagnaneswari, Christybapita & Michael 2007; Khanna, Sethi, Ahn, Pandey, Kunnumakkara, Sung, Aqqarwal & Aqqarwal 2007). Second, Chinese herbs work via a multitude of mechanisms. A variety of complex components are found in Chinese herbs, such as polysaccharides, proteins, alkaloids and/or flavonoids, vitamin E, minerals and fatty acids, which can all play a series of important roles in nutrition, antiviral and bactericidal activities and immune defence (Jian & Wu 2003; Horvath, Martos & Saxena 2005; Ardó, Yin, Xu, Váradi, Szigeti, Jeney & Jeney 2008; Cao, Ding, Zhang, Jeney & Yin 2008; Castro, Lamas, Morais, Sanmartin, Orallo & Leiro 2008).

Herbal extracts show potential for application as immunostimulants in fish culture primarily because they can be easily obtained and act against a broad spectrum of pathogens. Most herbs and herb extracts can be administered orally, which is the most convenient method of inducing immunostimulation. However, the effect is dose dependent, and the potential for overdosing is always present (Yin, Jeney, Racz, Xu, Jun & Jeney 2006). Consequently, dosage optimization is strongly recommended. Several studies in which manually prepared herbs or extracts were used reported difficulties in their practical application. The use of immunostimulants in combination with fish vaccines exhibits great potential as a means for increasing the protective capabilities of the immune systems of fish while decreasing vaccine dosages (i.e. the immunostimulant boosts the potency of the vaccine, thereby decreasing the dose necessary to achieve the same effect) (Jeney & Anderson 1993).

Fish treated with herbs typically exhibit enhanced phagocytosis. For example, the phagocytic activity of blood leucocytes was increased in crucian carp fed Chinese herbs (*R. officinale, A. paniculata, I. indigotica, L. japonica*) only at weeks 3 and 4. In vaccinated fish, elevated phagocytic activities relative to those of control fish were found on week 5 (Chen, Wu, Yin & Li 2003). Astragalus has been reported to increase the phagocytosis of the blood cells of soft-shelled turtles (*Pelodiscus sinensis*) (Cao, Sun & Sheng 1999; Zhou, Niu & Sun 2003; Misra, Das, Mukherjee & Meher 2006; Misra *et al.* 2006; Yin, Ardó, Thompson, Adams, Jeney & Jeney 2009). The extract of Scutellaria root has antimicrobial activity and is effective against many types of bacteria, such as *Streptococcus spp.*, *Mycobacterium spp.* and

Pseudomonas spp. (Tan & Vanitha 2004). Ardó *et al.* (2008) reported that Nile tilapia fed a mixture of Astragalus and Lonicera extracts showed increases in the respiratory burst and phagocytic activities of blood phagocytes and in plasma lysozyme activity. These fish also showed increased survival relative to controls when experimentally infected with *A. hydrophila* (Ardó *et al.* 2008). Yuan, Li, Chen, Sun, Wu, Gong, Tang, Shen and Han (2007) fed carp diets containing a mixture of *A. membranaceus* (roots and stems), *Polygonum multiflorum*, *Isatis tinctoria* and *Glycyrrhiza glabra* (0.5 and 1%) for 30 days and observed that both concentrations significantly increased phagocytosis, RBA and total protein levels in the serum. The herbs *Angelica membranaceus* and *A. sinensis* are commonly used to enhance the immune systems of common carp, large yellow croaker (Jian & Wu 2003, 2004), rainbow trout, catla carp (Dey & Chandra 1995) and Mozambique tilapia (Logambal & Michael 2000; Dügenci *et al.* 2003). The addition of green tea ethanol extract to the diet of black rockfish improved lipid utilization, lysozyme activity and stress recovery, and reduced total cholesterol levels (Hwang, Lee, Rha, Yoon, Park, Han & Kim 2013). A mixture of equal proportions of six herbs and plant materials enhanced or impaired enzyme activity in white-leg prawns (Lin, Li, Chen, Zheng & Yang 2006). Green tea, cinnamon and American ginseng improved the resistance of Nile tilapia against *A. hydrophila* infection (Abdel-Tawwab, Ahmad, Sakr & Seden 2010; Ahmad, El Mesallamy, Samir & Zahran 2011; Abdel-Tawwab 2012). *Azadirachta indica* (neem) plant extract can be used *in vivo* at a concentration of 150 mg L^{-1} as an alternative to antibiotics for treating bacterial infection (*Citrobacter freundii*) in *Oreochromis mossambicus* (Thanigaivel, Vijayakumar, Gopinath, Mukherjee, Chandrasekaran & Thomas 2015). A traditional Chinese medicine formulation of four herbs has been used as a prophylactic approach for disease control and can be used instead of antibiotics for treating enteritis in grass carp (Choi, Mo, Wu, Mak, Bian, Nie & Wong 2014). Three herbs (*Alternanthera sessilis*, *Eclipta alba* and *Cissus quadrangularis*) have been shown to act as appetite stimulants and to enhance the activities of digestive enzymes (protease, amylase and lipase) in freshwater prawns (Radhakrishnan, Saravana, Seenivasan, Shanthi & Poongodi 2014). American ginseng, green tea and cinnamon can enhance the growth performance and feed utilization of Nile tilapia (Abdel-Tawwab *et al.* 2010; Ahmad *et al.* 2011; Abdel-Tawwab 2012).

Further investigations into the stability of plant materials in the aquatic environment and their digestibility by fish, as well as *in vitro* and *in vivo* toxicological tests, are required to determine the safety of applying herbs to aquaculture (Bulfon, Volpatti & Galeotti 2015; Hai 2015). Moreover, the most effective substances/metabolites for use in formulating new natural feed additives for aquaculture still need to be identified (Bulfon *et al.* 2015; Hai 2015).

Vitamins

Vitamins are necessary for animal growth and development. Vitamins usually must be provided with food as they are only rarely synthesized *in vivo*. Growth retardation, susceptibility to declines in health or death can occur if long-term vitamin intake is not adequate. At present, VC (vitamin C) and VE (vitamin E) have become popular for use as vitamin immunostimulants in aquatic animals.

Vitamin C

VC, also known as ascorbic acid, can be used as a hydrogen acceptor or hydrogen donor in animals. VC cannot be synthesized in aquatic animals or obtained from food. Aquatic animals fed high doses of VC exhibit improved immunity and resistance to disease. Gao, Wang, Yang, Qu, Liang, Chang, Zhu and Ma (2008) observed that feed supplemented with 250 mg kg^{-1} of VC caused significantly increased lysozyme activity and increased the total number of white blood cells in the blood of Turbot. Cao, Luo and Yu (2009) reported that VC promotes the growth and early-stage development of *Monopterus alba*, and improved stress and disease resistance in late stage *M. alba.* Additionally, Hu and Li (2008) demonstrated that adding 150 mg kg^{-1} of VC to feed promotes growth, improves the quality of meat and increases the non-specific immunity of grass carp (*Ctenopharyngodon idellus*). Trenzado, de la Higuera and Morales (2007) demonstrated that VC enhances the antistress and pressure resistance of salmon. Rohu (*L. rohita*) fed a VC-supplemented diet showed higher specific growth rates (SGRs) (up to 1000 mg kg^{-1}) than control fish. Different

haematological and serological parameters, as well as non-specific immune parameters, are influenced by VC supplementation. Among the non-specific immune parameters affected, phagocytic activity (PR and PI) and RBA (as assessed via cellular NBT assay) have been shown to be significantly ($P \leq 0.05$) enhanced in response to increased doses of VC. Higher levels of dietary VC have also been shown to significantly ($P \leq 0.05$) enhance protection against *A. hydrophila* (AH1) infection relative to the rate of infection in controls (Tewary & Patra 2008). An 8-week feeding trial was conducted to determine the ability of two VC derivatives, L-ascorbyl-2-sulphate (C2S) and L-ascorbyl-2-polyphosphate (C2PP), to satisfy VC requirements and to assess their use as a supplement to support the non-specific immune responses of the grouper *Epinephelus malabaricus* (Lin & Shiau 2005a,b). A basal diet supplemented with emodin (60 mg kg^{-1}) or VC (700 mg kg^{-1}) has been shown to improve the non-specific immunity, antioxidant capacity and the mRNA expression levels of two (heat shock proteins) HSP70s, and to enhance resistance to crowding stress in Wuchang bream, *Megalobrama amblycephala* (Ming, Xie, Xu, Liu, Ge, Liu & Zhou 2011). Oxidized dietary lipids increase oxidative stress in fish, but more than 400 mg VC per kg as a dietary supplement has been shown to improve the growth and health of juvenile red sea bream (Gao, Koshio, Ishikawa, Yokoyama, Nguyen & Mamauag 2013). In addition, Emata, Borlongan and Damaso (2000) showed that in milkfish, *Chanos chanos,* broodstock given VC as a dietary supplement alone or in combination with VE had a higher percentage of spawning, with a higher (>90%) percentage of egg viability and hatching and a higher cumulative survival rate than the control group.

Vitamin E

VE, also known as tocopherols, is a group of biologically active phenolic compounds. The appropriate dose of VE can enhance the generation of antibodies and complement activity in response to antigens, promote the proliferation and differentiation of lymphocytes and cytokine production and improve cytotoxicity and phagocytosis. Lee and Shiau (2004) reported that feed containing different doses of VE significantly increased the total number of haemocytes in *Penaeus monodon* relative to the number in the control group. Zhou, Niu and Sun (2004) mixed VC and VE in feed given to *Trionyx sinensis* and found that the majority of physiological indexes were significantly improved, including the phagocytosis rate of blood cells, serum bacteriolytic activity, bactericidal activity and levels of the complement proteins C3 and C4. Ai, Chen, Liu, Gao and Wen (2008) demonstrated that 200–400 mg kg^{-1} VE effectively enhances the non-specific immunity of Chinese mitten-handed crab (*Eriocheir sinensis*). Galaz, Kim and Lee (2010) suggested that parrot fish require exogenous VE and that the optimum dietary level is approximately 38 mg α-TA per kg diet for normal growth and physiological function. Dietary α-TA concentrations over 500 mg kg^{-1} may be required to enhance non-specific immune responses and improve the resistance of juvenile parrot fish against *V. anguillarum*. Dietary supplementation with 60 mg kg^{-1} emodin or 500 mg kg^{-1} VE has been shown to improve HSP70 mRNA levels, antioxidant capacities, resistance to crowding stress and growth in *M. amblycephala* (Liu, Xu, Xie, Ge, Xia, Song, Zhou, Miao, Ren, Pan & Chen 2014). Amlashi, Falahatkar, Sattari and Tolouei Gilani (2011) indicated that VE has a direct effect on growth performance and is an essential nutrient that is required for normal growth in beluga sturgeon. Grouper, *E. malabaricus*, fed diets supplemented with VE have been shown to have lower hepatic and muscle thiobarbituric acid-reactive substance (TBARS) levels and higher white blood cell counts, leucocyte RBA, plasma lysozyme levels and alternative complement activity than are found in fish fed an unsupplemented control diet (Lin & Shiau 2005a,b). Gao, Koshio, Ishikawa, Yokoyama, Mamauag and Han (2012), Gao, He, Liu, Su, Gao, Li and Liu (2012) demonstrated that dietary oxidized fish oil (OFO) increases oxidative stress in fish, but a supplement of more than 100 mg kg^{-1} VE appears to protect tissues from lipid oxidation and improve the growth and health of juvenile red sea bream, *Pagrus major*.

Microorganisms

Probiotics, beneficial microorganisms that enhance the utilization of food and the disease resistance of the host, optimize the surroundings of aquatic animals through the colonization of the microflora in the areas aquatic animals live (Verschuere, Rombaut, Sorgeloos & Verstraete 2000). Numerous

types of probiotics have been applied in aquaculture, including *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Carnobacterium, Shewanella, Bacillus, Aeromonas, Vibrio, Enterobacter, Pseudomonas, Clostridium* and *Saccharomyces spp*. Screening probiotics is the first step for their application in aquaculture, and probiotics should have the following characteristics (Farzanfar 2006): (i) antagonism to pathogens, which is one property of probiotic bacteria (Ali 2000; Verschuere *et al.* 2000; Chang & Liu 2002; Irianto & Austin 2002, 2003); (ii) beneficial to the host animal; (iii) capable of surviving in or colonizing the gut of an aquatic organism via adhesion (Ali 2000; Verschuere *et al.* 2000); (iv) long-term stability under storage and field conditions; (v) non-pathogenic and non-toxic so that undesirable side effects are avoided when administered to aquatic organisms; and (vi) of an animal-species origin.

The application of probiotics has been widely investigated in aquaculture, and probiotics are typically used as feed additives or are directly added to the water. Furthermore, probiotics improve the balance of digestive tract flora and the utilization rate of feed, promote the growth of aquatic animals, enhance immune function and disease resistance and optimize the quality of water by absorbing or degrading organisms or toxic substances (Liu, Li *et al.* 2009; Liu, Song *et al.* 2009). Cao, Li, Yang, Wen and Huang (2010) demonstrated that periodically releasing *Bacillus licheniformis* into ponds with Yellow sea bream (*Sparus latus*) can significantly reduce the concentration of nitrites and phosphates in the water, as well as the mass fraction of organic carbon in the sediment. Additionally, Dalmin, Kathiresan and Purushothaman (2001) demonstrated that *Bacillus* can optimize the quality of water (Wang & Han 2007). Furthermore, *Bacillus* S11 can improve the survival rate, health and disease resistance in the breeding process of *P. monodon*.

Bacillus

Bacillus is a genus of gram-positive, aerobic or facultative anaerobic, endospore-forming, rod-shaped bacteria commonly found in soil and water and in association with plants. *Bacillus subtilis* is currently being used for aquaculture, terrestrial livestock and in human consumption for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders (Farzanfar 2006). Ghosh, Sen and Ray (2003) showed that adding 1.5×10^3 CFU g^{-1} of *B. circulans* to the feed of *L. rohita* for 18 days promoted the growth, decreased the feed coefficient and improved the protein utilization efficiency. El-Dakar, Shalaby and Saoud (2007) demonstrated that the commercial probiotics containing *B. subtilis* can reduce the feed coefficient and promote the growth of *Siganus rivulatus*. Bagheri, Hedayati, Yavari, Alizade and Farzanfar (2008) found that feed supplemented with a 3.8×10^9 CFU g^{-1} and 6.1×10^9 CFU g^{-1} *Bacillus* preparation significantly increases the specific growth and survival rate, improves the protein conversion efficiency and reduces the feed coefficient of rainbow trout. Gullian, Thompson and Rodriguez (2004) reported that adding *Vibrio* P62, *Vibrio* P63 and *Bacillus* P64 to the water promotes the growth of *L. vannamei*. Balcázar, Rojas-Luna and Cunningham (2007) added 10^5 CFU g^{-1} of *Vibrio alginolyticus* UTM102, *B. subtilis* UTM126, *Roseobacter gallaeciensis* SLV03 and *Pseudomonas aestumarina* SLV22 to the feed of *L. vannamei* for 28 days significantly reduces the feed coefficient and promotes growth. Wang (2007) added a mixture of *Photosynthetic bacteria* and *B. subtilis* to the feed of *L. vannamei* for 28 days and found that this also promotes the growth of *L. vannamei*, as well as significantly improving its protease, amylase, cellulase and lipase activities.

Nayak, Swain and Mukherjee (2007) showed that feed supplemented with 10^8 CFU g^{-1} of *B. subtilis* improves the immunity of carp. Newja-Fyzul *et al.* (2007) reported that feed containing 10^7 cell g^{-1} of *B. subtilis* AB1 given to rainbow trout for 14 days significantly improves the respiratory burst, antibacterial, the lysozyme activities in the serum and intestine and significantly reduces the mortality of rainbow trout after being infected with *Aeromonas*. Aly, Mohamed *et al.* (2008) found that feed supplemented with 10^7 CFU g^{-1} of *Bacillus firmus*, *Bacillus pumilus* and *C. freundii* significantly improves the survival rate of *O. niloticus* when challenged with *A. hydrophila*. Kumar, Mukherjee, Ranjan and Nayak (2008) demonstrated that feeding *L. rohita* for 2 weeks with 0.5×10^7, 1.0×10^7 and 1.5×10^7 CFU g^{-1} of *B. subtilis* can improve the respiratory burst and *A. hydrophila*-killing activity in their serum. In *C. catla*, significantly improved immune function against *A. hydrophila* was found after being provided feed supplemented with 2×10^3 CFU g^{-1} of *Bacillus circulans* PB7 for

60 days (Bandyopadhyay & Mohapatra 2009). Feeding *Bacillus* S11 to *P. monodon* can increase the number of blood cells and the antibacterial and phenoloxidase activity and can significantly improve survival rates after a challenge with *Vibrio harveyi* (Rengpipat, Rukpratanporn, Piyatiratitivorakul & Menasaveta 2000). Vaseeharan and Ramasamy (2003) found that feeding *B. subtilis* BT23 to *P. monodon* improves their resistance to *V. harveyi*. Tseng, Ho, Huang, Cheng, Shiu, Chiu and Liu (2009) observed that feed supplemented with 10^6, 10^7 and 10^8 CFU kg^{-1} of *B. subtilis* E20 significantly increases the phenoloxidase and phagocytic activity and the bacterial clearance rate and improves the survival rate of *L. vannamei* challenged with *V. alginolyticus* and *Lactobacillus.*

This group of gram-positive bacteria usually has no mobility, is non-sporulating and produces lactic acid. Some members of this group contain both rods (lactobacilli and carnobacteria) and cocci (streptococci). Different species of lactic acid producing bacteria (such as *Streptococcus*, *Leuconostoc*, *Pediococcus, Aerococcus*, *Enterococcus*, *Vagococcus*, *Lactobacillus*, *Carnobacterium*) have adapted to grow under a wide variety of environmental conditions. They are found in the gastro-intestinal tracts of various endothermic animals, milk and other dairy products, seafood products and on some plant surfaces (Ringø & Gatesoupe 1998). Although lactic acid bacteria are not dominant in the normal intestinal micro-biota of larval or growing fish, several studies have been undertaken to induce an artificial dominance of lactic acid bacteria in aquatic animals (Verschuere *et al.* 2000).

Byun, Park, Benno and Oh (1997) proved that adding 10^9 CFU g^{-1} of *Lactobacillus sp.* the feed of *Paralichthys olivaceus* for 30 days can promote growth. Suzer, Coban, Kamaci, Saka, Firat, Otgucuoğlu and Küçüksari (2008) feed *Sparus aurata* with Rotifers and Artemia enhanced with *Lactobacillus,* and the results showed improved digestive enzyme activity and fish growth. Apún-Molina, Santamaría-Miranda, Luna-González, Martínez-Díaz and Rojas-Contreras (2009) demonstrated that adding *Lactobacillus* to feed can significantly improve the growth of tilapia. Venkat, Sahu and Jain (2004) reported that *Lactobacillus acidophilus* and *Lactobacillus sporogenes* can significantly improve the growth of *Macrobrachium rosenbergii*.

Salinas, Cuesta, Esteban and Meseguer (2005) found that feeding 10^7 CFU g^{-1} of both *Lactobacillus* and *Bacillus* for 3 weeks stimulates phagocytic and cytotoxic activities in the fish. Aly, Abd-El-Rahman *et al.* (2008) demonstrated that adding a mixture of *Lactobacillus* and *Bacillus* to the feed of *Nile tilapia* for 8 weeks increases the growth of the fish, the RBA of macrophages and serum lysozyme activity and significantly improves the resistance of tilapia against *A. hydrophila, Pseudomonas fluorescens* and *Streptococcus iniae*. The resistance of rainbow trout against *Lactococcus garvieae* has been shown to be improved when they are fed for 30 days with 10^7 CFU g^{-1} of *Lactobacillus plantarum* CLFP 238 and *Leuconostoc mesenteroides* CLFP196 (Vendrell, Balcázar, de Blas, Ruiz-Zarzuela, Grionés & Luis Múzquiz 2008). In Grouper, feed supplementation significantly improves the phagocytic index of leucocytes, increases the respiratory burst, peroxidase and SOD activities and reduces the lethality of *Streptococcus* sp. (Son, Chang, Wu, Guu, Chiu & Cheng 2009).

Other probiotic

With interest in treatments using beneficial bacterial candidates increasing rapidly in aquaculture, several research projects that address the growth and survival of fish larvae, crustaceans and oysters have been undertaken (Ali 2000). Feed supplemented with 10^8 cells g^{-1} of *Clostridium butyrium* CB2 can improve phagocytosis in head kidney macrophages, serum and intestinal lysozyme activities, immunoglobulin content and the survival rate of *Miichthys miiuy* after a challenged with *Vibrio anguillarum* and *A. hydrophila* (Pan, Wu & Song 2008). El-Rhman, Khattab and Shalaby (2009) found that the addition of *Micrococcus luteus* to the feed of Nile tilapia (*O. niloticus*) for 90 days improves the immune function and disease resistance of the fish. The probiotic *Kocuria* SM1 has been shown to improve the phagocytic activity of macrophages and serum lysozyme activity and to significantly decrease mortality in rainbow trout after a challenged with *V. anguillarum* (Sharifuzzaman & Austin 2009). Hai, Buller and Fotedar (2009) demonstrated that feed supplemented with *Pseudomonas synxantha* and *Pseudomonas aeruginosa* can improve the immune function of *Penaeus latisulcatus Kishinouye.*

Secondary metabolites of bacteria

Different bacterial strains produce diverse secondary metabolites with a wide spectrum of

antibiotic activities that are valuable to medicine and agriculture. Recently, microbial secondary metabolites have received increasing interest for disease control in fish. Chen, Lu, Niu, Wang and Zhang (2015) isolated two active secondary metabolites from *B. licheniformis* XY-52 that positively influence the immune response of *C. carpio* and enhance its resistance against *A. hydrophila* infection. Yuan, Pan, Gong, Xia, Wu, Tang and Han (2008) studied the effects of *Astragalus* polysaccharide injections on the gene expression of IL-1β in the head kidney of common carp and demonstrated that IL-1β mRNA levels increased in a concentration-dependent manner. Wu, Liu, Zhou, Wang, Xia and Liu (2012) isolated three active secondary metabolites from *Alcaligenes faecalis* FY-3 that induce immune-related gene expression in *C. idellus* kidney cells. Oviya, Giri, Sukumaran and Natarajan (2012) and Giri, Sen, Jun, Sukumaran and Park (2016) demonstrated that *B. subtilis* VSG4, isolated from tropical soil, produces carbonic anhydrase, and the secondary metabolite (a biosurfactant) isolated from this strain (200 μg mL^{-1}) positively influences immune responses, enhances disease resistance and stimulates immune-related gene expression in *L. rohita.* The results of Giri *et al.* (2015a,b,c) indicate that the cellular components of *B. subtilis* VSG1, *P. aeruginosa* VSG2 and *L. plantarum* VSG3 can influence immune responses, enhance disease protection and stimulate immune-related gene expression in rohu. In a recent study, *L. rohita* immunized with cellular components of *Bacillus* species were shown to have improved protection against *A. hydrophila* (Ramesh, Vinothkanna, Rai & Vignesh 2015). The administration of the cellular components of the probiotic *Kocuria* SM1 and *Rhodococcus* SM2 resulted in notably higher survival rates in rainbow trout against *V. anguillarum* (Sharifuzzaman, Abbass, Tinsley & Austin 2011). The effects of three strains of intestinal autochthonous bacteria and their extracellular products on the immune response and disease resistance of common carp were also demonstrated by Chi, Bing, Xiao, Tian, Lei and Gao (2014). Probiotic extracellular products contain many immune boosting substances, such as the secondary metabolites *cyclo*-(Gly-L-Pro), *cyclo*-(L-Ala-4-hydroxyl-L-Pro), 4-trans-hydroxy-L-proline and *cyclo*-(L-Pro-Gly)$_2$, obtained from thermophilic *Anoxybacillus kamchatkensis* XA-1 and *A. faecalis* FY-3, which stimulate immune responses (Wang, Liu, Li, Gao, Lei & Liu 2010; Wang, Wang, Wu, Jiang, Dong, Li & Liu 2011; Wu *et al.* 2012). Therefore, screening for immunostimulants among bacterial secondary metabolites (biosurfactants) may lead to the identification of an eco-friendly way to strengthen fish immune systems.

Prebiotics

Prebiotics are indigestible fibres that enhance beneficial commensal gut bacteria, resulting in improved health of the host. The beneficial effects of prebiotics are due to by-products derived from the fermentation of intestinal commensal bacteria.

Carotenoids

Carotenoids, the precursors of vitamin A, have received increasing attention in recent years due to their health promoting effects in humans and in other animal models (Krinski 1991). Although some pure carotenoids can be synthesized, natural carotenoids are abundantly available and cheaper to obtain, making them an attractive pigment source for feed supplementation.

Carotenoids play a vital role in the cell-mediated host defence and humoral immune mechanisms in fish, such as phagocytosis, non-specific cytotoxicity, serum lysozyme activity, serum complement activity that promotes larval growth and survival, and disease resistance (Torrissen 1984; Tachibana, Yagi, Hara, Mishima & Tsuchimoto 1997; Amar, Kiron, Okamoto, Satoh & Watanabe 2000; Amar, Kiron, Satoh & Watanabe 2001, 2004; Yanar, Buyukchapar, Yanar & Gocer 2007). In contrast to the extensive studies on the effect of these pigments in mammals, there are only limited studies on the role of carotenoids in immune function in fish. In rainbow trout, dietary β-synthetic carotenoids and astaxanthin have been shown to enhance non-specific immune defence (Amar *et al.* 2000, 2001, 2004). A mixture of vitamin A and astaxanthin as a dietary supplement does not appear to increase growth rate or immune indices, except for serum antiprotease activity (Yanar *et al.* 2007). Amar *et al.* (2004) also found no differences in growth and feeding rates among rainbow trout fed b-carotenoids and astaxanthin. Based on these findings, other studies aimed to determine the effects of a carotenoid enriched diet on the innate immune response and disease protection in *C. carpio* against *A. hydrophila.* Enhanced or elevated phagocytic,

complement and lysozyme activities against pathogens have been reported in several fish and shrimp after treatment with different carotenoid enriched diets (Tachibana *et al.* 1997; Amar *et al.* 2000, 2001; Yanar *et al.* 2007). Anbazahan, Mari, Yogeshwari, Jagruthi, Thirumurugan, Arockiaraj, Velanganni, Krishnamoorthy, Balasundaram and Harikrishnan (2014) concluded that carotenoid-supplemented diets have the potential to modulate immune defence and confer disease protection in common carp against *A. hydrophila*.

Levamisole

Levamisole, a levo-isomer of tetramisole that has been widely used as an antihelminthic drug in humans and other animals, is a potential immunostimulant. Levamisole is known for exerting stimulatory effects on various functions of the immune system. It has been evaluated extensively in fish such as gilthead seabream (*S. aurata*), rainbow trout (*Oncorhynchus mykiss*) and carp (*C. carpio*) (Baba, Watase & Yoshinaga 1993; Jeney & Anderson 1993; Mulero, Esteban, Munoz & Meseguer 1998; Mulero, Esteban & Meseguer 1998). Levamisole stimulates immune responses not only *in vivo* but also *in vitro*. It has been demonstrated that levamisole can enhance the cytotoxic activity of leucocytes (Cuesta, Esteban & Meseguer 2002), phagocytosis, RBA (Mulero, Esteban, Munoz *et al.* 1998; Findlay & Munday 2000) and macrophage-activating factors (Mulero, Esteban *et al.* 1998). Baba *et al.* (1993) reported that carp immersed in a 10 μg mL^{-1} levamisole bath for 24 h show enhanced resistance against *A. hydrophila*, along with an increase in chemotactic ability, phagocytic activity and phagocyte chemiluminescence. Siwicki and Cossarini-Dunier (1990) demonstrated that the vitro immunization of spleen cells cultured with levamisole enhances phagocytic activity and RBA and increases the number of plaque-forming cells and cells producing antibodies against the O-antigen of *Yersinia ruckeri*. Li, Liu, Tan, Liu, Deng, Wan, Zhong and Chen (2011) showed that levamisole is a potent enhancer of macrophage activity in Barbel chub (*Squaliobarbus curriculus*) at 10^{-3} ng mL^{-1}, whereas levamisole at 10^{3} ng mL^{-1} has no effect.

Thyroxine

Hormones play pivotal roles in the regulation of growth and nutrient intake in fish. Thyroid hormone is required by all cells in the body to stimulate the enzyme synthesis required for cellular metabolism, especially for anabolic processes. There is a large body of evidence showing that thyroid hormones play an important role in the regulation of development, growth and reproduction in fish (Power *et al.* 2001).

Iromo, Zairin, Agus and Manalu (2015), Iromo, Junior, Agus and Manalu (2015) showed that low-dose thyroxine hormone supplementation for mud crab broodstock had a significant effect ($P < 0.05$), accelerating ovary maturation and survival. Treating mud crab (*Scylla serrata*) broodstock with thyroxine hormone supplementation at a dose of 0.1 μg BW^{-1} (body weight) during ovarian maturation has been shown to provide optimum results for vitellogenesis and hatching rate (Iromo, Zairin *et al.* 2015; Iromo, Junior *et al.* 2015). A combined treatment with thyroxine and recombinant growth hormone results in more efficient yolk utilization and faster development and growth rates in stripped catfish larvae (Sudrajat, Muttaqin & Alimuddin 2013). Kulczykowska, Sokolowska, Takvam, Stefansson and Ebbesson (2004) reported that a 2-week exposure to exogenous thyroxine (T4) causes a reduction in nighttime plasma melatonin level and, thus, likely inhibits melatonin-related circadian rhythms in juvenile salmon (*Salmo salar*). Moreover, several studies have demonstrated the beneficial effects of increased levels of maternal triiodothyronine (T3) on subsequent larval development and survival, for example in striped sea bass and rabbitfish (*S. guttatus*) (Brown, Doroshov, Nunez, Hadley, Vaneenennaam, Nishioka & Bern 1988; Brown, Doroshov, Cochran & Bern 1989; Ayson & Lam 1993);. In the Japanese flounder and summer flounder (*Paralichthys dentatus*), T4 treatment strongly stimulates the differentiation of the gastric gland and the production of pepsinogen, whereas thiourea (a potent inhibitor of TH synthesis) had the opposite effects (Miwa, Yamano & Inui 1992; Huang, Schreiber, Soffientino, Bengtson & Specker 1998).

The use of probiotics as a feed additive promotes the growth of aquatic animals, improves immune function and disease resistance and optimizes water quality in aquaculture systems. However, it is critical to study the endogenous probiotics of aquatic animals because few probiotics are commercially deployed for aquaculture. Additionally, the current methods of screening for probiotic are

relatively backward, and the details of probiotics in the guts of animals (e.g. their survival and adhesion) are unclear. Research on problems of ecological security regarding the application of probiotics is limited; therefore, the above issues should be researched and discussed in the future.

Biological factors

In past years, genes for numerous biological factors have been identified in many species. However, despite the fact that the use of biological factors as immunostimulants has been widely explored in mammals, few studies have focused on the possible use of biological factor gene products as immunostimulants in fish. Details concerning the immunological roles of the majority of these compounds are still lacking. Thus, until we understand the immune processes that the factors are regulating, their use would merely be a process of trial and error process. However, several attempts to explore their potential have been made in several fish species.

Lectin

Lectins are a large family of glycoproteins or carbohydrate-binding proteins (CBPs). Lectins have been shown to have a role in immunological recognition and host defence against pathogens. For example, lectins are up-regulated in response to microorganismal/viral infection in fish cells, inducing an antiviral state (Bayne 1990; Cooper, Rinkevich, Uhlenbruck & Valembois 1992; Gabius, Unverzagt & Kayser 1998; Gowda, Goswami & Islam Khan 2008a; Gowda, Goswami & Khan 2008b). In mammals, lectins have been widely used as adjuvants in vaccines against viral infections because they not only attract more cells to the site of inflammation but also regulates the immune functions of the recruited cells. In a recent study, the potential use of lectins as immunostimulants was investigated in Japanese flounder (Diesner, Wang, Jensen-Jarolim, Untersmayr & Gabor 2012). The holothuroid lectin CEL-III is a novel Ca^{2+}-dependent lectin that exhibits potent haemolytic activity and cytotoxicity and can be used as a cytotoxin to inhibit microbial activity *in vitro* (Hatakeyama, Suenaga, Eto, Niidome & Aoyagi 2004; Hatakeyama, Unno, Kouzuma, Uchida, Eto, Hidemura, Kato, Yonekura & Kusunoki 2007; Hisamatsu, Tsuda, Goda & Hatakeyama 2008; Hisamatsu, Unno, Goda & Hatakeyama 2009; Hisamatsu, Nagao, Unno, Goda & Hatakeyama 2013). Pufflectin, a mannose-binding lectin that is homologous to plant lectins, has been extracted from the mucus of the pufferfish, and subsequent studies have demonstrated that pufflectin has antibacterial and antiparasitic activities (de Santana Evangelista, Andrich, Figueiredo de Rezende, Niland, Cordeiro, Horlacher, Castelli, Schmidt-Hederich, Seeberger, Sanchez, Richardson, Gome de Figueiredo & Eble 2009). Despite their moderate protective effects, lectins are responsible for the activation of immunocytes, such as phagocytic cells and lymphocytes, and effectively interfere with pathogens. Therefore, lectins can be used as immunostimulants, playing a role in disease prevention in aquatic animals.

Antibacterial peptides (ABPs)

ABPs are a type of small molecular polypeptides that exhibit broad-spectrum antibacterial properties are present in a wide variety of organisms. ABPs are composed of 20–60 amino acid residues (Gordon, Romanowski & McDermott 2005; Fusetani 2010). ABPs cause membrane depolarization in bacteria, thus causing a loss of cell contents or interactions of the peptides with DNA, RNA and other targets, leading to the bacterial death. ABPs are small proteins derived from the animal and are easily digested by proteases after exerting their bactericidal effects. Low rejection of ABPs has been noted *in vivo*. ABPs are also non-toxic, harmless and environmentally safe products for use in the protection of aquaculture species.

It is difficult to extract the ABPs from animals due to their low concentrations and the high cost of the required technology, which limits the production scale. Genetic engineering is currently the only effective method to produce ABPs. Several ABPs have been cloned in aquatic animals such as catfish (Gao, Koshio *et al.* 2012; Gao, He *et al.* 2012; Ran, Carrias, Williams, Capps, Dan, Newton, Kloepper, Ooi, Browdy, Terhune & Liles 2012; Masso-Silva & Diamond 2014), salmon (Belhaj, Desor, Gleizes, Denis, Arab-Tehrany, Soulimani & Linder 2013; Neetoo & Mahomoodally 2014) and zebrafish (Lin, Liu, Hu & Zhang 2014; Zhao, Dai & Jin 2014; Jheng, Lee, Ting, Pan, Hui & Chen 2015; Wang, Meng *et al.* 2015; Wang, Chang *et al.* 2015). The sequence and structure of ABPs are complicated,

and several universal traits have been noted among different species. Many ABPs have bactericidal activity in fish and other animals (Smith, Desbois & Dyrynda 2010; Ryan, Ross, Bolton, Fitzgerald & Stanton 2011; Bhat, Kumar & Bhat 2015; Domalaon, Zhanel & Schweizer 2016), and the minimum inhibitory concentrations (MICs) of the ABPs from catfish and loach against fungi, and gram-positive and gram-negative bacteria range from 0.5~2 mmol L^{-1} (Liu, Tanner, Schumann, Weiss, Mckenzie, Janssen, Seviour, Lawson, Allen & Seviour 2002). The ABPs from perch have a MIC of 1.2 mmol L^{-1} against *A. salmonicida* and *A. hydrophila* (Weber, Moore & Sullivan 2007). The ABPs from rainbow trout and perch also exhibit activity against several parasites at higher concentrations. At present, the main application of antibacterial peptides in feed additives is the use of Cecropin AD-yeast preparation (CADYP), and Wang, Xie and Yu (2005) have demonstrated a significant improvement in disease resistance, as well as growth and survival rates, upon feeding *L. vannamei* (Boone) with ABP feed additives.

The mechanism of action underlying the effects of ABPs from aquatic animals and the structure of the target proteins on the surface of pathogens are related, as are pathogens and their corresponding targets in aquatic animals. Biological factors, such as immune molecules from aquatic animals, cannot effectively target the proteins on the surface of pathogens when immune function is decreased as decreased immune function interferes with pathogen recognition, making the animals susceptible to a variety of diseases. Therefore, biological factors used as immunostimulants can enhance immune defences and aid in avoiding diseases.

Factors affecting the efficiency of immunostimulants

Timing of immunostimulant administration

Immunostimulants may be administered separately or in conjunction with a vaccine. Fish could be prepared for predicted disease events, such as seasonal exposure to a pathogen, via treatment prior to the event. The easiest time to administer immunostimulants and vaccinations is at the same time as the exposure of the fish to a specific antigen. The two substances are mixed with feed or delivered through immersion. In several cases, immunostimulants are better administered prior to vaccination, thus helping the fish to prepare for antigen exposure. However, the dosage and timing for that administration of each substance should be determined.

Bagni, Romano, Finoia, Abelli, Scapigliati and Tiscar (2005) demonstrated that significant elevations ($P < 0.05$) in serum complement activity occurs in sea bass fed with alginic acid and glucans 15 days from the end of the first cycle of the treatment. Significant elevations ($P < 0.05$) in serum lysozyme and gill and liver HSP concentrations were observed in the same experimental groups 30 days from the end of treatment, whereas a significant increase ($P < 0.05$) in complement activity was only observed in fish that received an Ergosan diet. Forty-five days from the end of treatment, complement, lysozyme and HSP concentrations did not differ among groups. Over a long-term period, no significant differences were observed in innate or specific immune parameters, survival, growth performance and conversion indexes in treated and control fish. A dramatic decrease of both innate and acquired immune parameters was observed during the winter season in all groups, followed by a partial recovery when water temperatures increased. Reductions in complement and lysozyme activities were significantly correlated ($P < 0.01$) with water temperature variation (Bagni *et al.* 2005; Li, Li & Wang 2010).

Dosage of immunostimulant

Dosage profoundly influences the effects of immunostimulants. The evidence suggests that the optimal dosage of orally administered VE is 1200 mg kg^{-1} in gilthead seabream, while lower (<600 mg kg^{-1}) or higher (>1800 mg kg^{-1}) VE concentrations may all induce different patterns of immunostimulation (Ortuño *et al.* 2000). Another report demonstrates enhanced weight gain in fish fed diets supplemented with a low level (<500 mg kg^{-1}) of levamisole after a 3-week period. Dietary supplementation with levamisole at 100 mg kg^{-1} significantly ($P < 0.05$) enhances growth rate and feed efficiency compared with the patterns in fish fed basal diets. However, fish fed diets supplemented with 1000 mg levamisole per kg diet have been shown to exhibit signs of chronic toxicity, such as inferior growth and reduced feed intake and efficiency (Alvarez-Pellitero, Sitja-Bobadilla, Bermudez & Quiroga 2006). Regulating the dosage administered may affect the efficacy and potency of immunostimulatory compounds.

Mode of action

The oral administration or injection of immunostimulants could also benefit fish or stimulate immune protection and could be useful in preventing fish diseases in aquaculture. The oral administration of immunostimulants has already been reported for glucans, EF203, lactoferrin, levamisole and chitosan. This method is non-stressful and allows for mass administration regardless of fish size. The oral administration of these immunostimulants results in enhanced leucocyte function and protection against infectious diseases such as furunculosis, vibriosis and streptococcosis. The intravenous administration of chitin has been shown to have no effect of immune parameters; however, fish that were intraperitoneally injected with chitin exhibited increased humoral and cellular immune responses (Maqsood, Singh, Samoon & Munir 2011). Bathing fish in an immunostimulant solution is the third strategy have been studied for their delivery to fish. Baba *et al.* 1993 reported that carp immersed in a 10 μg mL^{-1} levamisole solution for 24 h showed activated phagocytosis and chemotaxis, the production of active oxygen in head kidney phagocytes and enhanced protection against *A. hydrophila*, which lasted for at least 2 weeks. Anderson, Siwicki and Rumsey (1995) demonstrated that rainbow trout immersed in glucan or chitosan solutions show increased protection against *A. salmonicida* after treatment for 3 days. Further research should be performed to assess the categories of suitable immunostimulants and their dosages for application in aquaculture.

Evaluating the efficacy of immunostimulants

There are two main procedures for evaluating the efficacy of an immunostimulant: (i) *in vivo*, such as a challenge test using fish pathogens, and (ii) *in vitro*, such as the measurement of the efficiency of cellular and humoral immune mechanisms. Knowledge of the immune system is often very limited for most fish species, and information on the mode of action of most immunostimulatory substances is even more restricted. The evaluation of an immunostimulants based on *in vitro* methods that test the effects of that substance on the immune system is preferred in preliminary studies. Nevertheless, *in vitro* tests should be performed together with *in vivo* experiments, if possible, to elucidate the basic mechanisms responsible for any protection provided. *In vitro* evaluations should be based on at least one of the following parameters: serum lysozyme activity, complement activation, total leucocyte and erythrocyte counts, RBA phagocytosis, chemotaxis, chemokinesis and lymphocyte proliferation. Several other recommendations of parameters to monitor include C-reactive protein levels, natural cytotoxic activity and macrophage-activating factor (MAF) levels. Techniques involved in these evaluations range from relatively simple and inexpensive methods to the use of immunoassays, flow cytometry and other bio-molecular approaches.

Concluding remarks and perspectives

The development of effective immunostimulants should be approached by combining the search for biological factors from animals together with the application of genetic engineering that maximizes the immunogenicity for a desired immune response. These immunostimulants may trigger specific immunological processes without producing a generalized response with strong side effects. The gene expression of biological factors used as immunostimulants within an animal has the following advantages: the factors (i) are stable and efficient immune-enhancing molecules; (i) they contain a specific antibacterial spectrum; (iii) they are harmless to the host; and (iv) drug resistance is unlikely to occur. New immunostimulants have the potential to become alternative bioactive agents, possibly replacing traditional antibiotics, and future research may provide new immunostimulant sources and ideas for their application. Therefore, immunostimulants developed via gene engineering may become an important new commodity in aquaculture as a candidate for use in new applications.

The search for the actual molecular correlates of the protection provided by immunostimulants should be vigorously pursued. In future research on immunostimulants, the immunostimulatory potential of a given substance should be unequivocally established, followed by studies on its potency and efficacy in the context of immunostimulants.

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