

**Tamil Nadu Dr. J. Jayalalithaa Fisheries University
Fisheries College and Research Institute
Thoothukudi – 628 008**



***Credit seminar on*
Prophylactics in Shrimp Aquaculture**



"Prevention is always better than cure"

Submitted by

P. SIVASANKAR

II Ph.D., ID. No. DPF 14007 (AQC)

Department of Fish Pathology and Health Management

School of Aquaculture

Prophylactics in shrimp aquaculture

1. Introduction

Shrimp production in India during 2012 and 2013 was very impressive. The overall export of shrimp during 2013-14 was to the tune of 3,01,435 MT worth US\$ 32210.94 million. The contribution of cultured shrimp to the total shrimp export is 73.31% in quantity and 92.29 % in dollar term. The export of vannamei has tremendously growth to 1,75,071 MT from 91,171 MT compared to 2012-2013. MPEDA reports that 130,947 hectares of shrimp and prawn farms in 2014-2015 producing about 4,34,557 metric tons of exportable products.

White shrimp (*Penaeus vannamei*) is farmed in 25,000 hectares of ponds, and giant tiger (*P. monodon*) shrimp is produced in 60,000 hectares of ponds, some of which are being converted to white shrimp.

A shrimp farm is an aquaculture business for the cultivation of marine shrimp for human consumption. Shrimp farming has changed from traditional, small-scale businesses in Southeast Asia into a global industry. Technological advances have led to growing shrimp at ever higher densities, and broodstock is shipped world-wide. Virtually all farmed shrimp are penaeids (family penaeidae) and two species of shrimp the *L. vannamei* and *P. monodon* account for roughly 80% of all farmed shrimp. Shrimp exports increased considerably during the year due to production of 10,000 t of the whiteleg shrimp *Litopenaeus vannamei*, in addition to the higher production of tiger shrimp *Penaeus monodon* (Ponniah *et al.*, 2011).

Disease outbreaks are a serious constraint to the development of intensive aquaculture systems and can have a major impact on production due to mortality and decreased growth. It has been recognized that disease is the most significant factor impacting the intensive production of shrimp.

Although many aquatic animal pathogens are well studied, unlike in terrestrial animals the spread of pathogens is easy through water and control is difficult due to high density culture in fluid environment. Disease occurs in all systems, from extensive to intensive, although heavy losses are always possible in intensive production systems (Bondad-Reantaso *et al.*, 2005).

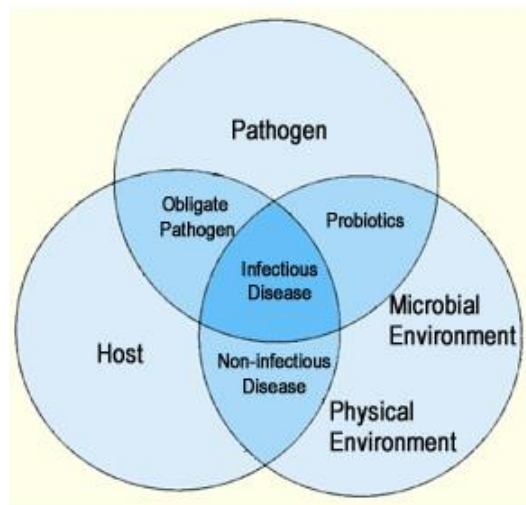
In the past decade, incidences of viral and bacterial diseases in shrimp aquaculture world-wide have been on the rise causing severe loss to the sector. Repeated incidence of diseases has led to increased use of chemicals, drugs and biological products as a measure to prevent disease. Indiscriminate use of the

chemical and biological (CB) products is one of the major concerns, considering the potential negative impacts on the environment and human health. Grasslund and Bengtsson (2001) documented the use of various CB products by shrimp farmers of South-east Asian countries. Though CB products are very widely used in Indian shrimp farming, there is lack of reliable field level data on the usage of these products.

“Chemicals or antibiotics used in preventing the disease in shrimp aquaculture could lead to development of resistance in the microorganism and reduce them unsuccessful. Immunization, a prophylactic measures and a protective management tool is designed to aid in the prevention of disease”.

2. Process of disease development

The development of disease in a particular aquaculture system involves several factors: the farmed animal (host), the disease-causing organisms (pathogens) and the surroundings (environment). A complex interaction exists among these three factors as represented in the diagram of three overlapping circles.



For a disease situation to exist, there should be a potential pathogen, a susceptible host, and environmental conditions that bring about either increased virulence of the pathogen, or decreased resistance of the host.

a. Hosts

A host (fish or shrimp) can be either resistant or susceptible to a given disease. Resistance or susceptibility of the host depends on (a) age or size of the

host organism, (b) species, (c) defence mechanisms employed and (d) the health of the animal, including its nutritional state

b. Pathogens

Agents of diseases or pathogens can be classified into physical, chemical, and biological agents. Extreme temperature changes and radiation, such as ultraviolet rays from the sun, are examples of physical agents. Chemical agents can cause illness in aquatic organisms in a variety of ways. Environmental contaminants, toxins, nutritional imbalances and drug and chemical overdose are classified as chemical agents of disease.

Biological agents may initiate disease. They are the primary focus of attention when dealing with infectious diseases. These agents include viruses, bacteria, fungi and parasites. They are often called infectious disease agents. They can be present in the water or in sediment as part of the normal flora. Their presence and number are largely influenced by environmental factors like temperature, dissolved gases, pH, and availability of food. The two major characteristics of an infectious agent are (a) capability for direct transmission and (b) ability to multiply in the host tissue. The mode of their transmission is either (a) vertical or (b) horizontal. In vertical transmission, infectious agents transfer from parent to offspring. The female or male broodstock may be carriers of diseases, and transfer them to their offspring through the egg or sperm. In horizontal transmission, infectious agents come in contact with the hosts through the water, the feeds or through carrier animals that are in the environment.

c. Environment

The environment of cultured shrimp is composed of the water and its holding system like tanks, ponds, cages, pens, etc. Stability of the environment, especially in the physico-chemical parameter of the water brought about by the shrimp culture activity itself or by natural causes, will determine the health of the shrimp. Fluctuations in temperature, pH, salinity or dissolved oxygen beyond the optimum range for the host may lead to stress and disease. The key to successful aquaculture is to understand and manage the environment of the host organism. Understanding the role of the environment in affecting the nature and cause of disease is essential to the prevention and control of disease.

3. Common diseases in shrimp aquaculture

a. Bacterial diseases

Bacterial diseases may cause a range of problems ranging from mass mortalities to growth retardation and sporadic mortalities. *Vibrio* spp are the most important bacterial pathogens of shrimp. *Vibrio* spp are aquatic bacteria that are widely distributed in fresh water, estuarine and marine environments. Over 20 species are recognized, some of these are human pathogens (eg. *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) while some species are pathogens of aquatic animals including shrimp (eg. *V. harveyi*, *V. splendidus*, *V. penaeicida*, *V. anguillarum*, *V. parahaemolyticus*, *V. vulnificus*). *Vibrio* spp are commonly observed in shrimp hatcheries, grow-out ponds and sediments (Otta *et al.* 1999a, 2001).

Though most *Vibrio* spp are regarded as opportunistic pathogens, some like *V. harveyi* could be primary pathogens. *V. harveyi* are luminous bacteria that are found in coastal and marine waters, in association with surface and gut of marine and estuarine organisms and also in shrimp pond water and sediment. *Vibrio parahaemolyticus* was consistently isolated from EMS/AHPNS-infected shrimp. Based on the work done in China (Zhang *et al.*, 2012), Chinese researchers reported on a virulent strain of *V. parahaemolyticus* isolated from *L. vannamei* suffering from this early mortality disease in 2010 in Guangxi Province. The susceptible species are *P. monodon*, *L. vannamei* and *P. chinensis*.

Filamentous bacteria such as *Leucothrix mucor*, *Thiothrix sp*, *Flexibacter sp*, *Flavobacterium*, *Cytophaga sp* may cause infection in penaeid shrimp larvae. Discolouration of gills, low growth and feeding, increased mortality, lethargy, empty gut white gut and damaged hepatopancreas are common signs of the disease. The disease is associated with poor water quality. Higher degree of infection may lead to necrosis in gill tissue. The disease can be diagnosed by microscopic examination of gills.

b. Viral diseases

White Spot Syndrome Virus (WSSV)

WSSV continues to be one of the most disease problems faced by the shrimp farming industry worldwide. Considering its virulent nature, wide host range, wide geographic distribution, high mortality (up to 100%), catastrophic economic losses, WSSV has become the single most–dangerous virus to the penaeid shrimp farming industry. WSSV is known to affect most commercially important species of penaeid

shrimp including *P. monodon*, *P. japonicus*, *P. indicus*, *P. chinensis*, *P. merguensis*, *P. aztecus*, *P. stylirostris*, *P. vannamei*, *P. duorarum* and *P. setiferus* (Lightner 1996a). Wild marine shrimp such as *P. semisulcatus*, *Metapenaeus dobsoni*, *M. monoceros*, *M. elegans*, *Heterocarpus sp.*, *Aristeus sp.*, *Parapanaeopsis stylifera*, *Solenocera indica*, *Squilla mantis* and fresh water cultured species *Macrobrachium rosenbergii* have also been found to harbor this virus (Lo *et al.* 1996a, Hossain *et al.* 2001a; Chakraborty *et al.* 2002).

Taura syndrome virus (TSV)

Taura syndrome was reported as a new disease in 1992 in commercial penaeid shrimp farms located near the mouth of River Taura in the Gulf of Guayaquil, Ecuador (Jimenez 1992). Since its discovery, this lethal shrimp disease has spread into major shrimp growing region in the Americas by mid-1996 (Lightner 1996; Lightner *et al.* 1997). This disease represents a serious problem in the culture of *L. vannamei* due to the high level of mortality (up to 80%) and the economic losses (Lightner *et al.* 1997). TSV causes 3 distinct disease phases in infected shrimp. The peracute/acute phase of the disease is characterized by moribund shrimp displaying an overall pale reddish coloration caused by the expansion of the red chromatophores. Shrimp in this phase usually die during the process of moulting. If the shrimp survive through the peracute/ acute phase, the recovery phase begins. Multifocal, melanized cuticular lesions are the major distinguishing characteristics of the recovery phase (Lightner 1996). In the chronic phase of TSV infection, infected shrimp appear and behave normally, but remain persistently infected perhaps for life (Hasson *et al.* 1997b). TSV infected shrimp display histological lesions characteristic of the disease, which are necrosis, and nuclear pyknosis of the cuticular epithelium of the general body surface, appendages, gills, mouth, esophagus, stomach and hindgut (Brock *et al.* 1995, Lightner, 1996b).

Yellow Head Virus (YHV)

YHV was first detected in central Thailand in 1990 in pond reared black tiger prawns *Penaeus monodon*. It is a positive-sense, single stranded RNA virus (genus Okavirus) in a new family Roniviridae of the order Nidovirales (Cowley *et al.* 2000, Mayo 2002). Yellow head disease is usually characterized by light yellow coloration of the dorsal cephalothorax area and generally pale or bleached appearance of affected shrimp. The yellow color in the cephalothorax region results from the

underlying yellow hepatopancreas visible through the translucent carapace in moribund shrimp (Chantanachookin *et al.* 1993).

YHV is widespread in cultured stocks of *P. monodon* in Thailand. Shrimps infected with YHV die within a few hours of developing color and the whole crop can be lost within 3-5 days after the first appearance of affected shrimp (Flegel *et al.* 1995b) YHV has also been shown to infect and cause disease in *L. vannamei* and *P. stylirostris* (Lu *et al.* 1994). *Palaemon syliferus* and *Acetes* sp. have been recorded as carriers of YHV (Flegel *et al.* 1995b).

Monodon Baculovirus (MBV)

MBV is the first reported virus of *P. monodon* and the second virus of penaeid shrimp (Lightner and Redman 1981). It is a nuclear polyhedrosis virus (NPV) of the family Baculoviridae (Lightner and Redman 1981). MBV has been identified and reported in *P. monodon*, *P. merguensis*, *P. semisulcatus*, *P. kerathurus*, *P. vannamei*, *P. esculentus*, *P. penicillatus*, *P. indicus*, *Metapenaeus ensis*. MBV is a common, widespread pathogen and despite its wide distribution, is not a highly virulent pathogen of *P. monodon* (Nash *et al.* 1988). However, occasionally, severe mortalities may be observed in post-larval (over 90%) and juvenile stages (70%).

Hepatopancreatic Parvovirus (HPV)

The HPV was first reported by Lightner and Redman (1985) in postlarvae of *Penaeus chinensis*. In Thailand, HPV in the black tiger shrimp, *P. monodon*, was first reported in 1992 by Flegel and Sriurairatana (1993, 1994). Shrimps affected by HPV usually show non-specific gross signs, including atrophy of the hepatopancreas, anorexia, poor growth rate, reduced preening activities and as a consequence increased tendency for surface and gill fouling by epibiotic organisms.

Baculovirus penaei (BP)

The BP was first detected in 1974 in the pink shrimp, *Penaeus duorarum* (Couch 1974a, b). BP has been reported to cause significant mortalities in the larval, post-larval and early juvenile stages of *P. aztecus*, *P. stylirostris*, *P. vannamei* and *P. penicillatus* (Couch 1991, Lightner and Redman 1991, 1992). BP infects only the hepatopancreas and midgut epithelial cells, and it is transmitted from shrimp to shrimp (Lightner and Redman 1998b). The typical route of infection of shrimp larvae is via fecal contamination of spawned eggs from BP infected adult spawners (Johnson and Lightner 1988, Lightner 1996a) fecal oral contamination through feces

from infected larvae or from cannibalism of diseased larvae (Overstreet *et al.* 1988, LeBlanc and Overstreet 1990, 1991).

Infectious hypodermal haematopoietic necrosis virus (IHHNV)

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is widespread in cultured *Penaeus monodon* and *L. vannamei* in Thailand. It causes runt-deformity syndrome that is characterized by physical abnormalities and stunted growth in *P. vannamei*, but causes no apparent disease in *P. monodon*. In both species, the virus may produce Cowdry Type A inclusions in tissues of ectodermal and mesodermal origin, but these are common in *L. vannamei* and rare in *P. monodon*.

c. Fungal diseases

Fungi occur in aquatic environment and about 500 fungal species have been isolated from marine and estuarine environment. Some of the aquatic fungi are opportunistic pathogens of shrimp. Mostly larval stages are affected and the common causative agents are *Lagenidium callinectes* and *Serolpidium* spp. The protozoa and mysis states are generally affected with clinical signs such as lethargy and mortality. Fungal spores and mycelia are observed in affected tissue, particularly gill and appendages. Larval mycosis is a problem in many hatcheries in India. Gopalan *et al.* (1980) reported *Lagenidium marina* and *Serolpidium parasitica* infection in *P. monodon*. Ramasamy *et al.* (1996) reported mortalities in *P. monodon* larvae at nauplii, zoea and mysis stages. Fusariosis and black gill disease caused by *Fusarium* spp may affect all developmental stages of penaeid shrimp. *Fusarium* spp (*F. solani*, *F. moniliformae*) are opportunistic pathogens that may lead to high mortalities (90%). Disease is noticed in ponds where water quality management is poor. Fungal hyphae can be detected in affected animal tissue using light microscopy.

d. Parasitic diseases

A number of parasites, particularly protozoa, may affect shrimp at different developmental stages. Epi and endo-commensal protozoa may be found adhering to gills, cephalothorax, periopod and other appendices and also internal organs. At high levels of infection, these protozoa may induce gill obstruction (brown gill) leading to anorexia, reduced growth, locomotion and increased susceptibility to infection by opportunistic pathogens. Protozoa such as *Zoothamnium*, *Epistylis*, *Vorticella*,

Anophrys, *Acineta* sp, *Lagenophrys* and *Ephelota* may be encountered as external parasites.

Ciliates such as *Paranophrys* spp and *Parauronema* sp may cause mortalities in larvae and juveniles. Ciliates may enter shrimp body through wounds and invade hemolymph and gills. This may lead to mass mortalities, particularly in conjunction with other parasitic flagellates such as *Leptomonas* sp. Diagnosis can be achieved through examination of hemolymph which appears turbid, does not clot and shows reduced hemocyte count and numerous ciliates.

Gregarians are endoparasitic protozoa infecting shrimp. These parasites generally have two hosts – usually a mollusk or an annelid worm and crustaceans. Gregarians found in shrimp include *Nematopsis* spp, *N. litopenaeus*, *Paraphioidina scolecoide*, *Cephalobolus litopenaeus*, *C. petiti* and *Cephaloidophoridae stenai*. *Trophozoites* and *gametocytes* may be found attached to the intestinal wall or may occur in the lumen. The parasites may cause reduced absorption of food from the gut and occasionally intestinal blockage, but these diseases seem to have little impact on aquaculture.

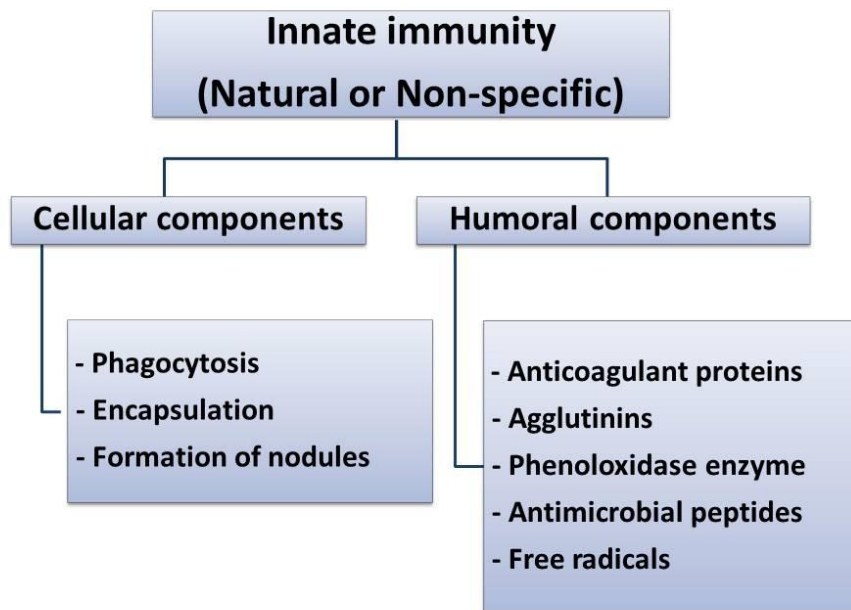
Microsporidia such as *Agmasoma* sp, *Microsporidium* sp may invade the muscle, heart, gonads, gills or hepatopancreas. The common microsporidian disease is cotton shrimp disease. The infection leads to opacity of the affected tissue and shrimp may appear crooked. Though infection may not be lethal, the appearance affects marketability. The diagnosis can be established by demonstrating microsporidian spores in the affected muscle tissue.

A new microsporidian parasite called *Enterocytozoon hepatopenaei* (EHP) has been identified and characterised from *litopenaeus vannamei* in India (Rajendren *et al.*, 2016). Shrimp infected with EHP shows retarded growth and white faecal syndrome (WSF). Aquaculture experts say there is no drug to control EHP infection in shrimp as on date. The only way out is better management practices and proper bio-security measures.

4. Immune system of shrimp

Shrimp solely depend on the innate defense system to defend themselves against the invasion of foreign micro-organisms as they don't possess the adaptive immune system. The innate immune system, also known as natural or non-specific defense system can be categorized in cellular and humoral reactions. Cellular components are directly performed by hemocytes, and include phagocytosis,

encapsulation and nodulation. Humoral defense refers to processes related to activation comprised of the prophenoloxidase (proPO) system, the clotting cascade, a wide array of antimicrobial peptides, free radicals, and the synthesis and release of several immune proteins, such as antimicrobial peptides, proteinase inhibitors, cytokinelike factors, and others.

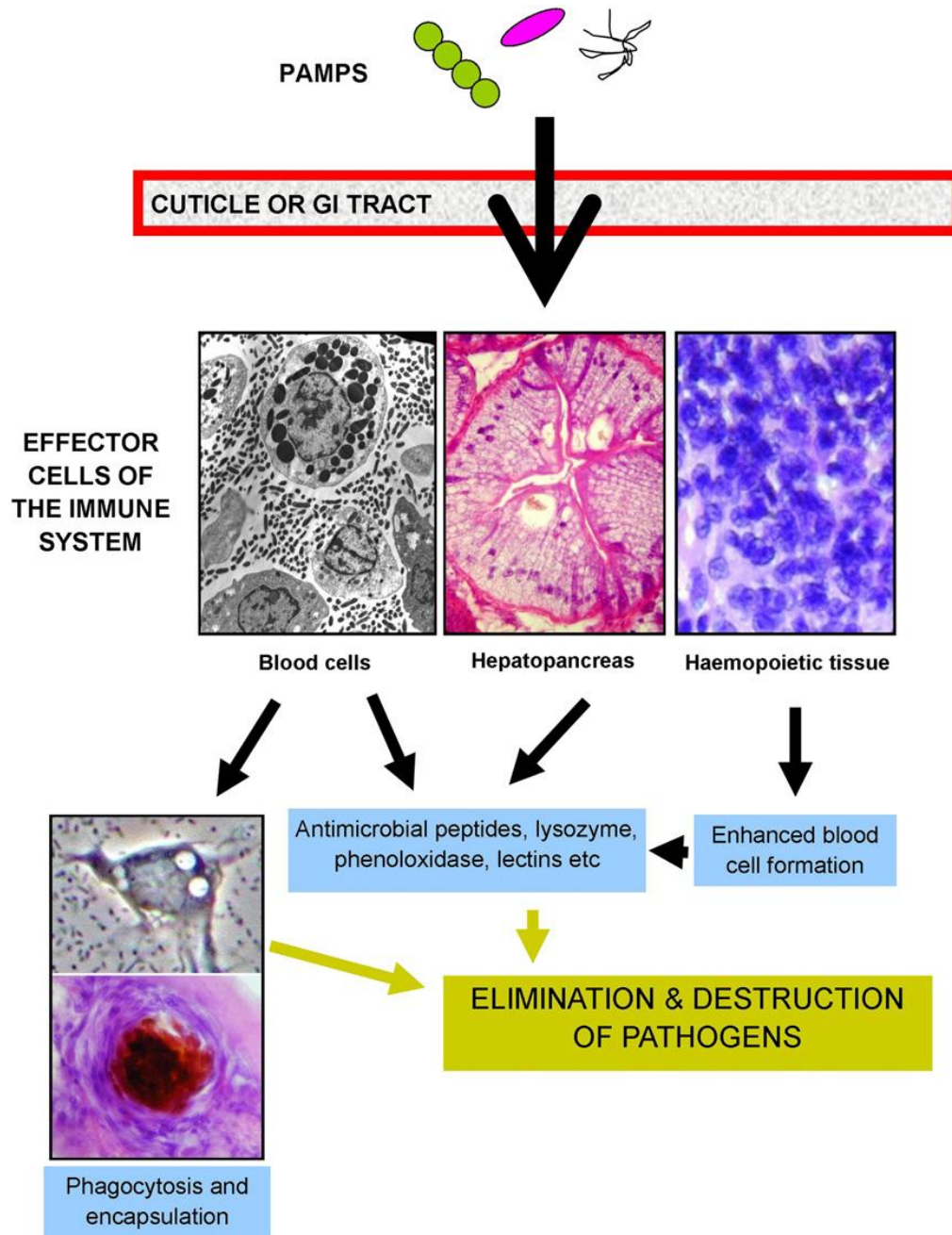


Cellular and humoral components of crustacean immune system

. The invertebrate immune system comprises both cellular and humoral arms that are intimately linked. The main effector cells of this immune system in crustaceans are the blood cells or haemocytes whilst the hepatopancreas is responsible for the biosynthesis of some humoral factors. In shrimp, as in other crustaceans, there appears to be three main types of haemocytes (blood cells), the hyaline cells, semi-granular cells and granular cells (termed granulocytes by some authors), all of which appear to participate in immune defences. Once potential pathogens invade through the gastrointestinal (GI) tract or cuticle, they immediately come into contact with haemocytes in the underlying haemocoel. Haemocytes recognise pathogen-associated molecular patterns (PAMPs) associated with these invaders and respond with either phagocytosis or an encapsulation reaction that segregates the pathogen into nodules or capsules. At a later time, either these blood cells or cells in the hepatopancreatic tubules synthesise and release a host of factors

including antimicrobial peptides (AMPs), such as penaeidins (in shrimp) and crustins, lysozyme and lectins.

In shrimp, lectins have recently been recognised as possess potent antibacterial, antifungal (Sun *et al.*, 2008) and antiviral activity (Zhao *et al.*, 2009). Similarly, the penaeidins synthesised and stored in the granular haemocytes of shrimp (Destoumieux *et al.*, 2000) have potent activity against Gram positive bacteria, bind vibrios (Gram negative bacteria) and contain a chitin-binding domain that presumably allows these molecules to be immobilised in the cuticle as part of the external defences against invasion. Fragments of the respiratory pigment, haemocyanin, may act as an alternative phenoloxidase and have both antifungal and antiviral activity. Finally, the prophenoloxidase activating system not only results in the formation of melanin (a hallmark of many cellular events such as encapsulation) but also generates a series of cytotoxic factors and is linked to wound healing and blood clotting (Cerenius *et al.*, 2010).



Diagrammatic representation of the cellular and humoral defence reactions of shrimp

5. Proactive disease management

Prevention of disease outbreaks is a major concern in shrimp farming. Antibiotics and other therapeutic agents are widely used as common therapies in post-infection practice. However, application of these therapeutic chemicals has caused the development and spread of antibiotic resistance, reduced the efficacy of antibiotic treatments, caused residue accumulations in tissues and potential environmental hazards, and also raised public health issues.

The World Health Organization (WHO) recommended the preventative (prophylactic) approaches to disease management rather than costly post-effect treatments (WHO 2002). The increasing political and environmental pressure to decrease the use of antibiotics and other therapeutic chemicals in agriculture and aquaculture has stimulated research into more environmentally friendly approaches for disease control (Hansen and Olafsen 1999; Verschuere *et al.* 2000).

The common tool for proactive (disease prevention) is by incorporating compounds that stimulates the host's immune system. The immune systems of shrimp are not developed as that of the vertebrates and no specific antibodies or proteins are produced in response to the structural components of a particular pathogen. Though mono and multivalent vaccines have been developed against several bacterial diseases in aquaculture, such vaccine preparations are not successful at times in the case of shrimps. In addition, as new diseases and pathogens may emerge from time to time, practically it is impossible to develop proactive strategies using vaccines. But the shrimp immune system could be stimulated in a 'non-specific' manner using a variety of cell wall fragments as well as components from various microorganisms.

The sustainability of production is dependent on the equilibrium between the environmental qualities, the disease prevention by prophylactics, epidemiological surveys of the pathogens, and the health status of the shrimp. Therefore, the prevention and the control of shrimp diseases (management) are emerging as an integrated approach. Considering the potential application of probiotics in animal husbandry, the antagonistic probiotics are being developed as a package of practice for the proactive management of mid-culture outbreaks, shrimp health and growth rate and maintenance of water and soil quality holistically.

The following components are undertaken in prophylactic measure for disease prevention in aquaculture

- a. Prebiotics**
- b. Probiotics**
- c. Vaccines**
- d. Immunostimulants**

A. Prebiotics in shrimp aquaculture

“A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health.”

Prebiotics are a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health (Gibson and Roberfroid, 1995). In different studies since 1999, many substances have been investigated as prebiotic. Three criteria were further stated for dietary carbohydrates to be considered as prebiotics: 1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; 2) fermentation by intestinal microflora; and 3) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being.

e.g. non-digestible carbohydrates, some peptides and proteins, as well as certain lipids, is a candidate prebiotic.

The prebiotics are typically carbohydrates derived from plant or yeast origins. They include resistant inulin and oligofructose, transgalactooligosaccharides (TOS), lactulose, isomalto oligosaccharides (IMO), lactosucrose, xylo-oligosaccharides (XOS), soyabean oligosaccharides and glucooligosaccharides. From in vivo and in vitro studies, inulin and oligofructose, TOS and lactulose are presently classified as prebiotics. Among those, inulin, fructooligosaccharide (FOS), mannanoligosaccharide (MOS), isomaltoligosaccharide (IMO) showed prebiotic characteristics in shrimp. Prebiotics are selectively fermented by Bifidobacteria, Lactobacillus and Bacteroides. Addition of prebiotic in the diet has been reported to increase the uptake of glucose (Breves *et al.*, 2001) and bioavailability of trace elements (Bongers and van den Heuvel, 2003). In later use of prebiotics, they have the binding capacity therefore increasing the absorption of mineral such as calcium, magnesium and iron.

The digestive tract is one of the common sites where pathogens could enter into the shrimp; colonization by microbiota of mucus in the gastrointestinal tract acts as the first line of defense against pathogenic, exogenous or opportunist

microorganisms, which establishes a barrier effect. A group of non-digestible food ingredients which could selectively stimulate the growth and/or the metabolism of health-promoting bacteria in the intestinal tract, and thus improve an organism's intestinal balance, is known as prebiotics.

The beneficial health effects of prebiotics are presumably due to the byproducts generated from their fermentation by gut commensal bacteria, which lead to promote the growth of health promoting bacteria and suppress the effects of harmful bacteria. Furthermore, some carbohydrates, such as short chain FOS (scFOS) and MOS could also be considered as immunosaccharides as they may directly activate certain innate immune responses, thus improving the host's health. It should be noted that β -glucan molecules, well accepted nonspecific immunostimulants, are not categorized as prebiotics in shrimp because crustaceans can digest glucan and use it as an energy source.

Inulin

Inulin belongs to a class of dietary fibers known as fructans, composed of a polymer of β -D-fructose (F) attached by β -2-1 linkages. The first monomer of the chain is either a β -D-glucopyranosyl or β -D-fructopyranosyl residue. D-fructose (F) link with D-glucose (G), with general structure of GF_n. "n" refers to the degree of polymerization of inulin, and it's usually 10 or so. Inulin is used by some plants as a means of storing energy and is typically found in roots or rhizomes. They constitute a group of oligosaccharides derived from sucrose that are isolated from natural vegetable sources.

Although inulin is not known as a natural fibre in shrimp diets, it has been suspected to contribute to balanced gut bacteria development, suppressing pathogens' presence and effects. *Bifidobacteria*, *lactic acid bacteria*, and *clostridia* are known to be able to ferment inulin. A recent study showed that dietary supplementation of inulin decreased the prevalence of WSSV in *Litopenaeus vannamei* and increased the phenoloxidase activity, but didn't affect hemocyte number, growth, survival, and lactic acid bacteria in shrimp. It seems unclear whether inulin alone can act as an immunostimulant or can work through its fermented by-products, such as short chain FOS, so that certain immune defense activities occur in shrimp.

FOS

Fructooligosaccharide is an inulin-like ingredient, having the same general formula of GF, with in ranging from 1 to 5. Two studies evaluated dietary effects of scFOS in recirculating systems using different sizes of *L. vannamei* juveniles (0.17 g versus 7.5 g), and both found that gut microbiota was affected by dietary supplementation of scFOS. It was reported that scFOS improved specific growth rates and feed conversions in younger shrimp juveniles, and significantly affected the counts of *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Lactobacillus sp.* and *Streptococcus faecalis* in shrimp's gut. Although another study using shrimp of bigger size observed no effect of dietary scFOS on weight gain, feed conversion or survival after a six week feeding trial, enhanced hemocyte respiratory bursts were observed in addition to increased colonization of a couple of gram positive aerobic microbes (*Alkalibacillus spp.* and *Micrococcus spp.*) and an unidentified seawater bacterium occurring in the digestive tract. However, whether these microbial shifts have any positive effects on the shrimp health, and through what mechanism, remains to be further investigated.

IMO

Isomaltooligosaccharides, specifically, are mixed glucose oligomers with α -D-(1,6)- linkages. Dietary inclusion of 0.2% isomaltooligosaccharides alone showed no beneficial effect on shrimp performance, immune response and disease resistance. However, positive synergistic effects on shrimp immune responses and disease resistance were indicated when the combination of 0.2% IMO and 10^8 CFU/g *Bacillus OJ* was administered in *L. vannamei* through feed.

Prebiotic Application in Shrimp Research.			
Prebiotic	Used for Shrimp Species (average size)	Dose and Duration	Major Effect
Inulin	<i>Litopenaeus vannamei</i> (1.1 g)	0, 1.25, 2.5, 5.0, and 10 g/kg (62 days, 73 days)	2.5-5.0g/kg decreased the prevalence of WSSV in shrimp, increased the phenoloxidase activity, but had no effect on hemocyte number, growth, survival, and lactic acid bacteria in shrimp.
scFOS	<i>Litopenaeus vannamei</i> (7.54 g)	0.25, 0.5, 0.75, 1, 2, 4, 8g/kg 42 days	1g/kg, 8g/kg affected gut microbiota, enhanced hemocyte respiratory burst, but not weight gain, feed conversion or survival
scFOS	<i>Litopenaeus vannamei</i> (0.17g)	0, 0.4, 0.8, 1.2, 1.6g/kg 56 days	≥0.4g/kg improved growth rate, feed intake, feed conversion, and affected gut microbiota
MOS	<i>Penaeus semisulcatus</i> (0.34g)	0, 1.5, 3, 4.5g/kg 48 days	3g/kg improved growth, feed conversion and survival. No detrimental effect was noted on hepatopancreas
MOS	<i>Litopenaeus vannamei</i> (1.35g)	3g/kg 75days	Enhanced shrimp survival, moulting rate, growth and FCR
MOS	<i>Litopenaeus vannamei</i> (1.65g)	0, 4, 6, 8, 10g/kg 30 days	≥0.4g/kg enhance shrimp growth, feed intake and FCR, affected gut microbiota.
MOS	<i>Litopenaeus vannamei</i> (2.52g)	0, 1, 2, 4, 6, 8g/kg 56 days	2-8g/kg enhanced growth rate and increased the intestinal microvilli length, 4-8g/kg improved resistance against NH ₃ stress, activities of phenoloxidase and superoxide dismutase in haemolymph
IMO	<i>Litopenaeus vannamei</i> (1.75g)	2g/kg 28days	No effect alone, but showed synbiotic effect on microbial population, immune responses and resistance to white spot syndrome virus

Source: (Gong, 2015, Aquaculture magazine)

B. Probiotics in shrimp aquaculture

"Live microorganisms that have a beneficial effect on the host by modifying the microbial community, associated with the host, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment".

The gastrointestinal microbiota of fish and shellfish are peculiarly dependent on the external environment, due to the water flow passing through the digestive tract. Most bacterial cells are transient in the gut, with continuous intrusion of microbes coming from water and food. Some commercial products are referred to as probiotics, though they were designed to treat the rearing medium, not to supplement the diet.

Most attempts to propose probiotics have been undertaken by isolating and selecting strains from aquatic environment. These microbes were Vibrionaceae, pseudomonads, lactic acid bacteria, *Bacillus* spp. and yeasts. Three main characteristics have been searched in microbes as candidates to improve the health of their host. (1) The antagonism to pathogens was shown in vitro in most cases. (2) The colonization potential of some candidate probionts was also studied. (3) Challenge tests confirmed that some strains could increase the resistance to disease of their host. Many other beneficial effects may be expected from probiotics, e.g., competition with pathogens for nutrients or for adhesion sites, and stimulation of the immune system. The most promising prospects are sketched out, but considerable efforts of research will be necessary to develop the applications to aquaculture.

When looking at probiotics intended for an aquatic usage it is important to consider certain influencing factors that are fundamentally different from terrestrial based probiotics. Aquatic animals have a much closer relationship with their external environment. There are the big differences between terrestrial and aquatic animals in the level of interaction between the intestinal microbiota and the surrounding environment. On the other hand, potential pathogens are able to maintain themselves in the external environment of the aquatic organisms and proliferate independently of the host (Hansen and Olafsen 1999; Verschuere *et al.* 2000; Kesarcodi-Watson *et al.* 2008).

The bacterial community composition of the intestinal tract of aquatic animals is different from that found in terrestrial animals. The larval forms of most fish and shellfish are released in the external environment at an early ontogenetic stage. These larvae are highly exposed to gastrointestinal microbiota-associated disorders, because they start feeding even though the digestive tract is not yet fully developed (Timmermans 1987), and though the immune system is still incomplete (Vadstein 1997). Thus, probiotic treatments are particularly desirable during the larval stages (Gatesoupe 1999).

a. Evaluation of probiotics

The use of probiotic bacteria, based on the principle of competitive exclusion, and the use of immunostimulants are two of the most promising preventive methods developed in the fight against diseases during the last few years (Verschuere 2000). One of the main challenges in developing probiotic bacteria is using appropriate selection and colonization methods. The selection criteria for probiotic bacteria should evaluate the colonization methods, competition ability against pathogens and the immunostimulatory growth effect on shrimp (Gatesoupe 1999; Gomez-Gil *et al.* 2000). By applying these bacteria in shrimp farms, a biological equilibrium between competing beneficial and deleterious microorganisms could be produced.

Probiotic bacteria directly uptake or decompose the organic matter or toxic material in the water and sediment thereby improving the quality of water (Wang *et al.* 2005). The addition of bacteria into the water, they could decompose the excreta of shrimps, remaining food materials, remains of the plankton and other organic materials to CO₂, nitrate, and phosphate.

The selection and application technologies of probiotics must be based on thorough understanding of the mechanisms involved and the putative consequences. An essential part of that understanding can be provided by studies looking in detail at host-microbial interactions. The development of suitable vehicles / delivery mechanism for the effective introduction of probiotics in the shrimp gut is inevitable for the attainment of successful probiotics in the “in captivity” environments. Although still a matter of debate, several authors has indicated that a minimal concentration of 1×10^6 (colony forming units) cfu g⁻¹ of a product is required to exert a probiotic effect (Ravula and Shah 1998; Shah 2000; Vinderola and Reinheimer 2000; Roy 2001; Talwalker and Kailasapathy 2004) in higher animals.

Probiotics that currently used in aquaculture industry include a wide range of taxa from Lactobacillus, Bifidobacterium, Pediococcus, Streptococcus and Carnobacterium spp. to Bacillus, Flavobacterium, Cytophaga, Pseudomonas, Alteromonas, Aeromonas, Enterococcus, Nitrosomonas, Nitrobacter, and Vibrio spp., yeast (Saccharomyces, Debaryomyces) and etc. (Irianto and Austin 2002; Burr *et al.* 2005; Sahu *et al.* 2008).

b. Types of aquatic probiotics

i) Gut probiotics which can be blended with feed and administrated orally to enhance the useful microbial flora of the gut.

ii) Water probiotics which can proliferate in water medium and exclude the pathogenic bacteria by consuming all available nutrients. Thus, the pathogenic bacteria are eliminated through starvation (Nageswara and Babu 2006; Sahu *et al.* 2008).

The first type probiotics are using mainly in finfish aquaculture and the second type in shrimp aquaculture. Commercially available probiotics include pure strains, defined mixture of specific strains, but also consortia of strains and undefined mixtures. Generally, probiotics proposed as biological control agents in aquaculture are applied in the feed or as a water additive supplement.

Aquatic probiotics are marketed in two forms:

i) Dry forms: the dry probiotics that come in packets can be given with feed or applied to water. They have many benefits, such as safety, easy using, longer shelf life and etc. (Decamp and Moriarty 2007).

ii) Liquid forms: the hatcheries generally use liquid forms which are live and ready to act. These liquid forms are directly added to hatchery tanks or blended with farm feed. The liquid forms can be applied any time of the day in indoor hatchery tanks, while it should be applied either in the morning or in the evening in outdoor tanks. Liquid forms give positive results in lesser time when compared to the dry and spore form bacteria, though they are lower in density (Nageswara and Babu 2006).

d. Guidelines and regulations

A number of definitions for the term 'probiotic' have been used over the years but the one derived by the Food and Agriculture Organization of the United Nations-World Health Organization (FAO-WHO) (FAO/WHO 2001) are endorsed by the International Scientific Association for Probiotics and Prebiotics (Reid *et al.* 2003). As per the FAO-WHO guidelines, the scope of probiotics could be defined as "live

microorganisms, which when administered in adequate amounts, confer a health benefit on the host". This definition retains the historical elements for the use of living organisms for health purposes but does not restrict the application of the term only to oral probiotics with intestinal outcomes (Reid and Bruce 2006). The guidelines that stipulate what is required for a product to be called a probiotic were published by FAO-WHO in 2002 (FAO/WHO Guidelines 2002). The requirements include, the strains must be designated individually, speciated appropriately and should retain a viable count at the end of their shelf life in the designated product formulation that confers a proven clinical end-point.

The selection and evaluation of potential probiotic candidates is a multistep process focusing on functional, safety, and technological aspects (Sanders and Huis in't Veld 1999; Saarela *et al.* 2000; Reid 2006). There is a growing awareness that the correct identification of a probiotic strain is one of the first prerequisites documenting its microbiological safety (Huys *et al.* 2006). Documentations of proven clinical efficacy and known mechanisms of action in addition to clearly outlined dosage, duration of use, and safety parameters will enable caregivers to recommend products and enable consumers to purchase probiotic foods and over-the-counter products with a high level of confidence.

As new probiotics emerge alongside genetically modified organisms (GMOs) that are designed specifically to treat disease (Steidler *et al.* 2003), long-term monitoring will be important to ensure that safety issues and (in the case of GMOs) proper environmental containment issues are addressed. According to Henriksson *et al.* (2005), more clinical studies need to be performed, preferably comparing one probiotic product against another or against standard medical practice. In this way, the strengths and limitations of probiotics can be determined.

e. Application of probiotics

Probiotic activity is mediated by a variety of effects that are dependent on the probiotic itself, the dosage employed, treatment duration and route, and frequency of delivery. Some probiotics exert their beneficial effects by elaborating antibacterial molecules such as bacteriocins that directly inhibit other bacteria or viruses, actively participating in the fight against infections, whereas others inhibit bacterial movement across the gut wall (translocation), enhance the mucosal barrier function by increasing the production of innate immune molecules, or modulate the inflammatory/ immune response. Several studies have demonstrated that pattern

recognition receptors (PRPs), such as toll-like receptors (TLRs) signaling pathways, immune responses, and the secretion of antimicrobial peptides such as defensins and chemokines by the epithelium play important roles in these mechanisms.

Bacillus S11, previously isolated from the GIT of *P. monodon* brood stock caught in the gulf of Thailand, demonstrated effective probiotic protection with *P. monodon*. After a 100-day feeding trial with probiotic supplemented and non-supplemented (control) feeds, *P. monodon* (from PL30 onwards) exhibited significant differences in growth, survival, and external appearance between the two groups. After challenging shrimps with a shrimp pathogen, *Vibrio harveyi* by immersion for 10 days, all probiotic treated groups had 100% survival, whereas the control group had only 26% survival which suggested competitive exclusion by probiotic *Bacillus* S11. Probiotic *Bacillus subtilis* UTM 126 was known to produce antimicrobial activity against vibriosis in juvenile shrimp, *Litopenaeus vannamei*.

Probiotics also influence the immune system of the fish, shrimp, and other aquatic species. *Streptomyces* has been applied as a probiotic in the laboratory culture of *Penaeus monodon*, which showed better water quality parameters than the control tank and increased length and weight in terms of growth. Some probiotic products like Super-biotic, Super Ps, Zymetin, and Mutagen were reported to play a vital role in postlarvae of *P. monodon* by maintaining good water quality parameters throughout the culture period. It was reported that *Bacillus subtilis* E20, isolated from the human health food, was used for white shrimp *LitoLitopenaeus vannamei* larvae where it showed a significant decrease in the cumulative mortality and also increased gene expression of prophenoloxidase I, prophenoloxidase II, and lysozyme of larvae.

f. Beneficial effects of probiotic

Probiotic strain	Used on	Effect of probiotic strain
<i>Bacillus</i> S11	<i>Penaeus monodon</i>	Protection against <i>Vibrio harveyi</i> by stimulation of cellular and humoral immune defences
<i>Bacillus subtilis</i> UTM 126	<i>Litopenaeus vannamei</i>	Control vibriosis by producing bacitracin, gramicidin, polymyxin, tyrotricin, and competitive exclusion

Streptomyces	<i>Penaeus monodon</i>	Better water quality parameters, increased length and weight of the animal
<i>Bacillus subtilis E20</i>	<i>LitoPenaeus monodon</i>	Enhance humoral immune response

g. Probiotics in activation of shrimp immune defence

Shrimp has a poorly developed immune system and probiotics were known to play an important role in the enhancement of immune response in shrimp. The probiotic bacteria *Lactobacillus plantarum* was reported to enhance the immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, when given in diet. The bacteria influenced both the cellular and humoral immune defences in the shrimp. *L. plantarum* was known to enhance the phenoloxidase (PO) activity, prophenoloxidase (ProPO) activity, respiratory bursts, superoxide dismutase (SOD) activity and clearance efficiency of *Vibrio alginolyticus*, peroxinectin mRNA transcription, and survival rate after challenge with *V. alginolyticus*. These effects the immune defenses also maintain the defence levels in the shrimp offering a prolonged protection. Probiotics strains *Vibrio* P62, *Vibrio* P63, and *Bacillus* P64 were isolated from hepatopancreas of healthy wild shrimp *Penaeus vannamei*, and their immunostimulatory effect was studied. Among the three, P64 showed a significantly higher immunity index and showed immune response similar to that of *V. alginolyticus* whereas the other two only showed good probiotic properties. Here, the P64 gave the immune alert with a significant increase in the hyaline cell population.

Some *Vibrio* spp. was assessed for their probiotic potential for *L. vannamei*. Among the species tested *V. alginolyticus* (NCIMB 1339) and *V. gazogenes* (NCIMB 2250) showed antagonistic activity towards shrimp pathogens vibriosis. When the juvenile shrimps were fed with chitin and *V. gazogenes*, they caused a significant decline in the number of vibrio-like bacteria in the fore and hind gut. In this study, the *Vibrio* and chitin mixture caused significant changes in haemocyte numbers. This change in haemocyte number probably reflects the immunological status of shrimp because these are involved in both cellular and humoral defences of the shrimp.

h. Potential sources of antagonistic probiotics

Gram-negative bacteria are considered to cause the majority of bacterial problems associated with shrimp diseases (Bachere *et al.* 1995; Verschueren *et al.*

2000; Selvin *et al.* 2005). With the exception of terrestrially derived Gram positive bacteria particularly lactic acid bacteria, the selection of probiotics in marine aquaculture is dominated by Gram-negative species (Austin *et al.* 1995; Gibson *et al.* 1998; Gram *et al.* 1999; Spanggaard *et al.* 2001).

Sponge associated marine bacteria is a potential source of novel shrimp probiotics (Selvin *et al.* 2008, unpublished data). It was found that the endosymbiotic marine actinobacterium *Nocardiopsis alba* MSA10 isolated from the marine sponge *Fasciospongia cavernosa* showed potential antagonistic activity against prominent *Vibrio* pathogens of *Penaeus monodon* *in vitro* and *in vivo*. Sfanos *et al.* (2005) made a survey of bacterial samples isolated from wild marine sources including macroalgae, seawater, and sea sediment to screen potential probionts. Numerous bacterial community have been explored from unique marine environments, such as hydrothermal vents (Jeanthon 2000), marine sea sediments (Cifuentes *et al.* 2000; Llobet-Brossa *et al.* 1998), marine biofilms, microalgae blooms (Seibold *et al.* 2001), and marine sponges (Lafi *et al.* 2005).

i. Successful and commercial probiotics

The large majority of commercial probiotic products contain one or multiple strains of lactic acid bacteria primarily belonging to the genera *Lactobacillus* (Donkor *et al.* 2007; Geier *et al.* 2007), *Bifidobacterium*, *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus*. In addition, other bacterial taxa such as *Propionibacterium* spp., *Bacillus* spp. and *Escherichia coli* and the yeast *Saccharomyces boulardii* have also been used in probiotic products (Holzapfel *et al.* 1998; Klein *et al.* 1998; Mercenier *et al.* 2003). Some *Bacillus* sp. (*B. megaterium*, *B. Polymyxa*, *B. subtilis*, *B. licheniformis*), lactic acid bacteria (*Lactobacillus* sp., *Carnobacterium* sp., and *Streptococcus* sp.), *Pseudomonas* sp. (*P. fluorescens*) and *Vibrio* sp. (*V. alginolyticus*, *V. salmonicida*- like) have been proposed and tested as probiotics in aquaculture (Gatesoupe 1991; Verschueren *et al.* 2000).

Four species including, *B. pumilus*, *Micrococcus luteus*, *P. fluorescens* and *P. putida* are currently included in bacterial mixtures that are marketed as probiotics for aquaculture (Prowins Biotech Private Ltd., India). Additionally, *Bacillus* sp. have been successfully used as probiotics in the aquaculture of black tiger shrimp (*Penaeus monodon*) in Thailand, where there was an improvement in the growth rate (47%) and survival rate when challenged with *Vibrio harveyi* (Rengpipat *et al.* 1998).

Aeromonas media UTS strain A199 has been shown to be a potential probiotic for the management of bacterial (Gibson *et al.* 1998; Tan *et al.* 2003) and fungal pathogens (Lategan and Gibson 2003; Lategan *et al.* 2004a,b) in the aquaculture industry (Lategan *et al.* 2006). Rengpipat *et al.* (2000) showed that the survival and growth of the black tiger shrimp (*Penaeus monodon*), fed with probiont *Bacillus* S11 was increased when compared with non-treated shrimp.

Prospective probiotics evaluated for shrimp aquaculture applications

Strain	Source	Evaluated for	Effective dose / mode of application	Reference
<i>Bacillus</i> S11	Black tiger shrimp	Growth and survival of black tiger shrimp <i>Penaeus monodon</i>	1 kg wet wt (~100 g dry wt) of BS11 (~10 ¹⁰ CFU g ⁻¹) in 3 kg of feed (2.5% BS11/3 kg)	Rengpipat <i>et al.</i> 2003
<i>Bacillus subtilis</i> BT23	Shrimp culture ponds	Against the growth of <i>Vibrio harveyi</i> isolated by agar antagonism assay from <i>Penaeus monodon</i>	10 ⁶ –10 ⁸ CFU ml ⁻¹ for 6 d	Vaseeharan and Ramasamy 2003
<i>Pseudomonas</i> sp. PM11 <i>Vibrio fluvialis</i> PM17	Gut of farm reared sub-adult shrimp	Immunity indicators of <i>Penaeus monodon</i>	<i>Pseudomonas</i> sp. PM 11 @ 10 ³ bacterial cells ml ⁻¹ for 3 days and <i>V. fluvialis</i> PM 17 @ 10 ³ bacterial cells ml ⁻¹ for seven days	Alvandi <i>et al.</i> 200
<i>Arthrobacter</i> XE-7	Isolated from <i>Penaeus chinensis</i>	Protection of <i>Penaeus chinensis</i> post-larvae from pathogenic vibrios such as <i>Vibrio parahaemolyticus</i> , <i>Vibrio anguillarum</i> and <i>Vibrio</i>	10 ⁶ CFU/ml	Li <i>et al.</i> 2006

		<i>nereis</i>		
<i>Bacillus subtilis</i> and <i>B. megaterium</i>	Marine environment	Production of digestive enzymes proteases, carbohydrases and lipases	Potential application in shrimp feeds	Solano and Soto 2006
<i>Paenibacillus</i> spp., <i>B. cereus</i> and <i>Pa. polymyxa</i>	Seawater, sediment and marine fish-gut samples	Against pathogenic	Vibrios 10^4 and 10^5 CFU ml^{-1}	Ravi <i>et al.</i> 2007
<i>Synechocystis</i> MCCB 114 and 115	Seawater	Antagonism against <i>V. harveyi</i>	Post-larvae fed on the cyanobacterial cultures	Preetha <i>et al.</i> 200
<i>Bacillus licheniformis</i>	Shrimp pond	Intestinal microbiota and immunity of the white shrimp <i>Litopenaeus vannamei</i>	<i>B. licheniformis</i> suspension of 10^5 CFU ml^{-1} for 40 days	Li <i>et al.</i> 2007
Lactic-acid bacteria	Shrimp gut	Survival of marine shrimp, <i>Litopenaeus vannamei</i> challenged with <i>V. harveyi</i>	Liquid diet supplemented with B6 strain at $10(8)$ CFU/ mL	Vieira <i>et al.</i> 2007
<i>Lactobacillus plantarum</i>	Shrimp isolate	Immune response and microbiota of shrimp digestive tract of <i>Litopenaeus vannamei</i> challenged with <i>V. alginolyticus</i> and <i>V. harveyi</i>	10^{10} CFU /kg diet /108 CFU / kg feed	Chiu <i>et al.</i> 2007

<i>Vibrio alginolyticus</i> UTM 102, <i>Bacillus subtilis</i> UTM 126, <i>Roseobacter gallaeciensis</i> SLV03, and <i>Pseudomonas aestumarina</i> SLV22	Gastrointestinal tract of adult shrimp <i>Litopenaeus vannamei</i>	Antagonism against the shrimp-pathogenic bacterium, <i>Vibrio parahaemolyticus</i> PS-017	Feed supplement	Balcázar <i>et al.</i> 2007
<i>Bacillus subtilis</i> UTM 126	Shrimp culture pond	Protection against vibriosis in juvenile <i>Litopenaeus vannamei</i>	10 ⁵ CFU/g	Balcázar and Rojas
<i>Pediococcus acidilactici</i>	Strain MA 18/5M, CNCM	Survival of <i>Litopenaeus stylirostris</i> against Vibriosis caused by <i>Vibrio nigripulchritudo</i>	Probiotic-coated pellet feed	Mathieu <i>et al.</i> 2008
<i>B. subtilis</i> , <i>B. natto</i> , and <i>B. licheniformis</i>	Not available	Growth and digestive enzyme activity of <i>Litopenaeus vannamei</i>	1.5 to 7.5% supplemented to the feed	Gómez and Shen 2008

j. Probiotics: Improvement in water qualities

The newest attempt being made to improve water quality in aquaculture is the application of probiotics and/or enzymes to the ponds. This type of biotechnology is known as "bioremediation", which involves manipulation of microorganisms in ponds to enhance mineralization of organic matter and get rid of undesirable waste compounds.

The probiotics can decompose the excreta of fish or prawns, remaining food materials, remains of the plankton and other organic materials to CO₂, nitrate and phosphate. These inorganic salts provide the nutrition for the growth of micro algae, while the bacteria grow rapidly and become the dominant group in the water, inhibiting the growth of the pathogenic microorganisms. The photosynthesis of the micro algae provide dissolved oxygen for oxidation and decomposition of the organic materials and for the respiration of the microbes and cultured animals. This kind of cycle improves the nutrient cycle, and it can create a balance between bacteria and micro algae, and maintaining a good water quality environment for the cultured animals.

C. Vaccines in shrimp aquaculture

"Vaccinations provide protection against specific pathogens".

a. Concept of vaccines

Vaccination is a process of by which a protective immune response is induced in an animal by administration of vaccine. Vaccines are preparation of antigens derived from pathogenic organism, rendered non-pathogenic to the disease on natural encounter with the pathogen.

Vaccines work by exposing the immune system of an animal to an "antigen" - a piece of a pathogen or the entire pathogen - and then allowing time for the immune system to develop a response and a "memory" to accelerate this response in later infections by the targeted disease-causing organism. Vaccines are normally administered to healthy animals prior to a disease outbreak.

b. The ideal vaccines

- i. is safe for the shrimp, the person(s) vaccinating the animal, and the consumer;
- ii. protects against a broad strain or pathogen type and gives 100% protection;
- iii. provides long-lasting protection, at least as long as the production cycle;
- iv. is easily applied;
- v. is effective in a number of shrimp species;

- vi. is cost effective; and
- vii. is readily licensed and registered.

c. Role of vaccines

Vaccines on primary administration sensitises the host and builds up a protective immune response by way of memory cells which persist for a longer time – longevity varies. Natural encounter with the pathogen acts as a booster to the immunity already produced by vaccination. Development of immunity may be systemic or in integumentary organs. It is thus important to demarcate the route of administration of the vaccines.

d. Types of vaccines

- **Inactivated vaccines** are the most common in aquaculture. They are produced by inactivating the disease-causing micro-organism with chemicals or heat.
- **Live, attenuated vaccines.** To make a live, attenuated vaccine, the disease-causing microorganism is grown under special laboratory conditions that cause it to lose its virulence or disease-causing properties
- **Subunit vaccines** are a more modern type of vaccine, developed from antigenic fragments that are able to evoke an immune response. Subunit vaccines can be made by purification of parts of the actual micro-organism or they can be made in the laboratory using genetic engineering techniques.
- **Recombinant vector vaccines.** A vaccine vector or carrier is a weakened bacterium into which harmless parts of genetic material from another disease-causing micro-organism have been inserted.
- **DNA vaccines** are developed using plasmids containing information on the expression (of part) of the antigen of interest.
- **Synthetic/peptide vaccines** are synthetically prepared antigenic epitopes in a suitable carrier or adjuvant.

e. Mode of vaccination

Vaccines are administered to animal in one of three ways: by mouth, by immersion, or by injection. Each has its advantages and disadvantages. The most effective method will depend upon the pathogen and its natural route of infection, the life stage of the fish/shrimp, production techniques, and other logistical considerations.

i. Oral vaccination

Oral vaccination results in direct delivery of antigen via the digestive system of the animal. It is the easiest method logistically because feeding is a normal, ongoing

part of the production schedule. Stress on the shrimp is minimal, and no major changes in production are required. Prior to feeding, vaccine is mixed, top-dressed, or bioencapsulated into the feed. To reduce leaching into the water and/or to provide some protection against breakdown of the vaccine by the fish's digestive processes, a coating agent is often used. For small animal, bioencapsulation may be a preferred method of oral delivery. Live food (rotifers, brine shrimp) is added to a concentrated vaccine solution, and allowed to take up vaccine. This live food is then fed to fry or small fingerlings. Although oral vaccine is the most preferred method, it conveys relatively short immunity (compared to the other methods) such that additional vaccination may be required. In addition, because of the problems involved with getting the vaccine intact through the intestine and adequately stimulating the immune system, there are few commercial oral vaccines available (Komar *et al.* 2004).

ii. Immersion vaccination

Immersion vaccination permits immune cells located in the shrimp's skin and gills to become directly exposed to antigens. These immune cells may then mount a response (e.g., antibody production), thus protecting the shrimp from future infection. Other types of immune cells in the skin and gills carry antigens internally, where a more systemic response will also develop. Immersion vaccination occurs by dip or by bath. Dips are short, typically 30 seconds, in a high concentration of vaccine. Baths are of longer duration - an hour or more - and in a much lower concentration of vaccine. In practice, dips are logistically more practical for large numbers of small shrimp. Unfortunately, protection using immersion methods may not last long and a second vaccination may be required (Komar *et al.* 2004) because smaller, younger shrimp may have immature immune systems and because this is a more indirect route.

iii. Injection

Injection vaccination allows direct delivery of a small volume of antigen into the muscle (intramuscular (IM) injection) or into the body cavity (intracoelomic [ICe= intraperitoneal or IP] injection), allowing for more direct stimulation of a systemic immune response. Injection vaccines normally include an oil-based or water-based compound, known as an adjuvant, which serves to further stimulate the immune system. Injection is effective for many pathogens that cause systemic disease; and protection - 6 months to a year - is much longer than by other methods. Every shrimp in the population is injected, giving more assurance to the producer. Another

advantage is that multiple antigens (for different diseases) can be delivered at the same time. However, vaccination by injection is logistically the most demanding of all three methods. Shrimp must be anesthetized to minimize stress. Injection requires more time, labor, and skilled personnel. The correct needle size is important. The vaccine may incite a more severe reaction if it is injected into the wrong portion of the shrimp. And finally, smaller-sized shrimp (under 10 g) may not respond well to this method (Komar *et al* 2004).

f. Development of vaccines

- Of principal importance in the entire vaccine development process is the precise identification of the causative organism, including the existence and significance of serotypes, and a full understanding of the epidemiology of the disease.
- Knowledge on the prevailing diseases, their economic significance and the pathogens associated are key information required to support a vaccination program.
- Unfortunately such information are still lacking for most pathogens involved.
- Far too often, disease outbreaks are described based on disease signs and not on the isolation and characterization of the pathogen

g. Vaccination against shrimp diseases

Two studies have examined if changes in antibacterial activity are associated with the phenomenon of 'specific immune priming' in crustaceans (Pope *et al.*, 2011; Powell *et al.*, 2011). Powell *et al.* (2011) used post-larvae of *L. vannamei* exposed to a commercial anti-vibrio vaccine (Vibromax™) whilst Pope *et al.* (2011) used the larger juveniles of *L. vannamei* and a bacterin composed of formalin inactivated *V. harveyi*. In the former experiments, post-larvae were exposed to the commercial vaccine using the manufacturer's recommended method (via food, *Artemia salina*). They observed that post-larvae exposed to vaccine showed elevated levels of antibacterial activity against *V. anguillarum* (in the vaccine) up to 14 days post-exposure. However, in the case of *V. harveyi* (also in the vaccine) they found no antibacterial activity either in the naïve or vaccine-exposed animals.

In the following study, the experimental WSSV survivors become resistant against experimental re-challenge with WSSV at weeks 3 or 4 and the resistance continued up to 2months. From the results, they assumed that the neutralizing factors in the survivor's plasma are involved in this resistance against WSSV (Wu *et*

al., 2002). These results stimulated the researchers into the possibility of vaccinating shrimp against WSSV. Within a few years several independent studies have explored different anti-WSSV strategies to protect shrimp, including inactivated vaccines (Singh *et al.*, 2005), recombinant protein vaccines (Witteveldt *et al.*, 2004a; Fu *et al.*, 2010), DNA vaccines (Rout *et al.*, 2007; Li *et al.*, 2010) and dsRNA vaccines (Robalino *et al.*, 2004; Kim *et al.*, 2007; Sarathi *et al.*, 2008; Mejia-Ruiz *et al.*, 2011).

Two structural protein genes, VP19 and VP466 have been against White Spot Syndrome Virus (WSSV) in shrimp (*Penaeus chinensis*) (Ha *et al.*, 2008). The cumulative mortalities of the shrimps vaccinated by the injection of rVP19 and rVP466 at 15 days after the challenge with WSSV were 50.2% and 51.8%, respectively. For the vaccination by oral feeding of rVP19 and rVP466, the cumulative mortalities were 49.2% and 89.2%, respectively. These results show that protection against WSSV can be generated in the shrimp, using the viral structural protein as a protein vaccine (Ha *et al.*, 2008).

Truncated VP28 as oral vaccine candidate has been studied against WSSV infection in shrimp *Penaeus monodon*. The truncated VP28 can be considered suitable for oral vaccination, when the digestion in the foregut can be bypassed (Kulkarni *et al.*, 2013). A vaccination with formalin-inactivated *Vibrio* sp. was effective against vibriosis in larval culture of penaeid shrimp (Alabi *et al.*, 1999). Shrimp that received formalin-inactivated *V. harveyi* or a commercial vaccine that contained inactivated *Vibrio* exhibited enhanced phagocytosis (Pope *et al.*, 2011). Administration of formalin-inactivated white spot syndrome virus (WSSV) or the purified envelope protein of WSSV produced a protective response against the virus in shrimp. Lin *et al.* (2013) have been reported that vaccination Enhances Early Immune Responses in White Shrimp *L. vannamei* after Secondary Exposure to heat-killed *V. alginolyticus* (HVa) and formalin-inactivated *V. alginolyticus* (FVa) (Lin *et al.*, 2013). The results were showed that FVa functions as a vaccine, whereas HVa functions as an inducer and can be used as an immune adjuvant. A combined mixture of FVa and HVa can serve as a “vaccine component” to modulate the immunity of shrimp.

Different vaccines strategies

Sl. No.	Vaccine	Details of vaccine	Result	Reference
1.	Inactivated	Inactivated WSSV	Positive	Namikoshi <i>et al.</i> (2004)
		Inactivated WSSV	Positive	Singh <i>et al.</i> (2005)
2.	Recombinant protein	VP19, VP28 expressed in bacteria	Negative and Positive (respectively)	Witteveldt <i>et al.</i> (2004a,b)
		VP19, VP28 expressed in bacteria	Positive	Jha <i>et al.</i> (2006)
		VP28 expressed in <i>Bombyx mori</i>	Positive	Wei and Xu (2005)
		VP28 expressed in <i>Bacillus subtilis</i>	Positive	Fu <i>et al.</i> (2010)
		VP28 expressed in baculovirus Syed	Positive	Musthaq <i>et al.</i> (2009), Syed Musthaq and Kwang (2011)
3.	DNA vaccine	VP15, VP28, VP35 and VP281 DNA constructs	VP35 and VP281 DNA constructs	Rout <i>et al.</i> (2007)
		VP35 and VP281 DNA constructs	Negative	
		VP28 DNA construct with chitosan nanoparticle	Positive	RajeshKumar <i>et al.</i> (2009)
		VP28 DNA construct in <i>Salmonella typhimurium</i>	Positive	Ning <i>et al.</i> (2009)
		VP28 DNA construct	Positive	Li <i>et al.</i> (2010)

4.	double stranded RNA	Non-specific dsRNA	Positive	Robalino <i>et al.</i> (2004)
		VP28, VP281 specific dsRNAs	Positive	Kim <i>et al.</i> (2007)
		VP28 specific dsRNA	Positive	Sarathi <i>et al.</i> (2008)
		VP28, VP26 specific dsRNAs	Positive	Mejia-Ruiz <i>et al.</i> (2011)

Source: Musthaq and Kwang, (2015)

h. RNAi technology applications for cultured shrimp

RNAi technology is a useful tool for identifying the genes in shrimp genome. Several genes has been well-established using this technique such as the hyperglycemic hormone cDNA (Lugo, *et al.*, 2006), ion transport peptide (Tiu, *et al.*, 2007), transglutaminase and clotting protein (Maningas, *et. al.*, 2008), proPO (precursor to phenoloxidase) (Fagutao, *et al.*, 2009) and proPO activating enzyme (PPAE) (Charoensapsri, *et al.*, 2009).

Most research using RNAi and is directly related to the shrimp industry is geared towards antiviral immunity, since shrimp plays a huge role economically, and most of its viral pathogens, such as White spot syndrome virus (WSSV), Taura syndrome virus (TSV) and Yellow head virus (YHV) where stocks infected result in mass mortality and great production and financial loss to the industry.

D. Immunostimulants in shrimp aquaculture

Generally, immunostimulants enhance individual components of the non-specific immune response but this does not always translate into increased survival. In addition, immunostimulants fed at too high dose or for too long can be immunosuppressive. The substances of capable of stimulating immune response are the compounds that promote release of from immune effector cells.

Immunostimulants enhance the humoral and cellular response in both specific and non-specific ways. The use of immunostimulants in aquaculture of other species for prevention of diseases is a promising new development. In general, immunostimulants comprise a group of biological and synthetic compounds that enhance the non-specific defense mechanisms in animals, thereby imparting generalized protection.

a. Classification of Immunostimulants

Immunostimulants can be classified into several categories by their origin and mode of action

- i. bacteria and bacterial products,
- ii. complex carbohydrates,
- iii. vaccines,
- iv. immunity enhancing drugs,
- v. nutritional factors,
- vi. animal extracts,
- vii. cytokines, and
- viii. Lectins, plant extracts.

b. Specific and non-specific Immunostimulants

Specific immunostimulation is related to the potentiation of the host's immune system towards a unique specific antigen. Vaccination is perhaps the best example of producing specific immunity.

Non-specific immunostimulation generally is an attempt to upgrade immunologic capabilities at a time when an animal may be exposed to one or several pathogens and/or be immuno-compromised.

c. Characteristics of an ideal immunostimulants

These can be described as:

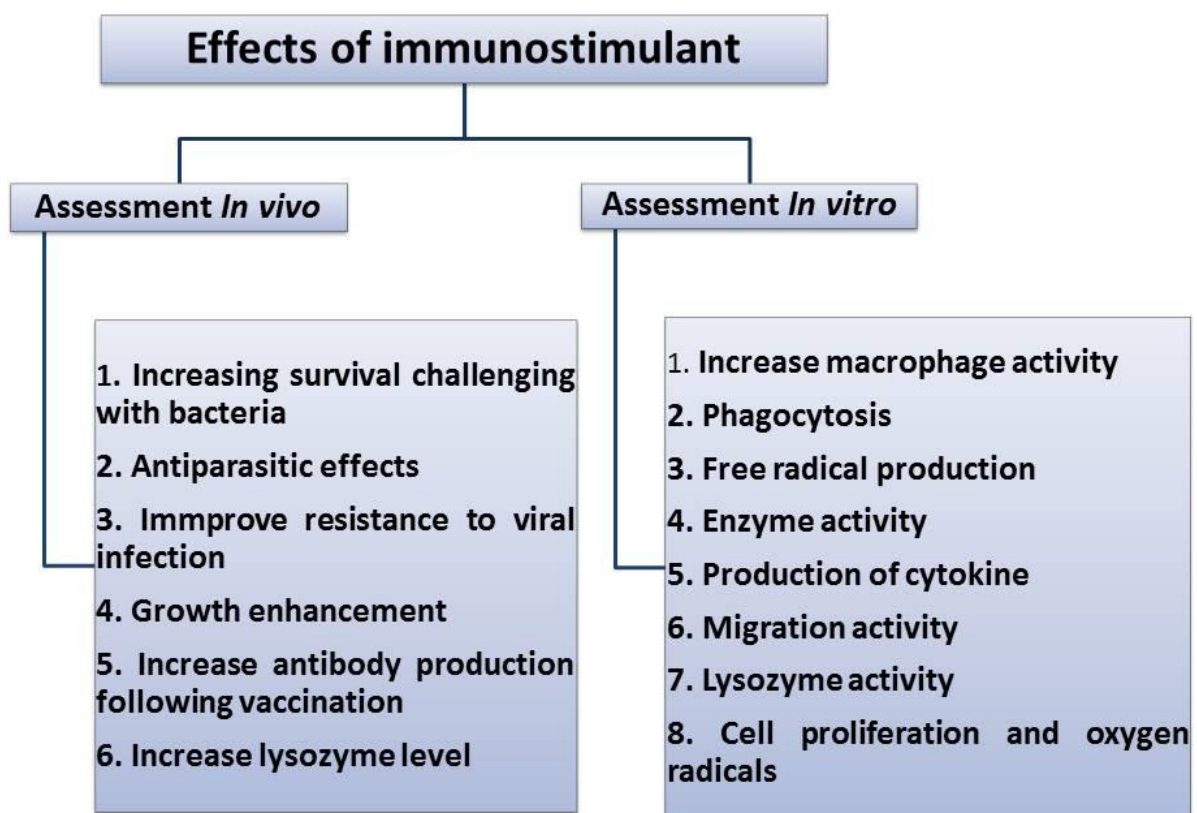
- i. It should be non-toxic, even at a high dose rate.
- ii. It should be non-carcinogenic or have long term side effects.
- iii. At therapeutic levels, it should have a short withdrawal period with low tissue residues.
- iv. It should stimulate a wide range of non-specific immune responses against bacteria, fungi, virus, protozoa and helminthes.
- v. It should be capable of amplifying primary and secondary immune responses to infectious agents.
- vi. Breakdown products of compound concerned should be either inactive or readily biodegradable in the environment.
- vii. It should be having defined chemical composition or biological activity.
- viii. It should be active by oral route and should be stable both in its native state and after incorporation into food and water.
- ix. It should be compatible with arrange of drugs including antibiotics and anthelmintics, and
- x. It should be inexpensive and either tasteless or palatable.

d. Objectives of immunostimulation

- i. Promoting a greater and more effective sustained immune response to those infectious agents producing subclinical disease without risks of toxicity, carcinogenicity or tissue residues.
- ii. Hastening the maturation of non-specific and specific immunity in young susceptible animals.
- iii. Enhancing the level of duration of specific immune response, both cell mediated and humoral, following vaccination.

- iv. Overcoming of immunosuppressive effects of stress and of those infectious agents that damage or interface with the functioning of cells of immune system.
- v. Selectively stimulating the relevant components of the immune system or non-specific immune mechanism that preferentially confer protection against micro-organisms. For example via interferon release, especially for those infectious agents for which no vaccines currently exists; and
- vi. Maintaining immune surveillance at hightened level to ensure early recognition and elimination of neoplastic changes in tissues.

e. Effects of immunostimulant on shrimp



f. Method of administration

There are mainly three ways to deliver immunostimulants: injection, immersion and oral uptake. Injection of immunostimulants can produce strong nonspecific response, but it is time consuming and labour intensive. It has been reported that injection has wide protection against a range of pathogens.

g. Mode of action

The mode of action of immunostimulants is to enhance the immunity level of organisms against invading pathogens. The approach is very diverse in nature depending factors such as the type of immunostimulants, dose, route of administration, time and length of exposure. In general immunostimulants activate the phagocytosis and bacterial killing ability of macrophages, complement cells, lymphocytes and nonspecific cytotoxic cells, resulting in resistance and protection to various diseases and invading microorganisms

h. Common immunostimulants

Several antigens (vibrio cells, yeast glucans or their derivatives) have been experimentally tested to elucidate the innate immune mechanisms in shrimp (Bohnel *et al.*, 1999; Sakai, 1999; Vici *et al.*, 2000). Astaxanthins, chitosan, fucoidan, 1-3 glucan, herbal extracts, laminaria, LPS, PG, saponins, and vitamin C are the main antigens experimentally tested in shrimp (Newman, 1999). These substances can be administered by injection, immersion, bioencapsulation, per os intubation, and in the feed in marine organisms (Robles *et al.*, 1998). The results suggested that they can be an important element in the control of disease.

Glucans: These molecules are non-specific immunostimulants in crustaceans, inducing resistance against bacterial pathogens (Vargas-Albores *et al.*, 1998). However, crustaceans can digest glucans and use them as sources of energy, losing their function in the animal immune system. Interestingly, the use of LPS together with yeast glucan acts synergistically inducing a better stimulation of the crustacean immune system than when they are used separately (Newman, 1999).

Peptidoglycans: PGs are a mix of amino acids and carbohydrates from the cell wall of many bacteria and have been deemed as potent immunostimulants for the immune system (Lee *et al.*, 2004). These molecules are recognized as immunogen by the shrimp immune system, and *Bifidobacterium thermophilum*, *Brevibacterium lactofermentum* and *Bacillus sp.* PGs have been tested against yellow head virus (YHV) and white spot syndrome virus (WSSV). The results showed that the PG exposed shrimp exhibit a higher survival rate than non-exposed shrimp (Itami *et al.*, 1998; Lee *et al.*, 2004).

Fucoidan: These molecules are sulphated polysaccharides from microalgae cell walls which have been used as immunostimulants for shrimp. These products have been somewhat successful against pathogens like WSSV, *Vibrio* sp. and other bacterial species. Experimental administration of fucoidan mixed in the diet has resulted in a 93% increase in survival compared to controls (Chotigeat *et al.*, 2004). However, the action mechanisms are not well understood and more research with these products is necessary.

Lipopolysaccharides: LPS affect the specific and nonspecific immune system of many animals, including crustaceans (Vargas-Albores *et al.*, 1998; Newman, 1999). LPS are part of the cell wall of Gram (-) bacteria and are the first molecules recognized by the host's immune system, thus they have been used in shrimp as a potential tool to prevent diseases. However, most of the work has been done under controlled laboratory conditions and results may vary when performed in the field.

Conclusion

Increased use of antibiotics has led to the high proportion of antibiotic-resistant bacteria which provide threat to shrimp and man through consumption of the infected shrimp. Inefficiencies in antibiotic treatment of shrimp illnesses lead to significant economic losses. But the use of prophylactics in shrimp aquaculture has shown to have beneficial impact on animal health and thereby economic performance of shrimp farming. Addition of prebiotics in Shrimp diets, more research efforts are needed to provide the shrimp aquaculture industry. The use of probiotics has also important environmental benefits. By reducing the risk of diseases, the necessity of medication and thereby the risk of residues left in the environment is reduced. Though the probiotics in shrimp health management has been well documented, its bioremediation potential and biocontrol (antagonism) strategies need to be resolved. In order to obtain sustained probiotic effect in the field conditions, it is obligatory to screen the vast marine microbes instead of utilizing terrestrial strains. On other hand, application of Immunostimulants only ensures the shrimp health while the vaccine is providing specific protection against specific pathogen infection. Injection of dsRNA is practical difficult in shrimp farming at the field level therefore simple and effective routes for dsRNA and siRNA delivery into shrimp are developed such as production of pellet feed using edible dsRNA

producing bacteria. Additional research is needed to define the specific dosage rates and efficacy of various compounds for a variety of aquatic species and their pathogens and to decrease costs of the vaccines and immunostimulants.

References

1. Aguirre-Guzman G, Sanchez-Martinez J G, Campa-Cordova A I, Luna-Gonzalez A F, (2009) Ascencio Penaeid Shrimp Immune System, Thai J. Vet. Med., 39(3): 205-215.
2. Alabi A.O., Jones D.A., Latchford J.W., (1999). The efficacy of immersion as opposed to oral vaccination of *Penaeus indicus* larvae against *Vibrio harveyi*. Aquaculture, 178: 1–11.
3. Davies O. A., Ugwumba O. A and Abolude D. S. (2012). Ribonucleic Acid Interference (RNAi) technology: promising solution to shrimp viral diseases, Journal of Research in Environmental Science and Toxicology, (ISSN: 2315-5698) 1(11): 275-278
4. Farzanfar A (2006) The use of probiotics in shrimp aquaculture, FEMS Immunol Med Microbiol 48 149–158
5. Gatesoupe F-J (2005) Probiotics and prebiotics for fish culture, at the parting of the ways, Aqua Feeds: Formulation & Beyond, Volume 2 (3) :3-5
6. Gong H (2015) Prebiotics in shrimp aquaculture, Aquaculture Magazine, <http://www.aquaculturemag.com/magazine/decemberjanuary2014/2015/01/01/prebioticsinshrimpaquaculture>
7. Ha, Y-M, Gong S-J, Nguyen T-H, Ra C H, Kim K-H, Nam Y-K, and Kim, S-K, (2008). Vaccination of Shrimp (*Penaeus chinensis*) against White Spot Syndrome Virus (WSSV), J. Microbiol. Biotechnol., 18(5), 964–967.
8. Hemaiswarya S, R. Raja, R. Ravikumar and Isabel S. C. (2013) Mechanism of Action of Probiotics, Braz. Arch. Biol. Technol. Vol.56, n.1: pp. 113-119
9. Johnsona K N, van Hultena M.C.W, Barnesa A C. (2008) "Vaccination" of shrimp against viral pathogens: Phenomenology and underlying mechanisms, Vaccine, 26: 4885–4892.
10. Kulkarni A., Rombout J.H.W.M., Singhi S.B. Sudheer N.S., Vlak J.M., Caipang C.M.A., Brinchmann M.F., Kiro V., (2013). Truncated VP28 as oral vaccine candidate against WSSV infection in shrimp: An uptake and processing study in the midgut of *Penaeus monodon*, Fish & Shellfish Immunology 34: 159-166

11. Labh SN and Shakya S R, (2014) Application of immunostimulants as an alternative to vaccines for health management in aquaculture, *International Journal of Fisheries and Aquatic Studies*; 2(1): 153-156
12. Lightner, D.V. 2011. Status of shrimp diseases and advances in shrimp health management, pp. 121-134. *In* Bondad-Reantaso, M.G., Jones, J.B., Corsin, F. and Aoki, T. (eds.). *Diseases in Asian Aquaculture VII*. Fish Health Section, Asian Fisheries Society, Selangor, Malaysia. 385 pp.
13. Lin Y-C, Chen J-C, Morni W. Z. W. , Putra D. F., Huang, C-L., Li C-C, Hsieh J-F, (2013). Vaccination Enhances Early Immune Responses in White Shrimp *Litopenaeus vannamei* after Secondary Exposure to *Vibrio alginolyticus*, *Volume*, 8:7, e69722.
14. Lio-Po G D, Lavilla C R, Cruz-Lacierda E R, (2006) Health Management in Aquaculture, Aquaculture Department, Southeast Asian fisheries development center Tigbauan, Iloilo, Philippines.
15. Maningas M. B. B., and Tare M V R (2015). RNAi technology applications for cultured shrimp, 123-134, ISBN: 978-81-308-0558-0
16. Michael ET, Amos SO, Hussaini LT (2014) A Review on Probiotics Application in Aquaculture. *Fish Aquac J* 5: 111. doi: 10.4172/2150-3508.1000111
17. Moriarty D J. W. (1999) Disease control in shrimp aquaculture with probiotic bacteria, *Microbial Interactions in Aquaculture*
18. Ninawe and Selvin, (2009) Probiotics in shrimp aquaculture: Avenues and challenges, *Critical Reviews in Microbiology*, 35(1): 43–66.
19. Pope E.C., Powell A., Roberts E.C., Shields R.J., Wardle R., et al. (2011) Enhanced cellular immunity in shrimp (*Litopenaeus vannamei*) after ‘vaccination’. *PLoS ONE* 6: e20960.
20. Rowley A F., Pope E. C. (2012). Vaccines and crustacean aquaculture—A mechanistic exploration, *Aquaculture* 334-337 1–11.
21. Seibert, C. H. and Pinto, A. R. (2012) Challenges in shrimp aquaculture due to viral diseases: distribution and biology of the five major penaeid viruses and interventions to avoid viral incidence and dispersion, *Brazilian Journal of Microbiology*: 857-864.
22. Musthaq, S. K. and Kwang, J. (2015) Reprint of “Evolution of specific immunity in shrimp – A vaccination perspective against white spot syndrome virus” *Developmental and Comparative Immunology*, 48 342–353.