# Control of Pathogenic Vibrios in Shrimp Aquaculture using Antiinfectives from Marine Natural Products

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## Abstract

Mid-culture outbreaks due to pathogenic *Vibrio* spp. are most common and frequent disease problems encountered in shrimp aquaculture. Literature showed that the decades old shell disease to recent early mortality syndrome are caused by vibrios as primary and/or secondary etiological agents. Poor water quality, deteriorated pond bottom due to over feeding, inadequate farm management, rapid intensification of stocking approaches etc. are major predisposing factors of disease outbreaks. Both reactive and proactive treatment methods are being progressed through R&D efforts, but tangible solution is remain to prevent/control mid-culture outbreaks in shrimp aquaculture. Alternate approaches to replace the use of antibiotics in aquaculture is a highly prioritized research area. Alternate approaches are being widened to explore marine natural products, smart biomolecules such as biosurfactants and poly-hydroxy butyrates (PHB), nanoparticles, quorum quenching molecules, small bioactive peptides and probiotics. The oceans are the single principal bio-resource of halo-metabolites produced by a range of marine organisms such as seaweeds, corals, sponges, molluscs, coelenterates, marine worms, tunicates, bacteria etc. but their utilization as aquaculture drugs are not being exploited. Application of seaweed secondary metabolites in treating shrimp bacterial diseases represents an easy, cost effective and environmentally benign venture for equitable and sustainable shrimp farming. Recently we reported antiadhesive activity of PHB biopolymer against *Vibrio alginolyticus* and *V. harveyi*, which were considered as the most significant pathogenic vibrios in the grow-out ponds of giant black tiger shrimp *Penaeus monodon*. In order to reduce the use of antibiotics, pesticides and other chemicals and to improve the ecological environment of shrimp farms, research is being focused on the potential use of marine probiotic bacteria in shrimp farms to improve water quality by balancing bacterial population in water and reducing pathogenic bacterial load.

Keywords: Shrimp, marine natural products, anti infectives

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## Introduction

Shrimp Aquaculture is a profitable industry in several countries of Asia particularly Indonesia, Taiwan, China and India. However, diseases are recognized as a major constraint as well as a limiting factor for sustainable shrimp farming. Estimates of economic bases indicate that developing countries in Asia lost at least US$ 1.4 thousand million due to diseases in 1990 alone. Since then, losses due to diseases have been increasing. According to the 1996 World Bank report, global losses due to shrimp disease are around US$3 thousands million and the Bank recommended investment to the tune of US$275 million in shrimp disease research in the ensuing 15 years (Lundin, 1996). It is well known that aquatic organisms come in direct contact with the ambient microbes continuously, which may act as opportunistic pathogens (Raa *et al.*, 1992). Therefore, it is very difficult to prevent diseases caused by opportunistic or secondary pathogens during the entire culture period. Medication of aquatic organisms cannot be restricted to the diseased individuals and as a result, resistant microbial strains may develop, which change the normal microbial composition leading to massive outbreaks of the disease.

Environmental factors and poor water quality, resulting from increased effluent discharge, movement of aquatic animals, inadequate farm management, rapid proliferation of farms etc., have been implicated in major disease outbreaks occurring in epizootic proportion. However, the underlying courses of such epizootics are highly complex and difficult to pinpoint. Viral outbreaks have been damaging the shrimp culture in Southeast Asia and South and Central America. At present, over 20 viruses have been identified as important to shrimp, the most threatening being White Spot Syndrome Virus (WSSV) (Wang *et al.*, 1995), which was previously known as Systemic Ectodermal and Mesodermal Baculovirus (SEMBV) (Wongteerasupaya *et al.*, 1995) in Asia and Taura Syndrome Virus (TSV) in USA. Diseases caused by bacteria are also considered as equally important in causing mass mortalities wherever shrimp are cultured. Among bacteria, vibrio has been implicated as the causative organism, which may trigger mortalities up to 100% (Nash, 1990). Such bacterial epizootics concomitantly emerge as facultative to shrimp due to

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primary viral infections or environmental stress (Lightner, 1988; Karunasagar *et al.*, 1996). The short generation time of bacteria ensures massive population, which develops rapidly in the infected host as well as in the host's environment.

## Diseases of Shrimps

Although a wide variety of bacterial causative agents were reported in cultured penaeid shrimps, from the beginning, the most common group was being non-filamentaous, motile, Gram-negative, oxidase positive and fermentative rods (Bell and Lightner, 1987). Based on the bacterial aetiology and external clinical symptoms, the major bacterial epizootics reported could be grouped under the following seven categories:

### 1. Bacterial Septicemia

'White pleura' disease, diagnosed as bacterial septicemia, cause mass mortality in the postlarvae and grow-out phase of *Penaeus indicus* and *Penaeus merguiensis*. The causative bacteria were initially identified as Gram-positive cocci arranged in tetrads (*Micrococcus* sp.). 'Red Vien' disease in *Penaeus monodon* was reported as bacterial septicemic condition in hatcheries (Chong and Chao 1986). Penaeid bacterial septicemia in juvenile *Penaeus monodon* in Malaysia was reported to be caused by *Vibrio alginolyticus*, *V. parahaemolyticus* and *Pseudomonas* sp. (Anderson *et al.*, 1988). Earlier the same authors (Anderson *et al.*, 1987) reported that Gram-negative, rod-shaped bacteria were responsible for tissue level changes in *Penaeus monodon*. A new Vibrio pathogen, *V. gazogenes* was isolated from the blood of *Penaeus chinensis* affected by epizootic septicemia (Zhan *et al.*, 1997). In addition, the epizootic bacterial septicemia in *Penaeus chinensis* was reported to be caused by yet another new pathogen identified as *Providencia rettegeri*. This was considered as the first report of *Providencia rettegeri* infection in shrimp (Zhan *et al.*, 1997).

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## 2. Red Leg Disease

The acute bacterial infectious disease, 'red leg disease' was first described for an epizootic occurred in shrimp farm of Fujian province, China during 1987. The heart and nearby origin became light orange and the pleopods became red with reduced swimming activity. The causative bacterium, *V. alginolyticus* was isolated and reported as the causative pathogen for the first time (Zheng *et al.,* 1990). Subsequently, it was reported to be the most frequently isolated bacterium of diseased penaeid shrimp in Italy (Giorgetti, 1990). Xu *et al.,* (1992) characterized 'red leg disease' as expansion of chromatophores on the pereiopods and pleopods, giving these appendages reddish colouration, yellow pigmentation on the branchial region of cephalothorax and reduced swimming. Two strains of bacteria isolated from the haemolymph of moribund shrimps were identified as *Proteus vulgaris. Vibrio parahaemolytics* and *V. alginolytius* isolated from *Penaeus japonicus* and *Penaeus monodon* were also described as causative agents of 'red leg disease' and 'yellow gill disease' respectively (Su *et al.,* 1994). The 'Red disease syndrome' was characterized by the reddening of the shrimp body. However, Alapide-Tedenzia and Dureza (1997) first isolated four *Vibrio* phenotypes, namely, *V. harveyi*, *V. parahaemolytics*, *V. fluvialis* and *Vibrio* sp. from shrimp with red disease. Both *V. parahaemolyticus* and *V. harveyi* produced the characteristic red colouration in healthy shrimp, when administered experimentally.

## 3. Necrotizing Hepatopancreatitis (NHP)

Texas necrotizing hepatopancreatitis (TNHP) was an economically significant disease of the marine shrimp *Penaeus vannamei* cultured in Texas, USA. It was first recognized since 1985, and was reported as seasonal (Lightner *et al.,* 1992). Frelier *et al.* (1992) noted the symptom as granulomatous hepatopancreatitis' and the aetiological agent was characterized as Gram-negative, double-enveloped, intra-cytoplasmic bacteria. The NHP required specific environmental conditions for transmission (Frelier *et al.,* 1993) and it causes serious economic impact in the western hemisphere (Jory, 1997). The NHP bacterium infected only the epithelial cell lining of the hepatopancreatic tubules, and to date no other cell type have been shown to become infected. The hepatopancreas in shrimp was a critical organ involved in food digestion, nutrient absorption and storage and any infection leads to serious consequences from reduced growth to death. In penaeid shrimp, the hepatopancreas was also known to be affected by various viruses in addition to vibrio bacteria. During 1993, the epizootic NHP affected a number of commercial shrimp farms in the northwestern Peru and adjacent area of Ecuador (Lightner and Redman, 1994). TEM examination revealed that the causative was a pleomorphic intracellular Gram-negative bacterium. The agent was named Peru NHP (PNHP) for its geographical occurrence and it was very similar to the TNHP bacterium.

## 4. Shell Disease

Shell disease, the degradation of exokeleton is a common disease. The diseases were reported to be seasonal and size-related. Prevalence of ‘cuticular lesion’ (black spot disease) in brown shrimp *Crangon crangon* was reported to be most common in larger female specimen during late summer and autumn (Knust, 1990; Dyrynda, 1998). SEM observations of diseased shell showed the shell was destroyed to various extents and the epicuticle structure composed of phenolic compounds was damaged totally. Exocuticle and calcified endocuticle were destroyed seriously in most of the affected area (Yang *et al.*, 1992).

Vibriosis and shell disease were reported to be the major bacterial diseases in India. Black lesions (brown spot disease) were observed on abdominal appendages and telson of larvae and adult *Penaeus indicus* and *Panulirus homarus* (Hameed, 1994). Bacterial isolates belonging to the genus *Vibrio*, especially *V. alginolyticus* was isolated. The *V. alginolyticus* bacterium caused black lesion on abdominal segment of larvae in experimental transmission (Hameed, 1994). Luminous and non-luminous *V. harveyi* were associated with ‘shell disease’ in cultured *P. indicus* (Abraham and Manley, 1995). Several species of bacterial genera *Vibrio*, *Aeromonas* and *Pseudomonas* were commonly cited as causative agents of ‘shell disease’ of lobsters and shrimp (Aguado and Bashirsullah, 1996).

Shell disease also prevailed among the wild caught *C. franciscor* and *C. nigricauda* (Arnold and Hendrickson, 1997). The causative bacteria associated with lesions were identified as *Vibrio* sp. and *Pseudomonas* sp. Song *et al.*, (1997) reported that the exoskeleton adherent and fouling bacteria degrade the chitin-based surface cuticle by chitinase, resulting in development of vibrio disease or generalized bacterial septicemia. Due to the destruction of surface cuticle, the opportunistic bacteria provided a route of entry for secondary pathogens such as *Leucotrix mucor* (Yang and Wu, 1992).

## 5. Black-gill Disease/other backening diseases

Although black-gill disease was well established as a fungal disease caused by *Fusarium* sp., Yang *et al.* (1992a) reported it as an epizootic syndrome with multiple aetiology. Bacteriological isolation made from the penaeid shrimps infected with epizootic black-gill and brown-spot of shell disease syndrome showed *V. pelagicus* and *V. alginolyticus* the major isolates. Subsequent experimental transmission in healthy host revealed that among these, *V. pelagicus* was the major pathogen among *P. chinensis*. Infections of *V. alginolyticus* together with *V. pelagicus* resulted in septicemia and high mortality.

Histological investigations revealed that bacterial gill-rot disease in *P. monodon* was caused by *Bacilli* infection. Moreover, the mortality occurred due to the infection of bacteria and bacteriotoxemia that caused gill and hepatopancreal functional blockages (Chen *et al.*, 1993). Alfaro *et al.* (1993) reported blackening disease in the reproductive system of *Penaeus setiferus* maintained for controlled maturation and reproduction. A progressive, melanised condition of the male reproductive tract was shown to be associated with bacterial infection. Three different species (*V. alginolyticus*, *Pseudomonas putrefaciens* and an unclassified strain) were isolated from the damaged tissues, which later successfully developed similar disease signs in challenge experiments. The authors concluded that the condition could be a progressive syndrome with bacterial invasion or could be of more than one aetiology.

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## 6. Vibriosis

Members of the genus *Vibrio* are autochthonous bacterial flora in the aquatic ecosystem and quite few of them are associated with infections in humans and aquatic animals. They are the normal bacterial flora of shrimp and the culture environment (Jiravanichpaisal *et al.*, 1994; Otta *et al.*, 1999), but often act as secondary or opportunistic pathogens that cause mortality ranging from few to 100% in affected populations under stress (Lightner, 1988). Vibriosis has been implicated as the cause of major mortality in juvenile penaeid shrimp (Lightner and Redman, 1994). Outbreaks of vibriosis occur only when fish/ shrimp are immunocompromised or under stress due to overcrowding. Reports on the epizootic luminescent bacterial diseases, especially in the shrimp farms of Asian countries have been connected to an increase in the shrimp production and intensive rearing systems (Karunasagar *et al.,* 1994). Luminescent vibriosis is mainly caused by *V. harveyi*, *V. campbellii,* and occasionally *V. splendidus* which can infect larval juveniles and adult stages of penaeid shrimp (Gomez-Gil *et al.*, 1998; Lavilla-pitogo *et al.*, 1998). In the Philippines, virulent *V. harveyi* strains have caused 100% loses in the larval production of *Penaeus monodon* with bacterial cell densities as low as $10^2$ cells/ml (Lavilla-pitogo *et al.,* 1990). Bacterial infections related to *V. harveyi* luminescent strains have also been reported to cause major losses in the shrimp larviculture in Australia (Pizzutto and Hirst, 1995), South America (Alvarez *et al.*, 1998; Robertson *et al.,* 1998) and Mexico (Vandenberghe *et al.,* 1999). In India, luminous *V. harveyi* and *V. alginolyticus* were reported as prominent opportunistic and secondary shrimp pathogens of highly devastative mid culture outbreaks (Selvin and Lipton, 2003; Selvin *et al.,* 2005).

Species of *Vibrio* were among the most important bacterial agents known and formed typical normal microflora of the penaeids. They become opportunistic pathogen when culture conditions favour their growth at the expense of the shrimp host (Lightner *et al.,* 1992a). Infection with opportunistic bacteria of the genus *Vibrio* has been found to be a serious disease problem in the intensive brackish water culture of the giant tiger shrimp *P. monodon*. According to Nash *et al.,* (1992), in general infections are secondary and related

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to stress caused by high stocking density and inadequate management. *Vibrio parahaemolyticus, V. anguillarum, V. vulnificus, V. damsela* and *V. alginolyticus* were major pathogens. Lu *et al.,* (1992) reported the mass mortalities due to vibriosis caused by *V. alginolyticus*. *Vibrio* epidemics cause mortality over 90%. Guzman-Murillo *et al.* (1994) have developed a rapid membrane filter method to detect and quantify marine *Vibrio*s. The pathogenic *V. splendidus* I was reported to cause the epizootic disease, 'photobacteriosis' and consequent mass mortality in shrimp. Pathological studies indicated that, it was pathogenic to all stages of shrimp (Chen *et al.,* 1995). According to Ma *et al.,* 1995, an outbreak of explosive epidemic disease occurred in *Penaeus chinensis* due to the combined infection of *Vibrio* and *Micrococcus*. The challenge experiments demonstrated the virulence of *Vibrio* as very strong than that of *Micrococcus*. As seen above vibriosis affects all developmental stages, i.e., from larvae in hatchery tanks to juveniles and brood stock in grow out ponds. However, bacterial strain responsible for vibriosis in the successive stages may be different and virulence specificity was reported to change in the species and stage levels. Accordingly the 'syndrome 93' was considered as a seasonal juvenile vibriosis caused by *V. penaeicida* which affected *Penaeus stylirostris* in grow-out ponds and broodstock tanks. This pathogen did not cause any mortality in hatchery or nursery phases (Goarant *et al.,* 1998). However, some of the *V. penaeicida* strains demonstrated a very high virulence (Costa *et al.,* 1998). *V. cholerae* was found to be pathogenic to *Penaeus chinensis* larvae, which caused swellings in the intestine. The pathogenicity of the isolates was proved in the challenge experiments (Wang *et al.,* 1997).

In penaeid larviculture, frequent larval mortalities were reported to be caused by bacteria with external and internal necrosis (Palanisamy, 1993). Apart from *Vibrio* sp., the causative bacteria for external necrosis were *Myxobacterium* sp., *Aeromonas* sp. and *Psuedomonas* sp. The genus *Leucotrix*, though, non-pathogenic, caused surface fouling in gills or other external organs. Internal necrosis of larvae led to destruction of internal organs, especially the mid-gut gland, resulting in mortality of larvae. Main species of bacteria associated with internal necrosis were *Vibrio* sp., *Pseudomonas* sp. and *Aeromonas* sp. The pathogenic role of *Pseudomonas* sp. and *Aeromonas* sp. in shrimp was also reported by Yang *et al.* (1995).

Sung *et al.* (1999) studied changes in the composition of vibrio communities in pond water during *Penaeus monodon* cultivation and in the hepatopancreas of healthy and diseased shrimp. Results indicated that the diversity of vibrio decreased in the culture ponds prior to outbreak of vibriosis. During disease outbreak, *V. furnisii* was the major component of *Vibrio* community in pond water and hepatopancreas. However biotype studies indicated that 68.2% of the isolates were *V. harveyi* or *V. carchariae*. The characterization of extra-cellular products (ECP) from *V. harveyi* and *V. carchariae* was carried out by Montero *et al.* (1999). The ECP demonstrated a range of biological activities including the presence of caesinase, gelatinase and haemolysins.

Vibriosis generally manifests as a hemorrhagic septicaemia with extensive skin lesions, and focal necrosis (Hjeltner and Roberts, 1993). Many factors have been implicated in the pathogenesis of vibriosis. These include, the production of hemolysins (Munn, 1978), proteases (Norqvist *et al*., 1990), a capsule (Yoshida *et al*., 1985; Wright *et al*., 1990), iron binding proteins (Actis *et al*., 1985) and the presence of a 40 KDa hydrophobic surface Ag, VS-P1 (Espelid *et al*., 1987). Invasion of host cells by most pathogens requires penetration and damage of the cell membrane, which is mediated by either physical or enzymatic means, or a combination of two. Phospholipids and proteins represent the major chemical constituents of the host cell envelope. Therefore phospholipases are likely to be involved in the membrane disruption process that often occurs during host cell invasion (Waite, 1996).

## 7. Luminesent vibriosis

Luminescence in shrimp *Penaeus monodon* larvae was reported to cause mass mortalities in hatcheries in Indonesia (Sunaryanto and Mariam, 1987). Results from initial test had led to the authors to suspect the luminous vibrio as *V. albensis*. In India, luminous bacteria cause serious concern to the hatchery operations (Jayabalan *et al.,* 1996). Epibiotic infestation of luminous bacteria was observed in hatchery reared mysis larvae of *Penaeus indicus*. About 28.37% and 58.98% of total viable counts (TVC) in larvae and rearing water respectively. The luminous bacteria isolated from larvae and rearing water were identified as *V. harveyi* (Abraham *et al.,* 1997). The strain of *V. harveyi* isolated from diseased *Penaeus vannamei* was pathogenic in penaeid shrimp larvae, when given as a bath at $10^5$ cfu/ml for 2 h. Koch's postulates were confirmed by reisolation and identification (Robertson *et al.,* 1998). These studies confirmed that the occurrence of luminous vibrios was mainly due to the high vibrio load in the rearing water.

The shift in bacterial profile of the rearing water, notably the dominance of luminous vibrio was observed preceding to the occurrence of mortalities (Lavilla-Pitogo *et al.,* 1998). The hepatopancreas (hp) load of luminescent vibrio was considerably increased ($9x10^4$ cfu/hp) during the outbreak in cultured *Penaeus monodon* when compared to the normal shrimp ($7.0x10^1$ cfu/hp) (Leano, *et al.,* 1998). High *V. harveyi* numbers (upto $10^5$ cfu/larva) in the larvae were correlated with larval weakness (*Penaeus chinensis*) and mass mortalities (Vandenberghe *et al.,* 1998). According to Pillai and Jayabalan (1993), advanced postlarvae of *P. indicus* challenged with *V. harveyi* inoculum levels of $10^3$, $10^4$ and $10^5$ cfu/l for 96 h did not induce luminescence or other clinical signs of luminous vibriosis. Though the exposed larvae were normal and accepted feed till the termination of experiments, the isolation of bacteria from haemolymph suggested their opportunistic pathogenicity.

One of the major problems in otherwise highly successful *P. monodon* hatchery in the Philippines was the occurrence of luminescent bacterial disease caused by *V. harveyi* (Lavilla-Pitogo *et al.,* 1992). Plate counts of the exoskeleton from all sampled female broodstocks revealed that *V. harveyi* was a minor component of the exoskeletal-associated microflora. Moreover, the authors stressed mid-gut content of mother was the massive source for luminescent vibrio. During 1996, in the southern Thailand, intensive luminescence was encountered in many shrimp ponds accompanied by massive mortality resulting in total crop loss within 3 or 4 days. The farmers named "tea-brown gill syndrome' (TBGS) due to the external signs. The causative bacteria were identified as *V.*

*harveyi* (Ruangpan *et al.,* 1999). Recently, non-luminous *V. harveyi* biotypes were also reported to cause mass mortalities among *Penaeus indicus* (Abdel-Aziz and Dass, 2001).

**Early Mortality Syndrome Outbreaks**

Early Mortality Syndrome (EMS) also termed as Acute Hepatopancreatic Necrosis Disease or AHPND is considered as a new emerging shrimp disease that has attacked the shrimp farms in Southeast Asia (Zorriehzahra and Banaederakhshan, 2015). This disease had caused mass mortality in China (2009) as first time and then in Vietnam (2010), afterward in Malaysia (2011) and finally in Thailand (2012). It was named as EMS due to mass mortality during few days after shrimp post larvae stoking, especially within 20–30 days. The causative agent of EMS has been reported to be a bacterium—more specifically a pathogenic Vibrio belonging to the Harveyi clade, presumably *Vibrio parahaemolyticus* (De Schryver *et al*., 2014). There were some conversations about the possible presence of a bacterial phage or plasmid affecting the virulence of *V. parahaemolyticus*. Earlier, Lightner's team proposed that EMS pathogen has a unique strain of a relatively common bacterium, *V. parahaemolyticus*, that is infected by a virus known as a phage, which causes it to release a potent toxin, but later they denied it.

However, Ung Eng Huan *et al*. (2013) considered that the EMS-causing strains of *V. parahaemolyticus* were given virulence associated DNA from another species by lysogenic phage mediated lateral transfer. They even proposed a transmission mechanism of EMS pathogens. Ung Eng Huan (2014) stated that his group has successfully isolated a lytic phage that can help by killing 50% of all the Malaysian EMS-causing isolates, and the production of phage-probiotic combinations that change every month will not allow the pathogens to build up resistance easily.

## Marine natural products (MNPs)

Marine ecosystem is considered to harbor diverse communities of organisms and it serves as source of chemical diversity structures with promising biological activities. In addition to their uniqueness associated with those compounds, some of them possess unique mechanisms of action as well. So far, eight marine drugs have been approved by FDA or EMEA. It includes Cephalosporin C, Cytarabine (Cytosar-U ® ; Depocyt®), Vidrabine (Vira-A ®), Ziconotide (Prial®), omega-3-acid ethyl esters (Lovaza® ), ET-743 (Yondelis®), E7389 (Halaven®), Brentuximab vendotin (SGN-35, Adcetris®), and other marine drugs such as Iota-carrageenan (Carragelose®), Pliditepsin (Aplidin®), PM00104 (Zalypsis®), DMXBA (GTS -21), Lurbinectedin (PM01183), CDX -011, SGN -75, PM060184, Marizomib, ASG -5ME are under clinical trials (Martins *et al*., 2014). Within the last four decades, over 20,000 marine natural products have been isolated from marine micro and macro organisms, many of which have demonstrated potent biological activities (www.grc.org; Marinlit, 2007). A number of these compounds, or synthetic analogues based on natural compounds, have entered clinical trials and some are currently administered as therapeutics. The "pseudopterosins" from the Caribbean sea whip *Pseudopterogorgia elisabethae* was the first clinically validated "cosmeceutical" derived from a marine source (Look *et al.*, 1986).

The exploration for novel bioactive compounds has been taking place in the terrestrial organisms long back, but, recently concern has cockeyed towards the marine biota and its products (Faulkner, 2000; Zhang *et al*., 2005). Marine organisms are the rich source of bioactive compounds, which are reported to have antibacterial, antifungal, cytotoxic, neurotoxic, immunosuppressive, antiviral, and anti-inflammatory activities (Blunt *et al*., 2007; Faulkner, 2002). There has been extensive study on medicinal properties of terrestrial plants as compared to their marine counterparts.

**MNPs in shrimp diseases**

At present, application of bioactive natural products from marine source in mariculture industry appeared to be an alternate strategy for this knotty problem (Selvin and Lipton, 2003). Albeit, the bioactive potential of marine algae has been established long before, the application of algal-based products in shrimp disease management is a recently emerged approach (Selvin and Lipton, 2003; Huang *et al.,* 2006).

Among the diverse variety of marine natural products, the halogenated natural products have been the focus of attention in recent years (Gribble, 1998; Kladi *et al.*, 2004). The oceans are the single principal bio-resource of halo-metabolites produced by a range of marine organisms including algae, corals, sponges, molluscs, coelenterates, several marine worms, tunicates, bacteria and other marine life (Gribble, 1998). These compounds are rare in terrestrial plants. Halogenated compounds are biosynthesized mainly from marine red and brown algae and these compounds are dispersed in several different classes of primary and secondary metabolites, including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons (Taskin *et al.*, 2010). These were reported as having biological activities including antibacterial and antitumoral (Cardozo *et al.*, 2007).

Oral administration of organic extracts from several species of marine algae and sponges has been reported to increase the propagation of haemocytes in *P. monodon* (Selvin, 2002; Huxley, 2002; Jose *et al.,* 2008). In addition, it has been known that treatment with hot water extract and polysaccharides from different marine algae progressively elevated the THCs in different species of shrimps (Huang *et al.,* 2006; Yeh *et al.,* 2006; Hou and Chen, 2005; Fu *et al.,* 2007).

**1. Seaweeds**

Among the diverse marine flora, marine algae (including microalgae) represents one of the most primitive photosynthesizing (contribute nearly 40 percent of global photosynthesis) autotrophic groups of ecologically and economically important vegetation of oceanic ecosystem with unique life-cycle and physiology. Nearly fifty thousand species of seaweeds have been discovered in the marine environment (Filho-Lima *et al*., 2002) and none is known to be poisonous (Zemke-White and Ohno, 1999). A relatively small percentage (1 to 5%) of seaweeds available is used as food by both humans and animal. About 221 seaweeds are utilized commercially world-wide of which 65% are consumed human (Zemke White and Ohno, 1999). Historically, seaweeds provide essential economic, environmental, aesthetic, and cultural benefits to humanity (Dhargalkar and Neelam, 2005). In contrast to terrestrial vegetation, the marine flora constitutes valuable source for drug development (De Vries and Beart, 1995). For centuries, many of the seaweed secondary metabolites (SSM) have been used for traditional medicines due to their therapeutic potentials (Fitton, 2006). Recent studies have shown that marine algae are a tremendous source of marine secondary metabolites (Williams *et al.*, 1989; Williams and Maplestone, 1992). Marine algae are continuously exposed to many biotic and abiotic pressures which influence the organism's physiology, which in turn leads to the production of multifunctional natural secondary metabolites (Schmitt *et al*., 1995). So far, more than 2,400 seaweed secondary metabolites (SSM) are described and many of the SSM are natural blueprints for the development of new drugs (Munro and Blunt, 1999; Faulkner, 2001 and previous authors). Several of these compounds exist in biologically active forms in healthy seaweeds. The major secondary metabolites produced by seaweeds are halogenated compounds (Blunt *et al*., 2007) displaying antibacterial, antifungal, antiviral, antifouling and antifeedent properties. The abundance and diversity of secondary metabolites in seaweeds elevated as the prime material for pharmaceutical Industry. Albeit thousands of bioactive compounds have been discovered, the need for novel therapeutic compounds is still urgent in concern of number of new diseases and resistant strains of microorganisms.

Moreover, the Indian red algal species belong to 136 genera, 36 families and 16 orders. The structural diversity of secondary metabolites from Indian red algal species were

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well reviewed by Sarma *et al.* (2006). Approximately 40 numbers of secondary metabolites have been reported from the Indian red algal species (Sarma *et al.*, 2006).

Application of seaweed secondary metabolites in treating shrimp bacterial diseases represents an easy, cost effective and environmentally benign venture for equitable and sustainable shrimp farming (Selvin *et al*., 2009). Currently, studies have been commenced to validate the efficacy of using algal metabolites in shrimp disease management (Selvin *et al*., 2009). Moreover, the range of bioactive compounds produced by the marine algae makes it an excellent source for the detection and characterization of bioactive compounds.

Dietary seaweed extracts "Vivanatural' prepared from an edible seaweed *Undoria pinnantifida*, demonstrated definite prophylactic activity against virus infection (Furusawa *et al*., 1991). The hot water extract of edible brown alga *Hijikia fusiforme* exhibited immunoenhancing activities and this property was associated with algal polysaccharides (Okai *et al*., 1997). Arsenosugar (AsSug) present in seaweed has induced different and interesting cellular responses in macrophages at high concentration (1-10 mM) (Sakurai *et al*., 1997).

The extracts of *Hijikia fusiforme* and *Meristotheca populosa* markedly stimulated human lymphophytes to proliferate whereas *Eucheuma musicatum* and *M. papulosa* gave weak stimulation of proliferation (Shan *et al*., 1999). The phosphate buffered extract of the red algae *Gracilaria verrucosa* and *Papenfum gigartinales* from Japan was known to contain lectin-based haemoglutinins (Kakita *et al*., 1999).

The efficacy of natural products from marine algae against various shrimp bacterial pathogens has been demonstrated in previous studies (Lipton *et al.*, 2009; Jose *et al.*, 2008; Kanjana *et al.*, 2011). Efficacy has also been demonstrated against other shrimp pathogens including virus such as WSSV in *P. monodon* (Witvrouw and De Clercqe, 1997; Chotigeat *et al.*, 2004; Manilal *et al.*, 2009), vibriosis in *Fenneropenaeus chinensis* (Huang *et al.*, 2006) *Litopenaeus vannamei* (Yeh *et al.*, 2006) and *P. indicus* (Immanuel *et al.*, 2004).

Oral administration of natural antimicrobials is the preferred route of chemotherapy in shrimp aquaculture owing to the ease of use and lack of any additional stress to the shrimp during treatment. Furthermore, it is impossible to isolate infected shrimp for treatment purposes as done in mammals.The application of seaweed-based feed may be an effective means for increasing the immune-proficiency and disease resistance/control in shrimp. The disease resistance of shrimp has been found to be induced by feeding with an algal-based medicated feed that had been a successful strategy for disease management (Selvin, 2002). There has been only limited research effort in the development of therapeutics from natural products for shrimp disease management. This has included work on the marine sponges, mangroves, microalgae, terrestrial plants (Selvin, 2002). It is noteworthy that, apart from Selvin (2002) all work published to date, originated as a consequence of screening for diffusible inhibitory substances/extract *in vitro*. Algal-based medicated feed is a valuable vehicle for oral collective antibiotic treatments in shrimps provided that an adequate amount of the active ingredient is available for the animals. Marine red algae *Asparagopsis taxiformis* was found to be a highly active alga from the southwest coast of India. *A. taxiformis* showed 100% inhibition against pathogenic Vibrio strains isolated from moribund shrimps and MTCC culture of shrimp Vibrio pathogens. Considering the broad anti-vibrio potency, less *in vitro* shrimp toxicity and huge biomass availability, *A. taxiformis* is well suited for the development of potential therapeutic agent (Manilal *et al*., 2010).

There are no reports available on the role of Marine Secondary Metabolites (MSMs) in the shrimp disease management. However, some of the seaweed-based products were reported to act as immunomodulators in fishes. A commercially obtained spray-dried preparation of microalgae, *Tetraselmis sueccica* showed promise in controlling prawn pathogenic strains such as *Vibrio* sp., *V. alginolyticus, V. anguillarum, V. parahaemolyticus* and *V. vulnificus* (Austin and Day, 1990). The survival rate and growth of larvae of kuruma prawn *Penaeus japonicus* fed with *Ulva pertusa* was increased (Yamsaki *et al*., 1997). It was reported that epibiotic marine bacteria present on the larvae of some crustacean protected them from fungal infection by production of low molecular antibiotics (Boyd *et al*., 1998).

In a study conducted by Chen *et al*. (2015), the immune parameters of ammonia-stressed white shrimp *Litopenaeus vannamei* like hemocyte counts, phenoloxidase activity, respiratory bursts, and superoxide dismutase activity significantly increased, when they were immersed in seawater containing *Gracilaria tenuistipitata* extract. Several scientists including Smith *et al*. (1984), Kitikiew *et al*. (2013) and Chen *et al*. (2014) specified that shrimp hemocytes incubated with β-1,3-glucan, fucoidan, and carrageenan exhibit degranulation, alterations in cell size and viability, and better phenoloxidase activity and respiratory bursts. Chen *et al*. (2014) reported that white shrimp receiving carrageenan via immersion revealed increase in hemocyte count, and higher number of mitotic cells in hematopoietic tissue.

## 2. Sponges and its associated bacteria

During the 40 years of extensive investigations, more than 6000 compounds were discovered from the marine source (Davidson, 1995). A series of arabinosyl nucleosides, including spongothymidine and spongouridine, isolated from the Caribbean sponge *Cryptotethia* crypta were the first compounds discovered from marine source (Bergmann and Feeney, 1951). The bioactivities to some of these metabolites include ichthyotoxicity, phytotoxicity, cytoxicity, antibiosis, antiviral, insecticidal, antifeedant and pharmacological activities. Despite the vast bioactive potential found in the MSMs, only a few have reached preclinical/commercial level. The difficulties associated with the collection and isolation of marine samples in comparison to terrestrial samples has also hampered the progress (Naya *et al*., 1993). In addition, the synthesis of the active principles has only recently entered a similar growth phase (Albizati, 1991). According to Rinehart, (1991) only three marine-derived compounds have reached preclinical trails. One of these 'didemin - B' in phase II trials as an anticancer agent. 'Ecteinasidins'- another group of compounds was proved to have potent solid-tumour activity. An analysis of phyletic distribution of MSMs revealed that the majority (93%) was confined to four groups (macroalgae, coelenterates, echinoderm and sponges), largely a reflection of the abundance and easy collection of these organisms (Attaway and Zaborsky, 1993). However, in the beginning of last decade, the contribution from macroalgae decreased significantly, whereas sponges become the dominant source for novel compounds. The increased studies on sponges could be attributed to their wider range of biosynthetic capabilities than any other group of marine invertebrates. Recently, marine microbes also received much attention as a renewable source of bioactive molecules for biomedical research (Bremer, 1998).

The marine bacterium *Pseudomonas* isolated from its host sponge *Suberea creba* from the Coral Sea of New Caledonia produced potent antibacterial quinones. However, the host sponge contained dibromo-verangiaquinol showing strong antibacterial activity. Moreover, it was promising for mariculture, as an antibacterial agent in cultures of *Pecten maximus* larvae, which was nontoxic in Artemia salina test (Debitus *et al.,* 1998).

Perusal of literature clearly indicated that the use of MSMs in shrimp disease management is an unexplored area, albeit it has a vast potential of developing potent safe antimicrobial/ immunostimulating drugs for shrimp disease management.

## 3. Marine microbe-associated products

As reported by Rattanachuay *et al*. (2010), marine microbes have the potential to produce many extracellular compounds against various shrimp pathogenic vibrios. The anti-vibrio compound produced by marine *Pseudomonas* is found to be pyocyanin (Priyaja *et al*., 2014). Likewise, many other products of marine microbes are under trial for application in shrimp farming. They are as follows:

## a. PHB

Poly-β-hydroxybutyric acid (PHB) is a natural, ecofriendly polymer accrued in the form of intracellular granules by a large variety of bacteria (Luzier, 1992). Kiran *et al.* (2014) reported the antiadhesive activity of PHB biopolymer from a marine bacterium *Brevibacterium casei* MSI04 isolated from a marine sponge *Dendrilla nigra*. PHB showed antibacterial activity against *Vibrio alginolyticus* and *V. harveyi*, which were considered as the most significant pathogenic vibrios in the grow-out ponds of giant black tiger shrimp *Penaeus monodon* in India. Also, Laranja *et al.* (2014) reported a PHB-accumulating *Bacillus* spp. isolated from the marine sediment improves the survival, growth and robustness of *Penaeus monodon* (Fabricius, 1798) postlarvae challenged with pathogenic *Vibrio campbellii*, which was in agreement with several reports (Halet *et al*., 2007; Defoirdt *et al.*, 2007).

## b. Biosurfactants

Biosurfactants are surface active compounds having both hydrophilic and hydrophobic domain that allows them to exist preferentially at the interface between polar and non-polar media, thereby reducing surface and interface tension (Banat *et al*. 2010). Donio *et al*. (2013) reported a halophilic bacterium *Bacillus* sp. BS3 producing pharmacologically important biosurfactants that has antiviral property against the shrimp white spot syndrome virus by subduing the viral replication and considerably outstretched shrimp survival. In the review of Dinamarca *et al.* (2013), it has been mentioned that biosurfactants produced by marine bacteria can act as immunostimulating molecules that strengthen fish immune system and, thus, reduce the quantity of antibiotics required to control infectious outbreaks in aquaculture ponds. Biosurfactants produced by a hydrocarbon-degrading marine bacterium *Cobetia* sp. inhibits the quorum sensing ability of *Vibrio* species (Ibacache-Quiroga *et al*., 2013). This evidences that biosurfactants are quorum quenching molecules that may in turn neutralize the virulence of shrimp pathogenic vibrios. Hence,

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biosurfactants from marine biota is one of the potential MNPs in the control of aquaculture diseases.

### c. Nanoparticles

Due to rapid disease outbreaks in the aquaculture industry, it is believed that many aquaculture experts are turning to an emerging technology termed as nanotechnology (Handy, 2012). Dominguez (2014) proposed that nanotechnology possibly will aid in aquaculture production by improving feeding formulation, controlling diseases and biofouling, etc. The synthesis of metal nanoparticles using the bioflocculant produced by a marine sponge associated bacteria could reveal improved safety and stability over existing methods (Sathiyanarayanan *et al*., 2013). Leaf extract of the coastal plant *Prosopis chilensis* was exploited by Kandasamy *et al*. (2013) for the synthesis of silver nanoparticles, which showed potential anti-vibrio activity in protecting the shrimp, *Penaeus monodon* from vibriosis. Rajeshkumar *et al*. (2009) attempted the synthesis of DNA-Nano vaccine made of VP28 gene of WSSV encapsulated within chitosan nanoparticle and succeeded in attaining the protective efficacy of oral delivery of that nanoparticle in black tiger shrimp (*Penaeus monodon*) challenged with WSSV. Perhaps their benefits, nanoparticles also reported to have considerable toxic effects on shrimps (Arulvasu *et al*., 2014).

## Probiotics in shrimp aquaculture

In order to reduce the use of antibiotics, pesticides and other chemicals and to improve the ecological environment of shrimp farms, research is being focused on the potential use of probiotic bacteria in shrimp farms to improve water quality by balancing bacterial population in water and reducing pathogenic bacterial load. In addition, the use of probiotics can increase the population of food organisms, improve the nutrition level of aquacultural animals and improve immunity of cultured animals to pathogenic microorganisms. By applying these bacteria in shrimp farms, a biological equilibrium

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between competing beneficial and deleterious microorganisms could be produced. Reports indicated that addition of probiotic bacterial strains will repress the growth of *Vibrio* spp., fungi and other pathogenic microorganisms. It also noticed that probiotic bacteria could produce some digestive enzymes, which might have improve the digestion of shrimp, thus enhancing the ability of stress resistance and health of the shrimp (Selvin *et al*., 2009). According to some recent publications, the mechanism of action of the probiotic bacteria may have several aspects:

1. Probiotic bacteria may competitively exclude the pathogenic bacteria or produce substances that inhibit the growth of the pathogenic bacteria.
2. Provide essential nutrients to enhance the nutrition of the cultured animals.
3. Provide digestive enzymes to enhance the digestion of the cultured animals.
4. Probiotic bacteria directly uptake or decompose the organic matter or toxic material in the water improving the quality of the water.

According to the findings of Chinese researchers, when these bacteria were added into the water, they could decompose the excreta of shrimps, remaining food materials, remains of the plankton and other organic materials to $CO_2$, 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 may improve the nutrient cycle, and it can create a balance between bacteria and micro algae, and maintaining a good water quality environment for the cultured shrimps (Selvin *et al*., 2009).

Microorganisms have a critical role in aquaculture systems because water quality and disease control are directly related and closely affected by microbial activity (Pillay, 1992). Intensive cultivation systems obviously led to a change in the composition of environment and indigenous protective flora of the cultured organisms. This leads to an

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increase in the susceptibility of the host animal to opportunistic / secondary pathogens as well as reduced feed conversion ratio due to the imbalanced microflora in the intestinal tract. However, it has been reported that both the health and survival of organisms in intensive rearing systems could be improved substantially by manipulating the gut / environmental microflora with “probiotic” microorganisms and/or prebiotics, which can be added to the diet and/or to the environment to promote the growth of beneficial bacteria in the gastrointestinal tract of the animal as well as in the detritivorous microbes in the pond bottom (Olafsen, 2001; Lin, 1995; Rengpipat *et al*. 1998a,b). The use of probiotics for disease prevention and improved nutrition in aquaculture is becoming increasingly popular due to an increasing demand for environment-friendly aquaculture. Probiotics are, in general, defined as live microbial feed supplements, which beneficially affect the host (Fuller, 1989). Probiotics for aquatic organisms have been defined as “microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health” (Gatesoupe, 1999). There have been many studies involving probiotics for use in aquaculture (e.g. Moriarty, 1998; Gatesoupe, 1994; Gram *et al*., 1999; Nikoskelainen *et al*., 2001; Panigrahi *et al*., 2004; Salinas *et al*., 2005), but the mode of action is incompletely understood. However, it is widely accepted that the mechanism of probiotics include inhibitory interaction (antagonism), production of inhibitory compounds competition for chemicals and adhesion sites, improving the microbial balance, immune modulation and stimulation, and bioremediation of accumulated organic lead in the pond bottom (McCracken and Gaskins, 1999; Verschuere *et al*., 2000; Lin, 1995; Rengpipat *et al*. 1998a,b).

Albeit the potential of probiotics has been well established, the constraints in probiotic development need to be considered in the evaluation of novel as well as commercially available probiotics (Fig. 1).

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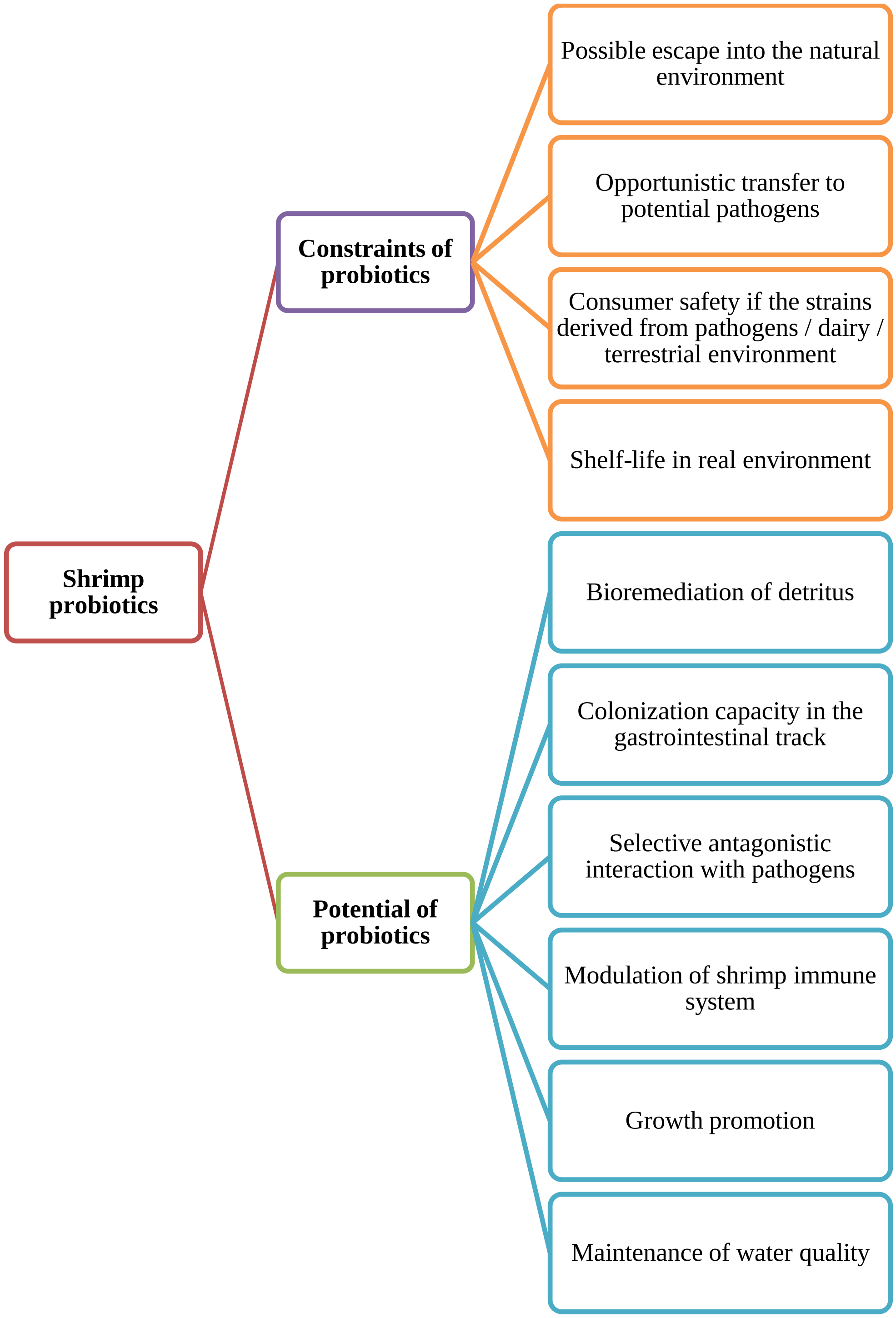


Fig. 1. Potentials and constraints of probiotics in shrimp aquaculture

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**Potential sources of antagonistic probiotics**

The marine environment has been mined for novel microorganisms used in drug development (Selvin *et al*., 2004) and is likely the largest contributor of bacteria to the aquaculture. Sfanos *et al*., (2005) made a survey of bacterial samples isolated from wild marine sources including macroalgae, sea water, 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). The representation of bacterial groups uncovered were mostly gamma proteobacteria tending to dominate in most communities (Eilers *et al*., 2000). Recently marine bacterial endosymbionts are emerging as potential source for the development of novel probiotics (Selvin *et al*., 2004: Selvin *et al*., 2007 unpublished data).

Bacteria in the aquatic environment and certainly those in the diet influence the composition of the fish intestinal tract where they can positively affect the health of the organism (Verschuere *et al*., 2000). In addition to gut microflora which can exclude the adhesion of other species to the intestinal wall, bacteria that can out-compete pathogens for carbon and energy sources in the aquatic environment may also be good candidates for probiotic mixtures (Verschuere *et al*., 2000). Furthermore, microorganisms displaying antibacterial properties have often been discovered associated with macroalgae and other sources in the marine environment (Sfanos *et al*., 2005), and these offer a third source of potential probionts. Literature evidenced that a wide variety of probiotics strains could be developed from different sources.

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## Successful and commercial probiotics in aquaculture

In parallel to the growth of probiotic application ranging from food supplements to biotherapeutics, the biodiversity of strains exhibiting potentially probiotic functionalities has increased remarkably in recent years. 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., Streptococcus sp.,*), *Pseudomonas* sp. (*Pseudomonas fluorescens)* and *Vibrio* sp. (*V. alginolyticus, V. salmonicida*-like) have been proposed and tested as probiotics in aquaculture (Gatesoupe, 1991; Verschuere *et al*., 2000). Although studies have shown that lactic acid bacteria effective in inhibiting the growth of various vibrio species in Atlantic cod fry *Gadus morhua* (Gildberg *et al*., 1997) and turbot larvae (Gatesoupe, 1994), the probiotic effects lasted only for a brief time after feeding was discontinued. Lactic acid bacteria are known to produce growth inhibiting factors such as bacteriocins that are particularly useful against other gram positive bacteria (Stoffels *et al*., 1992), however, since most of the known pathogens in aquaculture are gram negative, and lactic acid bacteria account for only a small part of the gut microbiota of fish, their usefulness in aquaculture is debatable (Verschuere *et al*., 2000). *Pseudoalteromonas* have been found to synthesize biologically active compounds with antibacterial, algicidal, anti-algal and bacteriolytic properties (Holstrom and Kjelleberg, 1999). This relatively new genus has been exclusively isolated from marine environments throughout the world (Enger *et al*., 1987), and species within this genus are often found associated with eukaryotes (Holstrom and Kjelleberg, 1999). One isolate has even produced a bactericidal antibiotic against methicillin-resistant *Staphylococcus aureus* (Isnansetyo and Kamei, 2003). Maeda *et al*.

(1997) showed that the addition of an anti-microbial strain of *Pseudoalteromonas undina* repressed the growth of pathogenic bacteria and viruses in fish and crustacean farming.

Four species including, *B. pumilus, Micrococcus luteus, Pseudomonas fluorescens* and *Pseudomonas 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*., 1998a,b). *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). *Pseudomonas fluorescens* (AH2) was shown to be strongly inhibitory against *Vibrio anguillarum* in model systems and it was found that this effect could be transferred to an in vivo situation where the mortality rate in rainbow trout infected with *V. anguillarum* was significantly reduced by the addition of the probiotic bacterium to the tank water (Gram *et al*., 1999). 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. The addition of bacterium CA2 as a food supplement to auxenic cultures of *Crassostrea gigas* larvae was found to consistently enhance the growth of the oyster larvae regardless of the season of the year (Douillet and Langdon, 1994). Thus, probiotics have been shown to be effective in a wide range of species for the promotion of growth, enhanced nutrition, immunity and survival.

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