Chapter

Bacterial Disease Control Methods in Shrimp (*Penaeus*, 1798) Farming Sector in Asian Countries

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Abstract

Aquaculture industry produces the enormous amount of sea foods (fish, shrimp, planktons, etc.) with enriched quantity of proteins, essential amino acids, essential fatty acids, and micronutrients and also possesses the medicinal values. This production industry is very important to meet out the need of the global population. Recently, different culture practices for aquatic culturing organisms were developed in practices, where the risk of infection and diseases outbreak also increased which leads to the production loss to the aquatic sector. Several conventional methods are used to prevent the diseases probiotics, antibiotics, plants, immmunostimulants, proteins, immune proteins enhancement, nanoparticles, etc. At the same time, these treatment techniques also have merits and demerits to execute into the practical platform. For instance, chemical or antibiotics treatment into the culture system leads to the some adverse effects in culturing organisms, environment, and also consumer. In this chapter, various diseases caused by the bacterial strains and its control strategies in the shrimp farming industry to enhance the aquaculture are discussed.

Keywords: aquaculture, pathogens, plants, disease management, immunostimulant

1. Introduction

Shrimp farming plays the major role in aquaculture industry globally; due to its proteinaceous nature increased export viability and high profit yield the enhanced economy to the country. Penaeid (Rafinesque, 1815) shrimp aquaculture is one of the major industries which have rapidly grown during the past three decades in tropical and subtropical areas of the world (FAO 2019). Global production of shrimp increased from 1,564,563 metric tonnes in 2017 to 2,002,449 metric tonnes in 2019 (FAO 2019). The black tiger shrimp, *Penaeus monodon* (Fabricius, 1798), Indian white shrimp, *Fenneropenaeus indicus* (H. Milne Edwards, 1837) and Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) are important commercial species of the Penaeidae family. *F. indicus* supports commercial fisheries in both marine and estuarine environments on the east and west coasts of India. India has been a major supplier of shrimp to Japan, Europe, and USA. In India, Indian white shrimp *F. indicus*, Pacific white shrimp *L. vannamei*, black tiger shrimp *P. monodon*, white shrimp

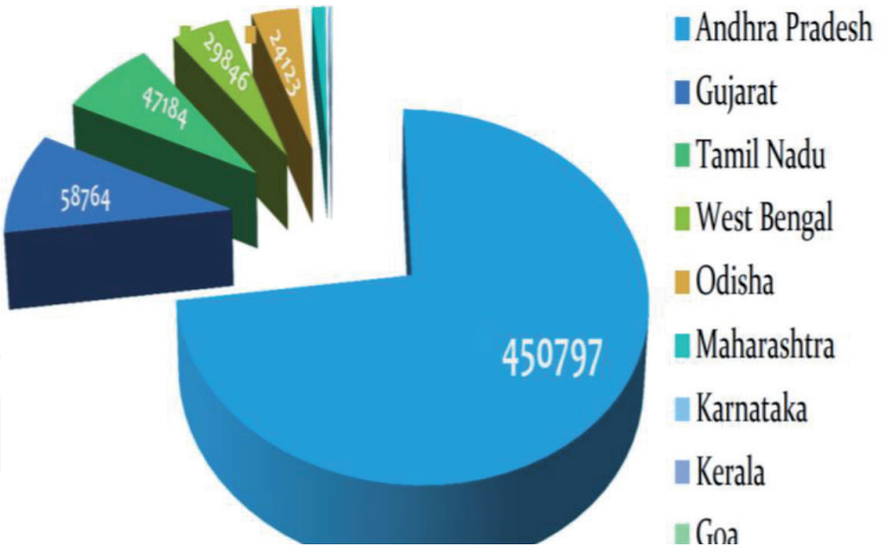

Figure 1.
Aquaculture sector contribution towards economy throughout India.

P. penicillatus (Olivier, 1791), green tiger shrimp *P. semisulcatus* (De Hann, 1844) and banana shrimp *P. merguiensis* (De Man, 1888) are farmed along the coastal areas. Although the production of cultured shrimp has increased, there have been considerable periodic losses due to disease of the farmed shrimp. Global Aquaculture Alliance (GAA) survey with respect to agents responsible for diseases has already revealed that 50% of losses due to diseases were attributed to viruses and about 22% to bacteria [1]. The global loss of shrimp production leads to research against the control of diseases required for the stability of aquaculture industry. In Indian aquaculture industry produces at the Global Outlook in Aquaculture Leadership (GOAL) conference, held in October in Chennai, India, the forecast was for Indian production to drop in 2019. The GOAL prediction has India flat at around 600,000 tonnes in 2019 and 2020, down from as much as (**Figure 1**).

The shrimp immune system, like other invertebrates lacks an adaptive immune system and relies solely on its innate immunity against invading pathogens. Innate immunity is an ancient protective mechanism that appeared early in the evolution of metazoans and is divided into humoral and cellular responses [2], which work in jointly coordination for the detection/elimination of all foreign organisms potentially hazardous for the host [3]. The cellular response mediated by haemocytes in hemolymph involves nodule formation, phagocytosis, encapsulation of pathogens and coagulation [4, 5]. The humoral components include the activation and release of molecules stored within haemocytes, such as anticoagulant proteins, agglutinins, phenoloxidase enzyme, antimicrobial peptides and protease inhibitors [3].

2. Commercially important shrimps in India

2.1 *Fenneropenaeus indicus*

The Indian white shrimp, *F. indicus* formerly known as *P. indicus* is a marine shrimp, prefers mud or sandy-mud bottom and can be found from 2 to 90 m depth. It attains up to 228 mm (nearly 9 inches) in length up to 14–20 g in weight and can tolerate low water quality, high salinities and high temperatures. It is one of the most important Indian commercial species, especially for the inshore fisheries and for rice field culture in Kerala and also captured in the East African coast. The taxonomic position of the Indian white shrimp *F. indicus* is.

Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Suborder: Dendrobrachiata
Family: Penaeidae
Genus: *Fenneropenaeus*
Species: *indicus*

2.2 *Litopenaeus vannamei*

Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Suborder: Dendrobrachiata
Family: Penaeidae
Genus: *Litopenaeus*
Species: *vannamei*

In penaeid shrimp farming bacterial diseases are commonly associated with natural microbial flora of seawater, which possess enriched organic matter that supports the growth and multiplication of bacteria and other microorganisms. The most common shrimp pathogenic bacteria belong to the genus *Vibrio*. Other Gram-negative bacteria such as *Aeromonas* spp., *Pseudomonas* spp., and *Flavobacterium* spp., are also occasionally implicated in shrimp diseases.

3. Bacterial Septicaemia (*Vibrio* disease)

Acute hepatopancreatic necrosis disease (AHPND) is one of the severe systemic diseases caused by bacteria *Vibrio parahaemolytics* especially in shrimps such *P. monodon* and *P. vannamei*. In this disease the infected animals periopods are red color in nature owing to its chromatophores, sometimes the severe infections of bacterial diseases in shrimps may occur, while gills looks eroded and melanization took place to form the black blisters can be seen on the carapace and abdomen. Apart from *V. parahaemolyticus* some other bacterial pathogens such as *V. alginolyticus, V. anguillarum, V. parahaemolyticus, Vibrio* spp. also involved in this disease pathogenesis.

3.1 Luminescent bacterial disease

This bacteria also causes the dangerous problems in aquaculture farms and heavy losses due to its infections it leads to the economy downfall and production rate loss too. These luminescent bacteria infected shrimps could be look like a fluorescent or luminescent producing nature in darkness. *V. harveyi* are the major pathogens creating this problem in hatcheries. The luminescent bacteria could be isolated using Zobell's Marine Agar, followed by morphological and biochemical characteristics.

3.2 Brown spot disease (Shell disease or rust disease)

Infected animals showed the brown and black erosions on the surface of the body and whole body appendages, this could be caused through *Vibrio* spp., *Aeromonas* spp., and *Flavobacterium* spp., with chitinolytic activity. Diagnosis could be achieved by simple observations such as gross signs and confirmed by isolation

of the bacteria from the site of infection on Zobell's Marine Agar and identification of the pathogen.

3.3 Necrosis of appendages

The tips of walking legs, swimmerets and uropods of affected shrimp undergo necrosis and become brownish and black. The setae, antennae and appendages may be broken and melanised. The epibiotic bacteria such as *Vibrio* spp., *Pseudomona*s spp., *Aeromonas* spp. and *Flavobacterium* spp., produced the gross signs in infected shrimps.

3.3.1 Vibriosis in shrimp larvae

The affected larvae show necrosis of appendages, expanded chromatophores, empty gut, absence of fecal strands and poor feeding. Cumulative mortalities may be very high reaching up to 80% within few days. *V. alginolyticus, V. parahaemolyticus, and V. anguillarum* caused this disease.

3.4 Filamentous bacterial disease

The affected shrimp larvae show fouling of gills, setae, appendages and body surface. Molting of affected shrimps is impaired and may die due to hypoxia. *Filamentous* bacteria, such as *Leucothrix mucor* are the causative agent for this disease. Diagnosis of filamentous bacterial disease could be achieved based on gross signs and symptoms and by microscopically demonstrating filamentous bacterial fouling of body surface and appendages of shrimp larvae.

4. Control measure of shrimp disease

Most common pathogenic bacteria of penaeid shrimp include *Vibrio* sp., *Aeromonas* sp., *Photobacterium* sp., *Citinoclastic* sp., *Leucothrix* sp. and *Thiothrix* sp. The loss of a stable microbial balance through disinfection leads an environment wide open for the proliferation of any opportunistic bacteria [6]. Therefore, disease control strategies ought to be a priority in the aquaculture practices. Several antibiotics are used in the aquaculture practices for treatment and to control diseases. The prolonged application of antimicrobial agents at sublethal concentrations may provoke the adaptation of microorganisms to antimicrobial agents [7].

5. Antibiotics

Antibiotics are potential molecules for the initial treatments, though it has its own demerits such as continues usages of antibiotics in the environment like farms, aquatic systems might be causes the pollution and also leads to the development of multiple drugs resistant strains in the environment [8]. For instance, adequate usage of chloramphenicol in shrimp farming sector in Myanmar, India, Pakistan, and Vietnam, paves the way to abuse of drugs resulting heavy loss in farming sector [9]. Considering the high promising results obtained in the in vitro screening of commercial antibiotics, the post-infection therapy using antibiotics remain the method of choice for many farmers [10]. Use of microbes for beneficial purposes is

increasingly recognized as a valuable input for sustainable aquaculture. Nowadays, several environmental-friendly prophylactic and preventive methods like probiotics, immunostimulants, antimicrobial peptides and quorum sensing interference are developed to control aquatic organism diseases. Therefore, novel antimicrobials with increased potency and least residual accumulation in shrimp tissue are required in lieu of conventional antibiotics for the management of bacterial epizootics. To keep the shrimp farming as a sustainable venture, new health management strategies must be used instead of the traditional methods like the abuse of antibiotics and chemotherapeutics.

5.1 Herbs as antibiotics

Herbs act as antibiotic for controlling or reduce the infection of pathogen in aquaculture sector and also increase the survival rate of organisms, during outbreak of disease managements. In *Fenneropenaeus indicus*, the anti-vibrio disease controlled by garlic extract [11]. Hot water extracts of brown seaweeds *Sargassum* sp. act as antibiotic against white spot syndrome virus in shrimp *P. monodon* [12]. *Azadirachta indica* plant extract act as antibiotics for treating *Citrobacter freundii* bacterial infection in *Oreochromis mossambicus*. Castro [13] observed, methanoic extract of Brazil herbs act as disinfectant against fish pathogens such as *Streptococcus agalactiae, Flavobacterium columnare* and *Aeromonas hydrophila.* In *Catla catla* disease resistance developed by fish immersed in extract of three herbs namely *Allium sativum, Azadirachta indica* and *Curcuma longa* [14]. The majority of herbs act as anti-pathogenic agent, acts as antibiotic due to strengthen the immune system of organisms prevent from disease or forming disease resistance variety in aquaculture sector.

6. Vaccination

Vaccination is the practice of administering weakened or dead pathogenic bacteria, in order to confer long lasting protection through immunological memory [15]. Adaptive secondary memory immune response of vertebrates depends on immunoglobulins (Igs), T-cell receptors (TCRS), major histocompatibility complex (MHC) and memory T cells. Memory cells and adaptive immunity differentiates the vertebrate and invertebrates immunity. Hence the several strategies are used to improve the adaptive immune system of invertebrates. Vaccination strategy must be designed with the key considerations of minimizing immunomodulatory stresses and stimulates the host defenses by triggering specific immune responses against infectious diseases.

7. Immunostimulants

Immunostimulants are chemical or natural source compounds that activate the immune system of aquatic animals and make them more resistant to infections by viruses, bacteria, fungi, and parasites. Stimulation of the non–specific immune system can improve the animal's response to challenges from pathogenic bacteria. Immunostimulants used to control vibriosis in shrimp increased the survival rate [16, 17]. The potential of immunostimulants is to reduce the effects of bacterial diseases and to improve larval growth. Nowadays commercial immunostimulants are produced in the aquaculture sector to reduce the microbial diseases, through potential activity, immunostimulating performance are not in satisfied level.

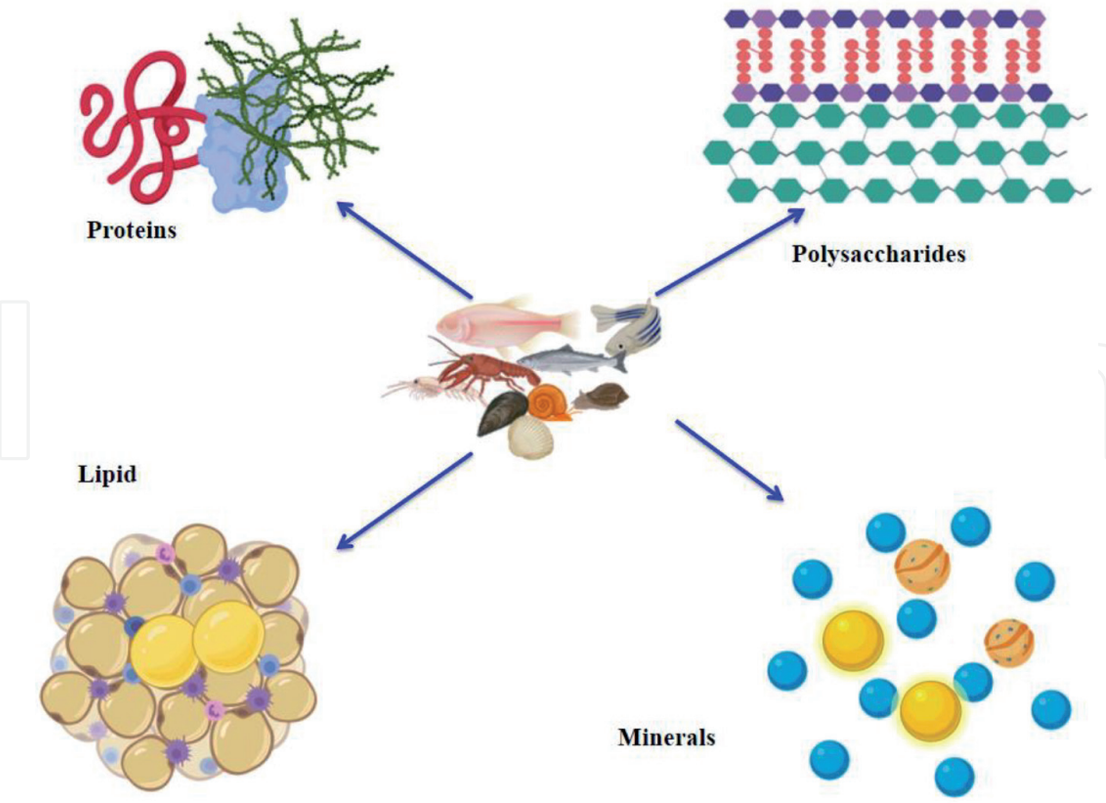

Figure 2.
Marine animal possessing the distinctive level of biomolecules.

Immuno stimulation might be too drastic and harm or even kill the host. Because there is no memory component involved, the response is likely to be short in duration, and hence immunostimulants have to be administered repeatedly. In addition, long term administration of such agents seems to decrease the immune stimulatory effect and does not always promote disease resistance [18]. Several bioactive compounds are isolated through various marine animals' body components (**Figure 2**).

8. Probiotics

During the past two decades, the use of probiotics as an alternative to antibiotics has shown to be promising in aquaculture, particularly in fish and shellfish larviculture hatcheries [19]. Probiotics could be used for the inhibitory studies because of its versatile nature such as inhibitory compounds production, competition for nutrients, competition for adhesion sites in the gastrointestinal tract, enhancement of the immune response, production of essential nutrients such as vitamins, fatty acids, and enzymatic contribution to digestion [20, 21]. Bacteria that are able to improve the water quality by removing toxic inorganic nitrogen or by mineralizing organic matter are also considered as probiotics. Bacterial strains dominantly present in culture water at high densities are also assumed to have the ability to compete efficiently for nutrients with possibly deleterious strains [20]. The development of drug resistant bacteria and the reduced efficiency of antibiotic resistant for human and animal diseases, have led to suggestions of the use of nonpathogenic bacteria as probiotic agents to control diseases (**Figure 3**).

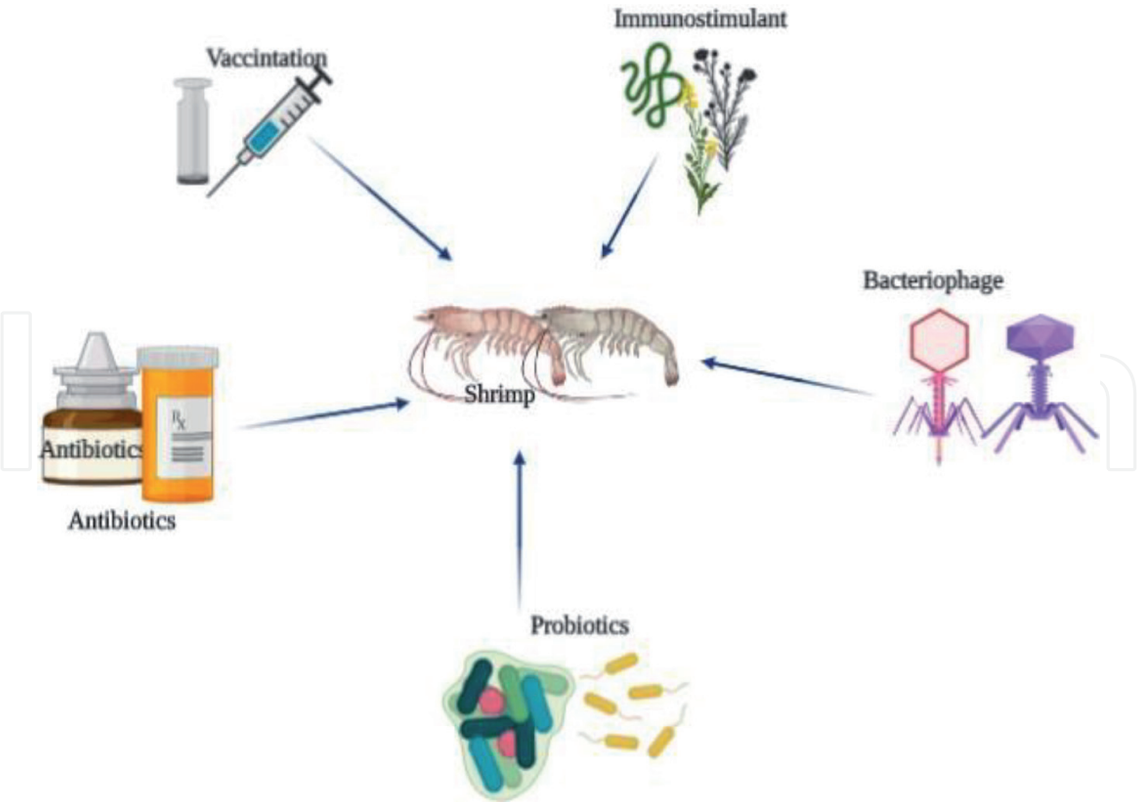

Figure 3.
Various strategies for the shrimp bacterial disease.

9. Bacteriophages

Recently, bacteriophages (phages) are proposed as candidate therapeutics for aquaculture [22]. They could reduce pathogenic bacteria safely, effectively, and ecofriendly, as they are the natural enemies of bacteria. A major advantage of phage therapy is that non–target microbiota is not affected because the phages usually have a narrow host range [18]. However, phages can transfer virulence factors rapidly and selective pressure on the *Vibrio* population might select for strains that are non–sensitive to the phage. Biofilm formation and control A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material. A biofilm is defined as a bacterial population in which the cells adhere to each other and to surfaces or interfaces with architectural complexity [23]. Bacteria are known to colonize the surfaces immediately after the formation of a film of organic molecules. A bacteria cell is grouped together and forms the microbial aggregation on the surface of various materials such as pipes and tanks and form biofilms [24]. All bacterial strains does not have the capacity to produce biofilm layers, particular group of bacterial strains has the potential activity to produce the biofilm layers. In aquaculture system the biofilms are produced by the important bacterial pathogen *Vibrio* sp., which causes large-scale mortalities in shrimp and prawn hatcheries [24]. Biofilm formation may be an important mechanism for host immune modulation and virulence factor for down regulation [25]. Bacterial proliferation in the intestines is thought to induce quorum sensing, which down regulates extracellular matrix production and adhesions required for biofilm formation [26]. The primary quorum sensing system in bacteria that appears to control important adhesins for in vitro biofilm formation is AI-3, an alternative class of quorum sensing molecule distinct from previously

reported AHLs and AI-2 [27]. AI-3 also controls other virulence factors such as the locus of enterocyte effacement (LEE) pathogenicity, which encodes for type III secretion system (T3SS) and toxins secreted by T3SS. AI-3 receptor quorum sensing also binds to epinephrine and norepinephrine [28]. Very few biofilm models of animal infections have been established, and even fewer have tested virulence of quorum sensing mutants in such models. Many research focuses towards the bacterial biofilm inhibition and eradication strategies, the potential antimicrobial agent production is very crucial to treat these biofilm problems in aquaculture industry. Some of the studies already proved the cleavage of bacterial extrapolysachharide layers and successfully inhibit the bacterial biofilms; however these compounds are not work well in all bacterial biofilms. Hence, the researches towards the development the biofilm inhibition through various approaches are enhanced. The use of enzyme-based detergents as biocleaners, also known as "green chemicals," can serve as a viable option to overcome the biofilm problems [29].

10. N-acyl homoserine lactonase

The first AHL-degrading enzyme was identified from Bacillus sp. expression of its gene in *Erwinia carotovora*, pathogenicity in plants has been reported [30]. Many AHL enzymes identified in bacteria, fungi, and mammals [31]. Paraoxonases (PONs) from mammalian sera also have lactonase-like activities in addition to their involvement in the hydrolysis of organophosphates [32]. AHL-degrading enzyme, with both short and long chain AHLs as substrates and little activity with other chemicals was documented earlier [33]. Screening of bacteria capable of producing enzymes, which inactivate the signal compound, blocking the quorum sensing systems of their competitors, has potential for disease control in aquaculture [18]. Diverse aquatic bacteria employ signal molecules to regulate the production of virulence factors [34]. Disruption of these signal molecules can significantly decrease virulence factor production in bacteria without interfering with their growth and it may be a particularly useful method in aquaculture [19, 35, 36]. One of the approaches proposed for quorum sensing disruption is the isolation of bacteria that degrade signal molecules involved in quorum sensing. Bacteria capable of utilizing N–acyl homoserine lactone (AHL) molecules as sole source of carbon and nitrogen can be used as potential quenchers of quorum sensing regulated functions in pathogenic bacteria. Bacteria capable of degrading AHL–type signal molecules have been reported extensively in the literature [37]. Hence, it is of interest to investigate whether these types of bacteria could be used as a new General Introduction: a report on N-acyl homoserine lactonase from quorum quenching *Bacillus licheniformis* and its control of *Vibrio parahaemolyticus* colonization in *Fenneropenaeus indicus* type of probiotic, a live microbial adjunct that is beneficial to the host [18]. Enrichment cultures of AHL degrading bacteria controlled the overall microbial activity in aquaculture. The addition of AHL presumably stimulates the virulence of opportunistic pathogenic bacteria. Assuming that in more intensive aquaculture systems, the often observed high mortality is related to the presence of quorum sensing molecules and quorum sensing induced virulence factors, the addition of an N-acyl homoserine lactonase could be beneficial [38]. The ability to degrade AHLs is widely distributed in the bacterial kingdom, isolated from soil by enrichment culture, is able to utilize AHL compounds as sole carbon, nitrogen and energy source. Bacterial species in natural environments that can metabolize AHLs and disrupt quorum sensing regulation in nearby bacteria was indicated [39]. Bacterial species that interfere with quorum sensing regulation in

another species were reported [40]. The AHL inactivation activity in *Bacillus cereus* isolate was due to its synthesis and secretion of a lactonase capable of opening the homoserine lactone ring of AHLs, thereby reducing the effectiveness of the signal molecules [41]. Some bacteria, especially Bacillus sp. may use AHL-lactonases in quorum-quenching to boost competitive strength in soil. Metallo-β-lactamase superfamily (MBL) family-type enzymes have been characterized from a variety of soil-associated *Bacillus* spp. and other bacteria [42, 43]. Metallo-lactamases consist of conserved motif HXHXDH and a zinc binding motif, and dinuclear zinc binding center bridged by an aspartate and an oxygen species [44, 45]. AHL lactonase AiiB from *Agrobacterium tumefaciens* also posses the similar active sites [46]. AHL lactonase expression in the pathogens *Erwinia amylovora*, *Pseudomonas aeruginosa* PAO1 and *Burkholderia cepacia* reduced their virulence by degrading AHLs [47]. In addition, AHL lactonases have also been expressed in *Escherichia coli* and *Pichia pastoris* [48]. Green fluorescent protein The introduction of green fluorescent protein (GFP) as an endogenous fluorescent tag provides a mean of rendering the bacteria visible and also tracing their activity in living host cells [49]. Green fluorescent protein is a small protein (27 KDa) found in the jellyfish, *Aequorea victoria*. It has the property of fluorescing when excited by ultraviolet light [50]. This gene codes for a fluorescent protein, when excited with UV light (470 nm), emits a wavelength of 502 nm. The GFP fluoresce is independent of cofactors, substrates or any additional gene products, sensitive, stable, specific, non-toxic and does not interfere with cell growth and function. GFP-marked fish pathogens have been constructed to study the invasion pathway in fish models [51]. The fate of *Vibrio parahaemolyticus* once filtered by oysters and its capacity to proliferate in different post-harvest conditions was defined, using a strain of *V. parahaemolyticus* with a plasmid that contains the GFP gene [52]. Recently, GFP was used to study colonization and pathogenesis of *Vibrio parahaemolyticus* in different tissues and hemolymph of *F. indicus* [53].

11. The prophenoloxidase activating system (proPO system)

The proPO system is an efficient part of the innate immune response and consists of several proteins, which are involved in pattern recognition proteins, proteases, protease inhibitors, antioxidants proteins and melanisation as represented in **Figure 4**.

In addition, it also associated with the cytotoxic reactions, cell adhesion encapsulation, and phagocytosis, which is present in many invertebrate groups, such as ascidians, mollusks, echinoderms, millipedes, bivalves, brachiopods and insects [54, 55]. In invertebrates the humoral mediated immune system is triggered through several hemolymph proteins amongst the prophenoloxidase plays the vital role against the invading pathogens. At the same time, this immune pathway is stimulated through microbial pathogens such as bacteria, fungi and virus. The stimuli are derived from the outer membrane components of microbes, those molecules are termed as pathogen-associated molecular pattern (PAMP) which are lipopolysaccharide (LPS) and peptidoglycans (PG) from bacteria and β-glucans from fungi. This proPO cadcade consist of pattern-recognition proteins (PRPs) including LPS and β-1,3-glucan-binding protein (LGBP), β-1,3 glucan binding protein (βGBP), and peptidoglycan binding protein (PGBP), several serine protease and zymogens, proPO as well as proteinase inhibitors, which are important regulatory factors to avoid activation of the system where it is not appropriate [56].

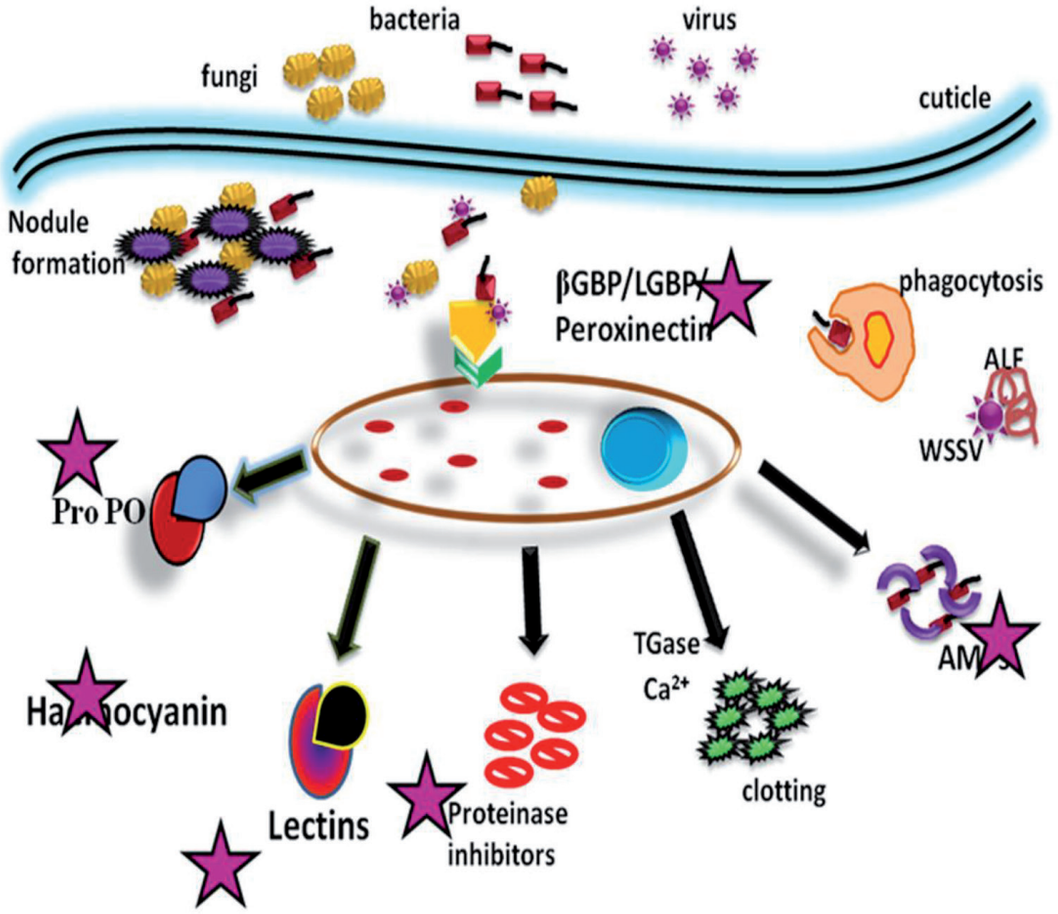

Figure 4.
Schematic outline of the principle components in the prophenoloxidase (proPO)-activating system in arthropods.

12. Protein mediated nanoparticles

Alternative approaches to treat bacterial infections are urgently needed in aquaculture worldwide. Nanobiotechnology and nanotechnology products have a wide usage potential in aquaculture and seafood industries. For instance, production of more effective fish feed for aquaculture species by the application of nanotechnology is possible. New materials obtained by the nanosciences can be used in the different aspects of fisheries and aquaculture. Nanotechnology may have the potential to provide aquaculture that is safe from disease and pollution. Use of quorum quenching enzymes as antimicrobial agents is nature-inspired and has recently attracted much attention as an antibiotic-free approach to treat bacterial infections. The use of antimicrobial enzymes covalently attached to nanoparticles is of special interest because of enhanced stability of protein-nanoparticle conjugates and the possibility of targeted delivery.

13. Antimicrobial peptides

AMPs are effectors of the innate immune system and function as a first line of defense to fight against invading microorganisms [57] are represented in **Figure 5**. Therefore, AMPs are critical for shrimp to fight against the pathogenic invasion. AMPs are typically small in size, are naturally derived or synthetic and are active against a wide range of microorganisms, such as bacteria, virus, yeast, parasite and fungi,

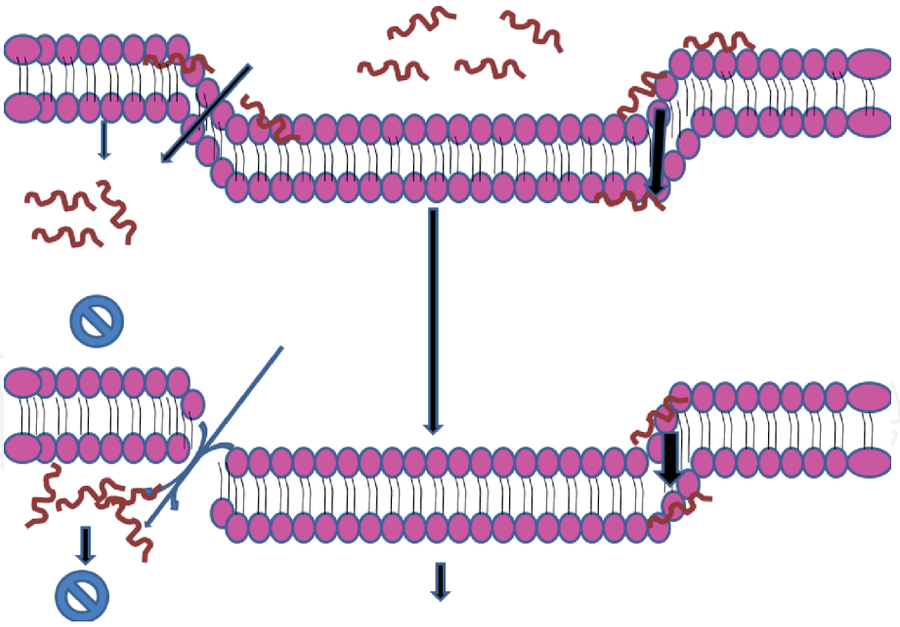

Figure 5.
Antimicrobial peptides intrusion mechanism inside the bacterial cell wall.

and they may also exhibit an anti-tumor activity [57, 58]. Generally, it has less than 150–200 amino acid residues, and it has an amphipathic structure with cationic or anionic properties. Several families of shrimp AMPs, such as penaeidins, lysozymes, crustins, ALFs and stylicins, have been identified and characterized [59, 60]. They are produced by and stored in the hemocytes; these are key cells in the crustacean immune system [61]. Various methods are discussed in the introduction section to eradicate the bacterial inhibition and bacteria causing disease management. However, in this chapter, we used the antimicrobial peptides to inhibit the bacterial causing biofilms and using probiotic bacteria, we attempted to reduce the bacterial disease.

13.1 Crustins

Crustins are generally defined as multi-domain cationic antibacterial polypeptides (7–14 kDa) containing a whey acidic protein (WAP) domain at the C-terminus (**Figure 5**) [62]. The first identified crustin member is an 11.5 kDa protein purified from the granular haemocytes of the shore crab, *Carcinus maenas* that exhibits specific activity towards Gram-positive marine or salt-tolerant bacteria [63, 64]. Over 50 crustins and crustin-like sequences have been identified in numerous crustacean species, including crayfish, shrimp, freshwater prawn, crab, lobster, and also in non-decapod crustaceans, such as amphipods, (through EST-based approaches) [65].

14. Materials and methods

14.1 Collection and maintenance of bacterial strains

Fenneropenaeus indicus, Penaeus monodon, Litopenaeus vannamei, and *Penaeus semisulcatus* were collected from different sea shore area in and around Tamil Nadu. Live *P. semisulcatus* was acclimatized in lab for two week before the experimentation. All the tanks received continuous aeration, and 50% of the water was exchanged daily to maintain quality. Pathogenic strains and probiotics bacteria were isolated from the whole intestinal tract and hepatopancreas of wild caught *P. semisulcatus* larvae.

14.2 Replica plating method

The shrimp intestinal tract, hepatopancreas content was aseptically removed from a live healthy prawn were homogenized and serially diluted with sterilized normal saline solution. Suspensions (0.1 ml) were spread on different media like nutrient agar (with 1% w/v NaCl) and thiosulfate/citrate/bile/salt (TCBS), Zobell Marine agar (ZMA), bacillus medium (HiMedia, India), and incubated under an aerobic atmosphere overnight at 37°C for 24 h. After incubation, predominant bacterial colonies were selected based on their morphological characters including color, shape, size colonies from all media were replica plated on the Muller Hinton agar medium (Hi Media, India) with target bacterial strains and incubated at room temperature for 24 h. After incubation viable colonies showing the zone of clearance against the target *Vibrio* strains were streaked on the nutrient medium to check purity of the isolate. All the purified strain was maintained in Zobell Marine Broth (HiMedia) at −20°C with 15% glycerol.

14.3 Antagonism assay

To evaluate the potential antagonistic activity of the isolated probionts by well diffusion assay on solid medium and eight *Vibrio* spp. was used for our study. *Vibrio* spp. was precultured in broth for 24 h and incubated at 28°C. Lawns were prepared by spreading 50 μl of each target strain (*Vibrio* spp.). Wells were cut in LA plates with a sterile 5-mm cork borer and filled with 50 μl (10^8 CFU) of the 72 h old each and probiotics cell free extract were carefully pipetted into each well. Two probiotics were tested per plate in triplicate. The diameter of the inhibition zones around the wells were recorded in millimeters after incubating the plates for 24 h at 25°C.

14.4 Characterization of strains

Isolated strains were subjected to standard morphological, biochemical assay followed by bacterial genomic DNA was extracted using the method [66]. Bacterial strains were cultivated in 10 mL of Luria-Bertani broth (LB) at 29°C in agitation for 18 h. culture was centrifuged for 5000 rpm for 5 min suspended pellet with Sucrose TE buffer 10 $mmol^{-1}$ lysozyme was added and incubated for 30 min. After incubation, add 100 μl of 0.5 M EDTA (pH 8) and 60 μl of 10% SDS added with 250 μl of equilibrated Phenol and 250 μl of Chloroform and mixed gently mixture was centrifuged. An equal volume of chloroform and isoamyl alcohol mixture (24:1) was added with shaking the mixture. Collect the aqueous phase in a sterile tube and precipitate it with 2 volumes of 100% ethanol and 3 M Sodium Acetate. Store at −20°C for 30 min. Followed by this addition, the sample was centrifuged with at 12000 rpm and the isolated DNA was precipitated with 70% ethanol. DNA was suspended with 30 μl of TE Buffer pH (8.0) and DNA was extracted for 16S rRNA sequence determination & RAPD analysis.

14.5 rRNA gene amplification

The region of the 16S ribosomal gene (rRNA) of the DNA extracted from each bacterial strain was amplified by the polymerase chain reaction (PCR). The reagent mixture was prepared with the universal 16S rRNA Fp 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 16S rRNA Rp 5′-ACG GCT ACC TTG TTA CGA CTT-3′ [67], samples were amplified by PCR in Std buffer 2.5 μl, dNTPs 0.5 μl, forward and reverse primers each 1.0 μl and Taq 0.2 μl and template DNA 1.0 μl condition

consist of 40 cycles of 95°C (5 min), 55°C (1 min), and 72°C for (2 min) and with final 72°C for 10 min for elongation process were performed with four bacteria strains, yielding positive amplification for all DNA tested, as determined by visualization on agarose gel electrophoresis. The amplification products were purified by using Real genomics kit, by following the specifications of the manufacturer.

14.6 Co-culture method

The co-culture method was performed to observe the antagonistic potential and reproductive effect of the isolated bacteria, when grown with the *Vibrio* spp. in a same medium. Culture broth of the prospective probiont and the target organism were inoculated into LB broth to check the probiotic activity of our isolated culture. Two Probiotic strains (DMP4 & DMB3) were used. Eight selected *Vibrio* spp., (v1, v2, v3, v4, v5, v6, v7, v8) visibly different from each other in size, shape and color of the colony morphology were used for co-culture method. The initial cell density of selected *Vibrio* strains was approximately 10^3 CFU ml^{-1}, whereas the initial concentration of probiotic cell free extract was 10^5, 10^6, 10^7, and 10^8 CFU ml^{-1}. All inoculums were prepared with LB broth each *Vibrio* inoculum and each probiotic inoculum was mixed together, and then incubated for 24 h days at 25°C and reading was taken in every 2 h intervals.

14.7 Artemia hatchability test

Probiotic activity of chosen strains were further tested with Artemia hatchability test, 160 mg of dried cysts were hatched in 80 ml of sterile sea water under the conditions of strong aeration and constant illumination, at 28°C *Vibrio* sp. was grown in leuria broth incorporated with NaCl enrichment (3%) and up to 10^8 CFU ml^{-1} was obtained within 24 h. The concentration of *Vibrio* sp. was dispensed in 10^3 CFU ml^{-1} in hatching unit. Six experimental group along with one control without any pathogen or probiotic bacteria was subjected for *hatchability test*. Five experimental groups namely *Vibrio* sp. + *Pseudomonas* sp. (vp), *Vibrio* sp. + *Bacillus* sp. (vb).positive control group *Bacillus* sp. (vb) (dahb) and *Pseudomonas* sp.(vp) (dahp) were taken for the experiment pathogenic *Vibrio* sp. was inoculated at conc. 10^3 CFU ml^{-1} and Probiotics bacteria dahp and dahb were inoculated in 10^8 CFU ml^{-1} to the corresponding experimental set up along with prehydrated cyst the hatchability was assessed for 24 h [68]. After 24 h exposure, the free nauplii were counted under microscope. Five replicates were calculated for the control and each treatment measures. The hatching percentage (%h) was calculated with following formula:

$$\%H = \frac{N}{N + C + U} \times 100 \quad (1)$$

where N = Nauplii, C = Unhatched cysts, and U = Umbrella stage.

14.8 Challenge studies in *Artemia nauplii*

Five experimental groups namely *Vibrio* sp. + *Pseudomonas* sp. (Vp), *Vibrio* sp. + *Bacillus* sp. (Vb).positive control group *Bacillus* sp. (b) and *Pseudomonas* sp. (p) were taken for the experiment *Vibrio* sp. was inoculated at conc. 10^3 CFU ml^{-1} and Probiotics bacteria *Pseudomonas* sp. and *Bacillus* sp. (p&b)

were inoculated in 10^8 CFU ml^{-1} The survival rate was determined in every 2 h intervals of exposure for 12 h. The percentage of survival was calculated by the formula

$$\text{Survival rate}(\%) = \frac{\text{Number of live nauplii } at \text{ every 2 h interval}}{\text{Number of nauplii } at \text{ the time of inoculation}} \quad (2)$$

The active nauplii were considered as live and counted under microscope.

15. Discussion

Various approaches are applied to eradicate the bacterial and other microbial diseases in aquaculture, however this chapter deals with the biofilm inhibitory mechanism through two different ways probiotic isolation from aquaculture farms or the specific animals such as shrimp or crab form the body part. For this many microbiology techniques are used to isolate the bacteria and identify, then inhibit the *Vibrio* causing biofilm in these probiotic strains. Another approach of treating bacterial biofilms via antimicrobial peptides that also derived from marine sources could be used here. Such kinds of peptides easily penetrates into the layer of bacterial cell membranes and quickly disrupt and dissolve the polysaccharide layer, which leads to the inhibition of biofilms in the surface of the layer.

16. The potential use of antimicrobial peptides for disease control in aquaculture

Antimicrobial peptides provide a good therapeutic alternative for the treatment of diseases in aquaculture. Several antimicrobial peptides from various sources are already in clinical and commercial use [69]. However, it is quite promising that the shrimp AMPs could be potential candidates as an alternative to antibiotics in shrimp farming. Besides their antimicrobial function, AMPs are also known to act as mediators of inflammation influencing diverse processes such as cell proliferation, wound healing, cytokine release and immune induction [70].

The significance of aquaculture in the context of global food production sector, the management of aquatic resources and the socio-economic development of coastal rural areas is now fully appreciated world-wide. In the last decade, a series of papers describing shrimp immunity were published and a batch of related data accumulated, which are very useful for understanding the interaction between shrimp and pathogens to enrich the immune theory of invertebrates. Recently, several review papers summarize the achievements in shrimp immunity including expressed sequenced tag and database construction [71], microarray analysis of shrimp immune response [72], shrimp molecular responses to viral pathogen [73] and the cationic antimicrobial peptides in penaeid shrimp [60]. Obviously understanding the shrimp immunology is necessary to develop an effective strategy for disease control. Indian white shrimp *Fenneropenaeus indicus* is an important crustacean for aquaculture and has brought significant revenue to rural economy. The epidemics, however, caused a dramatic mortality and resulted in a severe economic loss. Therefore, the understanding of the immune ability of shrimp and their defense mechanisms has become a primary concern in shrimp aquaculture.

17. Conclusions

Recent advances gives the notions to incorporate the different techniques and utilized in the aquaculture practical system as a combined mode to enhance the potential activity of the strategies towards the microbial pathogens, this would be efficient method when compare to conventional techniques alone. In addition this way of microbial eradication will helps to improve the aquaculture production as well as cost effective.

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Conflict of interest

The authors declare no conflict of interest.

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