

Chapter 1

Literature Review

1.1 Introduction

The term ‘marine environment’ covers a vast number of habitats, from deep-sea regions to the salty water of estuaries. These habitats are different in both biotic and abiotic characteristics (Kis-Papo, 2005). The oceans cover more than 70% of the Earth’s surface (Colwell, 2002; Fenical, 1993; Masuma *et al.*, 2001; Proksch *et al.*, 2003). One millilitre of seawater contains somewhere in the order of 10^3 fungal cells, 10^6 bacteria, and 10^7 viruses, including pathogens that cause extensive mortality, and microorganisms that induce host surface fouling (Kubanek *et al.*, 2003). There are many similarities between terrestrial and marine bacteria, but living in the marine environment requires the exploitation of different compounds, necessitating specific adaptations and genotypic changes in marine microorganisms (Běhal, 2003; Imamura *et al.*, 1997; Towse, 2005). Marine microbes have developed unique metabolic and physiological capabilities to be able to survive in extreme environments, and may therefore produce metabolites which are not produced by terrestrial bacteria and fungi (Fenical, 1993). The oceans have proven to be a rich source of a wide variety of potentially active agents that are mostly accumulated in invertebrates such as molluscs, sponges, bryozoans, and tunicates (Proksch *et al.*, 2002). Microbiologists have known that antimicrobial producing marine bacteria are partially responsible for the natural antimicrobial activities of seawater, in addition to playing a most important role in the population dynamics of marine microorganisms (Rosenfeld & ZoBell, 1947).

Preliminary studies of marine bacteria indicated that the bacterial population in seawater is composed mainly of Gram-negative bacteria, while Gram-positive bacteria represented less than 10% of the total. Today the evidence suggests that Gram-positive bacteria are found in higher proportions in biotic and abiotic surfaces, sea sediments, and inner spaces of invertebrate animals (Fenical, 1993; Zheng *et al.*, 2000). These commensal or symbiotic bacteria, in many cases, make up the non-pathogenic microorganisms associated with the host. They chemically protect their microhabitat while defending their host from pathogenic microbes by the production of secondary metabolite compounds (Zheng *et al.*, 2000).

Host-microbe interactions are often qualitatively as well as quantitatively different in aquatic and terrestrial species. In the marine milieu hosts and microbes share the ecosystem, in

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contrast, most terrestrial habitat interactions occur at the gut, which represents a moist habitat in an otherwise water-limited environment. In some sense, marine microorganisms have the option of living in association with the potential host's intestinal tract, gills, or skin (Harris, 1993; Verschuere *et al.*, 2000 a).

Most of the important antibiotics in human history were isolated from terrestrial microorganisms, while the suppressive effects of aquatic microorganisms against human pathogens has only recently been investigated (Lo Giudice *et al.*, 2007 b, p. 496). Nowadays it has been proved that aquatic bacteria are capable of producing a wide-range of biologically active compounds which might be used in conjunction with those recovered from terrestrial microorganisms (Isnansetyo & Kamei, 2003; Miao & Qian, 2005)(Lo Giudice *et al.*, 2007 b; Nair, 2005; Zheng *et al.*, 2000).

Aquaculture is an important food production industry, providing an alternative source of seafood products to wild fisheries. However, widespread infectious disease in aquaculture hatcheries causes significant economic losses to this industry. Traditionally antibiotics have been used to combat larval loss due to pathogen infection, however, there are increasing concerns about the development of antibiotic resistance which can render antibiotic treatments ineffective. This has created an urgent need for molecular biology and gene therapy research into the development of new drugs to reduce antibiotic resistance, in addition to enhanced technology in computer-assisted drug design (Munro *et al.*, 1999). New methods for controlling infectious diseases are needed in order to sustain the aquaculture industry. In recent years beneficial microorganisms have been applied in aquaculture industries. Such microbes improve the immune system of cultured marine animals, in addition to producing secondary metabolites that can also improve marine animal's resistance capability (Defoirdt *et al.*, 2007; Zhou *et al.*, 2009). Using probiotic bacteria as a disease controlling measure in aquaculture may replace or improve upon the traditional treatments, such as the use of antibiotics.

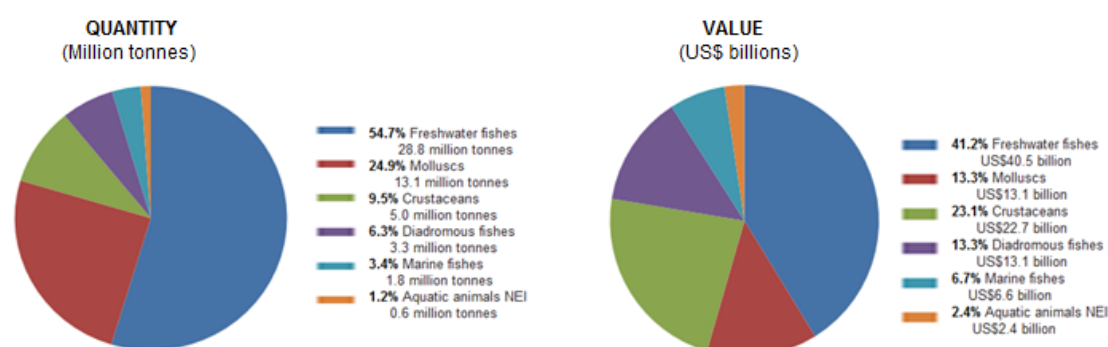
The aims of this work were to identify potential probiotic microbes from culture collections of marine microorganisms that may be used as biocontrol agents against the pathogenic bacteria *Vibrio owensii* that affects lobster larval culture. Once such potential probiotic microbes were found, molecular analysis was employed to identify the gene(s) involved in antimicrobial production.

1.2 Impact of pathogens on the aquaculture industry

1.2.1 Importance of Aquaculture

The demand for seafood products has increased alongside the world population. However, due to over-exploitation of aquatic environments worldwide there is a growing search for alternative food products (Anand *et al.*, 2011; Martinez-Porchas & Martinez-Cordova, 2012). Aquaculture is being viewed as the only alternative method of enhanced fish production (Anand *et al.*, 2011) and is considered a promising solution to meet the increasing demands for marine products at a global level (Martinez-Porchas & Martinez-Cordova, 2012). Aquaculture is the farming of aquatic organisms including fish, molluscs and crustaceans. “It is an emerging industrial sector which requires continued research and development to maximise efficiency and production” (Toranzo *et al.*, 2005, p. 37).

Worldwide fish consumption has increased gradually over the last five decades, with consumption of fish increasing at an average annual rate of 3.2%, above the 1.6% rise in world population growth per annum. Aquaculture production globally continues to grow slowly. The worldwide fishing production in 2008 was dominated by freshwater fish at 28.8 million tonnes representing 54.7% and worth 40.5 billion US dollars (41.2% value). This was followed by molluscs at 13.1 million tonnes, crustaceans at 5 million tonnes, diadromous fish at 3.3 million tonnes, marine fish at 1.8 million tonnes and other aquatic animals at 0.6 million tonnes, as shown in Figure 1.1 (FAO, 2010). In 2012, the world aquaculture production reached a peak of 90.4 million tonnes with a 144.4 billion US dollar value, including 66.6 million tonnes of food fish valued at 137.7 billion US dollars and 23.8 million tonnes of marine algae, being mainly seaweed valued at 6.4 billion US dollars (FAO, 2014).



Note: NEI = not elsewhere included.

Figure 1.1 Main species clusters aquaculture production worldwide in 2008
adapted from (FAO, 2010)

The Food and Agriculture Organization of the United Nations has estimated that production from aquaculture in 2020 will cover half of the worldwide need for seafood (Moriarty, 1999). However, the spread of infectious disease is considered as one of the main restrictions to this development.

1.2.2 Aquaculture and diseases

Populations of all organisms are determined partially or completely by diseases in their environments (Real, 1996). Aquaculture is the fastest growing industry in the production of animal protein worldwide (Bondad-Reantaso *et al.*, 2005; Mohapatra *et al.*, 2013; Subasinghe, 2005), however, the spread of disease effects the development of the aquaculture industry. Diseases are being increasingly documented as a substantial restriction on trade and the aquaculture industry globally, particularly diseases caused by microorganisms such as bacteria, viruses, parasites, fungi, and other emerging and undiagnosed pathogens (Bondad-Reantaso *et al.*, 2005; Defoirdt *et al.*, 2007; Desriac *et al.*, 2010; Muñoz-Atienza *et al.*, 2013; Sanmukh *et al.*, 2012; Subasinghe, 2005). The disease causing agents present in aquaculture tanks also usually exist in natural environments, though they rarely cause mortality in wild fish populations. It is thought that the reason for this is because of the more stressful conditions that are generally present in aquaculture tanks (Toranzo *et al.*, 2005).

Consequently, the spread of diseases in aquaculture is one of the most important causes of serious economic loss in aquaculture of many marine species in several countries (Bondad-Reantaso *et al.*, 2005; Meyer, 1991). For example, according to World Bank statistics, worldwide losses of approximately 3 billion US dollars occurred due to shrimp diseases (Subasinghe, 1997). In 1988 more than 20 million trout were lost, costing the trout industry around 2.5 million dollars, and in the same year, channel catfish producers reported losses of over 100 million fish, valued at almost 11 million dollars (Meyer, 1991).

Bacterial pathogens are the leading cause of loss due to disease in all types of seafood production, while fungal diseases and external protozoan parasites are responsible for epizootics and large losses of finfish fingerlings, fry and juvenile (larva) shellfish (Meyer, 1991). There have been major global economic losses in cultured fish as a result of opportunistic pathogenic bacteria; in particular Gram-negative bacteria are responsible for epizootics in almost all cultured species (Toranzo *et al.*, 2005).

1.2.3 *Vibrio* spp.

Family *Vibrionaceae* belong to the class Gammaproteobacteria, members of which are widespread in the marine environment. Genus *Vibrio* are motile, facultative anaerobic, Gram-negative bacteria (Mansson *et al.*, 2011; Simidu & Tsukamoto, 1985). It is understood that strains belonging to *Vibrio* species can exhibit different virulence patterns ranging from non-virulent to highly pathogenic (HP)(Goarant *et al.*, 2006; Juiz-Rio *et al.*, 2005).

Within the *Vibrionaceae* family, some species are known pathogens in animal systems including humans. However, species of the family *Vibrionaceae* are frequently prevalent in the microbial community of seawater, fish and plankton (Khandeparker *et al.*, 2011) and represent a major group of microorganisms found in the intestinal flora of aquatic fish and crustaceans (Kita-Tsukamoto *et al.*, 1993; Simidu *et al.*, 1977).

In a review conducted in the West Pacific Ocean, *Vibrio* represented nearly 75% to 100% of the heterotrophic bacterial inhabitants in surface seawater (Simidu *et al.*, 1980). The species *Vibrio anguillarum*, *V. salmonicida*, *V. ordalii*, and *V. vulnificus* biotype 2 caused the most economically serious diseases in marine culture (Toranzo *et al.*, 2005).

Vibrio vulnificus is naturally present in estuarine environments and may contaminate shellfish, causing potentially fatal sepsis and devastating wound infections (Gulig *et al.*, 2005; Gulig *et al.*, 2009). Few bacterial species belonging to the genus *Vibrio* are recognized to be fish pathogens. Of these, *Vibrio anguillarum* has caused serious losses worldwide in fish farms (Valla *et al.*, 1992). *Vibrio* species are also known to cause disease in humans, and such *Vibrio* species are considered to be seriously pathogenic from the perspective of public health (Nishibuchi & Kaper, 1995). These species currently include *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. metschnikovii*, and the non-O group 1 portion of the *Vibrio cholerae* species (Blake *et al.*, 1980). *V. parahaemolyticus* has been recognized as an agent of gastroenteritis, associated with consumption of seafood; however not all strains of this species are considered to be pathogenic (Nishibuchi & Kaper, 1995). For instance, *V. parahaemolyticus* is one of the most important food-borne disease causative agents in Asia, causing almost 50% of the food poisoning epidemics in Japan, Taiwan, and Southeast Asian countries (Joseph *et al.*, 1982; Wong *et al.*, 2000). However, the relationship between the bacterial isolates from seafood, estuarine environments, and clinical isolates of humans are not clear (DePaola *et al.*, 2003).

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Diseases of marine organisms caused by luminescent vibrios such as *Vibrio harveyi* and the other related bacteria including *V. parahaemolyticus* and *Vibrio campbellii* are a serious threat to marine organisms. Severe disease caused by luminescent vibriosis has become a major obstruction to penaeid shrimp aquaculture production in Asia and South America (Austin & Zhang, 2006). Diseases caused by species and strains belonging to *Vibrio* have become a serious threat to the crustacean aquaculture industry such as lobster, crab, and shrimp (Bäck *et al.*, 1974). For example, the American lobster *Homarus americanus* the subgroup *Vibrio fluvialis* has been involved in limp lobster disease (Tall *et al.*, 2003). Another example, more relevant to the research undertaken in this thesis, is *V. owensii* (DY05), a potential pathogen of spiny lobster *Panulirus ornatus* pelyosoma, which causes severe larval mortality (Goulden *et al.*, 2012).

1.2.4 Lobster and Disease

Spiny rock lobsters belonging to the family Palinuridae are an important aquatic resource for many countries (Phillips, 1985). The ornate spiny lobster, *Panulirus ornatus* (Decapoda: Palinuridae), is a tropical species with an Indo-West Pacific distribution. It is most abundant in the north-east of Australia (Jones *et al.*, 2001; Pitcher *et al.*, 1997). Thus, Australia is the world's largest producer and exporter of these animals (Phillips, 1985). Australia has nine species of rock lobster, with six tropical species of the genus *Panulirus* being found in northern areas of Australia (Phillips, 1985). *P. ornatus* is the most abundant of these species in the far north eastern coast of Queensland and the Torres Strait (Pitcher *et al.*, 1997). The Australian ornate rock lobster, *P. ornatus* has a short life cycle compared to other Palinurid lobsters and there is a high demand for the animal in the overseas markets. For these reasons *P. ornatus* was identified as a good candidate for commercialisation as an aquaculture species (Zhang *et al.*, 2009).

Rock lobster species are among the most valuable wild fisheries species. Wild populations are therefore under increasing pressure from overfishing. This pressure has led to research efforts aimed primarily at more effective management of stocks in the ocean to offer greater opportunity for wide dispersal of larvae. The market demand for rock lobster combined with recent research investment has led to an increased interest in the breeding of the tropical ornate rock lobster *P. ornatus*. Nevertheless, a number of limitations have prevented the success of this aquaculture industry, including the long pelyosoma phase of *P. ornatus*, spread of infectious diseases, lack of suitable live feed and food-borne diseases (Bourne *et al.*, 2004; Kittaka & Abrunhosa, 1997; Payne *et al.*, 2006b).

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1.2.4.1 Larval cycle and disease

Rock lobsters of the family Palinuridae represent the most highly valued fisheries sector in Australia, and are nowadays considered a target aquaculture species. However, larval stage diseases such as the high rate of larval attrition caused by inadequate nutrition and microbial management issues represent the most important challenges to overcome for commercial production success (Bourne *et al.*, 2007; Handler *et al.*, 1999; Ritar *et al.*, 2006). It is important to understand the relationship between the tropical ornate rocky lobster, *P. ornatus* phyllosoma and associated diseases, in order to control infectious diseases that currently prevent aquaculture success (Payne *et al.*, 2007). The extended larval stage of lobster phyllosomas emphasizes problems correlated with disease control. Through the molt phases lobster phyllosomas are similar to other crustacean larvae, which are especially sensitive to microbial infection before the new shell hardens and provides some protection against pathogen invasion (Payne *et al.*, 2006a; Payne *et al.*, 2007).

The development of lobster larvae differs between species in moult frequency and duration as well as morphology (Kittaka & Abrunhosa, 1997). For example, spiny lobsters (Decapoda: Palinuridae) and other closely related species, and coral lobsters (Scyllaridae and Synaxidae) pass through a sequence of unusual phyllosoma larval phases during development that are characteristic of these groups. The tropical rock lobster has a complex life cycle with long migrations and many growth phases as shown in Figure 1.2. The ornate tropical rock lobster diseases, particularly bacterial diseases, are currently considered the main problem in successful larval culture for most aquaculture of crustaceans (Johnston & Aqua, 2006; Payne *et al.*, 2006a).

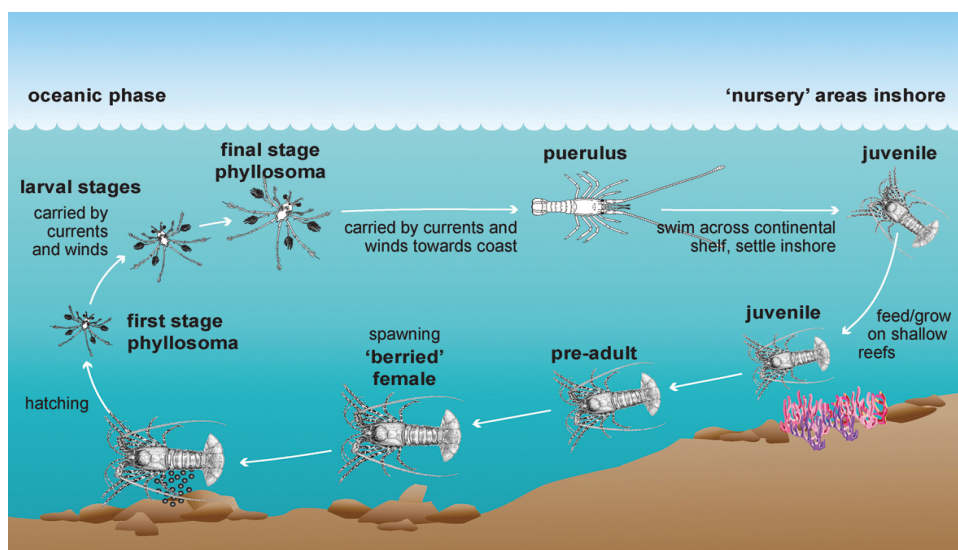


Figure 1.2. Lobster life cycle showing the larval stages (CSIRO 2014).

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Vibriosis causes severe losses of phyllosoma in lobster hatcheries. It affects both brine shrimp, raised to feed the lobsters, and the lobster larvae themselves (Diggles *et al.*, 2000). Several important studies investigating the microbiological community of crustacean larval breeding tanks have concentrated on the isolation and identification of disease pathogens, with species of *Vibrio* being the most frequently documented pathogen (Ferris *et al.*, 1996; Hameed & Rao, 1994). Specific bacterial species including *V. harveyi*, *V. anguillarum*, *V. alginolyticus*, and *V. tubiashii* are involved in causing disease in larval and juvenile rock lobster (Diggles *et al.*, 2000). Filamentous bacteria have also been found to be correlated with disease of larval and juvenile rocky lobsters. Filamentous bacteria, such as *Leucothrix mucor*, are usually considered an indicator of stress or bad water quality and have been observed on the eggs and gills of *Jasus edwardsii* (Shields & Behringer, 2004).

Another pathogenic bacterial species affecting lobster is *Leucothrix*, which causes an infestation on the surface of *Homarus americanus* larvae culture (Johnson, *et al.*, 1971). “*Leucothrix*-like” bacteria have also been found by Handler et al. (1999) to cause disease in *Jasus* species or southern rock lobsters. In a study by Bourne, et al. (2004) many bacterial species associated with phyllosoma larvae were isolated from the microbial community of the *P. ornatus* larval nursing system. These species included *Pseudoalteromonas* sp., *Desulfobulbus mediterraneus*, *Alteromonas* sp., *Pirulella* sp., *V. parahaemolyticus* and an uncultured bacterial species. Species of *Vibrio* were also reported to proliferate within the hepatopancreas of *P. ornatus* phyllosomas and proliferation of internal bacteria was associated with larval mortality (Webster *et al.*, 2006).

1.3 Biocontrol

1.3.1 Diseases Management and control Strategies

Control of harmful microbes is an important measure to ensure survival and health. Disease caused by microorganisms should be treated in humans, plants and animals (Gram *et al.*, 2010). Management of the health of aquatic species has recently been given a high priority in aquaculture production in many regions throughout the world. This increased focus on animal health has been stimulated by serious economic losses, and environmental impacts of disease

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in cultured aquatic organisms. A number of countries have enhanced their laboratory services, control and therapeutic strategies and diagnostic expertise, in order to efficiently control infectious disease in aquaculture (Bondad-Reantaso *et al.*, 2005).

Some progress has been made in the control of disease spread in marine animals by increased awareness, improved research, effective legislation and policy (Bondad-Reantaso *et al.*, 2005).

1.3.2 Traditional methods

Microbial diseases are one of the most significant problems that affect aquaculture commercialization (Natrah *et al.*, 2011). Traditionally the first response to control disease spread in aquaculture is the use of antibiotics (Defoirdt *et al.*, 2007; Kesarcodi-Watson *et al.*, 2008). This is similar in other industries; for example, certain antimicrobial drugs have demonstrated positive effects on the growth of livestock and are used extensively (Acar *et al.*, 2000; Phillips *et al.*, 2004; Wierup, 2001). However, conventional approaches, including the use of antimicrobial drugs and disinfectants, have had limited success in curing or preventing disease in marine organisms (Oliveira *et al.*, 2012; Subasinghe, 1997). The use and misuse of antimicrobial drugs for disease control in aquaculture and agriculture, and as growth promoters in animals, has led to the spread of antibiotic resistance, and therefore results in reduced efficiency of antimicrobial treatment of animal and human diseases (Aarestrup, 1999; Akinbowale *et al.*, 2006; Defoirdt *et al.*, 2007; Hjelm *et al.*, 2004a; Lim *et al.*, 2011; Moriarty, 1997; Witte, 2001). In addition to the spread of resistant pathogens, resistance genes, or genes for virulence are transferred via R plasmids and transposons to other bacteria which have not previously had been exposed to antibiotics (Moriarty, 1999; Witte, 2001). Luminous vibrios isolated from a shrimp breeding farm off Java island, Indonesia, have showed multi antibiotic resistance such as tetracycline, ampicillin, amoxicillin, and streptomycin (Tjahjadi *et al.*, 1994). Thus, β -lactam resistance is now common in *Vibrio* spp. isolated from different locations and sources. It is documented that antimicrobial drugs have been widely overused (Aarestrup, 1999; Schwarz *et al.*, 2001) and therefore a number of alternative strategies have been suggested to reduce antimicrobial use as a disease control which have previously been applied successfully in aquaculture (Lamari *et al.*, 2014; Subasinghe, 1997; Zhang *et al.*, 2009). These alternative approaches include the use of immunostimulants for the improvement of the innate immunity mechanisms of the host, the use of bacteriophages or phage therapy, the use of probiotic bacteria or vaccines, microalgae

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or green–water, short chain fatty acids and poly- β -hydroxybutyrate (Garriques & Arevalo, 1995; Lunestad, 1998; Zhang *et al.*, 2009). The use of probiotics to control diseases in aquaculture is an interesting area, particularly in light of the increasing concern about the development of bacterial resistance with increasing use of chemical methods such as antibiotics and/or disinfectants (Hjelm *et al.*, 2004 b ; Kapareiko *et al.*, 2011; Lim *et al.*, 2011).

1.3.3 Probiotic Bacteria

The aquaculture industry is increasingly interested in the control or reduction of antimicrobial use. Therefore, alternative strategies should be developed to preserve a healthy microbial environment in larval hatcheries. In aquaculture industries the use of probiotic bacteria has become an accepted alternative method to control the spread of disease (Gomez-Gil *et al.*, 2000). This has resulted in a greater amount of research in order to achieve substantial progress in our understanding and ability to distinguish specific probiotic organisms, in addition to efforts to prove their reputed health benefits (Kapareiko *et al.*, 2011; Senok *et al.*, 2005). It is important to remember that different probiotic strains provide different health benefits (Senok *et al.*, 2005).

1.3.3.1 Definition

Fuller (1989) defines a probiotic “as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 1989, p. 366). This definition emphasizes the importance of living microbes and of the application of the probiotic to the host as a feed supplement (Irianto & Austin, 2002). (Gram *et al.* 1999, p. 972) proposed that a probiotic is ‘a live microbial supplement, which beneficially affects the host animal by improving its microbial balance’. This is a wider definition that does not restrict probiotics to application in food. Whole microorganisms are usually used as probiotics, but parts of microbial cells have also been observed to be capable of improving the hosts’ health. However, metabolites are excluded as part of the definition, and antibiotics are thus excluded. A proposed definition by Salminen *et al.* (1999) is based on several factors that include mechanisms of action, viability and non-viability, selection criteria, and scientifically documented health effects. They define probiotics “as viable microbial cultures that influence the health of the host by balancing the intestinal microflora and thus preventing and correcting the microbial dysfunctions”. Verschuere *et al.* (2000 a, p. 657) defined probiotic bacteria as “a live microbial adjunct which has a beneficial effect on the host by modifying

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the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". The spectrum of probiotics studied for use in aquaculture has comprised both Gram-positive and Gram-negative bacteria, yeasts, bacteriophages and unicellular algae (Irianto & Austin, 2002). When considering probiotics for marine usage it is important to examine the differences from terrestrial environments. There is a closer relationship between marine animals and their external environment than terrestrial animals and their environment. Potential pathogens are capable of surviving in the water column and may spread independent of the host animal (Hansen & Olafsen, 1999; Verschuere *et al.*, 2000 a). Marine animals are continuously in contact with pathogenic agents through the processes of feeding and drinking (Kesarcodi-Watson *et al.*, 2008).

1.3.3.2 Modes of action

Bacteria are closely linked with all life stages of marine organisms (Bergh, 2000) and marine plants and animals are constantly at risk from a wide variety of pathogenic microorganisms. It seems reasonable then to assume that potential hosts might produce bioactive compounds to prevent microbial attack (Engel *et al.*, 2002). Proksch *et al.* (2003) suggested that microorganisms living in their invertebrate hosts could be the real producers of secondary metabolites often ascribed to the host itself. In order to design protocols to select potential probiotics it is important to understand the mechanisms by which probiotic organisms compete either with pathogens or with other microbes, including other probiotics (Vine *et al.*, 2006). Probiotic isolates have successfully inhibited potential pathogens both *in vivo* and *in vitro* by a number of different mechanisms (Balcázar *et al.*, 2006; Jayaprakash *et al.*, 2006). These mechanisms include production of antibacterial and growth-inhibiting compounds, inhibition or suppression of virulence mechanisms, competition with pathogens for adhesion sites, enhancement of water quality, and improvement of host immune response and digestive enzymes (Balcázar *et al.*, 2006; Irianto & Austin, 2002; Kesarcodi-Watson *et al.*, 2008; Tinh *et al.*, 2008; Verschuere *et al.*, 2000 a; Vine *et al.*, 2006). Additionally, bacteria that are able to improve water quality by mineralizing organic matter or by removing toxic inorganic nitrogen are also considered probiotics. Probiotic bacteria could inhibit virulence gene expression, for example by obstructing quorum sensing (Defoirdt *et al.*, 2007; Zhou *et al.*, 2009).

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Antagonisms

The ability of microorganisms to survive in natural environments is related to the ability to produce toxic compounds to deter other organisms, and the capacity to resist the effects of such toxic compounds (Del Sorbo *et al.*, 2000). Some microorganisms present in marine environments inhibit growth of other microorganisms by producing secondary metabolites that have a bactericidal or bacteriostatic effect, such as bacteriocins, bacteriolytic enzymes, toxins, pigments, pheromones, pesticides, siderophores, antitumor agents and antibiotics (Bryers & Characklis, 1982; Burgess *et al.*, 1999; Pandey *et al.*, 2010; Pandey *et al.*, 2011; Verschuere *et al.*, 2000 a). Antagonism between microorganisms is a natural phenomenon by which pathogens in the aquaculture environment may be killed or reduced in number. This phenomenon is called biological control, or biocontrol (Maeda *et al.*, 1997). Bacterial strains have been known to produce a diversity of antibacterial compounds. For example, a *Roseobacter* sp. Strain (BS107) secretes an antimicrobial compound that actively inhibited *Vibrio anguillarum* growth. The antimicrobial activity was shown to be highest after 48 h of incubation of *V. anguillarum* pathogen in the BS107 supernatant. *In vivo*, filtered extracts of the BS107 strain dramatically improved the survival of scallop larvae (Ruiz-Ponte *et al.* 1999). Many authors have suggested that marine bacteria of the *Roseobacter* clade be used as a probiotic treatment in aquaculture (Hjelm *et al.*, 2004 a; Planas *et al.*, 2006; Ruiz-Ponte *et al.*, 1998; Ruiz-Ponte *et al.*, 1999) because these bacteria may produce a variety of antibacterial compounds (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005 b; Buchan *et al.*, 2005). One such strain is *Roseobacter* strain 27-4, which has been shown to improve the survival rates of turbot larvae infected with *Vibrio anguillarum*, and also inhibits fish pathogenic bacteria via a sulphur-containing antimicrobial substance (Bruhn *et al.*, 2005 b; Hjelm *et al.*, 2004 b ; Planas *et al.*, 2006).

Aeromonas media strain A199 produces a bacteriocin-like antimicrobial compound (BLIS), which, *in vitro*, displays antagonistic activity inhibiting a widerange of fish and shellfish pathogens. For instance, this strain successfully protected the Pacific oyster (*Crassostrea gigas*) larvae from pathogenicity of *Vibrio tubiashii* and inhibited growth of *Saprolegnia* species (Gibson *et al.*, 1998b; Lategan & Gibson, 2003). The active substance was later identified by Lategan *et al.* (2006) as an indole (2,3-benzopyrrole). This compound demonstrated a wide range of antifungal and antibacterial activity. Furthermore, growth of *Vibrio alginolyticus* has been inhibited *in vitro* using extracellular products from

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Lactobacillus brevis. For example, a 10^8 bacteria/ml concentration of inclusive culture of the same species was able to control heavy growth of *V. alginolyticus* in *Artemia* culture water (Villamil *et al.*, 2003).

Ajitha *et al.* (2004) demonstrated that free cell extracts from lactic acid bacteria strains such as *Lactobacillus acidophilus*, *Streptococcus cremoris*, *Lactobacillus bulgaricus*-56, and *Lactobacillus bulgaricus*-57 inhibited growth of *Vibrio alginolyticus* in culture media. In addition, the sea water bacterium, *Micrococcus* MCCB 104, isolated from a hatchery water tank, showed extracellular antagonistic activity that inhibited *Vibrio alginolyticus*, *V. vulnificus*, *V. parahaemolyticus*, *Vibriofluviallis*, *Vibrio proteolyticus*, *Vibrio nereis*, *V. cholerae*, *Vibrio mediterranei* and *Aeromonas* species associated with *Macrobrachium rosenbergii* larval breeding tanks. This isolate also inhibited the growth of *V. alginolyticus* through co-culture (Jayaprakash *et al.*, 2006).

Competition

Competition between microorganisms for nutrients and space in the aquatic milieu is a strong selective criteria which has led to the development of a diversity of effective strategies involving inoculation of specific beneficial microbes into the ecosystem (Burgess *et al.*, 1999). Different bacterial species use different mechanisms to outcompete or dominate other organisms for the same pool of resources (Hibbing *et al.*, 2010).

Competition is a phenomenon where the species was established to reduce or inhibit the bacterial pathogens colonization by competing for nutrients, attachment sites on the mucous membrane, or by producing inhibitory substances including antimicrobial compounds which prevent growth or destroy putative pathogens (Geovanny *et al.*, 2007; Nair, 2005; Patterson & Bolis, 1997; Vine *et al.*, 2006). For example, competition for availability of energy or nutrients may play a role in establishing the microbial structure of the gut flora or culture water of marine organisms (Tinh *et al.*, 2008).

Competition for attachment sites can serve as the first defence barrier against invasion by bacterial pathogens (Vine *et al.*, 2006). Microbes use a number of mechanisms to assist in attachment or adhesion to intestinal epithelial cells, including specific structural lipoteichoic acids, hydrophobic and steric forces, electrostatic interaction, passive forces, and production

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of inhibitory substances (Geovanny *et al.*, 2007). The most well known mechanism is siderophores, which are iron-complex chemical compounds produced by microorganisms (Braun & Braun, 2002). Gatesoupe (1997) found that *Pseudomonas fluorescens* strain AH2, which produces siderophores, has been effectively used as a biological control factor against the fish pathogen *Vibrio anguillarum*. Furthermore, *Vibrio E* secreted siderophores that increased the turbot larvae resistance against *Vibrio splendidus* pathogen, and enhanced larval growth. It also reduced the mortality of rainbow trout (*Oncorhynchus mykiss*) that had been infected with *Vibrio anguillarum* *in vitro*. The authors show the relationship between the production of siderophores and the mode of action of *P. fluorescens* and suggested that competition for free iron is involved in the protective activity (Gram *et al.*, 1999).

The bacterial strain *Bacillus subtilis* UTM 126 exhibits antibacterial activity against several pathogenic species of *Vibrio*, such as *V. parahaemolyticus*, *V. harveyi* and *V. alginolyticus*, (Balcázar & Rojas-Luna, 2007).

Suppression of Virulence

Quorum Sensing (QS)

Bacteria species communicate with each other using chemical signalling molecules called auto inducers (Schauder & Bassler, 2001; Waters & Bassler, 2005). quorum sensing (QS) is a mechanism of bacteria-to-bacteria communication by which bacteria establish specific gene expression in response to the presence or absence of small signalling molecules (Nazzaro *et al.*, 2013; Schauder & Bassler, 2001; Zhu & Sun, 2008).

Bacterial behaviour regulated by QS is involved in a wide spectrum of host-associated phenotypes, including antibiotic biosynthesis, the production of virulence determinants in animal, plant, human, and fish pathogens, symbiosis, swarming, bioluminescence competence and sporulation, secondary metabolism, plasmid transfer, development of fruiting bodies and biofilm formation (Beck von Bodman & Farrand, 1995; Bosgelmez-Tinaz *et al.*, 2007; J. Bruhn *et al.*, 2005 a; Hardman *et al.*, 1998; Hentzer *et al.*, 2002 ; Lewenza *et al.*, 1999; Ni *et al.*, 2009; Schauder & Bassler, 2001; Williams *et al.*, 2000). In addition biofilm formation is considered a pathogenicity trait modulated by quorum sensing (Costerton *et al.*, 1999). Several pathogenic microorganisms of plants and animals are capable of forming a biofilm in certain environments (Choi & Kim, 2009).

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Different bacterial species might use different pathways and autoinducers. Gram-positive and Gram-negative bacteria use at least six quorum-sensing pathways, as shown in Table 1.1. Among Gram-negative bacteria the cognate signal molecules: *N*-acyl-homoserine lactones (AHLs) and LuxR-LuxI homologous system represented a pattern on most well studied quorum-sensing systems (Eberl, 1999; Hentzer *et al.*, 2002; Swem *et al.*, 2009; Waters *et al.*, 2010).

Table 1.1. Different quorum sensing pathways in Gram-positive and Gram-negative bacteria.

Pathway	Signal molecules	Bacteria	References
AHL (A1-1) pathway	Various AHLs	Gram-negative	(Salmond <i>et al.</i> , 1995; Zvilgelsky & Manukhov, 2001)
4Qs pathway	PQS and AHLs	Gram-negative	(Diggle <i>et al.</i> , 2006)
AI-3 pathway	AI-3 (unknown structure)	Gram-negative	(Kendall <i>et al.</i> , 2007; Sperandio <i>et al.</i> , 2003)
AI-2 pathway	Two different forms	Gram-negative and Gram-positive	(Chen <i>et al.</i> , 2002; Surette <i>et al.</i> , 1999)
AIP pathway	Various oligopeptides	Gram-positive	(MDowell <i>et al.</i> , 2001)
CAI-1	Hydroxyketons	Gram-negative (<i>V. cholera</i>)	(Henke & Bassler, 2004; Higgins <i>et al.</i> , 2007)

Adapted from (Ni *et al.*, 2009).

Organisms' immune response to pathogenic bacterial invasion may be controlled by cell-cell communication AHLs QS which might effect host protein expression and broader sensing (Chhabra *et al.*, 2003; Mathesius *et al.*, 2003; Telford *et al.*, 1998).

AHLs are produced by a large number of Gram-negative bacterial species belonging to the alpha, beta, and gamma subclasses of proteobacteria, including strains of *Aeromonas salmonicida*, *Yersinia ruckeri*, *Aeromonas hydrophila*, *Vibrio vulnificus*, *Vibrio salmonicida*, *Burkholderia*, *Agrobacterium*, *Chromobacterium*, *Citrobacter*, *Enterobacter*, *Pseudomonas*, *Erwinia*, *Nitrosomonas*, *Hafnia*, *Obesumbacterium*, *Pantoea*, *Rahnella*, *Ralstonia*, *Rhizobium*, *Rhodobacter*, *Serratia*, and *Xenorhabdus* (Bruhn *et al.*, 2005 a; Eberl,

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1999; Freeman & Bassler, 1999; Rasch *et al.*, 2004). The first description of the quorum sensing processing was in a bioluminescent marine bacterium *Vibrio fischeri* (Nealson & Hastings, 1979). Using quorum sensing to regulate population density behaviour makes good biological sense for pathogens, allowing them to keep a low profile of virulence gene expression until they reach an adequate number required for effective attack on the host. Quorum sensing may well make sense in swapping from the appropriate physiological behaviour of the free-living status to behaviour corresponding to cells in a biofilm or a colony (Bauer & Robinson, 2002).

Quorum sensing inhibition (QSI)

Quorum-sensing mechanisms have a significant role in influencing settlement and biofilm formation. However, it is unclear to what extent quorum sensing molecules effect antibacterial production (Bowman, 2007; Dobretsov *et al.*, 2007). It is reasonable that inhibitors of bacterial quorum sensing might have pharmacological applications as bacterial quorum sensing is implicated in various pathologically relevant events. Firstly, quorum sensing assists in organizing bacterial community behaviour, nonetheless it is not critical for microbial survival. However, quorum sensing inhibitors can aid in the attenuation of biofilm formation, reduce virulence and increase bacterial sensitivity to antimicrobial treatment. However, they can only be useful as adjuvants in complementing other inhibitory mechanisms, since they do not display bacteriostatic or bactericidal activity (Ni *et al.*, 2009). *Vibrio anguillarum* is a Gram-negative human, fish and plant pathogenic bacterium, which regulates the expression of virulence factors by producing quorum sensing signal molecules called AHLs. Production of these molecules may be prohibited using specific quorum-sensing inhibitors (Rasch *et al.*, 2004). Some quorum sensing inhibitors may specifically block the AHL-regulated systems, and thus the expression of virulence factors, at concentrations where growth of the bacteria is not affected. An example of such inhibitors are the halogenated furanones from the red algae *Delisea pulchra* (Givskov *et al.*, 1996) which specifically block expression of virulence factors in *Erwinia carotovora*, *Vibrio harveyi* and *Pseudomonas aeruginosa* (Hentzer *et al.*, 2003; Manefield *et al.*, 2000). These compounds have therefore been suggested as a new treatment for controlling bacterial disease. The halogenated furanone C30 was added to the water during a trout co-habitant challenge experiment with *V. anguillarum* and was reported to substantially reduce mortality in comparison with untreated or negative controls (Rasch *et al.*, 2004).

Immunostimulation

Crustaceans, similar to other invertebrates, do not have specific immunity but display innate-wide-spectrum protection mechanisms including encapsulation, phagocytosis, and a number of antimicrobial factors circulating in plasma (Bachère, 2000; Bachère *et al.*, 1995; Roch, 1999; Smith & Chisholm, 1992). As an alternative, crustaceans fight pathogenic microbes through the innate immune system, which is a composite of humoral and cellular immune responses. The humoral response comprises production of antimicrobial peptides (AMPs) (Söderhäll & Cerenius, 1998). Stimulation of host defences using vaccination can enhance infectious disease resistance by activating acquired immune responses, in addition to improving innate immune systems by immunostimulation. Vaccines comprise living and dead bacteria, glucans, peptidoglycans, and lipopolysaccharides (Sakai, 1999; Smith *et al.*, 2003). When mortality is often high due to opportunistic pathogens the prophylactic use of probiotics and immunostimulants has many advantages as they can be applied during larval and early fry stages (Gildberg *et al.*, 1995). For example shrimp do not have antibodies and the immune response of shellfish is broadly non-specific. In spite of this fact there is some proof for limited specificity (Browdy, 1998). Stimulation of the non-specific immune system may enhance the animal's response to challenges from pathogenic bacteria. The use of immunostimulants to control luminescent vibriosis in shrimp is mentioned in several reports, where it is shown that different immunostimulants significantly increased survival when shrimp were infected experimentally with luminescent *Vibrio* spp. (Alabi *et al.*, 1999; Marques *et al.*, 2006; Thanardkit *et al.*, 2002).

Daniels *et al.* (2006) studied the effect of different concentrations of mannan oligosaccharide in the diet on growth and survival of *H. gammarus*. The results showed that the larvae fed with the supplemented diets had higher survival rates to stage IV in comparison to larvae fed with the control diet. The intensive culture of Atlantic halibut, *Hippoglossus hippoglossus*, as well as success of many other marine species, is hard to predict as a result of suboptimal growth and poor resistance to disease of juveniles and larvae (Bergh *et al.*, 2001). Opportunistic pathogenic bacteria and down-regulation of the non-specific immune response due to stress in larvae in industrial culture conditions are problems. Therefore, stimulation of the non-specific immune response to deal with microbial problems in juvenile production is attractive (Vadstein, 1997).

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Probiotic species

Probiotics are microorganisms or their products, which give health benefits to the host. They are used in aquaculture to control the spread of disease, and to complement or in several cases provide an alternative to using antibacterial drugs (Irianto & Austin, 2002). Despite there being many examples of microorganisms being used for biocontrol of pathogens, until recently only a few commercial probiotics have been used in the culture of larvae of marine organisms (Zhang *et al.*, 2009). According to Vine *et al.* (2006), probiotics have rarely been tested on a commercial scale. A wide range of yeasts (*Debaryomyces*, *Saccharomyces* and *Phaffia*), microalgae (*Tetraselmis*), Gram-negative bacteria (*Aeromonas*, *Photobacterium*, *Pseudomonas*, *Alteromonas*, and *Vibrio*) and Gram-positive bacteria (*Bacillus*, *Enterococcus*, *Carnobacterium*, *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Micrococcus*, and *Weissella*) have been evaluated (Irianto & Austin, 2002). Several bacterial species, including *Bacillus* species and lactic acid bacteria, that comprise a major part of the microflora on skin, gills and intestinal tracts of shrimp, are used as probiotics against fish and shellfish pathogenic bacteria (Rengpipat *et al.*, 2000; Skjermo & Vadstein, 1999). *Lactobacillus* sp., the lactic acid producing bacteria, was one of the first probiotics discovered (Sahu *et al.*, 2008). In warm-blooded animals lactic acid bacteria has been successfully tested as a probiotic bacteria, and this bacteria has also been tested with shrimp to antagonize shrimp pathogens (Gatesoupe, 1999; Skjermo & Vadstein, 1999). *Bacillus* strains, including *Bacillus* strain S11 (BS11) and *Bacillus cereus* have also been shown to reduce mortality of black tiger shrimp *Penaeus monodon* exposed to *V. harveyi* (Ravi *et al.*, 2007; Rengpipat *et al.*, 2003).

Dang and Lovell (2000) reported that bacteria belonging to the *Roseobacter* subclass were ubiquitous and rapidly colonized marine habitats, and have shown strong antibacterial activity *in vitro* against marine pathogens such as *Vibrio* spp. (Hjelm *et al.*, 2004b). *Roseobacter* spp. have been implicated in improving the survival of prawn *Litopenaeus setiferus* and pathogen-challenged turbot (*Scophthalmus maximus*) larvae (Balcázar *et al.*, 2007; Planas *et al.*, 2006). Fjellheim *et al.* (2007) and Hjelm *et al.* (2004 a) documented that many *Vibrio* spp. isolated from the intestinal regions of turbot (*Scophthalmus maximus*) and Atlantic cod (*Gadus morhua*) in hatcheries were antagonistic to *Vibrio* pathogens. In

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particular *V. alginolyticus*, has been revealed to efficiently reduce mortality in Atlantic salmon exposed to *Aeromonas salmonicida* (Austin *et al.*, 1995). Vandenberghe *et al.* (1998) implicated this bacterium in enhancing host resistance to disease caused by *V. harveyi* in the larval stage of cultured *Penaeuschinesis*.

According to Gomez-Gil *et al.* (2000) *V. alginolyticus* is a commonly occurring bacterium which is considered a promising probiotic in shrimp hatcheries. Gatesoupe (1990) discovered *V. alginolyticus* in healthy rotifers and documented a positive relationship between the proportion of *V. alginolyticus* and the survival rate of turbot larvae in the rearing environment. Austin *et al.* (1995) showed that *V. alginolyticus* serotype 1 VIB235 was capable of conferring some degree of protection to shrimp against disease, as detailed in Table 1.2.

Table 1.2. Bacterial probiotics employed in the larval culture of the aquatic organism

Species of bacteria	Target organism	Reference
<i>V. alginolyticus</i>	Shrimp (<i>P. vannamei</i>)	(Garriques & Arevalo, 1995)
<i>T. utilis</i> (PM-4)	Shrimp (<i>P. monodon</i>)	(Maeda & Liao, 1992)
<i>V. Harvey</i> , <i>Pseudomonas</i> sp., <i>Nitrobacter</i> sp., <i>Nitromonas</i> sp. and <i>Bacillus</i> sp.	Shrimp (<i>P. Monodon</i> and <i>Penaeus penicillatus</i>)	(Anonymous, 1991)
<i>T. utilis</i> (PM-4)	Crab (<i>Portusustrituberculatus</i>)	(Nogami <i>et al.</i> , 1997; Nogami & Maeda, 1992)(Maeda & Liao, 1994)
<i>V. Pelagius</i>	Turbot (<i>Scophthalmus maximus</i>)	(Ringo & Vadstein, 1998)
<i>Bacillus toyoi</i> and <i>Bacillus</i> sp. spores	Turbot via rotifers (<i>Brachionus plicatilis</i>)	(Gatesoupe, 1989)(Gatesoupe, 1991)
Lactic bacteria	Turbot via rotifers	(Gatesoupe, 1990)
<i>Lactobacillus plantarum</i> and <i>L. helveticus</i>	Turbot via rotifers	(Gatesoupe, 1991)
<i>L. bulgarius</i> <i>Streptococcus lactis</i>	Turbot via <i>Artemia</i>	(Garcia-de-la-Banda <i>et al.</i> , 1992)
<i>Alteromonas</i> sp.	Oyster (<i>Crassostrea gigas</i>)	(Douillet & Langdon 1993, 1994)
<i>A. media</i>	Oyster	(Gibson <i>et al.</i> , 1998)
<i>Roseobacter</i> sp.(BS107)	Scallop (<i>Pecten maximus</i>)	(Ruiz-Ponte <i>et al.</i> , 1999)
<i>Vibrio</i> sp.	Chilean scallop (<i>Argopecten purpuratus</i>)	(Riquelme <i>et al.</i> , 1997)
<i>Enterococcus faecium</i> SF68	<i>Anguilla Anguilla</i>	(Chang & Liu, 2002)
<i>L. rhamnosus</i> ATCC53103	<i>Oncorhynchus mykiss</i>	(Nikoskelainen <i>et al.</i> , 2001)
<i>Micrococcus luteus</i> A1-6	<i>O. mykiss</i>	(Irianto & Austin, 2002)
<i>Carnobacterium</i> sp.	Hg4-03 <i>Hepialus gonggaensis</i> larvae	(Youping <i>et al.</i> , 2011) Citation
<i>Lactobacillus acidophilus</i>	<i>Clarias gariepinus</i>	(Al-Dohail <i>et al.</i> , 2011)

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<i>Bacillus</i> spp., <i>Enterococcus</i> sp. and <i>Lactobacillus</i> spp	<i>Farfantepenaeus brasiliensis</i>	(de Souza <i>et al.</i> , 2012)
<i>B. stbtilis</i> BT23	<i>Pemaeus monodon</i>	(Vaseeharan & Ramasamy, 2003)
<i>A. media</i> , strain A199,	<i>Crassostrea gigas</i>	(Gibson <i>et al.</i> , 1998)
<i>Pseudomonas fluorescens</i> strain AH2	Fish	(Gram <i>et al.</i> , 1999)
<i>Saccharomyces cerevisiae</i>	<i>Penaeus lannamei</i>	(Scholz <i>et al.</i> , 1999)
<i>Vibrio alginolyticus</i>	Atlantic salmon	(Austin <i>et al.</i> , 1995)
<i>Lactococcus lactis</i>	<i>Epinephelus coioides</i>	(Sun <i>et al.</i> , 2012)
<i>Pseudoalteromonas</i> isolate PP107	Spiny lobster phyllosoma	(Goulden <i>et al.</i> , 2012)
<i>Vibrio</i> sp. isolate PP05	Spiny lobster phyllosoma	(Goulden <i>et al.</i> , 2012)

Parts of this table were adopted from Gomez-Gil *et al.* (2000).

1.3.4 *In vitro* screening

The first step in probiotic screening is to establish a collection of candidate probiotics. The most widespread method to screen for probiotic bacteria is through *in vitro* antagonism assays, in which known pathogens are exposed to the probiotic strains, or their extracellular secretions, in liquid or solid medium (Figure 1.3)(Balcázar *et al.*, 2006; Geovanny, *et al.*, 2007). Recently, several methods of *in vitro* screening for inhibitory compounds have been described. These methods include the well diffusion method, the double layer method, the disc diffusion method, the cross streaking method and the co culture method (Kesarodi-Watson *et al.*, 2008). These assays are based on the principle that the probiotic (the producer) secretes extracellular compounds that inhibit the other bacterial strain (the indicator). This inhibitory activity is indicated by inhibition of the indicator growth on agar medium (Kesarodi-Watson *et al.*, 2008).

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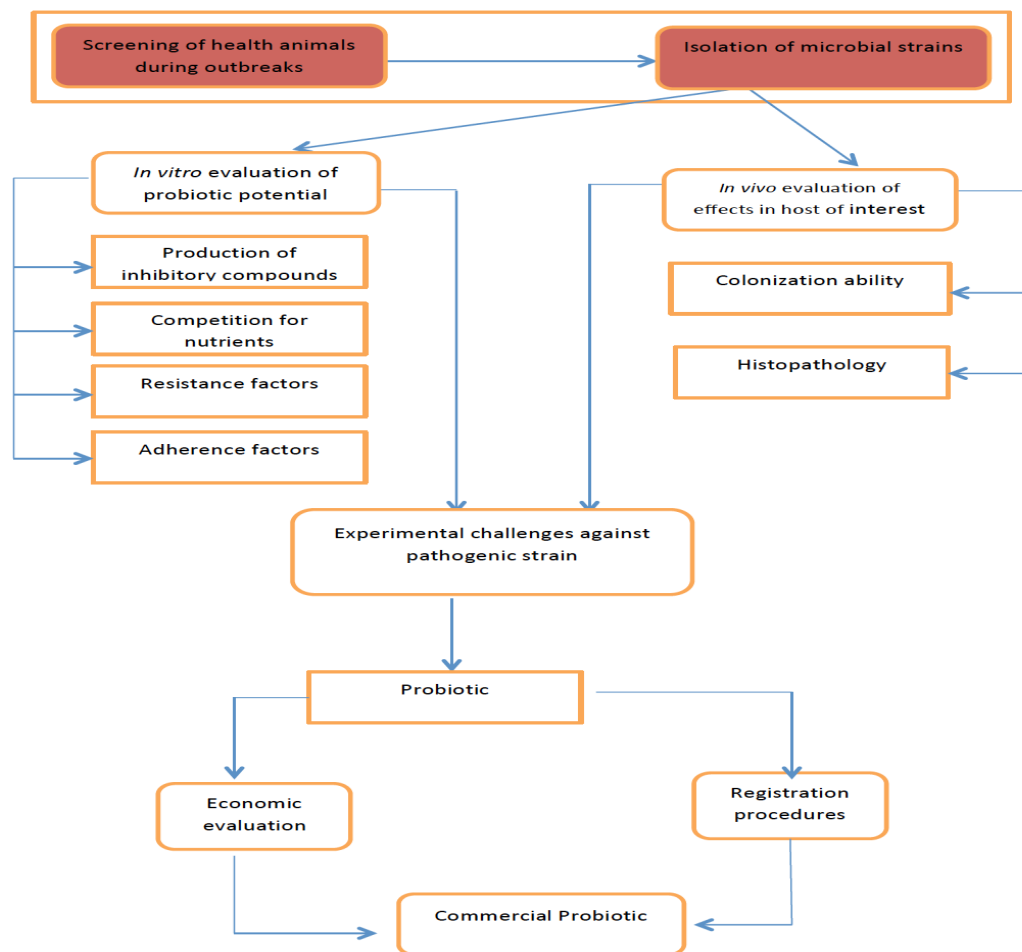


Figure 1.3. Scheme for selection of bacteria as biocontrol agents in aquaculture (Balcázar *et al.*, 2006).

In general, selection of probiotic candidates has usually been a multifaceted process supported by limited scientific evidence (Gomez-Gil *et al.*, 2000). The selection of probiotics is mostly determined by biosafety considerations, method of administration of the probiotic, the methods of production and processing, and the site in the host body where the probiotics are likely to be active (Veld *et al.*, 1994). Methods to select probiotic microorganisms for use in the larval culture of marine animals are multifaceted process involving (1) collection of background information, (2) acquisition of putative probiotics (PP), (3) assessment of the pathogenicity of the PP, (4) evaluation of the effect of the PP in larvae, (5) evaluation of the ability of the PP to out-compete pathogenic strains, and (6) an economic cost benefit analysis (Gomez-Gil *et al.*, 2000; Verschuere *et al.*, 2000 a).

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1.4 Aims and objectives of this study

P. ornatus also known as the ornate spiny lobster is an aquaculture species with great commercial potential. A significant obstacle to the commercialisation of *P. ornatus* are bacterial diseases that spread during the larval stage with *V. owensii* DY05, isolated during an epizootic of aquaculture-reared ornate spiny lobster emerging as a significant pathogen (Cano-Gómez *et al.*, 2010). *V. owensii* DY05 has been demonstrated to be transmitted through live feed vectors (artemia) and to proliferate in the hepatopancreas (midgut gland) of phyllosoma (the larval stage) resulting in extensive tissue necrosis and ultimately major systemic infection (Goulden *et al.*, 2012). In order to identify bacteria from natural prey that were antagonistic to *V. owensii* DY05, Goulden *et al.* (2012) used a multi-tiered probiotic screening strategy. This study identified *Pseudoalteromonas* sp. PP107 and *Vibrio* sp. PP05 two antagonistic bacterial strains from 500 candidates based on their ability to inhibit attached and planktonic forms of pathogenic *V. owensii* DY05. Inoculation of artemia with a combination of *Pseudoalteromonas* sp. PP107 and *Vibrio* sp. PP05 was found to provide significant protection to *P. ornatus* phyllosomas against *V. owensii* DY05 infection *in vivo*.

The antagonistic mechanisms through which *Pseudoalteromonas* and *Vibrio* spp. inhibit *V. owensii* DY05 are unknown. Implicated in the suppression of aquatic vibrios are the production of broad-spectrum anionic proteins and non-proteinaceous antibiotics by *Pseudoalteromonas* spp. and aliphatic hydroxyl ethers and andrimid antibiotics by *Vibrio* spp. produce.

The aim of the present study was to further isolate potential probiotic species from marine sources and to study the molecular factors and mechanisms employed by such antagonistic bacteria to suppress the growth or infection by the lobster phyllosoma pathogen *V. owensii* DY05.

A collection of bacterial cultures isolated from coral tissues and shrimp haemolymph was screened to identify potential probiotic candidates antagonistic to the phyllosoma pathogen DY05. Many bacterial isolates from this collection suppressed the pathogen *in vitro*, however, three bacterial isolates were found to be promising candidates for potential use as probiotic bacteria in lobster aquaculture hatcheries. Studies of these isolates were carried out to investigate the mechanisms by which they inhibited the pathogen. They were further subjected to molecular analysis in order to study the genetic background and other traits related to pathogen suppression by the *Pseudoalteromonas* strains.