2.2 Introduction

Aquaculture is the farming of aquatic organisms and is the fastest growing food-producing sector in the world. However, the spread of diseases is increasing as aquaculture activities grows and expand (Bondad-Reantaso *et al.*, 2005). Rock lobsters of the family *Palinuridae* are a target aquaculture species and are the most valuable wild fisheries sector in Australia. The extended larval stage of rock lobster acerbates a high rate of phyllosomas mortalitycaused by insufficient nutrition and the spread of microbial diseases, caused particularly by opportunistic pathogens. This presents a significant challenge to the production of commercial scale quantities of post-larvae stage lobster (Bourne *et al.*, 2007; Bourne *et al.*, 2004; Jeffs, 2010). *Vibrio* spp. are abundant in the aquatic environment, and are well documented opportunistic pathogens that are often correlated with larval mortalities in aquatic hatcheries (Forward *et al.*, 2011; Shields, 2011). Several pathogenic *Vibrio* species are known to cause serious disease in invertebratessuch as lobsters, crabs, shrimp, and fish and may even affect humans (Shields, 2011). These species include *V. alginolyticus, V. harveyi, V. parahaemolyticus* and *V. anguillarum* (Abraham *et al.*, 1996; Bowser *et al.*, 1981; Brinkley *et al.*, 1976; Forward *et al.*, 2011). *V. owensii* DY05 was selected as a model pathogen in our study since it has been demonstrated to be a significant pathogen causing mid-gut gland infection and mass mortalities of cultured ornate spiny lobster *Panulirus ornatus*phyllosoma (Goulden, *et al.*, 2012).

Antibiotic prophylaxis is commonly used in aquaculture industries to protect against substantial losses to bacterial pathogens such as *V. owensii* DY05 (Goulden, 2012). There is an increasing interest in the use of probiotic in aquaculture to control diseases since application of antibiotics is causing concern over the possible development of antibiotic-resistant bacteria (Hjelm *et al.*, 2004 b). Probiotic bacteria thatare capable of reducing opportunistic pathogens can improve larval health of marine organisms (Hjelm *et al.*, 2004 b ; Kesarcodi-Watson *et al.*, 2008). Understanding the mechanisms of antagonism by probiotic bacteria against pathogens, and the proliferation and/or infection site of marine pathogens is important in the selection of probiotic strains for use in a commercial setting (Hjelm *et al.*, 2004b). Several bacterial species have previously been used as biocontrols in aquaculture hatcheries (Brinkhoff *et al.*, 2004). Examples include *Vibrio alginolyticus*, *V. harveyi*, *Pseudomonas* spp., *Nitrobacter* spp., *Lactobacillusspp*, *Alteromonas* spp., *Roseobacter* spp. and *Bacillus* spp. (Douillet & Langdon 1994; Gatesoupe, 1991; Gibson *et al.*, 1998b).

Many surface-attached bacteria produce antimicrobial substances that obstruct the growth, attachment or survival of competing microbes in order to achieve an advantage in highly competitive environments (Long & Azam, 2001). Antagonistic interactions between bacterial species represent an interesting evolutionary strategy, which confers advantages in competition for space and food in the natural environment (Lo Giudice *et al.*, 2007b), and an effective control of microorganisms residing the same ecological niche (Hentschel *et al.*, 2001). There are few reports investigating inter-species interactions among microbes of the same or related marine habitat, but there is growing evidence to suggest that antagonistic effects, expressed by phylogenetically different bacterial groups, are a widespread feature in marine environments (Bhattarai *et al.*, 2006; Brinkhoff *et al.*, 2004; Grossart *et al.*, 2004; Long & Azam, 2001; Nair & Simidu, 1987). Many antagonistic marine bacteria are known to stimulate or increase production of antimicrobial compounds in the presence of competitors, and play an important role in preventing attack by bacteria, including potential pathogens (Burgess *et al.*, 1999; Slattery *et al.*, 2001; Trischman *et al.*, 2004).

Antimicrobial activity has been extensively reported for extracts of various groups of marine organisms. Many organisms, such as invertebrates that are associated with coral reef ecosystems, have long been known as the sources of structurally unique natural products, particularly antimicrobial compounds. These organisms include soft corals, sponges, tunicates; shrimp, algae, bryozoans and zooplankton (Kumaran *et al.*, 2012; Long & Azam, 2001; Ritchie, 2006; Shnit-Orland & Kushmaro, 2009; Wiese *et al.*, 2008). For example, sponge-associated bacteria were described a rich source of bioactive metabolites, with more than 6000 metabolites having been recovered from aquatic environments (Dieckmann *et al.*, 2005; Hentschel *et al.*, 2001; Mangano *et al.*, 2009; Santos *et al.*, 2010). Corals are sessile eukaryotic organisms, which represent unexploited sources of diversity of microorganisms of economic importance, and a unique surface for microorganisms' colonization (Nithyanand *et al.*, 2011; Ritchie, 2006; Shnit-Orland & Kushmaro, 2009). Corals harbour complex and diverse microorganisms, including bacteria, archaea, zooxanthellae, and viruses. Coral mucus layers are richer in nutrients than sediments or seawater (Carlos *et al.*, 2013; Karna *et al.*, 2004; Rosenberg *et al.*, 2007; Rosenfeld & ZoBell, 1947; Shnit-Orland & Kushmaro, 2009). Shrimp represent a good source of antimicrobial producing bacteria, and many bacteria with antimicrobial activity have been isolated from shrimp (Austin *et al.*, 1995; Chandrasekaran &Ashok Kumar, 2011; Kumaran *et al.*, 2012). For example, bacteria on the surface of eggs of

the shrimp *Palaeman macrodactylus* produce a metabolite that inhibits fungal infections that would be lethal to the eggs (Gil-Turnes *et al.*, 1989).

The aim of the present work was identify potential disease suppressive bacteria from marine collections that may act as probiotic agents against the lobster pathogen *V.owensii* DY05. In addition, the phylogeny and bioactivity of DY05-supressive strains, demonstrating pronounced antibacterial activity, were investigated.

2.3 Materials and methods

2.3.1 Bacterial strains and culture conditions

A total of 499 marine bacterial isolates from culture collections at the laboratory of L Pereg (UNE, Australia) were used in this study. These isolates originated from the corals *Turbinaria mesenterina, T. redicalis, T. frondens and Acropora solitaryensis* (Godwin, 2007; Harris, 2004) and from males and females of the estuarine ghost shrimp *Trypaea australiensis* (Goulden, 2006).

Coral samples were originally collected in 2004 and 2005 from rocky reefs at depths of 10–20 m at the Solitary Island Marine Park (SIMP) by Godwin et al. (2012). Some isolates were from the seawater adjacent to the coral surface (SW). Coral sources were either healthy ones or Australian Subtropical White Syndrome (ASWS)-affected corals (Figure 2.1) (Dalton *et al*., 2010). Particularly, bacteria were isolated from the coral surface mucus (Figure 2.1) and tissues of healthy corals (H), healthy looking tissue on ASWS-affected corals (HL-ASWS), disease margin (M-ASWS) or diseased tissue on ASWS-affected corals (D-ASWS).

Estuarine ghost shrimps from Coffs Creek and Wooli-Wooli River, NSW, Australia, were either freshly caught or exposed to severe stress that apparently cause them bacteriosis (vibriosis) before bacteria were isolated from their extracted haemolymph (Figure 2.2) using *Vibrio*-selective TCBS agar by Lily Pereg and Evan Goulden (Goulden, 2006).

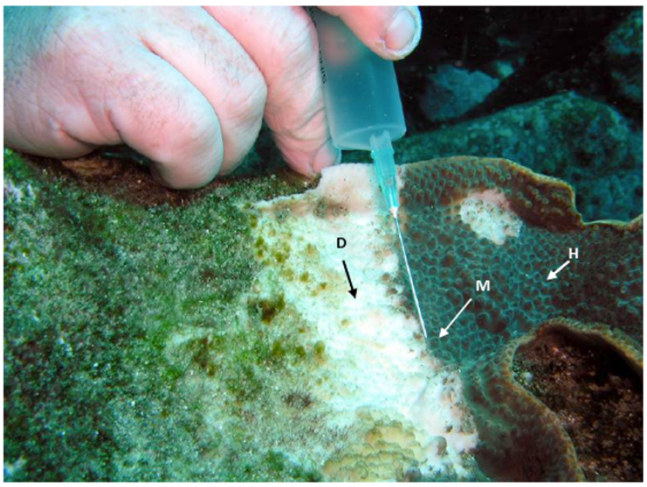

Figure 2.1. *T. mesenterina* colony displaying typical signs of ASWS. Arrows indicate the regions sampled for bacterial community analysis. Dark areas of the coral surface are covered in living tissue; white areas are recently exposed calcium carbonate skeleton. (H on photo) Apparently healthy tissue of disease colony (note – we designated it here as HL-ASWS), (M on photo) Margin of disease lesion (note – we designated it here as M-ASWS), (D on photo) Dead coral skeleton (note- we designated it here as D-ASWS). Healthy tissue from a nearby colony unaffected by disease was also collected (not visible in this photograph, we designated it here H). (Photograph by Steven Smith) (Godwin *et al.*, 2012).

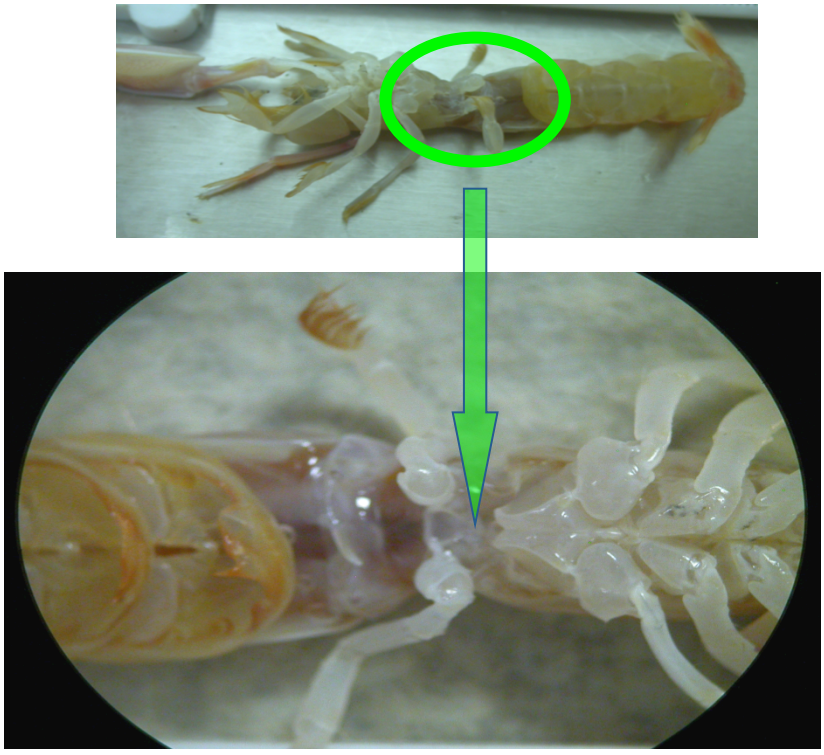

Figure 2.2. Extraction of Haemolymph from Ghost Shrimps. Extraction was performed using a syringe after surface cleaning with 70% ethanol (Photos by Evan Goulden and Lily Pereg).

The test pathogen *V. owensii* DY05 (Cano-Gómez *et al.*, 2010) and the broad spectrum *Vibrio*-suppressive bacterium *Phaeobacter* (formerly *Roseobacter*) strain 27-4 (Hjelm *et al.*, 2004 a) were obtained from Lone Hoj, the Australian Institute of Marine Science (AIMS).

All microorganisms were grown in marine broth 2216 (Difco) for cryopreservation at -80 ^{o}C in 25-50% (v/v) glycerol. Growth media were marine agar 2216 (MA, Difco) or LB10 (10 g/L Tryptone,(OXIOD) 5 g/L yeast extract (OXIOD), 10 g/L sodium chloride (Chem-supply) and 15 g/L of agar (Difco) was added to solidify the medium). Liquid cultures were in marine broth (MB, Becton) or LB10 at 28 ^{o}C, 150 rpm, unless otherwise stated.

2.3.2 In vitro antagonism assays

Two different assays were used as rapid qualitative methods to screen isolates for antimicrobial activities: a well-diffusion assay and a disk diffusion assay.

2.3.2.1 Well diffusion assay (WDA)

The isolates from both culture collections were screened for antagonistic activity against *V. owensii* DY05, a pathogen of the *P. ornatus* phyllosoma (Cano-Gómez *et al.*, 2010; Goulden, *et al.*, 2012), using well diffusion assay (WDA) which was performed as described by Goulden, *et al.* (2012). Briefly, an 18 hr culture of *V. owensii* DY05 was seeded (10 µL/mL) into molten (45 ^{o}C) minimal medium agar (MMA; 0.3% casamino acids; 0.4% glucose; 1% bacteriological agar in filtered seawater). We used 10 mL of the seeded medium per petri dish (85mm in diameter). Following solidification, wells (diameter 6 mm) were cut aseptically into the agar and loaded with 50 µL of dense tested isolate cultures, pre-grown for 18-24 hr period at 28 ^{o}C with 150 rpm shaking. Plates were incubated (28 ^{o}C) for 5 days and inspected every 24 hr for clearing zones signifying antagonistic activity against *V. owensii* DY05. The effect of temperature on the production of growth inhibition zone in well diffusion assays was initially examined at 25, 28 and 37 °C for the first 100 isolates. In all cases 28 °C gave the clearest and largest inhibition zone. Therefore we continued examining other isolates in this assay using this temperature.

The inoculum was sufficient to produce confluent growth on the assay plates and growth inhibition was assessed as the diameter of the zone of pathogen growth inhibition in mm (also called clearing zone).

Assays were performed at least in triplicate for each isolate using *Phaeobacter* sp. 27-4 (Hjelm *et al.*, 2004 a) and in some cases *Pseudoalteromonas* sp. S9.2.2 (an isolate found to inhibit pathogen growth early in this work) as positive antagonistic controls.

2.3.2.2 Disk diffusion assay

Disk preparation protocol was modified from Hayashida‑Soiza *et al.*(2008). The same 18-24 hr tested isolate cultures (see above) were used to produce cell-free supernatants by centrifugation at 14,000 rpm for 30 min at 4°C followed by filter sterilisation (FS; 0.2 µm Sartorius stedim (Minisart)). Sterile filter paper disks (6 mm diameter) were saturated with 50 µL bacterial-free culture supernatant. Paper disks treated with 50 µL of sterile medium were used as control. Disks were placed onto MMA seeded with pathogen as described above. Plates were incubated at 28 ^{o}C for 18-24 hr, monitored and inhibition zones measured as described above.

2.3.3 Identification of antagonistic isolates using 16S ribosomal DNA sequencing

Bacterial isolates were identified by 16S rRNA gene fragment sequence analysis. Genomic DNA was extracted using the freeze-boil method (Pereg-Gerk, 1997) from every bacterial strain, which demonstrated positive *in vitro* antagonistic activity. Briefly, isolates were revived from cryopreserved glycerol stocks by streaking onto MA plates and incubated at 28 ^{o}C for 18-24 hr. Bacterial subculture were grown on MA for 18 hr at 28 ^{o}C. A loop full of fresh colonies was suspended in 50-75 µL of elution buffer (Qiagen) and incubated at -70 ^{o}C for 30 min, then boiled for 2 min at 100 ^{o}C and centrifuged at 14,000 rpm for 5 min at room temperature. The supernatant containing genomic DNA was freshly used.

Preliminary experiments indicated that, while the freeze-boil method was suitable for extracting genomic DNA from most isolates, it was unsuitable for the extraction of genomic DNA from the genera *Pseudoalteromonas* and *Pseudovibrio* (data not shown). For these isolates, genomic DNA was extracted using DNeasy® blood and tissue kit (Qiagen-Hilden, Germany) according to the manufacturer's instructions, using the protocol recommended for Gram-negative bacteria.

2.3.4 PCR amplification of 16S rRNA gene fragment

PCR amplification, sequencing and phylogenetic analysis of 16S rRNA gene fragment from bacterial isolates were performed on genomic DNA extracts using the 16S rRNA gene universal primers 27F (5' GAGCTCAGAGTTTGATCMTGGCTCAG) and 1492R (5' CACGYTACCTTGTTACGACTT) (Valinsky *et al.*, 2002). 16S rRNA gene fragments were amplified from the genomic DNA extracts in PCR reaction mix composed of sterile Milli-Q water (12 µL), 10X PCR buffer (2.5 µL, Qiagen), 50 mM $MgCl_2$ (0.75 µL), 2 mM dNTP mix (2.5 µL), DMSO (2.5 µL), 10 pm of each primer (1.25 µL), Taq DNA polymerase (0.25 µL). PCR was performed using a thermal cycler (PTC-100^{TM}, MJ Research Inc.) using the following conditions: 10 min initial denaturation at 94°C, 35 cycles of 30 s at 94°C (denaturation), 30s at 48°C (annealing), 2 min at 72°C (extension); and final extension for 10 min at 72 °C.

PCR products (of expected size, 1.5 kb) were purified using QIAquick PCR Purification Kit (Qiagen-Hilden, Germany) eluted in 10 mM Tris/HCl (pH 8.0) and quantified on agarose gels against 1 kb standard DNA ladders (New England Biolabs) following the manufacturer's recommendations. Purified PCR products were sequenced by the Australian Genome Research Facility (AGRF, Sydney, Australia) using 8.8 to 43.8 ng of cleaned 16S rRNA gene product and 10 pmol of the forward primer 27F in a final volume of 12 µL.

2.3.5 Classification and identification

The nucleotide sequences were edited (ChromasLite v2.1) and submitted to the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) to determine nucleotide-nucleotide similarity to identify the nearest relative with sequences in the GenBank database. Sequences containing unread bases ('Ns') were trimmed from the 5' and 3' ends and misread bases were corrected where possible and submitted to GenBank database. Classification of isolates to genus level was achieved by submitting their 16SrRNA gene sequences to the RDP Classifier (Wang *et al.*, 2007)

2.4 Results

Potential probiotics bacteria showing various levels of antagonistic activity against the lobster phyllosoma pathogen *V.owensii* DY05 were identified from two marine culture collections, one from corals and the other from shrimp, originated from Northern NSW, Australia.
Bacterial isolates were tested over a range of growth temperatures. It was found that the optimal growth temperatures of all isolates were between 25 ^{o}C and 28 ^{o}C, and all isolates showed a decrease in or no growth at 37 ^{o}C. An isolate was considered suppressive if it showed any size of pathogen-clearing zone.

Out of a total of 499 bacterial isolates tested (400 from corals and 99 from shrimps) 111 isolates (80/400 (20%) of the corals isolates and 31/99 (31.3%) of the shrimp isolates) produced clearance zones, free of *V.owensii* DY05 pathogen growth, using the well-diffusion assay. However, only 46 of these strains showed clearance zones using the disk-diffusion assay. Moreover, the clearance zones using the well-diffusion assay were wider than those obtained in the disk-diffusion assay. Well-diffusion assays produced pronounced inhibition zones of 10-23 mm in diameter after incubation periods of 6 – 24 hr, whereas, clearing zones in disk-diffusion assays were under 10 mm in diameter. Therefore, the well-diffusion assay was selected for further bacterial antagonism test in this project. Examples of the results of both methods are shown in Figure 2.3. Isolates producing clearing zones are summarized in Table 2.3 for coral isolates and Table 2.4 for shrimp isolates.

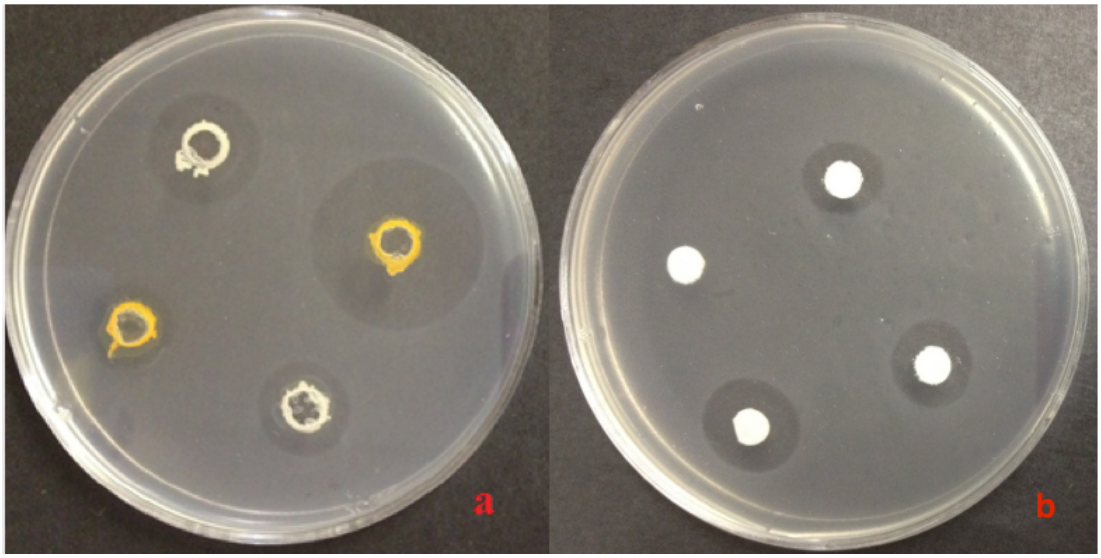

Figure 2.3. Typical *V.owensii* DY05 pathogen-suppression tests: well diffusion assay (a) disk diffusion assay (b). Clearing zones around the wells or the disks indicate that the tested bacterial isolate shows antagonistic activity against the pathogen *V. owensii* DY05.

Antagonistic activity was defined as weak (≤10 mm), moderate (11-20 mm) or strong (≥21 mm) according to the size in diameter of the inhibition zones (Goulden, *et al.*, 2012). The majority of the coral and shrimp antagonistic isolates showed weak activity (58/80 (72.5%), and 20/31 (64.5%) respectively). Other coral antagonistic isolates produced moderate (12/80 (15%)) or strong inhibition zone (10/80 (12.5%)). The reminder of shrimp antagonistic isolates showed moderate activity (10/31 (32.3 %)) and a single strain produced a strong inhibition zone (3.2 %). These results are summarised in Figure 2.4.

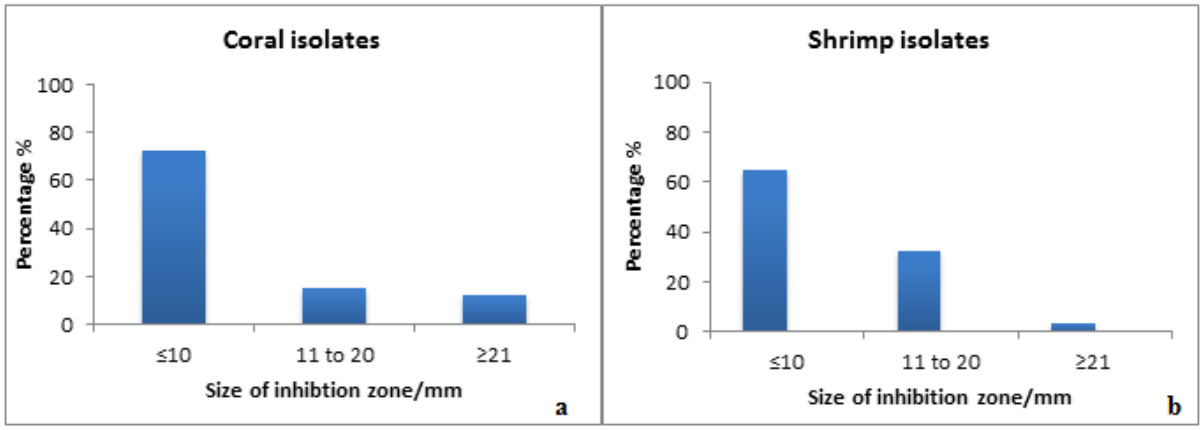

Figure 2.4. Percentage of bacterial isolates showing weak, moderate or strong antagonistic activity against *V. owensii* DY05 in a well diffusion assay. (a) Coral isolates, (b) Shrimp isolates.

2.4.1 **Coral isolates showing antagonistic activity**

Out of the 400 coral isolates from *Turbinaria* and *Acropora*spp., 125 were from healthy coral tissue (H), 57 were from healthy-looking tissue on ASWS-affected corals (HL-ASWS), 89 were from disease margin (M-ASWS) and 129 were from diseased tissue (D-ASWS). In total, 80/400 (20%) DY05-suppressive strains were isolated, with 19/125 (15.2%) from H, 18/57 (31.6%) from HL-ASWS, 20/89 (22.5%) from M-ASWS, and 23/129 (17.9%) from D-ASWS (Figure 2.5).

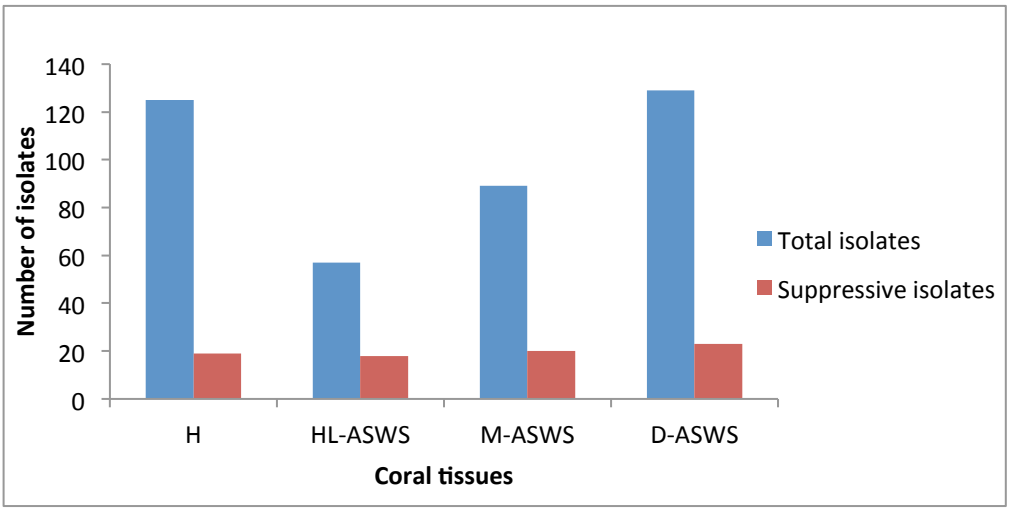

Figure 2.5. Distribution of *V. owensii* DY05-antagonistic isolates originated from coral species. Information is provided on the source of the samples i.e. whether they were obtained from a healthy coral (H), healthy looking tissue on ASWS-affected coral (HL-ASWS), diseased tissue on ASWS-affected coral (D-ASWS).

2.4.1.1 **Shrimp isolates showing antagonistic activity**

Out of the 99 isolates sourced from shrimps of the species *Trypaea australiensis* and their surrounding seawater, 34 were from severely stressed males, 3 from freshly collected males, 42 from severely stressed females and 20 from the surrounding seawater.

All pathogen-antagonistic bacteria identified in this work were from the haemolymph of stressed shrimps (Figure 2.6): 13/34 (38.2%) were from severely stressed male shrimps and 18/42 (42.9%) were from severely stressed females. There were no antagonistic isolates found from freshly collected shrimps (male or females or seawater).

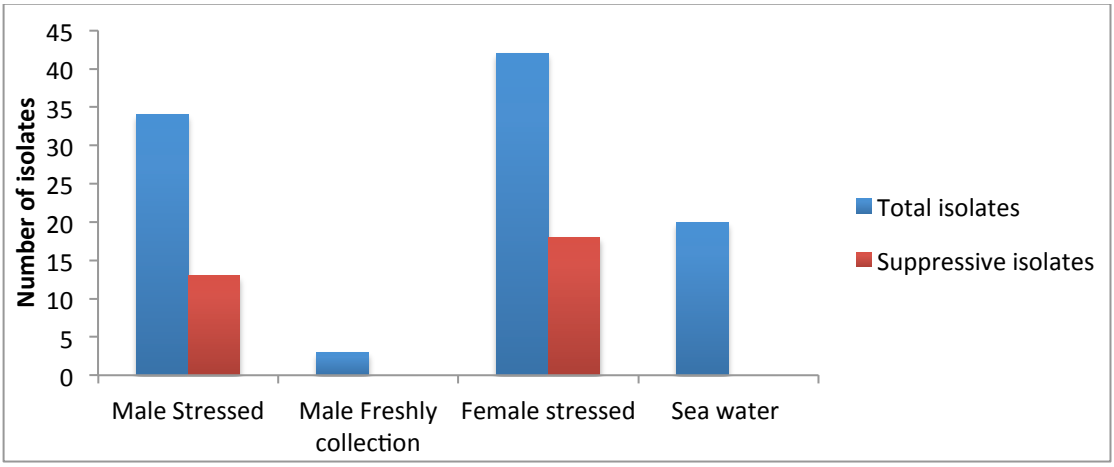

Figure 2.6. Distribution of *V. owensii* DY05-antagonistic isolates originated from shrimp *Trypaea australiensis*. Isolates originated from male and female shrimps that were either stressed,freshly collected or from the surrounding seawater.

2.4.2 Identification of strains

2.4.2.1 Coral isolates

The identity of coral isolates suppressive to *V. owensii* DY05 was determined by sequencing and analysis of the 16S rRNA gene fragment (submitted to BLAST database). RDP classifier was used to classify the bacteria to the genus level and the BLAST to identify species or the closet relative. Antagonistic isolates belonging to 4 classes and 9 different genera were identified, as shown in Figure 2.7.

Gram-negative bacteria comprised 90% of the antagonistic isolates and Gram-positive bacteria 10%. The majority of the isolates were identified as belonging to the classes Gammaproteobacteria within Gram-negative bacteria ((71/80) 88.8% of total antagonistic strains), while other well-represented classes included Bacilli ((5/80) 6.3%), Actinobacteria ((3/80) 3.8%) and single isolate from the class Alphaproteobacteria ((1/80) 1.3%). Within the Gammaproteobacteria, the genus *Vibrio* was the most prevalent, accounting for 50/71 (69.4%) of the isolates. Isolates belonging to genus *Psychrobacter* were also well represented, accounting for 19/72 (26.4%) of the isolates. They were isolated from all different parts of coral tissue similarly to the genus *Vibrio* as summarized in Table 2.1. A single isolate of each of *Photobacterium, Acinitobacter*, and *Pseudovibrio* was identified from bacterial collection from healthy coral.

Within the Gram-positive bacteria the genus *Staphylococcus* was the most predominant, accounting for 4/8 (50%) followed by genus *Micrococcus* 25%. Genus*Bacillus* and *Brachybacterium* represented 12.5 each.

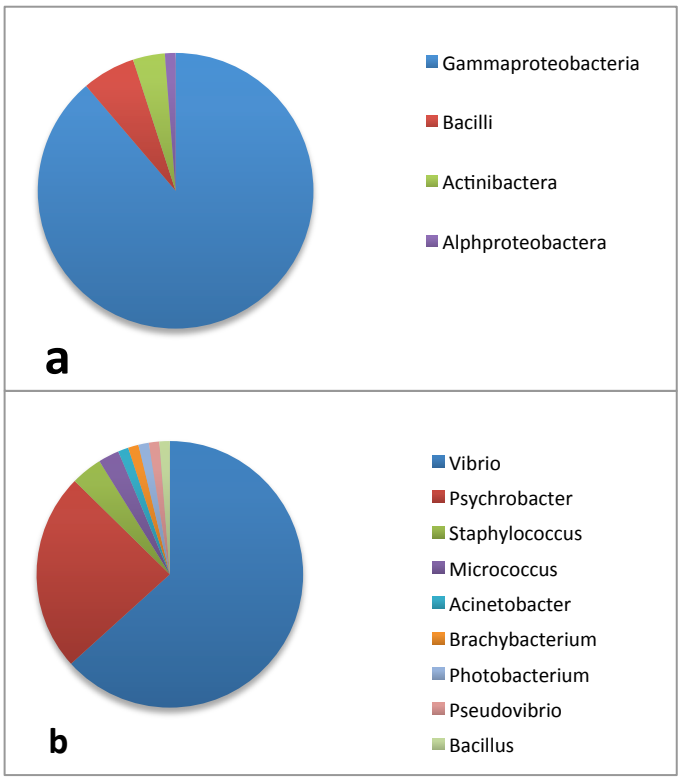

Figure 2.7. Coral Isolates having antagonistic activity against *V. owensii* DOY5 were identified as belonging to 4 major phylogenetic classes (a) and 9 main genera (b).

Table 2.1. The distribution of antagonistic isolates from coral collection identified on the bases of 16S rRNA gene fragment sequencing.

Identification based on 16S rRNA gene sequence	Number of isolates from H	Number of isolates from HL-ASWS	Number of isolates from D-ASWS	Number of isolates from M-ASWS	Total isolates	Percentage of the total isolates
Vibrio spp.	11	8	13	18	50	62.5
Psychrobacter spp.	2	8	7	2	19	23.75
Staphylococcus spp.	3	0	0	1	4	5.0
Micrococcus spp.	0	1	1	0	2	2.5
Bacillus sp.	0	0	1	0	1	1.25
Acinetobacter sp.	1	0	0	0	1	1.25
Photobacterium sp.	1	0	0	0	1	1.25
Pseudovibrio sp.	1	0	0	0	1	1.25
Brachybacterium sp.	0	0	1	0	1	1.25
Total	19	17	23	21	80	

2.4.2.2 Shrimp isolates

The shrimp antagonistic isolates, identified by 16S rRNA gene fragment sequncing, belonged to 3 classes and 6 genera (Figure 2.8). The antagonistic isolates included both Gram-negative and Gram-positive bacteria.

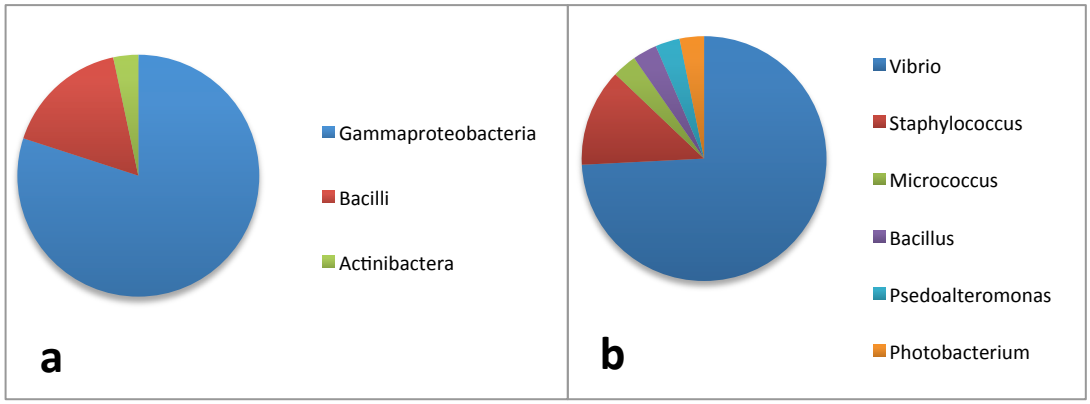

Figure 2.8. Shrimp isolates showing antagonistic activity against *V. owensii* DY05 belong to six genera (b) in three classes (a).

The majority of shrimp bacterialisolates were identified as belonging to the classes Gammaproteobacteria (25/31, 80.7% of a total antagonistic strains), and others were Bacilli (5/31, 16.1%) and Actinibacteria (1/31, 3.2%). Within the Gammaproteobacteria class, on the gene level, the genus *Vibrio* was the most prevalent, accounting 23/25 (92%) of the isolates that were isolated from stressed males and females. One isolate was found belongto the genus *Pseudoalteromonas*, representing 3.2% of the antagonistic isolates. Within the Gram-positive bacteria the genus *Staphylococcus* was the most predominant, accounting for 4/31 isolates (12.9%). Other Gram-positive bacterial isolates belong to the genera*Micrococcus*, *Photobacterium* and *Bacillus,* there was one isolate (3.2%) of each as shown in Table 2.2 and Figure 2.8.

Table 2.2. Antagonistic isolates originated from stressed shrimps.

Identification based on 16S rRNA gene sequence	Number of isolates from MS	Number of isolates from FS	Number of Total isolates	Total percentage
Vibrio spp.	10	13	23	74.19
Staphylococcus spp.	1	3	4	12.90
Micrococcus sp.	1	0	1	3.23
Bacillus sp.	1	0	1	3.23
Photobacterium sp.	0	1	1	3.23
Pseudoalteromonas sp.	0	1	1	3.23
Total	13	18	31	100.01

MS: Male stressed; FS: Female Stressed

2.4.3 Phylogenetic relationships of isolates

The 16SrRNA gene fragments of the bacterial isolates were sequenced and corrected using Chromas 2.1.1 software and submitted to the BALST to determine the identity of the closest relative as shown in Tables 2.3 and 2.4 for the coral isolate and shrimp isolates respectively.

Table 2.3. Isolates from corals exhibiting antimicrobial activity against lobster phyllosoma pathogen *V. owensii* DY05.

Isolate number	Taxonomic Group (Closest 16SrRNA gene BLAST match)	Clearance zone: diameter in mm		% Identity to nearest relative	Nearest accession number
		WDA	DDA		
Healthy coral (H)					
3CC1	*Vibrio* sp.S2-26	10	<10	99%	KM216237
1CC1	*Staphylococcus equorum* strain 179J/2012	10		99%	KM036089
2CC11	*Vibrio mediterranei* strain H025	10		99%	KJ577020
2CC8	*Vibrio coralliilyticus* strain RE98	23		100%	CP009617
2CC7	*Vibrio* sp. VibC-Oc-005	13		99%	KF577010
T2F	*Vibrio* sp.S7-74B	10	<10	99%	KM216245
2CS1	*Vibrio nereis* strain AP508	22		99%	GQ254512
1CS3	*Vibrio* sp. S1653	12	<10	99%	FJ457407
7C(1)	*Vibrio chagasii* strain HNS039	22	<10	99%	JN128273
6C(1)	*Vibrio* sp.S3142	10		99%	FJ457491
9C(4)	*Vibrio* sp. PaH2.24a	10		99%	GQ406770
12C(9)	*Vibrio* sp.S3942	10	<10	99%	FJ457581
1CC4	*Psychrobacter* sp. 2CpBB14	10		99%	JN602232
2CS2	*Psychrobacter piscatorii* strain VSD503	10		100%	KC534182
1CC2	*Staphylococcus* sp. ZWS13	10	<10	99%	KM051419
9C(8a)	*Staphylococcus* pasteuri strain C1PO1	10		99%	JQ689192
7C(6b)	*Acinetobacter* Cai-b1	23	<10	100%	(JX997899
6C(17)	*Photobacterium* sp.h5	22	<10	99%	EF187011
6C(16)	*Pseudovibrio* sp.P1MA3	10	<10	99%	JX477110T
Healthy Looking (HL- ASWS)					
15H(2)	*Vibrio jasicida* (AB562593)	10	<10	99%	AB562593
3.3(14)	*Vibrio coralliilyticus* strain OCN014	10		98%	CP009264
6H(4b)	*Vibrio rotiferianus* strain VSD813	10		99%	KC534399
9HS(1)	*Vibrio* sp. R1M3	10	<10		KC439183
6HS(4)	*Vibrio coralliilyticus* strain OCN014	10		99%	CP009264
9HS(6)	*Vibrio* sp. VibC-OC-067	10			KF576992
4.3(21)	*Vibrio* sp. vibc-co-053	10	<10	100%	KF577062
12HS(3)	*Psychrobacter* sp. SCSWC28	14	<10	95%	FJ461450
9HS(10)	*Psychrobacter celer* strain CUA-856	12		99%	KJ732920
6HS(1)	*Vibrio harveyi* strain 0012KARWAR	10		100%	KC345010
4.3(3)	*Psychrobacter* sp. PM8	23	<10	92%	KC195801

4.3(20)	*Psychrobacter celer* strain 91	21	<10	99%	JF710993
4.3(10)	*Psychrobacterceler* strain 91	10		99%	JF710993
4.3(15)	*Psychrobacter alimentarius* strain N-154	10	<10	100%	KJ735914
4.3(9)	*Psychrobacter* sp. 4Bb	16	<10	99%	HM771253
4.3(19)	*Psychrobacter* sp. PM8	22	<10	97%	KC195801
6H(1)	*Vibrio chagasii* strain HNS039	10		100%	JN128273
9HS(4)	*Micrococcus* sp. 5.22	10	<10	100%	KF681067
Disease coral (D-ASWS)					
15D(5)	*Psychrobacter nivimaris* strain PAMC 27111	10		99%	KJ475192
14D(7)	*Vibriochagasii*	10		99%	HM583983
14D(2)	*Vibrio jasicida*	10	<10	99%	AB562594
14D(3)	*Vibrio* S-3	10	<10	99%	KM201464
12DS(12)	*Vibrio campbellii* ATCC BAA-11	10	<10	96%	CP006606
12DS(3a)	*Vibrio* sp. B8D	10		99%	KF146218
12DS(2)	*Vibrio* sp. S3658	10		99%	FJ457531
6DS(1)	*Vibrio* sp. 2.3.053MS5	10	<10	99%	EU267670
9DS(5)	*Vibrio* sp. VibC-Oc-029	20	<10	99%	KF577114
7D(8)	Uncultured *Vibrio* sp. clone 17H-14	20		100%	KC917724
6DS(8)	*Vibrio* sp. S3942	10		99%	FJ457581
4.2(3)	*Vibrio crassostreae* strain CR-II-12	20		99%	KM014020
4.2(4a)	*Vibrio* sp. S2-26	10	<10	99%	KM216237
4.2(2)	*Vibrio* sp. S2-26	23		99%	KM216237
4.2(8)	*Psychrobacter adeliensis* strain SJ 14	17	<10	99%	NR_104882
4.2(9)	*Psychrobacter piscatorii* strain VSD503	20	<10	100%	KC534182
4.2(10)	*Psychrobacter celer* strain C5B1	10	<10	99%	JX501675
15D(4)	*Psychrobacter nivimaris* strain PAMC 27111	12	<10	99%	KJ475192
9DS(2)	*Psychrobacter adeliensis* strain SJ 14	10		99%	NR_104882
7D(10c)	Uncultured *Psychrobacter* sp. clone CI39	10	<10	99%	FJ695559
1.2(12)	*Brachybacterium* sp. IARI-AB-1	10	<10	100%	JN411299
14.2(4)	Unculturde *Bacillus* sp.clone 264AG8	10		100%	KF836540
4.2(8a)	*Micrococcus luteus*strain UASWS1011	12	<10	100%	KM251315
Disease Margin coral (M-ASWS)					
16M(8)	*Vibrio* sp.ps1	10	>10	100%	EU031648
15M(9)	*Vibrio chagasii* strain CAIM186	10		100%	HM583983
15M(1)	*Vibrio* sp.S2396	10	<10	100%	(FJ457442)
9MS(2)	*Vibrio harveyi*	10	<10	100%	HM771342
9MS(3)	*Vibrio* sp. VibC-Oc-043	10		99%	KF576999
9MS(4)	*Vibrio* sp. F15C3	10		99%	JX999958
12MS(3)	*Vibrio* sp. VibC-Oc-043	10		99%	KF576999
6MS(4)	*Vibrio* sp. VibC-Oc-043	10		99%	KF576999
7MS(8)	*Vibrio* sp. S2-24	10		99%	KM216248
6MS(1)	*Vibrio harveyi* strain CAIM 1792	22		100%	JQ434106
7MS(2)	*Vibrio harveyi* strain CAIM 1792	10		99%	JQ434106
4.1(15)	*Vibrio* sp. VibC-Oc-043	10	<10	99%	KF576999
7MS(12)	*Vibrio jasicida*	10	<10	99%	AB562593
TM(1)	*Vibrio* sp.j21431	10	<10	100%	FJ357689

16M(1)	*Vibrio chagasii* strain HNS039	10		99%	JN128273
15M(4)	*Vibrio* sp.S3661	10		99%	FJ457534
15M(5)	*Vibrio* sp.S7-74B	10		99%	KM216245
16M(3)	*Psychrobacter* sp.189	10	<10	99%	JQ012954
15M(6)	*Psychrobacter* sp.MF2-10b2	10		99%	JF800153
15M(2)	*Staphylococcusxylosus* strain S17	10		99%	KM269001

Table 2.4. Isolates from the shrimp (male and female) *Trypaea australiensis* exhibiting antimicrobial activity against the lobster phyllosoma pathogen *V. owensii* DY05.

Isolate number	Taxonomic Group (Closest 16SrRNA gene BLAST match)	Clearance zone: diameter in mm		% Identity to nearest relative	Nearest accession number
		WDA	DDA		
Shrimp isolates male stressed (MS)					
S6.12.12	*Vibrio* sp.S4074	12		99%	FJ457586
S6.14.14	*Vibrio jasicida*	10		99%	AB562593
S6.5.5	*Vibrio* sp. S7-74B	10		99%	KM216245
S4.6.6	*Vibrio brasiliensis* strain UST010723-012	9		99%	EU834004
SCC6.1	*Vibrio* sp.s2222	10		99%	FJ457425
SCC4.2	Uncultured *Vibrio* sp. clone 2_50	11	<10	99%	KF785711
SCC6.14	*Vibrio hepatarius* strain UST950701-002	10	10	99%	EU834019
SCC16.2	*Vibrio* sp. W-3	9		99%	KC777293
SCC16.1	*Vibrio coralliilyticus*strain ATCC BAA-450	10		99%	NR_117892
S4.2.2	*V. hepatarius* strain UST950701-002	10		99%	EU834019
SCC10.1	*Micrococcus luteus* strain SMR8	10		100%	KF600756
SCC12.14	*Bacillus thuringiensis* strain Dahb4	12	<10	99%	HQ693278
SCC4.1	*Staphylococcus equorum* Subsp.	10		99%	AB975354
Shrimp isolates female stressed (FS)					
S9.2.2	*Pseudoalteromonas sp* NC203	23	11	99%	KF146215
S91.1	*Vibrio* sp.B3K	10	<10	100%	KF146215
S1.9.9	*Vibrio* sp. EJY3 chromosome 1	10		83%	CP003241
SCC2.2	*Vibrio* sp.S3855	10		99%	FJ457563
SCC3.1	*Vibrio* sp.W-13	10		99%	KC777293
SCC1.14	*Vibrio* sp. M-137-19	9		100%	KF746900
SCC.1	*Vibrio* sp. VibC-Oc-012	11		99%	KF576989
CC1.7	*Vibrio* sp. VibC-Oc-012	11		97%	KF576989
CC1.2	*Vibrio harveyi* strain NIOT-sb1	10	<10	99%	KF607055
CC1.1	*Photobacterium damselae* strain SBTB24	10		75%	JX028545
SCC3.14	*Vibrio* sp.BBT47	10	<10	99%	FJ981869
SCC7.14	*Vibrio rotiferianus* strain KM30-12-2	10		99%	JQ670739
SCC2.34	*Vibrio azureus* strain S-41	10		99%	JF412237
SCC2.24	*Staphylococcus equorum* strain BPt-5	9		90%	KJ865579
CCS.1	*Staphylococcus* sp. XQW7	11	<10	99%	KM224521
CC5.1	*Vibrio* sp. VibC-Oc-043	10		100%	KF576999
SCC1.24	*Staphylococcus* sp. XQW7	10		99%	KM224521
SCC6.11	*Vibrio* sp. S2222	10		99%	FJ457425

2.5 Discussion

Vibrio spp. are important bacterial pathogens and the pathogenic effects of certain *Vibrio* spp. are critical for animals reared in aquaculture (Bergh *et al.*, 2001; Samuelsen *et al.*, 2006; Thompson *et al.*, 2004; Toranzo *et al.*, 2005). Members of the *Vibrio* genus may be commensal or symbiotic with different eukaryotic host (Thompson *et al.*, 2004). *Vibrio* species are the most common pathogen and constitute a large percentage of the microbial community associated with cultured larval invertebrates including *P. orantus* (Bourne *et al.*, 2004; Diggles *et al.*, 2000; Payne *et al.*, 2007; Webster *et al.*, 2006).

However, previous studies have suggested that some *Vibrio* species may be used (perform) as biocontrol agents in aquaculture, reducing the need for antibiotics and reducing effluent discharges (Douillet, 2000; Thompson *et al.*, 2003; Vaseeharan & Ramasamy, 2003). Isolation of bacteria that produce inhibitory substances has led to the identification of probiotics for potential use in aquaculture systems (Irianto & Austin, 2002). Antagonistic activity is considered as an important selection criterion for probiotic candidates.

To identify antagonistic bacteria from a culture collection of corals and shrimp two screening assays were used in present study, a well diffusion assay and disk diffusion assay. The well diffusion assay identified a much greater number of bacterial isolates as having probiotic activity, compared to the disk diffusion assay. The well diffusion assay was therefore considered as a more efficient tool for initial screening for antimicrobial production. A possible explanation for the difference between the two assays is that the disk diffusion assay was carried out using filtered supernatant, while the well diffusion assay was carried out using whole culture.The concentration of antibacterial compounds in the filtered supernatant may not be high enough to inhibit the pathogen or produce the same inhibition zone compared with the well diffusion assay as not all the active molecules pass through the filter. On the other hand, the bacteria continuously produce antagonistic substances on an agar plate. In addition it was noticed during the experiment that the antagonistic activity was increased with the presence of pathogen in the liquid culture. Similar results have been documented in previous studies with different explanations for the size of inhibition zones in disk diffusion assay in comparison with other assays. For example, in a study of the antagonistic activity of bacteria and marine fungi by Miao & Qian (2005) two different assays including an antifungal and disk diffusion assay were used. Based on their resultsthey

suggested that bacteria may only produce active antifungal compounds when cultured with target microorganisms on agar plates.Also, not all antimicrobial compounds may be released into the media and therefore may not be present in the filtered supernatant.However, they attributed a small inhibition zone produced using disk diffusion assays to the physical effects of the bacterial extracts and suggest it may not be due to the presence of antimicrobial compounds. It may also be due to the ability of active compounds on the paper disc to diffuse through the agar medium. This may affect the size of inhibition zone produced by the disk diffusion assay, thus leading to a possible underestimation of antimicrobial activity. In another study by Long & Azam (2001) they suggested that the inhibition may not be due to antimicrobial activity, it might be due to aphysiological reaction or bacterium-bacterium communication such as quorum sensing. Shnit-Orland & Kushmaro (2009) also found that whole cultures, but not cell-free supernatants, demonstrate antibacterial activity and raised the possibility that active bacteria may not necessarily secrete compounds to the environment, but may instead be active through alternative mechanisms. Long & Azam (2001) suggested that the percentage of strains identified as antibiotic-producing can be affected by the assay method as well as the species and the number of indicator microbes used in the screening.

In the study of chemical ecology of marine epibiotic bacteria by Brugress *et al.* (1999) they explained the deficiency of the disk diffusion assay in screening of antimicrobial compounds due to a reaction occurring between the chemical components of filter paper and the chemical structure of the antimicrobial compounds. Therefore, they suggested that using different assays to screen for antimicrobial compounds may increase the number of compounds to be discovered.

In brief there are different explanations of the disk diffusion assay efficiency of diagnosing antimicrobial activity of antagonistic bacteria compared with other antimicrobial identification assays. Therefore more than one assay should be used to increased diagnosis of a greater number of antagonistic isolates.

Marine invertebrates present good sources of microorganisms for screening for antimicrobial activity. Bacteria associated with living surfaces and/or particle-associated bacteria displayed a higher degree of antagonistic activity than free-living bacteria (Gram *et al.*, 2010; Hjelm *et al.*, 2004b; Long & Azam, 2001; Long *et al.*, 2005; Miao & Qian, 2005; Nair & Simidu, 1987). The current study was focused on isolation and identification of probiotic bacteria from a coral and shrimp collection. Previous studies found antibacterial activity among and

between coral-associated bacteria, in particular the microbial communities associated with the mucus of healthy corals (Chen *et al.*, 2012; Gantar *et al.*, 2011; Ritchie, 2006; Rypien *et al.*, 2010). Therefore, coral associated bacteria present likely candidates to be screened for antimicrobial production (Chen *et al.*, 2012; Rypien *et al.*, 2010). Godwin (2007) concluded that antimicrobial compounds are produced by the symbiotic bacteria community associated with living tissue of *T. mesenterina* which regulate bacterial densities and inhibit the growth of invasive bacteria, thereby reducing the total number of culturable bacteria in his study on coral disease.

Antagonism is a widespread attribute implicated in the competiveness and ecological success of many aquatic microbes. It is thus considered an important trait of aquaculture probionts (Fjellheim *et al.*, 2007; Goulden, M. Hall*, et al.*, 2012; Gram *et al.*, 2010). Several studies (Burgess *et al.*, 1999; Slattery *et al.*, 2001; Trischman *et al.*, 2004) suggest that antagonism plays a critical role in preventing invasion of bacteria, including potential pathogens. Likewise, the surfaces of the healthy embryos of the lobster *Homarus americanus* are covered almost exclusively by a single Gram-negative bacterium, that produces an antifungal compound highly effective against the fungus *Lagenidium callinectes*, a common pathogen of many crustaceans (Gil-Turnes & Fenical, 1992). In the current study 22.2% of all strains in the culture collections were shown to have antagonistic activity against lobster phyllosoma pathogen *V. owensii* DY05. 72.1% of these strains were from the coral collection and 27.9% from the shrimp collection. This is in agreement with Ritchie (2006), who found that almost 20% of the cultured bacteria from *Acropora palmata* coral in the Caribbean displayed suppressive activity, including towards the causative agent of white pox disease. A study using well-diffusion assays showed that 10 of the 104 isolates (9.6%) released antimicrobial molecules into culture supernatants (Wilson *et al.*, 2010). Burgess *et al.* (1999) found that 35% of surface-associated bacteria isolated from various species of seaweed and marine invertebrates produced antimicrobial compounds. Kelmanet al. (2006) and Motta *et al.* (2004) also demonstrated that the majority (83% and 70% respectively) of marine bacterial species tested exhibited appreciable antimicrobial activity against one or more indicator bacteria.

Most of the antagonistic bacteria identified in the current study were Gram-negative bacteria, while fewer Gram-positive bacteria showed antagonistic activity. This observation is consistent with a previous studies (Lo Giudice *et al.*, 2007 b; Moriarty & Hayward, 1982).

This result may beexplained by the prevalence of Gram-negative culturable bacteriain these aquatic environments (Lo Giudice *et al.*, 2007 b). Fjellheim *et al*. (2010) found only Gram-negative bacteria to have antagonistic properties when attempting to isolate probiotic bacteria with antagonistic activity against *Vibrio anguillarum*. In contrast Lauzon *et al.* (2008)found that 81% of the antagonistic bacteria obtained from cod rearing systems were Gram-positive. Grossart *et al.* (2004) attributed the reason for the predominance of Gram-positive bacteria in the study carried in German Wadden Seamay be related to specific features of the Wadden Sea environment.

It has been known for decades that marine microorganisms produce antibacterial compounds.The current and previous studies (Fjellheim *et al.*, 2007; Grossart *et al.*, 2004; Long & Azam, 2001) have shown that a range of marinebacterial genera produce antimicrobial compounds in *in vitro* tests.We isolated and identified bacteria antagonistic to *V. owensii* DY05 using the well diffusion assay in culture media from four different coral species including 19 isolates of H-ASWS, 17 isolates of HL-ASWS, 23 isolates of D-ASWS and 21 isolates of M-ASWS (Table 2.1). The most abundant antimicrobial-producing bacteria isolated from coral samples were members of Vibrionaceae and Moraxellaceae families belonging to the class Gammaproteobacteria 88.8% of total isolates. Long and Azam (2001) also reported that bacteria belonging to the Gammaproteobacteria (Alteromonadales and Vibrionales) were the most prolific producers of inhibitory substances. In a study on intestinal samples from marine fish all the bacteria found to have inhibitory activity were *Vibrio* species (Makridis *et al.*, 2005).

On the other hand, we found that the shrimp bacterial isolates exhibiting the highest levels of antimicrobial activity belonged to class Gammaproteobacteria and class Bacilli. *Vibrio* were the predominant genus frommale and female stressed shrimps shrimp isolates (74.2% and 92% of total isolates), which is not surprising considering that many of these isolates were selected on TCBS medium, a selective medium for the growth and identification of *Vibrio* (Goulden, 2016).

This results is similar to most earlier studies, that revealed that Gammaproteobacteria are the predominant producers of antimicrobial compounds among marine bacterial isolates (Fjellheim *et al.*, 2007; Long & Azam, 2001). Romanenko *et al.* (2008) also found that most antagonistic bacteria were members of the Gammaproteobacteria and Alphaproteobacteria, and less with Firmicutes, Actinobacteria, and Cytophaga-Flavobacterium-Bacteroides group. Members of the genera Psychrobacter and *Pseudoalteromonas* were found to dominate the microbiota of all shrimp samples regardless of processing procedures or storage conditions (Broekaert *et al.*, 2013). Bacteria belonging to Bacillus and Actinobacteria groups, as well as those isolated from marine environment, are well known for their ability to produce a wide range of antimicrobials and other secondary metabolites (Romanenko *et al.*, 2008).

The results of the current study confirm that shrimp and coral associated bacterial communities represent good sources of antagonistic bacteria. Such strains, particularly *Pseudoalteromonas*sp. Strain 80 may be promising for use as probiotics in lobster hatcheries. The genus *Pseudoalteromonas* is a marine group of bacteria belonging to the class Gammaproteobacteria that has come to attention in the natural product and microbial ecology science fields in the last decade. Pigmented species of the genus have been shown to produce an array of low and high molecular weight compounds with antimicrobial, anti-fouling, algicidal and various pharmaceutically-relevant activities. Compounds formed include toxic proteins, polyanionic exopolymers, substituted phenolic and pyrolle-containing alkaloids, cyclic peptides and a range of brominesubstituted compounds (Bowman, 2007).

Three strains that gave strong antagonistic activity against *V. owensii* in well diffusion assay were renamed and chosen for further work (Table 2.5), as one aim of this study was to choose potential probiotic bacteria from culture collections (coral and shrimp isolates). Different antagonistic bacteria produce antimicrobial compounds at different times when mixed with pathogen. The chosen strains showed strong inhibition zones after 6 hours of incubation.

Table 2.5. The isolates selected for future work and their new names following 16S rRNA gene fragment sequencing.

Original name	New name	Source
S9.2.2	*Pseudoalteromonas* sp. strain 80	Stressed female shrimp
9DS(5)	*Vibrio* sp. strain 34	Diseased region on ASWS-affected coral
4.2(9)	*Psychrobacter* sp. strain 62	Diseased region on ASWS-affected coral

The mechanisms used by the antagonistic bacteria isolated in this study to suppress the pathogen are unknown and were further investigated as presented in Chapters 3-4.