Introduction

Aquaculture is a fast growing sector in the world, including Malaysia, which is able to produce high protein resources to fulfill food demands for human consumption. In 2011, 32.38% of world’s aquaculture production was from mollusks and crustaceans, and the shellfish aquaculture industry is expected to expand due to decreasing natural resources and increased demand for shellfish derived products (FAO, 2013).

Black tiger shrimp (Penaeus monodon) is among the leading shellfish species produced in Malaysia, while demand for slipper cupped oyster (Crassostrea iridalei) is...
remarkably increasing year by year. Due to this, various culture practices have been explored in order to yield higher production and reducing mortalities in farms and hatcheries. However, the uncontrolled development of these culture practices have led to outbreaks of infectious diseases from microorganisms such as bacteria, parasites and viruses (Lightner et al., 1992).

Diseases have always been a major problem in aquaculture. In shellfish culture, vibriosis is the most common diseases that infect shrimp (Otta and Karunasagar 2001), as well as oysters (Gomez-Gil et al., 2000). Bacterial diseases caused by *Vibrio* spp. have caused high mortalities and major economic losses to some countries (Estes et al., 2004). *Vibrio* species, which is the most common organism that has been found in the shellfish farms and hatcheries, and in particular *V. harveyi* causes luminescent vibriosis, which is an important disease that initiated mass mortalities in shellfish hatcheries (Lavilla-Pitogo et al., 1990). *Vibrio* species such as *Vibrio parahaemolyticus* is a bacterial pathogen commonly found in oysters and *P. monodon* along with other strains of *Vibrio* spp. such as *Vibrio tubiashii, Vibrio alginolyticus* and *Vibrio anguillarum* (Najiah et al., 2008; Tubiash and Otto 1986; Castro et al., 2002). In Malaysia, there are almost 94 shrimp hatcheries and few oysters farms, which are mostly located in the Peninsular area and Sabah. Outbreaks of this disease are hard to control and consistently lead to high mortalities, especially at early stages (Hashim and Kathamuthu 2005).

Environmentally friendly methods, for controlling microbial pathogenesis in aquaculture using probiotic bacteria, have gained substantial research interest and are increasingly preferred as viable and alternative management practices for disease prevention. Fuller (1989) stated that the application of probiotic as a live microbial feed supplement beneficially affects the host animal by improving its intestinal balance. Moriarty (1998) later suggested that the definition of a probiotic in aquaculture needs the addition of live naturally occurring bacteria to tanks and ponds in which animals live.

The administration of probiotics appears to be a very promising research area for nutrition, biocontrol and disease prevention in aquaculture (Balcazar et al., 2006). It is of significant interest to identify potential probiotic bacteria that could be isolated from shellfish species and attempt to develop novel probiotic strains to improve survival and health of these species, especially to shrimp and oysters. Thus, this study aimed to isolate, screen and evaluate beneficial bacteria isolated from shellfish as probiotics against different *Vibrio* spp. pathogens.

**Materials and Methods**

**Collection of samples:** Twenty samples of healthy adult tiger shrimp (*P. monodon*) were obtained from a commercial hatchery in Banting, Selangor, Malaysia. Meanwhile, 10 samples of slipper cupped oyster ranging from 9 to 10 cm were obtained from a hatchery at FRI Pulau Sayak, Kedah, Malaysia.

**Isolation and purification of bacterial strains:** Potential bacteria were isolated from the internal parts of tiger shrimp (stomach, intestine and hind-gut) and oysters (gills and digestive tract) using Marine agar (Difco, USA). The internal organs were aseptically removed and mashed using a sterilized mortar and pestle. A one ml aliquot of the homogenate solution was serially diluted up to $10^5$ CFU ml$^{-1}$ cell density in sterile saline water (SSW) and plated onto MA. The plates were then incubated overnight at 30°C. Pure colonies were picked based on differences in their morphology and colors from each plate.

**Pathogenic strains:** *Vibrio alginolyticus* ATCC 33839 and *Vibrio harveyi* ATCC 35084 were obtained from the Faculty of Agriculture, UPM. The pathogens were cultured on Thiosulfate Citrate Bile Sucrose agar (TCBS) (Difco, USA). All the cultures were incubated overnight at 30°C.

**Preliminary in vitro screening for potential probionts:**

(a) **Spot lawn assay:** A total of 144 isolates from tiger shrimp and 136 isolates from oysters were screened for their antagonistic activity by using a spot lawn assay method against two strains of *Vibrio* spp.; *V. alginolyticus* and *V. harveyi*. All potential probionts and vibrios were grown in Marine Broth (MB) (Difco, USA) overnight. The plates were

<table>
<thead>
<tr>
<th>Potential probionts</th>
<th><em>V. harveyi</em></th>
<th><em>V. alginolyticus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>I24</td>
<td>5±2</td>
<td>7±2</td>
</tr>
<tr>
<td>G11</td>
<td>-</td>
<td>6±2</td>
</tr>
<tr>
<td>S66</td>
<td>18±2</td>
<td>-</td>
</tr>
</tbody>
</table>

The diameter of the inhibition zone in millimeters ± SEM, including the size of the colony for the candidate probiont (2 mm). —, no zone.
swabbed with the pathogen at concentration of $10^4$ to $10^5$ CFU ml$^{-1}$ and 2 ul of potential probiotics were spotted onto it. All the plates were incubated overnight at 30 °C. The inhibition zone was observed, and diameter of inhibitory zone was recorded to the nearest millimeter (mm).

(b) **Co-culture assay:** In this assay, the initial density of selected pathogens *V. alginolyticus* and *V. harveyi* were approximately $10^4$ CFU ml$^{-1}$. These pathogens were selected based on the previous spot lawn assay results, which the potential probiotics showed antagonistic activity against these vibrios. Each pathogen and probiotic inoculum were co-cultured in 10 ml of Tryptic Soy Broth (TSB) (Difco, USA), and incubated at 30°C. Samples were taken at 0, 6, 12, 24, 48 and 96 hr of incubation. A 100 µl culture from each inoculum was plated on TCBS in order to quantify the number of cells. All the plates were incubated overnight at 30°C. The number of colonies for each inoculum was recorded as CFU ml$^{-1}$ and counted by the following formula:

$$\text{CFU ml}^{-1} = \frac{(\text{No. of colonies} \times \text{dilution factor})}{\text{Volume of culture plate (ml)}}$$

**Artemia challenge:** *Artemia* cysts were hatched in filtered sterile seawater (FSSW) with aeration and intense light. After 24 hrs, 20 tails of newly hatched *Artemia* were distributed into sterile tubes each filled with 30 ml of FSSW. Potential isolates were added at three different concentrations ($10^6$, $10^7$, and $10^8$ CFU ml$^{-1}$) before being incubated at room temperature overnight with gentle rocking. Pathogens (*V. alginolyticus* and *V. harveyi*) were added, respectively, on the next day at $10^6$ CFU ml$^{-1}$ concentration and incubated for an additional 24 hrs. In this assay, the probiont I24 and G11 were pre-incubated with *V. alginolyticus* and probiont S66 were pre-incubated with *V. harveyi* based on the previous co-culture assay results (results not shown). Observations were made until 50% mortality occurred in tubes that contained *Artemia* and pathogen only. The survival and mortalities of *Artemia* cyst were recorded and analyzed.

### Identification of potential probiotics:

(a) **Bacterial identification and grouping:** The isolates were identified to a generic level using standard biochemical test. Catalase and oxidase reaction were determined, as well as the Gram-staining and morphological characteristics of the isolates.

(b) **Molecular identification using 16s rDNA gene analysis:** A total genomic DNA of potential candidates was isolated by using Geneaid™ Genomic DNA Mini Kit (Geneaid Biotech, Taiwan). The universal primers used to amplify the 16S rDNA gene sequence from each DNA template were; forward primer (5'-AGAGTTTGATCCTGTTGATCTGGAGCAGACACAG-3'), and reverse primer (5'-GGTATTACAGCCCGCCAAT-3').

### Table 2: A comparative data on basic generic characterization of probiotics and 16S rRNA gene analysis

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>I24</th>
<th>G11</th>
<th>S66</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Morphology</td>
<td>LR</td>
<td>LR</td>
<td>SR</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Swarming on TSA (+1.5% Nacl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on TCBS</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Identification based on 16S rRNA gene sequence</td>
<td>Bacillus megaterium</td>
<td>Virgibacillus halodenitrificans</td>
<td>Exiguobacterium acetylicum</td>
</tr>
<tr>
<td>Origin</td>
<td>Tiger shrimp</td>
<td>Tiger shrimp</td>
<td>Slipper cupped oyster</td>
</tr>
</tbody>
</table>

### Table 3: Identification of potential probiotics to the nucleotide sequences from GenBank database

<table>
<thead>
<tr>
<th>Probiotic</th>
<th>Description</th>
<th>Similarities (%)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G11</td>
<td><em>Virgibacillus halodenitrificans</em> strain DSM 10037 16S ribosomal RNA gene, partial sequence</td>
<td>98</td>
<td>NR_042967.1</td>
</tr>
<tr>
<td>I24</td>
<td><em>Bacillus megaterium</em> strain NY-3 16S ribosomal RNA gene, partial sequence</td>
<td>98</td>
<td>EU918562.1</td>
</tr>
<tr>
<td>S66</td>
<td><em>Exiguobacterium acetylicum</em> strain DSM 20416 16S ribosomal RNA gene, partial sequence</td>
<td>87</td>
<td>NR_043479.1</td>
</tr>
</tbody>
</table>

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Fig. 1: Growth pattern of *Vibrio alginolyticus* (VAL) at \(10^5\) CFU ml\(^{-1}\) incubated with different concentrations of potential probiont G11 \((10^7, 10^8\) and \(10^9\) CFU ml\(^{-1}\)) against time. *Different letter indicates significant different at p<0.05*

Fig. 2: Growth pattern of *Vibrio alginolyticus* (VAL) at \(10^5\) CFU ml\(^{-1}\) incubated with different concentrations of potential probiont I24 \((10^7, 10^8\) and \(10^9\) CFU ml\(^{-1}\)) against time. *Different letter indicates significant different at p<0.05*
Online Copy

Statistical analysis: All data were analyzed using SPSS 16 software. The compared mean data were evaluated and subjected to One-way ANOVA at the significant level of P<0.05 using Tukey's post hoc test.

Results and Discussion

Shrimp and oysters have close interaction with their surrounding environment. If the density of pathogens in cultured water increases, there are chances of disease outbreaks such as vibriosis. This is especially a problem in hatcheries that serve as source of seeds and high mortalities of these larvae can cause serious economic losses (Estes et al., 2004).

Vibrio spp. is highly pathogenic to shrimp and oyster larvae during their early stage that can lead to mass mortalities within 24 hours of infection, however, the application of antibiotics in disease prevention can cause disease resistance not only to the bacterial strains, but also to human pathogens (Van den Bogaard and Stobberingh 2000). In the present study, two potential probionts from tiger shrimp and one from the oysters were successfully isolated and identified as Bacillus megaterium (I24), Virgibacillus halodenitrificans (G11) and Exiguobacteria acetylicum (S66). The results demonstrated that the isolated probionts inhibited the growth of Vibrio spp. during in vitro assay and was able to confer protection to Artemia in the in vivo assay.

CTC AG-3') and reverse primer (5'-CGGTTACCTTGTAC GACTT-3'). The 16S rRNA amplification of DNA was performed using a Mastercycler Gradient PCR system (Eppendorf, Germany). Amplification was performed by initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing temperature of the primers at 55 °C for 15 sec, and extension at 72 °C for 90 sec. Negative control without template was included in every set of reactions.

Detection of PCR products was carried out by 0.8 % agarose gel electrophoresis (Fisher Scientific, USA) with IX TBE (Trisborate-EDTA) buffer. A 1 kb DNA ladder (Fermentas, USA) was used as DNA standard. The gel was viewed under a UV transilluminator gel imager and photographed. The purified DNAs were then sent for sequencing (First Base Laboratories Sdn. Bhd., Malaysia).

DNA sequences of potential probionts were compared to other known sequences from GenBank database using BLASTn (Nucleotide Basic Local Alignment Search Tool) searches of National Center for Biotechnology Institute (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi). According to Drancourt et al. (2000), bacteria of 99 to 100 % GenBank similarities on 16S rRNA sequences are members of the same species, while 97 to 99 % GenBank similarities are for members of the same genus.

Fig. 3: Growth pattern of Vibrio harveyi (VH) t 10⁷ CFU ml⁻¹ incubated with different concentrations of potential probiont S66 (10⁵, 10⁶ and 10⁷ CFU ml⁻¹) against time. *Different letter indicates significant different at p<0.05
A total of 144 isolates obtained from *P. monodon* were used in the study. All the isolates originated from the intestines (50 isolates), hind-guts (30 isolates) and stomach (64 isolates). On the other hand, 136 isolates were isolated from *C. iridalei*, which comprised of 46 isolates from gills, 36 isolates from digestive tract and 54 isolates obtained from swabbing inner shells.

Isolation process plays a pivotal role as a primary step in searching potential bacteria that can act as a probiotic. For tiger shrimp, bacteria's were isolated from the mid-gut (intestine), hind-gut (six segment to anus) and stomach of *P. monodon*, since these parts are known to contain high amount of microflora that helps the hosts to digest food. As for the oyster, this organism has close interaction with their surrounding environments. Due to filter feeding behavior of oysters, various microbial communities can be accumulated on the surface, gills and their digestive gland.

Meanwhile, as the first screening step when using a spot lawn assay, out of 136 strains isolated from the oysters, only 12 strains showed inhibitory activities against different strains of *Vibrio* spp.. As for tiger shrimp, 18 out of 144 strains displayed positive results (Table 1) and isolates with positive results were considered as potential candidates that were later subjected to broth co-culture assay.

In the co-culture assay, a growth pattern of *V. harveyi*...
A preliminary identification of these three isolates was done using Gram-staining, catalase, oxidase and pathogenic tests on TCBS agar. Based on Table 2, the results showed that all the three isolates were Gram-positive, positive for catalase test and both negative reactions in oxidase and pathogenic tests. All the isolates unable to grow on TCBS, which indicates that they did not belong to genus *Vibrio*.

PCR amplification of 16S rRNA gene on all the three isolates produced an expected 1500 bp amplicon each. The BLASTn results revealed that isolate G11 was identified as *V. halodenitrifican*, S66 as *Exiguobacterium acetylicum* and I24 was confirmed as *B. megaterium* (Table 3), respectively.

According to Ashokkumar and Mayavu (2013), *Bacillus* sp. are able to colonize both culture water and digestive tract of shrimp. They also reported that a strain of halophilic *Bacillus* sp. was effective in inhibiting shrimp pathogens, such as *V. parahaemolyticus* and *V. harveyi* both in *vitro* and in *vivo*. The present study showed that these two strains, *V. halodenitrifican* and *B. megaterium* had the ability to inhibit the growth of *V. harveyi*, *V. alginolyticus* and *V. anguillarum*. Both these potential probiotics showed their ability in inhibiting *Vibrio* spp. in motile (co-culture assay) and stagnant (spot and colony on top assay) conditions. Previous findings have disclosed that this halophilic *Bacillus* sp. significantly reduced mortality and also did not have any negative effects on the shrimp health.

The findings of the study revealed that the concentration of probiotics must be higher than vibrios in order for the probiotics to kill the pathogens. Moreover, it is suggested that these probiotics may need to be supplied at specific time in order to maintain their effectiveness. The study further postulates that the mechanism of competitive exclusion occurred between the potential isolates and *Vibrio* spp. They might be competing for nutrients, space, and/or release water-soluble substances that inhibit growth of other bacteria (Balcazar et al., 2006). This explains a strong antagonistic activity against *Vibrio* spp. tested in *in vitro* assay.
pathogenic effect on shrimp larvae (Ashokkumar and Mayavu, 2013). Based on the earlier study of Vary (1994), Bacillus megaterium was found in diverse environments like rice paddies to dried food, seawater, sediments, fish, normal flora, and even in bee honey. Taxonomically, B. megaterium is classified into B. subtilis group of Bacilli with an advantage on the absent of alkaline protease that enables excellent production and secretion of foreign proteins (Meinhardt et al., 1989; Rygus and Hillen 1991; Vary et al., 2007). Due to this reason, B. megaterium played crucial economic role as commercial enzyme, e.g., Penicillin amidase and steroid hydrolases (Vary et al., 2007).

The present study demonstrated that E. acetylicum as a rhizospheric, Gram-positive, rod shaped and yellow pigmented bacterium. The genus Exiguobacterium has emerged as a bacterium with an impressive diversity in its geographic distribution. Exiguobacterium strains are known for their enzymes production and extremophilic abilities (Wada et al., 2004; Kasana and Yadav 2007). Commonly known as a plant-growth promoting bacteria, E. acetylicum has an ability to inhibit different species of fungal pathogens by promoting antagonistic activity. To the best of our knowledge, there are no recent findings on the antagonistic ability of Exiguobacteria sp. on aquatic microbes (Selvakumar et al., 2008).

All the potential isolates conferred protection to Artemia against pathogens as shown in Fig. 4, Fig. 5 and Fig. 6. The survival rate of Artemia inoculated with both pathogens and potential isolates were higher as compared to Artemia with pathogen alone.

Fig 4 shows the survival of Artemia treated with probiont I24 after being challenged with V. alginolyticus. Meanwhile, Artemia treated with probiont I24 at 10^1 and 10^2 CFU ml^{-1} showed 40 % to 70 % increment in survival, respectively, as compared to Artemia with V. alginolyticus alone (5 %). Similarly, Artemia treated with probiont G11 showed 70 % to 80 % survival as compared to the control (40 %) and Artemia with V. alginolyticus only (20 %) (Fig 5). Both the probionts worked best at a bacterial concentration of 10^1 CFU ml^{-1}. On the other hand, Artemia treated with probiont S66 displayed highest and significant (p < 0.05) survival rate (85 %) after pre-incubation of probiont (10^1 CFU ml^{-1}) (Fig 6). Moreover, Artemia treated with 105 and 104 CFU ml^{-1} of probionts also showed significant different in survival (60% and 80% respectively) compared to V. harveyi alone (25%).

Pre-incubation of probionts in Artemia demonstrated their abilities to increase the survival of Artemia as compared to control without any probionts. Moreover, the survival of Artemia increased with increasing probionts concentrations. The results suggest that these probionts provide beneficial effects to Artemia and were able to confer protection against both V. alginolyticus and V. harveyi. Genus Bacillus was found prove to increase the survival of shrimp larvae along with enhancing some digestive enzyme activities (Zhou et al., 2009). It was able to provide protection against vibriosis in juvenile shrimp and enhance their immunity and disease resistance (Balcazar and Luna-Rojas 2007; Deng-Yu et al., 2009). However, to our knowledge, there are few reports emphasizing the use of Exiguobacteria sp. as a probiotic in aquaculture.

To date, probiotics or beneficial bacteria are widely used as an alternative method to control pathogens in aquaculture. However, more information is still needed to understand the mechanisms of action, which can lead to good selection of probiotics. The use of suitable probiotics from the natural environment will ensure healthy conditions for aquaculture, as well as food safety in shrimp industries.

In conclusion, the present study revealed that three isolates appeared as potential probiotics due to their ability to inhibit growth of different Vibrio spp. All the potential probionts successfully reduced the number of pathogen counts and increased the survival of Artemia in the in vivo assay. This study demonstrated a positive correlation between the in vitro and in vivo assay. Therefore, these isolates have potential of being probiotics, which can be applied in shellfish aquaculture industries. In order to confirm their potential, further studies should be carried out to strengthen the results of this research.

Acknowledgments

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