ISSN 1819-1894

Asian Journal of Agricultural Research

http://knowledgiascientific.com

Knowledgia SCIENTIFIC
A Place to Publish Outstanding Research

Asian Journal of Agricultural Research 9 (5): 223-236, 2015
ISSN 1819-1894 / DOI: 10.3923/ajar.2015.223.236

Elucidating the Probiotic Potential of Malaysian *Paenibacillus pabuli* Against *Vibrio alginolyticus* in *Artemia* Culture

[1,2]M.Y. Ina-Salwany, [1]H. Hishammuddin, [1]Z. Zulperi, [1]M. Salema, [1,2]M. Karim and [1,2]F.M.I. Natrah

[1]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia

[2]Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia

Corresponding Author: M.Y. Ina-Salwany, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia Tel: +60389474886 Fax: +60389408311

ABSTRACT

In the present study, two strains of Malaysian *Paenibacillus* spp. isolated from intestinal tract of cultured hybrid red tilapia were tested for their antagonistic activity against *Vibrio alginolyticus*, a destructive bacterial pathogen to marine fishes as probiotics in aquaculture. Homology searches of 16S rRNA and Internal Transcribes Spacer (ITS) genes sequencing with nucleotide Basic Local Alignment Sequence Tool (nBLAST) revealed that both strains were identical to *Paenibacillus* spp. and *Paenibacillus pabuli*, respectively. *In vitro* and *in vivo* experiments were conducted to study the efficacy of these *Paenibacillus* strains as probiotics. Co-culture assay demonstrated that the growth of *V. alginolyticus* was inhibited when grown with these probionts. A complete inhibition of the pathogen by *P. pabuli* strain D12 occurred at 48 h of incubation period. To qualify strains as probiotics, *Artemia* challenged with *V. alginolyticus* was performed, with or without these potential probiotics. The highest survival rate (72%) of *Artemia* was observed in *P. pabuli* strain D12 treatment after challenged with *V. alginolyticus*, followed by *P. pabuli* strain D14 at 68% survival rate. The lowest survival of *Artemia* (23% survival rate) was recorded when challenged with only *V. alginolyticus*. This study reported the ability of *Paenibacillus* D12 and D14 as potential probiotics to control *V. alginolyticus* in *Artemia* culture system.

Key words: Probiotics, antagonism, *Paenibacillus*, *Vibrio alginolyticus*, *Artemia*

INTRODUCTION

Over time, global fish aquaculture production will need to expand to cater the growing human population. This rapid intensification and commercialization of aquaculture production nevertheless faces a significant constraint due to disease outbreaks, particularly in marine and freshwater fish cultures. *Vibrio* species such as *V. anguillarum*, *V. alginolyticus, V. ordalii, V. salmonicida, V. vulnificus* and *V. harveyi* have been documented as causative agents for intestinal necrosis, anemia, septicaemia and haemorrhages in cultured aquaculture systems worldwide (Ransangan *et al*., 2012; Austin and Zhang, 2006; Hjeltnes and Roberts, 1993). These microorganisms were literally identified in several fish species e.g. salmonid, rainbow trout, turbot, burbot, carps, catfish and tilapia, with severe economic losses and environmental degradation have been recorded in numerous countries (Zheng *et al*., 2012; Parthasarathy and Ravi, 2011;

Al-Sunaiher *et al*., 2010; Spanggaard *et al*., 2000). As a promoter of health, probiotics could be beneficial through multiple ways, either by a single strain introduction or as a combination of several probionts, which include the inhibiting pathogens through production of antagonistic compounds, competition for adhesion sites and nutrients, alteration of enzymatic activities of pathogens, immunostimulatory functions, improving feed digestibility, promotion of growth and survival and also improvement to water quality (Utiswannakul *et al*., 2011; Nimrat *et al*., 2008; Balcazar *et al*., 2006; Bomba *et al*., 2002). As proposed by Merrifield *et al*. (2010), probiotics must not be pathogenic and should display antagonistic properties against one or multiple pathogens. They should be administered either by direct addition to the dry food, the culture water or via live food during the early stages of larval development (Verschuere *et al*., 2000).

During the initial stages of cultivated fish, survival is considered the most important factor compared to growth improvement. Verschuere *et al*. (1999) revealed the application of beneficial bacteria to prevent detrimental bacterial colonization in *Artemia* culture. *Artemia* in recent years have been applied as live food for larviculture since they possessed high nutritional value due to the high energy reserves during the first eight hours upon hatching (Sorgeloos *et al*., 2001). Hence, it is feasible to evaluate the impact of using *Artemia* to potentially improve the growth and survival of the host, especially in marine hatcheries system (Marques *et al*., 2006).

A previous study was performed in our laboratory to screen and characterize bacterial isolates from intestinal tract of red tilapia from different farms in Malaysia to reveal their potential in preventing mortality by fish pathogens of marine and freshwater fishes. Screening of bacterial isolates using cross streaking method for detection of antimicrobial activity against fish pathogens led to the isolation of probiont candidates, *Paenibacillus* spp. strains D12 and D14. Unfortunately, there are very limited publications disclosing *Paenibacillus* role as probiotic in aquaculture. In this study, we aimed to determine the antagonistic activity of *Paenibacillus* spp. against the pathogenic *Vibrio alginolyticus* under *in vitro* conditions and validation of their probiotic potential through *in vivo* assessment on *Artemia nauplii* culture.

MATERIALS AND METHODS

Bacterial strains and culture conditions: The strains used were *Paenibacillus* D12 and *Paenibacillus* D14, which were retrieved from the intestine of healthy red tilapias, while the pathogen used was *V. alginolyticus* strain V2 isolated from a diseased tiger grouper. All cultures were stored as stock suspensions in 15% glycerol at -80°C, retained in agar slant or were sub-cultured onto agar media.

Pure colonies of bacteria strains from glycerol stock were thawed and grown overnight on Trypticase Soy Agar (TSA) (Oxoid, UK) supplemented with 1.5% NaCl at 25°C. A sterile disposable loop was used to pick a colony from each culture plates, which was resuspended in 10 mL Trypticase Soy Broth (TSB) (Oxoid, UK) supplemented with 1.5% NaCl. All cultures were incubated again at 25°C for 24 h, with gentle shaking. The bacterial density was determined by measuring optical density at 550 nm using a spectrophotometer (BioPhotometer, Eppendorf, Germany) by assuming that an optical density of 1.000 corresponds to 1.2×10^9 CFU mL^{-1} of the McFarland standard (BioMerieux, France). The bacterial suspension was later diluted to the target concentration in sterile seawater (SSW). All strains were identified by their morphology and biochemical characterization.

Amplification of 16S rRNA and Internal Transcribed Region (ITS) gene and sequence analysis and phylogenetic analysis: The DNA was extracted from pure cultures using One-Tube

Bacterial Genomic DNA extraction kit (Bio Basic, Canada) according to the manufacturer's instruction. The partial DNA fragment of bacterial 16S rRNA gene was amplified by PCR using a forward primer fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and a reverse primer rp2 (5'- ACGGCTACCTTGTTACGACTT-3') (Allen *et al*., 2001). For the ITS region, the highly conserved sequences were designed based on published sequences with adjacent 3' end region of the 16S rRNA and 5' end region of the 23S rDNA using a forward primer ITS-16SF (5'- CGGTGAATACGTTCCCGGGYCTTG-3') and a reverse primer ITS-23SR (5'- TTTCRCCTTTCCCTCACGGTA-3'). The PCR amplification was performed as follows: 10 µL of 5X Green Go Taq® Flexi buffer (Promega, USA), 4 µL of 25 mM MgCl (Promega, USA), 2 µL of 10 mM dNTP, 2 µL of 20 pmol primers, one unit of Go Taq DNA polymerase (Promega, USA) and bacterial DNA as a template. The total volume was brought up to 50 µL with sterile pure water. The PCR was carried out in Mastercycler gradient (Eppendorf, Germany) using these following program: 95°C for 5 min, 40 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension of 72°C for 5 min. The amplified PCR products were subjected to electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The PCR product was purified using the QIAGEN Gel Purification kit (Qiagen, Australia) and sent for sequencing (First Base Laboratories, Malaysia. The DNA sequences were compared to known sequences from GenBank database using a nucleotide Basic Local Alignment Search Tool (nBLAST) searches of National Center for Biotechnology Institute (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi). All sequences were then analyzed using MEGA 5.1 software (Tamura *et al*., 2011). In order to obtain information on candidate probionts molecular phylogeny, the Neighbor-Joining (NJ) method with bootstrap analysis of 1000 replicates were applied to assess the reliability of nodes on trees (Tamura *et al*., 2011).

Competitive inhibition in co-culture assays: A broth co-culture assay was performed according to Vaseeharan and Ramasamy (2003) with minor modifications. Briefly, the probionts (D12 and D14) and the pathogenic strain (*V. alginolyticus*) were cultured separately in TSB+1.5% NaCl at 25°C for 24 h. The concentrations of probionts and pathogen were adjusted to reach the initial cell density of 10^6 and 10^5 CFU mL^{-1}, respectively. The probiotic inoculums were applied into 10 mL of TSB+1.5% NaCl separately followed by the pathogenic strain. One hundred microliter of co-culture samples were inoculated on Thiosulphate Citrate Bile Sucrose (TCBS) (Panreac Quimica, Spain) agar medium. The remaining co-culture samples were incubated at 25°C until 72 h, with gentle shaking. All six treatments was performed in triplicate (Table 1). Determination of the viable pathogen count was estimated by withdrawing 100 µL from the 24 h co-culture samples and 10-fold serial dilutions were prepared in triplicate and inoculating 0.1 mL from each dilution on TCBS agar plates. The steps were repeated for 48 and 72 h.

***Vibrio alginolyticus* challenge of *Artemia nauplii* enriched with probiotics:** *Artemia* cysts (Bio-Marine) were hatched for 24 h at 28°C in 35 ppt filtered and SSW. Continuous aeration and

Table 1: Different treatments of broth co-culture assay

Treatments	Description
T1	Control (TSB+1.5% NaCl only)
T2	D12 (10^6 CFU mL^{-1})
T3	D14 (10^6 CFU mL^{-1})
T4	Va (10^5 CFU mL^{-1})
T5	D12 (10^6 CFU mL^{-1})+Va (10^5 CFU mL^{-1})
T6	D14 (10^6 CFU mL^{-1})+Va (10^5 CFU mL^{-1})

TSB: Tryptone soya broth, Va: *Vibrio alginolyticus*

Table 2: Treatments for bacterial challenged of *Artemia*

Treatments	Description
T1	Control
T2	D12
T3	D14
T4	Va
T5	D12+Va
T6	D14+Va

Va: *Vibrio alginolyticus*

constant lighting was provided during hatching. After 24 h of incubation, *Artemia* nauplii (instar I) were harvested and stocked at the density of 20 nauplii per 30 mL SSW in each falcon tube for each treatment series under sterile conditions. Six different treatments were prepared in triplicates; with treatment 1 (T1) as control and treatment 2 to treatment 4 (T2, T3, T4) as monoculture treatments. For treatment T2, T3 and treatment T5, T6, *P. pabuli* strain D12 and *P. pabuli* strain D14 were added at a density of 10^6 CFU mL^{-1}, respectively (Table 2). After 24 h, T4, T5 and T6 were all challenged with the pathogen, *V. alginolyticus* at a final concentration of 10^5 CFU mL^{-1}. The experiment was run for seven days and nauplii were fed every day with yeast until the last day of experiment. All activities were performed under a laminar flow hood in order to maintain the sterility. Total amount of live and dead *Artemia* were counted at the end of the experiments.

Pathogen load in culture water and *Artemia*: In the end of the seven days experimental period, the *Artemia* were subjected to sample processing in order to determine the pathogen load in the culture water as well as test organism. The nauplii were separated from the culture water in each treatment by sieving onto a sterile 100 μm mesh. The trapped nauplii were rinsed and resuspended with SSW. Both nauplii and 1 mL of culture water were aseptically stored in sterile tubes. One hundred microliter of each sample was later inoculated and spread onto a TCBS (Panreac Quimica, Spain) agar medium plate. The plates were then further incubated for 24 h at 25°C. The colonies of Vibrios were counted using a ROCKER galaxy 230 colony counter and calculated as CFU mL^{-1} using the following formula:

$$\text{CFU mL}^{-1} = \frac{\text{No. of Colony Forming Unit (CFU)}}{\text{Volume planted (mL)} \times \text{Total dilution used}}$$

Water quality analysis: The pH, Dissolved Oxygen (DO) and temperature were monitored daily, while the total ammonia was measured in the beginning and end of the experiment. The pH and temperature were measured using a YSI 60 pH and Temperature, Milwaukee; DO (Smart D.O Meter, USA) and NH_3-N (HANNA Instruments HI93715, Italy), respectively.

Statistical analysis: All data was analyzed using a One-way Analysis of Variance (ANOVA) after prior confirmation of homogeneity of variance. When significant differences were detected, Tukey's post-hoc test was used to determine significant differences among treatments. Results were expressed as Mean±Standard Error and differences were considered significant at $p<0.05$. All data was analyzed using a statistical analysis SPSS Version 16 software.

RESULTS

Molecular identification of probionts and phylogenetic analysis: The PCR amplification of D12 and D14 strains with 16S rRNA and ITS primer pairs each produced ~1000 and 750 bp

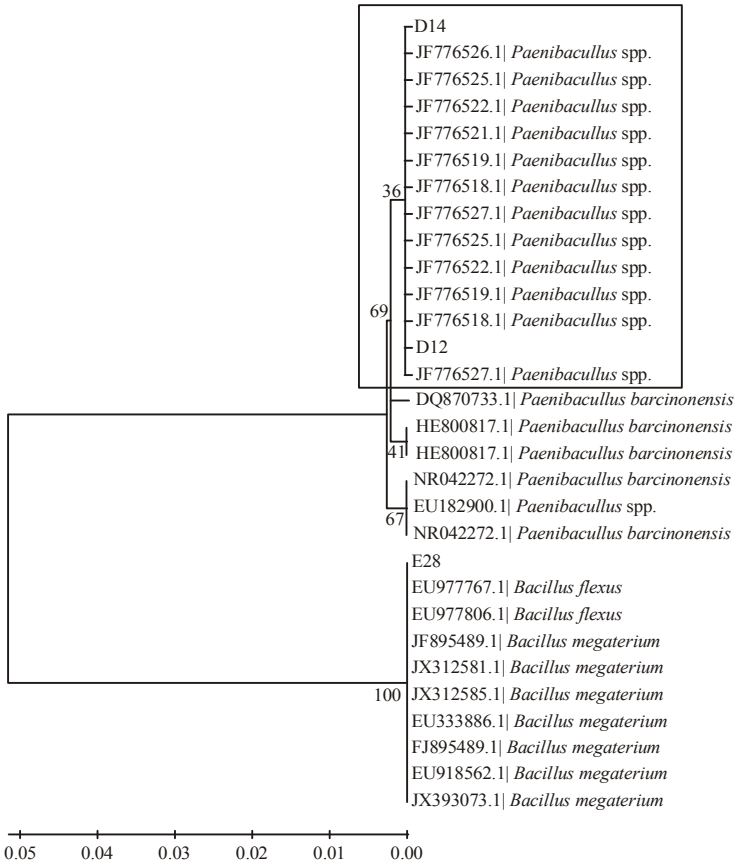

Fig. 1: Neighbour-joining tree inferred from 16S rRNA gene sequence of candidate probiotics D12, D14 and E28. Bootstrap values >30% derived from 500 replications are shown at nodes. The bar indicates 0.05% estimated sequence divergence. Strains classification is signified using colored rectangle

Table 3: Comparative analysis of phenotypic and genotypic identification of probiotic strains

	Taxonomic identification based on		
Strains	Biochemical test	16S rRNA gene sequencing	ITS gene sequencing
D12	*Bacillus circulans*	*Paenibacillus* spp.	*Paenibacillus pabuli*
D14	*Bacillus circulans*	*Paenibacillus* spp.	*Paenibacillus pabuli*

ITS: Internal transcribed region

amplicon, correspondingly. No amplicon was obtained in the control without template, indicating that there was no contamination during the PCR amplification. Homology searches of 16S rRNA and ITS genes sequencing with nBLAST revealed that both strains were identical to *Paenibacillus* spp. and *Paenibacillus pabuli*, respectively.

Based on the phylogenetic analysis of 16S rRNA gene sequences, our probiont strains D12 and D14 were clustered together with *Paenibacillus* spp. reference strains (Fig. 1). The ITS phylogenetic tree later revealed that both strains were in the same node with *P. pabuli* and *P. polymyxa* reference strains (AM087616 and EF451155), respectively (Fig. 2).

The comparative analysis of phenotypic and genotypic identification of probiotic strains D12 and D14 using biochemical test, 16S rRNA and ITS gene sequencing were represented in Table 3.

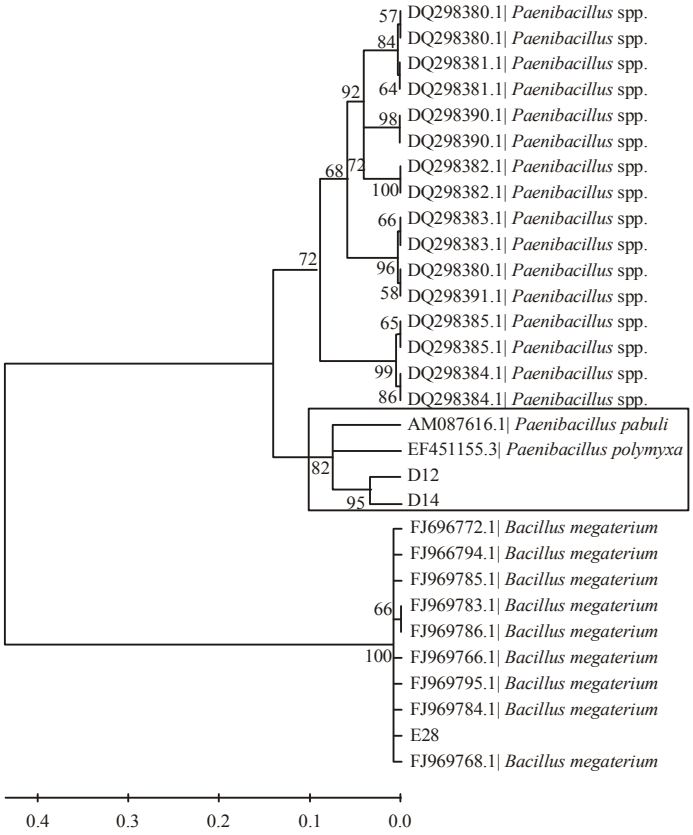

Fig. 2: Neighbour-joining tree inferred from ITS region sequence of candidate probiotics D12, D14 and E28. Bootstrap values>50% derived from 500 replications are shown at nodes. The bar indicates 0.1% estimated sequence divergence. Strains classification is signified using colored rectangle

Growth competition in co-culture assays: Present study showed *Paenibacillus* strains D12 and D14 were able to inhibit virulent strain *V. alginolyticus* (Va) in broth co-culture assay. This was evidence by a declining pattern of *V. alginolyticus* growth when they were grown concurrently with probiont strains D12 and D14 (Fig. 3). At 24 h interval, a similar degree of inhibition for treatments with both probionts (D12+Va) and (D14+Va) were observed, without any significant differences ($p>0.05$). The inhibitory activity of the *Paenibacillus* strain D12 against the pathogen was significantly accelerated from 10^5 CFU mL^{-1} after 24 h incubation period. A complete inhibition of strain D12 occurred at 48 h of incubation period. For *Paenibacillus* strain D14, a 72 h was required for a complete inhibition on *V. alginolyticus*. Moreover, strain D12 also disclosed a higher inhibitory level in a shorter period, as compared to strain D14. On the other hand, the monoculture treatment of *V. alginolyticus* (control) in fresh TSB supplemented with 1.5% NaCI imitated the normal growth of *V. alginolyticus* without any influence of probionts, with the period of incubation significantly affected the number of pathogens ($p<0.05$) (Fig. 3).

Effect of pre-treatment with probionts on survival rate of *Artemia* to bacterial challenge: The highest survival rate (72%) of *Artemia* when challenged with *V. alginolyticus* (Va) was observed in T5 (D12+Va) and a 68% survival rate was observed in T6 (D14+Va). For both non-challenged

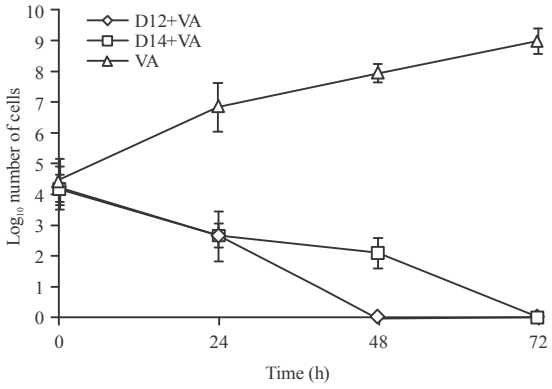

Fig. 3: Growth pattern of *Vibrio alginolyticus* at 25°C with and without *Paenibacillus pabuli* strain D12 and D14

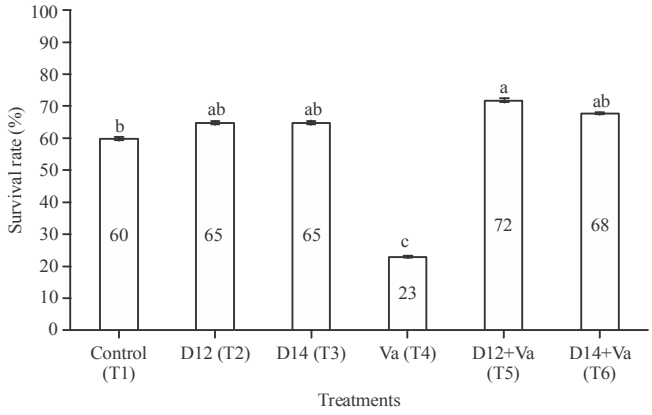

Fig. 4: Survival rate (%) of *Artemia* at the end of the experiment period in six different treatments. Vertical bars indicate standard error of the means. All values represents mean±standard error. Mean values with different superscripts were significantly different ($p<0.05$)

treatments T2 (D12 only) and T3 (D14 only), the *Artemia* could survive at 65% survival rate. The lowest survival rate was recorded at 23% of monoculture treatment with the pathogenic strain, T4 (Va only). Meanwhile, the control T1 (without probiotic treatment) showed a less survival rate (60%) compared to the non-challenged treatment of probionts and challenged treatments with probionts (Fig. 4).

Assessment of pathogen load inside *Artemia* and in culture water: Pre-treatment of probionts with addition into the culture water were able to improve the survival rate of *Artemia*. Pathogen counts were further applied to quantify and determine the pathogen load in the culture water and *Artemia*. Figure 5 and 6 illustrated pathogen load within culture water and in *Artemia* at the end of treatment. In general, *Artemia* treated with probionts, T5 (D12+Va) and T6 (D14+Va) showed no significance differences ($p<0.05$) in pathogen load. In culture water, the highest number of *V. alginolyticus* (1.2×10^2 CFU mL^{-1}) was recorded in T4 (Va only) since there was no probiotics applied. Challenged treatment with T5 (D12+Va) indicated the least pathogen load (4.47×10^1 CFU mL^{-1}) compared to T6 (D14+Va) with 5.13×10^1 CFU mL^{-1}. Treatment with *P. pabuli* strain D12 successfully reduced pathogen load to 3.3×10^1 CFU mL^{-1}, while *Artemia* treated with

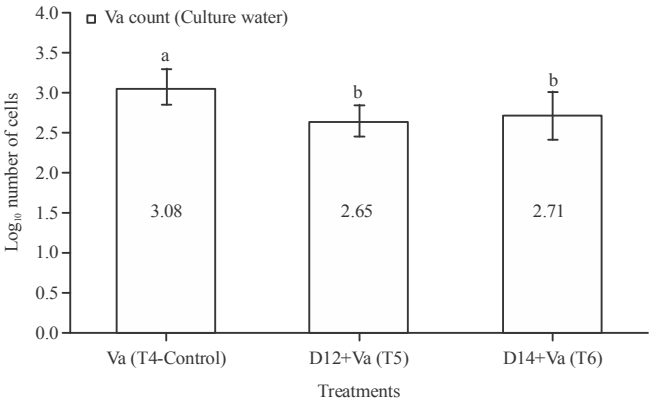

Fig. 5: Pathogen load in culture water at the end of experiment. All values represents Mean±standard error. Mean values with different superscripts were significantly different ($p<0.05$)

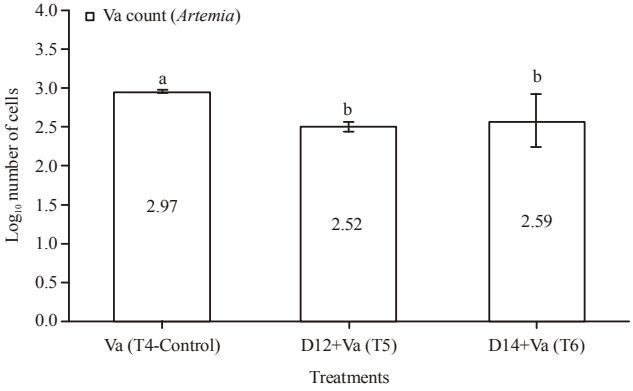

Fig. 6: Pathogen load in *Artemia* at the end of experiment. All values represents mean±standard error. Mean values with different superscripts were significantly different ($p<0.05$)

P. pabuli strain D14 was able to decrease the number of the virulence strain to 3.87×10^1 CFU mL^{-1}. Moreover, a much lower pathogen load in *Artemia* lead into suggestion that these probiont strains, D12 and D14 were able to penetrate in *Artemia* and successfully outcompete the pathogen, *V. alginolyticus* for attachment site or colonization space. The results revealed that the higher pathogen load in the culture water, the lesser survival rate of the *Artemia*. Survival rates were significantly higher in treated *Artemia* with probionts compared to those exposed to monoculture treatment of *V. alginolyticus*, which resulted in high mortalities (Fig. 7).

Water quality parameters: Water quality analysis produced results as follows; temperature ranged from 24.92±0.26-25.53±0.12°C, pH ranged between 7.89±0.03-8.05±0.03 and dissolved oxygen ranged between 4.29±0.05-4.47±0.04 mg L^{-1}, respectively (Table 4). For ammonia, there was a significant difference ($p<0.05$) between initial (Day 1) and final (Day 7) levels but still within an acceptable range (between 0.42-0.56 mg L^{-1}). Meanwhile, the ammonia level in the control treatment was significantly lower (0.42 mg L^{-1}) compared to other treatments.

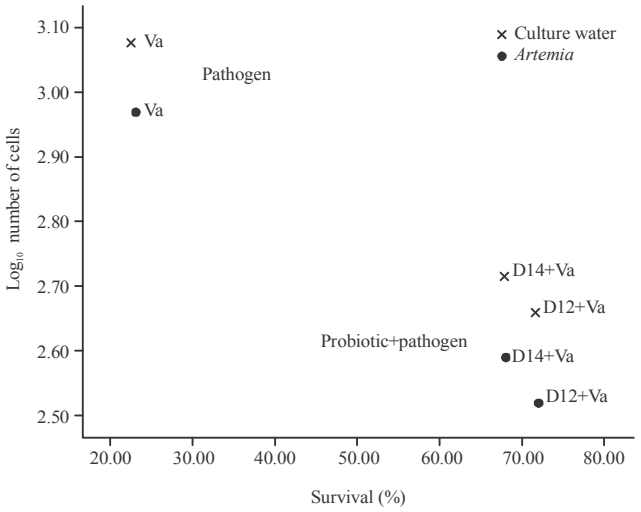

Fig. 7: Relationship between pathogen load (CFU mL^{-1} $\times 10^{1}$) in *Artemia* and culture water with the survival rate (%). All values in the table represents mean±standard error. Mean values with different superscripts were significantly different in column ($p<0.05$)

Table 4: Average of water quality parameters for all treatments during experiment

				NH_3-N (mg L^{-1})	
Treatments	Temperature (°C)	pH	DO (mg L^{-1})	Day 1	Day 7
Control	25.53±0.12^{a}	7.95±0.10^{ab}	4.33±0.05^{b}	0	0.42
D12	25.04±0.09^{b}	8.05±0.03^{a}	4.47±0.04^{a}	0	0.53
D14	24.92±0.26^{b}	8.02±0.02^{a}	4.37±0.10^{ab}	0	0.56
Va	25.36±0.07^{a}	7.89±0.03^{b}	4.36±0.06^{ab}	0	0.44
D12+Va	25.43±0.08^{a}	7.92±0.03^{b}	4.29±0.05^{b}	0	0.55
D14+Va	25.43±0.09^{a}	7.99±0.03^{a}	4.40±0.08^{ab}	0	0.46

DO: Dissolved oxygen, Va: *Vibrio alginolyticus*

DISCUSSION

An effective probiotic strain for aquaculture purposed required proper identification and characterization in order to access background information such as the optimal culture requirements and its pathogenicity (Vine *et al*., 2004). Phenotypic variability among bacterial strains belonging to the same species may result in misidentification thus, molecular analysis was proposed for identification of probionts down to their species level (Drancourt *et al*., 2000).

In present study, PCR amplification targeting 16S rRNA and ITS genes were employed to precisely identify our probionts. Despite commonly used in bacterial identification, 16S rRNA gene sequencing produces a low discriminatory ability at species level and between some genera (Mignard and Flandrois, 2006). On the contrary, the ITS region has been theoretically considered as a reliable monitoring tool for both bacterial identification and classification since, it offers higher resolution, contains multiple copies of rRNA operons, shows higher variability in length and sequences compared to those of 16S rRNA and 23S rRNA genes, as well as being species-specific among related organisms, or among different strains of the same species (Dang *et al*., 2012; Gonzalez *et al*., 2003; Berridge *et al*., 2001; Jensen *et al*., 1993). The PCR amplification with 16S rRNA primers revealed that our probiont strains D12 and D14 were 98% similar to at least five closely related strains namely *Paenibacillus* spp. (JF776525), *Paenibacillus* spp. (JF776522),

Paenibacillus spp. (JF776519), *Paenibacillus* spp. (JF776518) and *Paenibacillus* spp. (JF776527). Our early conclusion was these results might support the fact that 16S rRNA gene sequencing was unable to distinguish closely related species. However, the ITS region sequencing tends to be much more specific when referring to closely related strains. The nearest homolog species for our probiont strains D12 and D14 strain were found to be *Paenibacillus pabuli* (GenBank Accession Number: AM087616), both with 95% similarities. In fact, subsequent phylogenetic analyses of the 16S rRNA and ITS sequences allowed the generation of trees that grouped our strains into the same clade with *Paenibacillus* spp. and *Paenibacillus pabuli* reference strains (Fig. 1 and 2).

Numerous reports have used *in vitro* antagonistic activity, or production of inhibitory compounds toward known pathogens of a particular species as the first screening for selection of candidate probionts (Nurhidayu *et al*., 2012; Hjelm *et al*., 2004). Antagonistic compounds are best defined as chemical substances that can inhibit or eliminate other microorganism, usually produced as secondary metabolites by microorganism of interest (Madigan *et al*., 2003). Measuring the inhibition zone of the culture bacteria against pathogenic bacteria is one of the most prominent techniques to determine antibacterial activity of potential probionts (Du Toit and Rautenbach, 2000). Competitive inhibition between probionts and pathogen in liquid medium through co-culture assay proved that the probionts inoculums density should be of higher level than the pathogenic strains and sufficient incubation period are needed for their effective inhibitory activities (Vaseeharan and Ramasamy, 2003). Our study demonstrated that various incubation periods significantly affecting number of pathogens based on the log number of cells (Fig. 1). This finding was in accordance with a previous study reported by Spanggaard *et al*. (2001), which showed that a complete inhibition was discovered in *V. anguillarum* growth when co-cultured in TSB with five strains (E174, E156, AH2 and E102) of antagonistic *Pseudomonas* isolated from rainbow trout. Conversely, they also reported that two out of three respective *Pseudomonas* strains and *Carnobacterium* strains D4 and D5 were not able to inhibit target strains in M9GC broth and TSA diffusion assay, thus suggesting that substrate compositions significantly influenced the production of secondary metabolites. In our case, increasing probionts density resulted in a higher inhibitory activity towards the pathogen and eventually affected the degree of inhibition which were earlier reported by Van Hai *et al*. (2007) and Vaseeharan and Ramasamy (2003). However, the positive result gained in our is in contrast to a study by Domrongpokkaphan and Wanchaitanawong (2006), where they proposed that a low concentration of *B. amyloliquefaciens* (B17 and B21) and *B. megaterium* (B25) at 10^6 CFU mL^{-1} have no effect on the growth of *V. harveyi* VHG03 (10^2 CFU mL^{-1}), whereas a minimal effect was evident at an initial concentration of 10^7 CFU mL^{-1} and above of those potential strains. It is of notable acceptance that the ability of a bacterial strain to inhibit the growth of pathogenic bacteria has been widely used as one of the criteria for the selection of probiotics. However, an *in vitro* study does not truly imply the effectiveness of a probiotics *in vivo* (Vine *et al*., 2004).

The brine shrimp *Artemia* are the most commonly used live food in the larviculture of marine fishes due to their simple yet feasible production. Several publications have disclosed the application of probiotics supplemented via live carriers such as rotifers and *Artemia* to be able to stimulate the immunity of fish and shrimp, thus improving their growth and survival (Picchietti *et al*., 2009, 2007; Rengpipat *et al*., 1998). Our study discovered, the higher pathogen load in the culture water would result in a lower survival rate of *Artemia*. However, the survival of *Artemia* with our probiont strains D12 and D14 were significantly higher compared to those exposed to monoculture treatment of *V. alginolyticus* that resulted in high mortalities. Based on

previous studies, *Vibrio* species such as *V. alginolyticus*, *V. parahaemolyticus*, *V. anguillarum* and *V. proteolyticus* are common pathogenic strains recognized in *Artemia* culture. *Vibrio alginolyticus* and *V. parahaemolyticus* affected *Artemia* larvae by direct attachment to their body surface, hence imposing adverse effects towards their normal swimming behavior (Gunther and Catena, 1980). Apart from their role as food or feed additives, probionts may colonize the digestive tract and form an environment forming symbiotic association with *Artemia* and consequently conferred protection against pathogens. For example, a probiont may release beneficial digestive enzymes that could enhance digestion and nutrient assimilation, resulting in higher survival of *Artemia* (Lara-Flores, 2011; Sahu *et al*., 2008; Wang, 2007). During our study, we fed the *Artemia* with yeast, thus suggesting our probionts may also provide essential nutrients that by nature do not present in yeast (Vine *et al*., 2006).

In addition, probiotics have been reported to improve the health of the host organism by stimulating their immunities, therefore enhancing disease resistance (Merrifield *et al*., 2010; Nayak, 2010; Rengpipat *et al*., 2000). This may be one of potential mechanisms provided by our probionts in order to protect the *Artemia* against *V. alginolyticus* and hence promote higher survival rate, as observed in our *in vivo* assay. However, further research is necessary to elucidate the exact mode of action of the observed beneficial effects as well as to understand the possibilities and limitations of microbial control in aquaculture.

CONCLUSION

We proved that two *Paenibacillus* strains, D12 and D14 isolated in this study conferred protection to *Artemia* against pathogens in a bacterial challenge experiment. Application of these probionts significantly increased the survival of *Artemia* in all treatments over the controls and able to reduce the pathogen levels in the organism. It is evident that *P. pabuli* strain D12 displayed better performance than *P. pabuli* strain D14 in *in vivo* challenge test which were validated in the *in vitro* experiment.

ACKNOWLEDGMENTS

This study was supported by grants from FRGS (Vote no: 5524086), Ministry of Education Malaysia and Universiti Putra Malaysia (Vote no: 13001-DA). We would like to thank Dr. Nicholas Romano for his critical review of this manuscript. We also like to thank students and staffs from the Biotechnology Aquatics Laboratory Department of Aquaculture, Universiti Putra Malaysia for their kind assistance and technical support.

REFERENCES

Al-Sunaiher, A.E., A.S.S. Ibrahim and A. Al-Salamah, 2010. Association of *Vibrio* species with disease incidence in some cultured fishes in the Kingdom of Saudi Arabia. World Applied Sci. J., 8: 653-660.

Allen, A.E., M.G. Booth, M.E. Frischer, P.G. Verity, J.P. Zehr and S. Zani, 2001. Diversity and detection of nitrate assimilation genes in marine bacteria. Applied Environ. Microbiol., 67: 5343-5348.

Austin, B. and X.H. Zhang, 2006. *Vibrio harveyi*: A significant pathogen of marine vertebrates and invertebrates. Lett. Applied Microbiol., 43: 119-124.

Balcazar, J.L., I. de Blas, I. Ruiz-Zarzuela, D. Cunningham, D. Vendrell and J.L. Muzquiz, 2006. The role of probiotics in aquaculture. Vet. Microbiol., 114: 173-186.

Berridge, B.R., H. Bercovier and P.F. Frelier, 2001. *Streptococcus agalactiae* and *Streptococcus difficile* 16S-23S intergenic rDNA: Genetic homogeneity and species-specific PCR. Vet. Microbiol., 78: 165-173.

Bomba, A., R. Nemcova, D. Mudronova and P. Guba, 2002. The possibilities of potentiating the efficacy of probiotics. Trends Food Sci. Technol., 13: 121-126.

Dang, H.T., H.K. Park, S.C. Myung and W. Kim, 2012. Development of a novel PCR assay based on the 16S-23S rRNA internal transcribed spacer region for the detection of *Lactococcus garvieae*. J. Fish Dis., 35: 481-487.

Domrongpokkaphan, V. and P. Wanchaitanawong, 2006. *In vitro* antimicrobial activity of *Bacillus* spp. against pathogenic *Vibrio* spp. in black tiger shrimp (*Penaeus monodon*). Kasetsart J. (Nat. Sci.), 40: 949-957.

Drancourt, M., C. Bollet, A. Carlioz, R. Martelin, J.P. Gayral and D. Raoult, 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J. Clin. Microbiol., 38: 3623-3630.

Du Toit, E.A. and M. Rautenbach, 2000. A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. J. Microbiol. Methods, 42: 159-165.

Gonzalez, N., J. Romero and R.T. Espejo, 2003. Comprehensive detection of bacterial populations by PCR amplification of the 16S-23S rRNA spacer region. J. Microbiol. Methods, 55: 91-97.

Gunther, D.C. and A. Catena, 1980. The interaction of *Vibrio* with *Artemia nauplii*. The Brine Shrimp *Artemia*, Morphology, Genetics, Radiobiology, Toxicology, pp: 213-221.

Hjelm, M., O. Bergh, A. Riaza, J. Nielsen and J. Melchiorsen *et al*., 2004. Selection and identification of autochthonous potential probiotic bacteria from turbot larvae (*Scophthalmus maximus*) rearing units. Syst. Applied Microbiol., 27: 360-371.

Hjeltnes, B. and R.J. Roberts, 1993. Vibriosis. In: Bacterial Diseases of Fish, Roberts, R.J., N.R. Bromag and V. Inglis (Eds.). Blackwell Scientific, Oxford, UK., ISBN-13:9780632034970, pp: 109-121.

Jensen, M.A., J.A. Webster and N. Straus, 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appllied Envion. Microbiol., 59: 945-952.

Lara-Flores, M., 2011. The use of probiotic in aquaculture: An overview. Int. Res. J. Microbiol., 2: 471-478.

Madigan, M.T., J.M. Martinko and J. Parker, 2003. Biology of Microorganisms. Prentice Hall, New Jersey.

Marques, A., T.H. Thanh, W. Verstraete, J. Dhont, P. Sorgeloos and P. Bossier, 2006. Use of selected bacteria and yeast to protect gnotobiotic *Artemia* against different pathogens. J. Exp. Mar. Biol. Ecol., 334: 20-30.

Merrifield, D.L., A. Dimitroglou, A. Foey, S.J. Davies and R.T.M. Baker *et al*., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture, 302: 1-18.

Mignard, S. and J.P. Flandrois, 2006. 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. J. Microbiol. Methods, 67: 574-581.

Nayak, S.K., 2010. Probiotics and immunity: A fish perspective. Fish Shellfish Immunol., 29: 2-14.

Nimrat, S., S. Suksawat, P. Maleeweach and V. Vuthiphandchai, 2008. Effect of different shrimp pond bottom soil treatments on the change of physical characteristics and pathogenic bacteria in pond bottom soil. Aquaculture, 285: 123-129.

Nurhidayu, A., M.Y. Ina-Salwany, H. Mohd Daud and S.A. Harmin, 2012. Isolation, screening and characterization of potential probiotics from farmed tiger grouper (*Epinephelus fuscoguttatus*). Afr. J. Microbiol. Res., 6: 1924-1933.

Parthasarathy, R. and D. Ravi, 2011. Probiotic bacteria as growth promoter and biocontrol agent against *Aeromonas hydrophila* in Catla catla(Hamilton, 1822). Indian J. Fish., 58: 87-93.

Picchietti, S., M. Mazzini, A.R. Taddei, R. Renna and A.M. Fausto *et al*., 2007. Effects of administration of probiotic strains on GALT of larval gilthead seabream: Immunohistochemical and ultrastructural studies. Fish Shellfish Immunol., 22: 57-67.

Picchietti, S., A.M. Fausto, E. Randelli, O. Carnevali and A.R. Taddei *et al*., 2009. Early treatment with *Lactobacillus delbrueckii* strain induces an increase in intestinal T-cells and granulocytes and modulates immune-related genes of larval *Dicentrarchus labrax* (L.). Fish Shellfish Immunol., 26: 368-376.

Ransangan, J., T.M. Lal and A.H. Al-Harbi, 2012. Characterization and experimental infection of *Vibrio harveyi* isolated from diseased Asian seabass (*Lates calcarifer*). Malaysian J. Microbiol., 8: 104-115.

Rengpipat, S., W. Phianphak, S. Piyatiratitivorakul and P. Menasveta, 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture, 167: 301-313.

Rengpipat, S., S. Rukpratanporn, S. Piyatiratitivorakul and P. Menasaveta, 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture, 191: 271-288.

Sahu, M.K., N.S. Swarnakumar, K. Sivakumar, T. Thangaradjou and L. Kannan, 2008. Probiotics in aquaculture: Importance and future perspectives. Indian J. Microbiol., 48: 299-308.

Sorgeloos, P., P. Ghert and P. Candreva, 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. Aquaculture, 200: 147-159.

Spanggaard, B., I. Huber, J. Nielsen, T. Nielsen, K.F. Appel and L. Gram, 2000. The microflora of rainbow trout intestine: A comparison of traditional and molecular identification. Aquaculture, 182: 1-15.

Spanggaard, B., I. Huber, J. Nielsen, E.B. Sick and C.B. Pipper *et al*., 2001. The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. Environ. Microbiol., 3: 755-765.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol. Biol. Evol., 28: 2731-2739.

Utiswannakul, P., S. Sangchai and S. Rengpipat, 2011. Enhanced growth of black tiger shrimp *Penaeus monodon* by dietary supplementation with *Bacillus* (BP11) as a probiotic. J. Aquac. Res. Dev., 10.4172/2155-9546.S1-006

Van Hai, N., R. Fotedar and N. Buller, 2007. Selection of probiotics by various inhibition test methods for use in the culture of western king prawns, *Penaeus latisulcatus* (Kishinouye). Aquaculture, 272: 231-239.

Vaseeharan, B. and P. Ramasamy, 2003. Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett. Applied Microbiol., 36: 83-87.

Verschuere, L., G. Rombaut, G. Huys, J. Dhont, P. Sorgeloos and W. Verstraete, 1999. Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. Appl. Environ. Microbiol., 65: 2527-2533.

Verschuere, L., G. Rombaut, P. Sorgeloos and W. Verstraete, 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev., 64: 655-671.

Vine, N.G., W.D. Leukes, H. Kaiser, S. Daya, J. Baxter and T. Hecht, 2004. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. J. Fish Dis., 27: 319-326.

Vine, N.G., W.D. Leukes and H. Kaiser, 2006. Probiotics in marine larviculture. FEMS Microbiol. Rev., 30: 404-427.

Wang, Y.B., 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture, 269: 259-264.

Zheng, W., H. Cao and X. Yang, 2012. Grass carp (*Ctenopharyngodon idellus*) infected with multiple strains of *Aeromonas hydrophila*. Afr. J. Microbiol. Res., 6: 4512-4520002E.