CIBTech Journal of Biotechnology ISSN: 2319–3859 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/cjb.htm
2015 Vol. 4 (3) July-September, pp.25-35/Ina-Salwany and Nurhidayu
Research Article

A PROSPECT OF USING *VIBRIO HARVEYI* ISOLATED FROM THE INTESTINE OF HEALTHY FARMED TIGER GROUPER, *EPINEPHELUS FUSCOGUTTATUS* (FORSSKAL, 1775) AS PROBIOTICS

[*]Ina-Salwany M.Y. [1, 2] and Nurhidayu A.[1]
[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
**Author for Correspondence*

ABSTRACT
Two bacterial strains *Vibrio harveyi* JAQ01 and JAQ02 isolated from the intestine of farmed Tiger Grouper (*Epinephelus fuscoguttatus*) were studied to illustrate their in vitro characteristics as probiotics. Phenotypic characterization of the test strains was performed following a standard physiological test aided by commercial identification kits. A comparative phenotypic data between the test strain and a reference strain *V. harveyi* ATCC35084 was generated. These bacterial strains were eventually designated as *V. harveyi* JAQ01 and JAQ02 following 16S rRNA gene sequences analysis. Both strains were successfully exhibited a significance level of inhibition of target strains using a modified bacteriocin-like inhibitory substance (BLIS) method to fulfill their functional requirement as probiotics. Assessment on the safety aspects regarding their haemolytic activity and antibiotic resistance profile completed this study. Our investigation revealed the presence of two antagonists *V. harveyi* that may play a role in diseased control of farmed *E. fuscoguttatus*. This is the first report made to assess in vitro characterization of intestinal *V. harveyi* of farmed *E. fuscoguttatus* suggesting the prospect of using *V. harveyi*JAQ01 and JAQ02 in aquaculture is worth an advance research.

Keywords: *16S rRNA Gene, Antagonist, Epinephelusfuscoguttatus, Vibrio Harveyi, Probiotics*

INTRODUCTION
Despite being pathogenic to various fishes and other marine organisms (Saeed, 1995; Thakur *et al.*, 2003) the fact that *Vibrio* species are widely distributed in the marine environment (Sugita *et al.*, 2002; Schulze *et al.*, 2006) cannot be neglected. Comparative study on gut microflora of wild and cultured *Penaeus merguiensis* (Oxley *et al.*, 2002) revealed that the host may play a vital role in influencing the composition of their own gut microflora. Apart from intimate relationships between marine organisms and their environment, diverse roles offered by *Vibrio* spp. (Thompson *et al.*, 2004) may in turn contribute to the establishment of specific *Vibrio* sp. as one of their predominant gut microflora (Oxley *et al.*, 2002; Verner-Jeffreys *et al.*, 2003; Makridis *et al.*, 2005).
For common instance, certain *Vibrio* strains were proven to be effectively used as probiotics in aquaculture either use individually or in combination with other bacteria as experimented by Austin *et al.*, (1995), Gatesoupe (1997), Douillet (2000) and Riquelme *et al.*, (2001). The idea of using *Vibrio* sp. as probiotics however was precluded among aquaculturist partly due to their pathogenic characters and knowledge on the benefits offered by this genus are not well issued. Surprisingly, known opportunistic pathogens which lost its pathogenicity were said to possess other beneficial factors such as attachment ability or higher growth rate which formerly aided their success as pathogens may indirectly contribute to their success as probiotics (Vine, 2004).
In our knowledge, the prospect of using *V. harveyi* as probiotics in aquaculture has never been discussed before. However, from a microbial control point of view, *V. harveyi* are known to be able to exert intra- and/or interspecies control on growth and production of specific substances in marine environment by quorum sensing (Bassler *et al.*, 1997). Therefore, present study was performed merely to investigate the background information of two antagonists *V. harveyi* strains isolated from the intestine of farmed Tiger

Grouper (*E. fuscoguttatus*) in attempt to be used as probiotics. A set of bacterial probiotics criteria has been experimented herein focusing on phenotypic and genotypic characterization of test strains, there in vitro capacity to inhibit the growth of target strains and their antibiotic resistance capability. Such data may contribute in future attempts to correlate their potential usage as probiotics in vivo with present in vitro study.

MATERIALS AND METHODS

Bacterial Strains

The strains used in this study were originally isolated from the intestine of healthy farmed Tiger Grouper (*E. fuscoguttatus*) by dilution plating on marine agar 2216 (MA) (Difco, Becton Dickinson and Company, MD USA) and tryptic soy agar (TSA) (BBL, Becton Dickinson and Company, MD USA) supplemented with 1.5 % NaCl followed by an incubation at 25 °C for 24 h. The strains were then designated as JAQ01 and JAQ02 respectively. The pure culture of strains and *V. harveyi*ATCC35084 used to generate a comparative study were maintained onto agar slant and frozen at -80 °C in broth containing 80 % (v/v) glycerol throughout this study.

The target strains *V. alginolyticus*ATCC33839, *V. parahaemolyticus*ATCC43996, *V. harveyi*ATCC35084 and *Aeromonashydrophila* ATCC35654 were grown on tryptic soy broth (TSB) (Becto, Becton Dickinson and Company, MD USA) + 1.5 % NaCl and were incubated at 25 °C for 24 h prior antagonistic activity assay.

Morphological and Physiological Characteristics

The two strains investigated herein were characterized phenotypically on the basis of standard biochemical test as follows: Gram reaction was performed using both a potassium hydroxide reaction (3 % KOH) (Ryu, 1938) and a standard gram staining (Gram stain kit, Becton Dickinson and Company, MD USA), oxidase and indole reaction (Oxidase and Indole Dropper, Becton Dickinson and Company, MD USA), catalase reaction (3 % H_2O_2), swarming activity on TSA + 1.5 % NaCl and cell shape and cultural characteristics as they developed onto agar plates and broth.

Species level identification was performed using API 20E kit (API, BioMerieux, France) and BBL Crystal identification system (Becton Dickinson and Company, MD USA). Size measurement and photomicrographs of bacterial cell were taken using a Leica DM750 microscope fitted with Leica ICC50 digital camera (Leica Microsystems, Switzerland) by selecting five cells of each strain as representative. Purity of the selected isolates was regularly checked and maintained throughout the study.

DNA Extraction and 16S rRNA Gene Sequence Analysis

Overnight culture of the isolate was used for DNA extraction. The extraction was done using genomic DNA purification kit (Fermentas, Europe) according to the manufacturer's instruction. Amplification of the 16S rRNA gene was performed using a pair of primer Ecoli9 5' GAG TTT GAT CCT GGC TCA G 3' and Loop27rc 5' GAC TAC CAG GGT ATC TAA TC 3' (Sfanos *et al.*, 2005). A standard PCR was executed with a 50 µl reaction mixture containing 50 ng of template DNA, Taq polymerase (Fermentas, Europe) at 0.16 U/µl, 2.0 mMMgCl, 0.4 µM primers, 200 µM dNTP and 1X buffer as recommended by the manufacturer (Fermentas, Europe).

PCR parameters were carried out in Mastercycler gradient (Eppendorf, Germany) as follows: pre-denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min. The whole PCR parameters were terminated by a final extension step at 72 °C for 10 min and the PCR product was visualized by 1 % (v/v) agarose gel elctrophoresis.

The amplified products were then purified with GeneJET PCR purification kit (Fermentas, Europe) and subsequently sent for sequencing. 16S rRNA gene sequences of probionts were used as query to search for homologous sequences in the non-redundant collection (GenBank, RDP and DDBJ) of sequences. Sequences were aligned by using the ClustalW program and studied using MEGA 4.0 software (Tamura *et al.*, 2007) for phylogenic inference. *Vibrio rotiferianus* mj02-PW6-OH4 (HQ425687) was used as the out-group.

Antagonistic Activity Assay

Antagonistic activity assay was performed by means of BLIS or cross-streaking method as described by Hill *et al.,* (2009). Modification was made to assess the effect of the initial densities of isolates administered and different pre-incubation periods of isolates prior antagonism test to their antagonistic activity against the target strains. Briefly, overnight culture of the isolate was diluted in TSB + 1.5 % NaCl to achieve the final cell density of 10^6, 10^7,10^8 and10^9cfu ml^{-1}. A 1 cm streak of the diluted bacteria culture was made vertically at the centre of TSA + 1.5% NaCl plate using a sterile cotton swab. Four sets of plates were prepared for each test strain at four different cell densities. The first set of plates, denoted as 0 h pre-incubation period was cross- streaked with the 10^8cfu ml^{-1} active growing target strains (perpendicularly across the vertical streak). The plates were then incubated at 25 °C for 24 h to 48 h followed by observation of the inhibition zones. The width of inhibition zones were measure to the closest centimeters (cm) and recorded. Significant inhibition zones were considered for those greater than 1 cm. A set of plates for each strain was cross-streaked for every 24 h. The experiment was performed in triplicate for 72 h.

Data for the assay were analyzed in SAS statistical program (Version 9.2) (SAS Institute Inc., USA). Analysis of variance (ANOVA) and Duncan's multiple range test were performed at P = 0.05 on the basis of split plot design. Results were presented as mean ± SE (standard error) of the inhibition zone.

Antibiotic Susceptibility and Haemolytic Assay

Kirby-Bauer method as proposed by Lalitha (2004) was used to determine the antibiotic susceptibility of both strains tested. In brief, Chloramphenicol (30 µg), Tetracycline (30 µg), Streptomycin (10 µg), Kanamycin (30 µg), Gentamicin (10 µg) and Ampicilin (10 µg) impregnated discs were placed on the surface of Mueller-Hinton agar (Difco, Becton Dickinson and Company, MD USA) that has been seeded with strain to be tested. Following the incubation, the plates are examined for the presence of clear zone surrounding the antibiotic discs which directly signify the growth inhibition of bacteria tested. The sizes of the inhibition zone were made in millimeters and interpreted by referring to the approved standard as proposed by the Clinical and Laboratory Standards Institute (CLSI) which subsequently designated each strain as either susceptible, intermediate, or resistant to the antibiotic that have been tested.

Each strain was then subjected to a haemolytic assay by inoculating them onto blood agar base (ScharlauChemie, S.A., Spain) supplemented with 0.5 % defibrinated horse blood. The plates were incubated at 25 °C for 24 hours. The haemolytic zones were observed and subsequently classify the probionts as alpha, beta or gamma haemolysis.

RESULTS AND DISCUSSION

Results

Phenotypic Characteristics and Haemolysis Assay

A comparative data on the basic phenotypic and colony characteristics as well as cell morphology between test strains JAQ01 and JAQ02 anda reference *Vibrio harveyi*ATCC35084 was generated. Both JAQ01 and JAQ02 were identified as gram negative bacteria with smooth-edges circular colonies on TSA + 1.5 % NaCl measured 1.89 ± 0.233 mm and 1.02 ± 0.19 mm respectively. The colonies of reference strain on the other hand appeared larger with mean diameter 2.35 ± 0.16 mm. Furthermore, the cells of ATCC35084 and JAQ01 configured as single short rods with almost identical size of 2.40 ± 1.140 µm mean length with 1.04 ± 0.089 µm mean width and 2.23 ± 0.65 µm mean length with 0.88 ± 0.16 µm mean width respectively. In contrast, JAQ02 was found as single thin-long rod with 3.40 ± 1.140 µm mean length and 1.04 ± 0.089 µm mean width (Figure 1). All three bacteria have grown with uniform turbidity in TSB + 1.5 % NaCl.

Basic phenotypic characters of JAQ01, JAQ02 and ATCC35084 are described in Table 1. Comparatively, JAQ02 exhibits different phenotypic characters as negative indole reaction and swarming activity were observed. In addition to that, instead of forming yellow colonies on thiosulfate citrate bile salt sucrose agar (TCBS) (Difco, Becton Dickinson and Company, MD USA), colonies of JAQ02 were observed green.

Table 1: A comparative data on the basic phenotypic characters of ATCC35084, JAQ01 and JAQ02

Characteristics	ATCC35084	JAQ01	JAQ02
Gram staining	-	-	-
Morphology	SR	SR	LR
Swarming on TSA (+ 1.5 % NaCl)	+	+	-
Growth on TCBS[a]	Y	Y	G
Catalase	+	+	+
Oxidase	+	+	+
Indole	+	+	-

Note: [a] TCBS: thiosulfate citrate bile salt sucrose agar; +: posiive; -: negative; Y: yellow colonies formed; G: green colonies formed; SR: short rods; LR: long rods

Table 2: Phenotypic characteristics of JAQ01 and JAQ02 using API 20E kit and BBL Crystal identification system

Substrate	JAQ01	JAQ02
2-nitrophenyl-ßD-galactopyranoside	-	-
Arginine	-	+
Lysine	-	+
Ornithine	-	-
Trisodium citrate	-	-
Sodium thiosulfate	-	-
Urea	-	+
Tryptophane	-	-
Voges-Proskauer	-	-
Gelatinase	-	-
Glucose	-	-
Mannitol	+	-
Inositol	-	-
D-sorbitol	-	-
Rhamnose	-	-
Sucrose	+	-
Melibiose	-	-
Amygdalin	-	-
Arabinose	-	-
Acetyl glucosaminide	+	+
β-glucuronide	-	-
Proline and Leucine-p-nitroanilide	+	-
Phosphate	+	+
Esculin	-	-
Mannose	+	-
Adonitol	-	-
Galactose	-	-
α-β-glucoside	+	+
β-galactoside	-	-
bis-phosphate	+	+
Xyloside	-	-
Arabinoside	-	-
Phosphorylcholine	+	-
Glutamylnitroanilide	+	+
Nitro phenylalanine	-	-
Glycine	-	+
Citrate	-	+
Malonic acid	-	-
TriphenylTetrazolium chloride	-	-

+: positive colour change; -: negative colour change

Further characterization using API 20E suggested that they were *Pasteurellamultocida* and *Photobacteriumdamsela* correspondingly with *Vibrio* sp. as the next taxon in consideration. BBL Crystal identification system however managed to identify the JAQ01 as *Vibrio alginolyticus* and JAQ02 as *Chromobacteriumviolaceum*. Phenotypic characteristics of JAQ01 and JAQ02 as described by the two identification kits are summarized in Table 2. Both strains were categorized as gamma haemolysis.

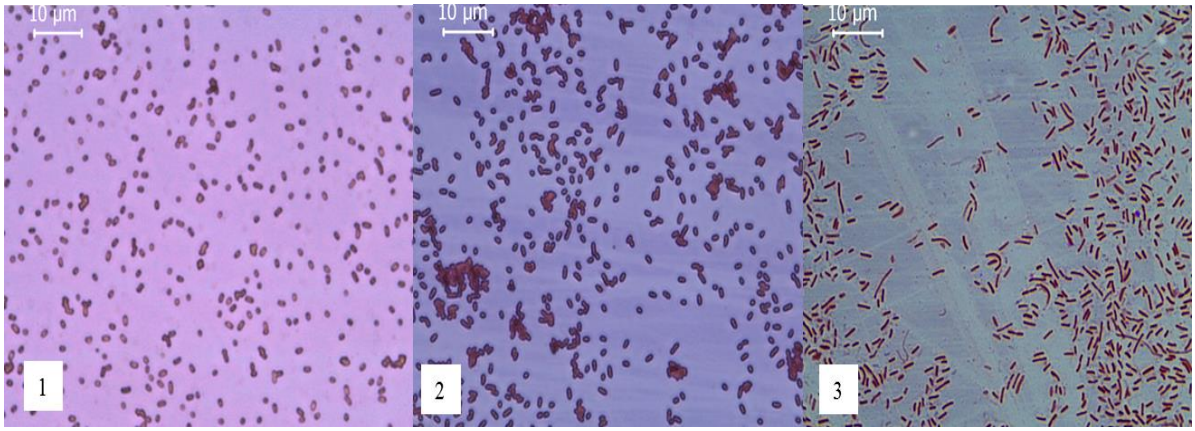

Figure 1: Photomicrographs of bacterial cell. 1:*Vibrio harveyi* ATCC35084, 2:*Vibrio harveyi*JAQ01, 3: *Vibrio harveyi*JAQ02

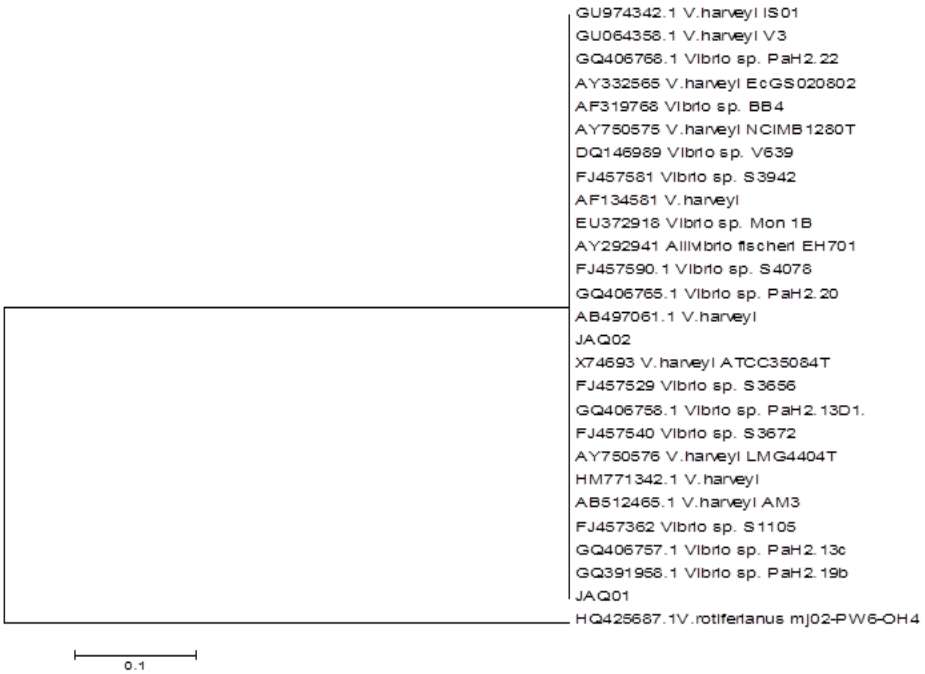

Figure 2: Evolutionary distances of 16S rRNA gene of JAQ01 and JAQ02 computed using Kimura-2 parameter method with 1000 bootstrap test. Reference sequences are labeled with their respective GenBank, DDBJ and RDP accession number. The tree is drawn to scale which the branch lengths represent the evolutionary distances used to infer the phylogenetic tree. There were a total of 601 positions in the final dataset.

16S rRNA Gene Sequence Analysis

The *V. harveyi*JAQ01 and JAQ02 strains were clearly classified in the genus of *Vibrio* with 80 % confidence threshold by RDP Classifier check program (http://rdp.cme.msu.edu).

Table 3: The mean ± SE of the inhibition zone (cm) of *Vibrio* spp. and *Aeromonashydrophila* tested against JAQ01 and JAQ02 at different pre-incubation periods prior antagonism test. The test strains were administered at the initial densities of 10^6, 10^7, 10^8 and 10^9cfu ml^{-1}.

Target strains	Pre-incubation period (h)	Test strains (cfu ml^{-1}) 10^6 JAQ01	10^6 JAQ02	10^7 JAQ01	10^7 JAQ02	10^8 JAQ01	10^8 JAQ02	10^9 JAQ01	10^9 JAQ02
Vibrio harveyi	0	-	-	-	-	-	-	-	-
	24	1.73 ± 0.11^{3a}	-	1.53 ± 0.11^{3c}	-	2.03 ± 0.11^{3b}	-	1.80 ± 0.11^{3b}	0.32 ± 0.11^{3b}
	48	3.03 ± 0.11^{2a}	1.03 ± 0.11^{2a}	2.10 ± 0.11^{2c}	1.07 ± 0.11^{2c}	2.57 ± 0.11^{2b}	1.50 ± 0.11^{2b}	2.43 ± 0.11^{2b}	0.47 ± 0.11^{2b}
	72	3.93 ± 0.11^{1a}	2.03 ± 0.11^{1a}	3.30 ± 0.11^{1c}	1.57 ± 0.11^{1c}	2.93 ± 0.11^{1b}	1.53 ± 0.11^{1b}	3.47 ± 0.11^{1b}	1.87 ± 0.11^{1b}
Vibrio parahaemolyticus	0	-	-	-	-	-	-	-	-
	24	1.27 ± 0.14^{3a}	1.00 ± 0.14^{3a}	1.10 ± 0.14^{3c}	-	1.40 ± 0.14^{3a}	0.43 ± 0.14^{3a}	1.33 ± 0.14^{3b}	1.13 ± 0.14^{3b}
	48	2.27 ± 0.14^{2a}	1.13 ± 0.14^{2a}	1.53 ± 0.14^{2c}	1.07 ± 0.14^{2c}	1.93 ± 0.14^{2a}	1.33 ± 0.14^{2a}	1.93 ± 0.14^{2b}	0.63 ± 0.14^{2b}
	72	2.27 ± 0.14^{1a}	1.73 ± 0.14^{1a}	2.37 ± 0.14^{1c}	1.57 ± 0.14^{1c}	2.73 ± 0.14^{1a}	1.80 ± 0.14^{1a}	2.40 ± 0.14^{1b}	1.27 ± 0.14^{1b}
Vibrio alginolyticus	0	-	-	-	-	-	-	-	-
	24	1.33 ± 0.09^{3b}	-	1.27 ± 0.09^{3b}	-	1.70 ± 0.09^{3a}	0.63 ± 0.09^{3a}	1.60 ± 0.09^{3b}	0.73 ± 0.09^{3b}
	48	2.27 ± 0.09^{2b}	1.03 ± 0.09^{2b}	1.53 ± 0.09^{2b}	0.93 ± 0.09^{2b}	2.30 ± 0.09^{2a}	1.40 ± 0.09^{2a}	1.97 ± 0.09^{2b}	1.03 ± 0.09^{2b}
	72	2.40 ± 0.09^{1b}	1.57 ± 0.09^{1b}	2.73 ± 0.09^{1b}	1.43 ± 0.09^{1b}	2.53 ± 0.09^{1a}	0.97 ± 0.09^{1a}	2.33 ± 0.09^{1b}	0.93 ± 0.09^{1b}
Aeromonas hydrophila	0	-	-	-	-	-	-	-	-
	24	1.63 ± 0.20^{3ab}	2.27 ± 0.20^{3ab}	1.33 ± 0.20^{3c}	-	2.30 ± 0.20^{3a}	0.77 ± 0.20^{3a}	1.83 ± 0.20^{3b}	1.37 ± 0.20^{3b}
	48	2.63 ± 0.20^{2ab}	1.87 ± 0.20^{2ab}	2.57 ± 0.20^{2c}	1.70 ± 0.20^{2c}	3.03 ± 0.20^{2a}	2.10 ± 0.20^{2a}	3.37 ± 0.20^{2b}	1.40 ± 0.20^{2b}
	72	3.60 ± 0.20^{1ab}	2.20 ± 0.20^{1ab}	4.07 ± 0.20^{1c}	1.87 ± 0.20^{1c}	4.27 ± 0.20^{1a}	2.37 ± 0.20^{1a}	3.70 ± 0.20^{1b}	2.07 ± 0.20^{1b}

Approximately 750 nucleotides of 16S rRNA gene sequence of strain JAQ01 and JAQ02 was revealed to their closest relative with 99 % similarity. A total of 24 reference sequences were retrieved from the databases occupied by a large cluster of *Vibrio* sp. and *V. harveyi*. Phylogenetic analysis clearly revealed

that the JAQ01 and JAQ02 specifically fall in the radiation of *Vibrionaceae*family (Figure 2) with the closest relative are *Vibrio* sp. PaH2.19b (GQ391958) and *V. harveyi* (AB497061) respectively. The phylogenetic tree presented depicted their distant relation with *V. rotiferianus* (HQ425687) as the out-group. These strains were eventually named *Vibrio harveyi*JAQ01 and JAQ02 respectively.

Antagonistic Activity Assays

Table 3 illustrates the mean ± SE of the inhibition zone of target strains when tested against JAQ01 and JAQ02 at different pre-incubation periods and initial cell densities. The ANOVA analysis revealed that the growth inhibition of target strains was significantly affected by pre-incubation periods and initial densities of test strains. Obvious significant difference was observed between both tested strains as JAQ01 was found to produce greater inhibitory effects against all four target strains than JAQ02. In most cases, the inhibition zones resulted by JAQ02 did not achieve or slightly higher than the standard significant level of 1 cm.

Among other target strains, *A. hydrophila* seems to be the most susceptible as the inhibition zones were observed up to 4.27 ± 0.20 cm. Comparatively, the growth of *V. alginolyticus* and *V. parahaemolyticus* were less inhibited by both test strains. A relatively high inter-strain inhibition was observed when *V. harveyi* strain JAQ01 and JAQ02 were tested against *V. harveyi* ATCC35084. For all target strains, no significant differences were observed when the tested strains were administered at the initial cell densities of 10^6 and 10^8cfu ml^{-1} with exception to the *V. alginolyticus* and *V. harveyi* (Table 3). No inhibition of target strains was observed at 0 h pre-incubation period of the test strains.

Values for each test strain initial cell density in any one row preceded by the same superscript numbers are not significantly different at $P<0.05$. Values in any one column followed by the same superscript letters are not significantly different at $P<0.05$. "-" is referring to no inhibition zones were observed.

Antibiotic Susceptibility Assay

Antibiotic susceptibility assay revealed that JAQ01 and JAQ02 were susceptible to both broad spectrum antibiotics (chloramphenicol and tetracycline) and two of the aminoglycosides (gentamicin and kanamycin) used. On the other hand, both strains showed a fairly high level of resistance to ampicilin. Results for the rest of this assay were recapitulated in Table 4.

Table 4: Antibiotic susceptibility *Vibrio* sp. JAQ01 and JAQ02 isolated from the intestine of healthy farmed *Epinephelusfuscoguttatus*

Antibiotics	**Test strains**	
	JAQ01	**JAQ02**
Chloramphenicol (30 ug)	S	S
Tetracycline (30 ug)	S	S
Ampicillin (10 ug)	R	R
Gentamycin (10 ug)	S	S
Kanamycin (30 ug)	S	S
Streptomycin (10 ug)	I	R

R: resistant; I: intermediate; S: susceptible

Discussion

Up to now, there is no clear cut approach has been suggested to categorize a specific *Vibrio* species as either probiotics or pathogens to marine organisms. This is partly due to dissemination of their tremendous amount of strains in marine environment. For instance, a strain of *V. alginolyticus* have been reported effectively reducing diseases caused by *Aeromonas salmonicida*, *V. anguillarum* and *V. ordalii* (Austin *et al.*, 1995) when in fact, another strain has been reported as grouper pathogens by Lee (1995). This ambiguous circumstance has generated the idea that many *Vibrio* species are best categorized as opportunistic pathogens (Thompson *et al.*, 2004) and might be useful as probiotics.Ideally, the putative probiotic strain should favorably be of host origin, from where they were isolated (Verschuere *et al.*, 2000; Vine, 2004) with special attention were given to those that were nonhaemolytic and were isolated

from healthy organisms (Schulze *et al.*, 2006). All these criteria have been executed wonderfully by both *V. harveyi* JAQ01 and JAQ02 studied herein. Haemolytic activity of a bacterial strain was regularly signifying their pathogenicity. Previously, a study on *Mycobacterium avium* by Maslow *et al.*, (1999) has proposed that their haemolysin expression may play a role in the pathogenesis of invasive disease. Alternatively, present strains were suggested harmless to *E. fuscoguttatus*as they were isolated from the intestine of healthy individuals with a good growth. Indeed, *V. harveyi* was postulated as the microflora of *E. fuscoguttatus* since both strains were isolated from two geographically unrelated farms which practicing different management system. Therefore, the ability of these strains to act as biocontrol agents in aquaculture is worth for an extensive research.

Prior use as probiotics, the strains introduced need to be well characterized phenotypically and genotypically to provide an adequate information as a backbone of subsequent steps. Considering their basic phenotypic characters (Table 1 and Figure 1) profile of JAQ01 resembled more that of ATCC35084 compared to JAQ02. Previously, Gatesoupe (1997) reported the presence of a *Vibrio* isolates that shown phenotypic characteristics that are different than those of known *Vibrio* species. Phenotypic variability among strains belonging to the same species may result in misidentification of the bacterial isolates. The 16S rRNA gene sequence analysis was proposed and study showed that JAQ01 and JAQ02 were successfully identified down to the species level aided by this powerful molecular tool. A study by Drancourt and acquaintances (2000) on a large collection of environmental and clinical bacterial isolates recommended the usage of 16S rRNA gene to recover the failure of conventional identification. Conclusively, our study demonstrated that a sequence of approximately 700 bp of 16S rRNA gene generated by a pair of universal primer is enough for an accurate identification of *V. harveyi*.

Present study uncovered the functional requirement of JAQ01 and JAQ02 as probiotics via in vitro antagonism assay against selected known fish pathogens. The ability of a bacterial strain to inhibit the growth of pathogenic bacteria has been proposed to be one of the criteria used for the selection of probiotics (Vine, 2004). *Vibrio alginolyticus* and *V. parahaemolyticus* were chosen as target organisms since they have been listed as the most significant causative agents of vibriosis in cultured *E. fuscoguttatus*. Vibriosis has been reported affecting all growth stages of *Epinephelus* sp. and causing up to 50 % severe mortality (Ali *et al.*, 2008). *Aeromonas hydrophila* on the other hand is the common opportunistic bacteria associated with diseased freshwater organisms. They were also naturally found in marine environment which have a close contact with fresh water. Recently, a study by Thayumanavan *et al.*, (2003) has directly supported the incidence of haemolysin-positive *A. hydrophila* in freshly caught marine fishes and prawn. They eventually concluded that the *A. hydrophila* was the frequent contaminating agent of fishes caught at the Indian coast. In combination, successful growth inhibition of these pathogenic bacteria by JAQ01 and JAQ02 suggesting their potential to control the detrimental effect offered by selected pathogens in *E. fuscoguttatus* culture.

For many researchers (Riquelme *et al.*, 2001; Alavandi *et al.*, 2004; Vine, 2004), in vitro antagonism assay does not truly imply the effectiveness of a probiotic in vivo. However, they did agree that this criterion may systematically be used as a screening process to reduce the number of candidates for relatively more costly and complicated in vivo trials. In contrast, two separate studies made by Austin *et al.*, (1995) and Thompson *et al.*, (2010) have successfully proved the positive correlation between in vitro antagonism assay of *Vibrio* spp. and there in vivo trials. Thus, our study was specifically designed based on a strong belief that the in vitro assay may wholly or at least partly reflect their in vivo effectiveness.

In our study, this BLIS method was modified to evaluate the suitable initial cell densities for use in field, to generate a comparative data on the inhibitory effectiveness of JAQ01 and JAQ02 against the same range of pathogens and finally to assess the degree of inhibition of the pathogens as proposed by Hai *et al.*, (2007). Similarly, our data suggested that the optimal inhibition was observed when an appropriate initial cell density of JAQ01 and JAQ02 are allowed to grow and produce the antimicrobial compound prior the introduction of pathogens. As low as 10^6cfu ml^{-1} initial cell densities of test strains are able to significantly inhibit the target strains. Nevertheless, 10^8cfu ml^{-1} cell densities with 72 h pre-incubation periods of test strains are optimally recommended for all inter-species inhibition. Our study was further

supported by Prasad and acquaintances (2005) as they published a scientifically significant data on a novel bacteriocin-like substance (BLIS) produced by a pathogenic *V. harveyi* strain VIB571. The ability of this substance in mediating an inter-strain and inter-species inhibition was strongly demonstrated herein. Prudent safety considerations should takes place when considering a new strain to be used as probiotics and these includes the assessment of their antibiotic resistance (Saarela *et al.,* 2000). In the present study, the pattern of ampicillin and streptomycin resistance showed by strains tested (Table 4) are in accordance to few studies done on *Vibrio* spp. (Thakur *et al.,* 2003; Manjusha *et al.,* 2005; Vaseeharan *et al.,* 2005).

Earlier study made by Roque *et al.,* (2001) revealed that 68 % of the 144 Vibrios isolated from healthy and diseased penaeid shrimp were ampicillin resistance. In different study, a solid figure was observed by Vaseeharan and associates (2005) when all the *Vibrio* spp. and *Aeromonas* spp. investigated were 100 % resistant to ampicillin. Conversely, similar intermediate and resistant to streptomycin showed by JAQ01 and JAQ02 respectively were figured in two different studies performed by Manjusha *et al.,* (2005) and Vaseeharan *et al.,* (2005). The fact that neither ampicillin nor streptomycin has been employed in the farms from where they were isolated has led to the theory that the ampicillin and streptomycin resistance in both JAQ01 and JAQ02 are intrinsic.

The β-lactamases are known to be responsible in mediating the bacterial resistance to ampicillin. Studies on the β-lactamase genes (Teo *et al.,* 2000; Weng *et al.,* 2004) elicited that the β-lactamase was chromosomally encoded which further supporting that their production might have appeared in response to natural selection and not the effect of antibiotics usage in aquaculture. However, there is incomprehensible evidence that the novel gene encoded for β-lactamase in *V. harveyi* might have been carried in a transposon or integron (a mobile genetic element which capable of inter-species transfer) before being incorporated into the bacterial chromosome (Teo *et al.,* 2000). Strains acquiring this element carrying their resistance genes should not be used as probiotics. Therefore, further studies on the genetics of *V. harveyi*strain JAQ01 and JAQ02 becoming a necessity to understand the mechanism of their antibiotic resistance.

Conclusion

The two bacterial strains in the present study possess in vitro desirable traits as described above and may potentially use as probiotics in aquaculture. Lack previous indication on pathogenicity of *V. harveyi*to humans provided a solid basis for current study. However, further in vivo effect and genetic study must be assessed properly prior their application in aquaculture as this species have previous pathogenicity records to marine organisms. Study on specific modes of action and type of inhibitors produced by these strains are advised for this attempt. Our results suggest that *V. harveyi* strain JAQ01 is more capable to inhibit the growth of tested *Vibrio* spp. and *A. hydrophila* than *V. harveyi* strain JAQ02. Collectively, the data presented have successfully issued the future prospect of *V. harveyi* as probiotics.

ACKNOWLEDGMENT

The authors wished to thank Mr. Lu Kien Chee and Mr. Hussin Mat Ali for their cooperation and contribution during specimen collection in their respective farms. Financial support from Agro-Biotechnology Institute (ABI), Ministry of Science, Technology and Innovation (MOSTI) Malaysia is greatly acknowledged.

REFERENCES

Alavandi SV, Vijayan KK, Santiago TC, Poornima M, Jithendran KP, Ali SA and Rajan JJS (2004). Evaluation of *Pseudomonas* sp. PM11 and *Vibrio fluvialis* PM17 on immune indices of tiger shrimp, *Penaeusmonodon*. *Fish Shellfish Immunology* **17** 115-120.

Ali HM, Yussoff NHN, Om AD, Musa CUC and Othman MF (2008). Status and Prospects of grouper aquaculture in Malaysia. In: *The Aquaculture of Grouper*, edited by Liao IC and Leaño EM, Asian Fisheries Society, World Aquaculture Society, the Fisheries Society of Taiwan and National Taiwan Ocean University 155-175.

Austin B, Stuckey LF, Robertson PAW, Effendi I and Griffith DRW (1995). A probiotic strain of *Vibrio alginolyticus* effective in reducing diseased caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Disease* **18** 93-96.

Bassler BL, Greenberg EP and Stevens AM (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *Journal of Bacteriology* **179**(12) 4043-4045.

Douillet PA (2000). Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions 2. Use of single and multiple bacterial probiotics. *Aquaculture* **182** 241-248.

Drancourt M, Bellet C, Carlioz A, Martelin R, Gayral JP and Raoult D (2000). 16S Ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *Journal of Clinical Microbiology* **38**(10) 3623-3630.

Gatesoupe FJ (1997). Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. *Aquatic Living Resources* **10** 239-246.

Hai NV, Fotedar R and Buller N (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.

Hill JE, Baiano JCF and Barnes AC (2009). Isolation of a novel strain of *Bacillus pumilus* from penaeid shrimp that is inhibitory against marine pathogens. *Journal of Fish Disease* **32** 1007-1016.

Lee KK (1995). Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus* Bloch et Schneider. *Microbial Pathogenesis* **19** 39-48.

Makridis P, Martins S, Tsalavouta M, Dionisio LC, Kotoulas G, Magoulas A and Dinis MT (2005). Antimicrobial activity in bacteria isolated from Senegalese sole, *Soleasenegalensis*, fed with natural prey. *Aquaculture Research* **36** 1619-1627.

Manjusha S, Sarita GB, Elyas KK and Chandrasekaran M (2005). Multiple antibiotic resistances of *Vibrio* isolates from coastal and brackish water areas. *American Journal of Biochemistry Biotechnology* **1**(4) 201-206.

Maslow JN, Dawson D, Carlin EA and Holland SM (1999). Haemolysin as a virulence factor for systemic infection with isolates of Mycobacterium avium complex. *Journal of Clinical Microbiology* **37**(2) 445-446.

Oxley APA, Shipton W, Owens L and McKay D (2002). Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*. *Journal of Applied Microbiology* **93** 214-223.

Prasad S, Morris PC, Hansen R, Meaden PG and Austin B (2005). A novel bacteriocin-like substance (BLIS) from a pathogenic strain of *Vibrio harveyi*. *Microbiology* **151** 3051-3058.

Riquelme CE, Jorquera MA, Rojas AI, Avendaño RE and Reyes N (2001). Addition of inhibitor-producing bacteria to mass cultures of *Argopecten purpuratus* larvae (Lamarck, 1819). *Aquaculture***192** 111-119.

Roque A, Molina-Aja A, Bolán-Mejía C and Gomez-Gil B (2001). In vitro susceptibility to 15 antibiotics of vibrios isolated from penaeid shrimps in Northwestern Mexico. *International Journal of Antimicrobial Agents* **17** 383-387.

Ryu E (1938). On the Gram-differentiation of bacteria by the simplest method. *Journal of Veterinary Medical Science* **17** 31.

Saarela M, Mogensen G, Fondén R, Mättö J and Mattila-Sandholm T (2000). Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology* **84** 197-215.

Saeed MO (1995). Association of *Vibrio harveyi* with mortalities in cultured marine fish in Kuwait. *Aquaculture* **136** 21 – 29.

Schulze AD, Alabi AO, Tattersall-Sheldrake AR and Miller KM (2006). Bacterial diversity in a marine hatchery: Balance between pathogenic and potentially probiotic bacterial strains. *Aquaculture* **256** 50-73.

Sfanos K, Harmody D, Dang P, Ledger A, Pomponi S, McCarthy P and Lopez J (2005). A molecular systematic survey of cultured microbial associates of deep-water marine invertebrates. *Systematic and Applied Microbiology* **28** 242 – 264.

Sugita H, Okano R, Suzuki Y, Iwai D, Mizukami M, Akiyama N and Matsuura S (2002). Antibacterial abilities of intestinal bacteria from larval and juvenile Japanese flounder against fish pathogens. *Fish Science* **68** 1004-1011.

Tamura K, Dudley J, Nei M and Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24** 1596-1599.

Teo JWP, Suwanto A and Poh CL (2000). Novel β-lactamase genes from two environmental isolates of *Vibrio harveyi*. *Antimicrobial Agents and Chemotherapy* **5** 1309-1314.

Thakur AB, Vaidya RB and Suryawanshi SA (2002). Pathogenicity and antibiotic susceptibility of *Vibrio* species isolated from moribund shrimps. *Indian Journal of Marine Science* **32** 71-75.

Thayumanavan T, Vivekanandhan G, Savithamani K, Subashkumar R and Lakshmanaperumalsamy P (2003). Incidence of haemolysin-positive and drug-resistant *Aeromonas hydrophila* in freshly caught finfish and prawn collected from major commercial fishes of coastal South India. *FEMS Immunology Medical Microbiology* **36** 41-45.

Thompson FL, Iida T and Swings J (2004). Biodiversity of Vibrios. *Microbiology and Molecular Biology Review* **68**(3) 403-431.

Thompson J, Gregory S, Plummer S, Shields RJ and Rowley AF (2010). An in vitro and in vivo assessment of the potential of *Vibrio* spp. as probiotics for the Pacific White shrimp, *Litopenaeusvannamei*. *Journal of Applied Microbiology* **109**(4) 1177-1187.

Vaseeharan B, Ramasamy P, Murugan T and Chen JC (2005). In vitro susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Panaeusmonodon* hatcheries and ponds. *International Journal of Antimicrobial Agents* **26** 285-291.

Verner-Jeffreys DW, Shields RJ, Bricknell IR and Birkbeck TH (2003). Changes in the gut-associated microflora during the development of Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae in three British hatcheries. *Aquaculture* **219** 21-42.

Verschuere L, Rombaut G, Sorgeloos P and Verstraete W (2000). Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Review* **64**(4) 655–671.

Vine NG (2004). Towards the Development of a Protocol for the Selection of Probiotics in Marine Fish Larviculture. Thesis of Doctor of Philosophy of Rhodes University.

Weng SF, Chao YF and Lin JW (2004). Identification and characteristic analysis of the *amp* C gene encoding β-lactamase from *Vibrio fischeri*. *Biochemical and Biophysical Research Communications* **314** 838-843.