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Full Length Research Paper

Isolation, screening and characterization of potential probiotics from farmed tiger grouper (*Epinephelus fuscoguttatus*)

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A study was conducted to isolate, screen and characterize some potential probiotics from tiger grouper (*Epinephelus fuscoguttatus*). Collection of bacterial strains was isolated from intestines of 51 juveniles tiger grouper. Preliminary screening on 123 isolates demonstrated that 40 (32.5%) bacteria displayed strong antagonistic activity when tested against four fish pathogens. Most of them (n=32; 80%) are Gram-positive. Only nine (22.5%) out of the 40 isolates showed gamma haemolysis and they were grouped based on 16S rRNA gene sequence analysis. This genotypic strategy and antibiotic susceptibility assay were used to eliminate another six isolates considering their potential pathogenic nature and sequences similarity. Lastly, a modified BLIS assay was performed on the three selected probionts and *Bacillus cereus* JAQ04 was shown as the most promising probiotic candidates. This study suggested three promising probiotic candidates' namely *B. cereus* JAQ04 and *Micrococcus luteus* JAQ06 and JAQ07. However, thorough studies on all the three bacteria including in-depth assessment on their effect *in vivo* are recommended.

Key words: *Epinephelus fuscoguttatus*, intestinal microflora, aerobes, facultative anaerobes, probiotics.

INTRODUCTION

Probiotics are lately gaining importance as a major mean for controlling aquaculture pathogens. The use of probiotics has successfully been demonstrated in various marine fish and shellfish culture (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Ringø and Birkbeck, 1999; Verschuere et al., 2000). Probiotics is defined as microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health (Gatesoupe, 1999). Studies (Gatesoupe, 1999; Irianto and Austin, 2002; Schulze et al., 2006; Vine et al., 2006) have proven that addition of probiotics into water and/or feed can enhance the vitality of fish larvae by preemptively colonizing them with selected beneficial bacteria.

Researches in selecting bacterial probiotics were initially focused to be administered to adult and juvenile organisms. However, current attention has been shifted towards the fish and shellfish larvae (Hjelm et al., 2004; Qi, 2009). It has been suggested that a particular probiotic strain is likely to be much effective in the host species from where it was originated (Verschuere et al., 2000). Therefore, many probiotic candidates for aquatic larviculture have been isolated from adults (Gram et al., 1999; Rengpipat et al., 2000) and healthy larvae (Gatesoupe et al., 1997; Hjelm et al., 2004). Successful candidates were varied and belonging to *Bacillus* sp. (Rengpipat et al., 2000) *Pseudomonas fluorescens* AH2 (Gram et al., 1999), *Vibrio* sp. (Gatesoupe et al., 1997), and *Roseobacter* sp. (Hjelm et al., 2004).

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Figure 1. The collection sites of fish specimens showed geographically unrelated distribution.

Groupers under family Serranidae, are among the most important commercial fishes in the tropics and representing 3.7% of the total marine and brackishwater aquaculture production in Malaysia (DoF, 2009). Locally known as tiger grouper, *Epinephelus fuscoguttatus* has been listed as one of the highly valued at RM40 per kilo and a major farmed Asian species (Tucker, 1999; Ali et al., 2008). A remarkable growth and demand of this species in many parts of Southeast Asia is partly due to its adaptability in captivity and rapid growth in culture conditions (Rimmer, 2000).

Generally, in most intensive aquaculture, low and unpredictable larval and juvenile survival during first feeding is claimed to be associated with establishment of opportunistic bacteria in various marine organisms and environment. In the case of grouper culture, a range of bacterial diseases have been reported in the past few decades and the most notable impact was caused by vibriosis (Yii et al., 1997; Ali et al., 2008). Therefore, prevention of diseases has become an important prerequisite in numerous intensive farms and hatcheries. Incompetent usage of vaccination for certain diseases and drawback effects caused by excessive usage of antibiotics and chemotherapeutics has led probiotics as an excellent alternative strategy. There are several selection criteria listed for choosing probiotics including safety, function and technology (Saarela et al., 2000). A systematic *in vitro* test on a large number of isolates promises an elimination of less-promising candidates, hence reducing the number of isolates for *in vivo* trials.

The main purpose of this study was to isolate, screen and characterize intestinal bacteria of healthy tiger grouper for potential use as probiotics. Following isolation process, bacterial identification and characterization was made by means of both genotypic and phenotypic strategies. A series of consecutive multi-level screening approach was selected namely, evaluation of the inhibitory potential towards four fish pathogens, haemolytic activity and antibiotic susceptibility assays.

MATERIALS AND METHODS

Fish samples

Healthy individuals of 51 juvenile groupers were randomly collected from three different farms namely Fish Landing Jetty, Semerak, Pasir Putih, Kelantan (A); Global Hi-Q Biotech Sdn. Bhd., Pangkor, Perak (B) and Tanjung Badak Breeding Centre, Tuaran, Sabah (C) (Figure 1). Fishes were weighed, measured and immediately sacrificed by pithing and collections of intestines were performed at the farm sites. The whole intestines were then aseptically removed (from anus to the pyloric caeca) and kept chilled (0 to 4°C) in individual scintillation vials containing 10 ml sterile phosphate buffer saline (PBS) (Merck, Germany). The specimens were transported to the laboratory within 24 h of collection.

Bacterial isolation

Upon arrival at laboratory, the intestines were washed with sterile PBS to remove excess fats and blood. The whole intestines were then minced individually with sterile scissor, weighed to 1 g and homogenized in 9 ml PBS. Serial dilutions of the homogenate were then performed up to 10^{-8} using PBS. All samples were plated simultaneously onto two non-selective media: 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 following the spread plate method and incubated at 25 ± 1°C for 4 days. After incubation period, colony-forming units (cfu) per gram of sample were counted after which 5 colonies were selected randomly from each plate and were streak on fresh media for isolation of pure colony. All the selected colonies were labeled accordingly, maintained onto agar slant and frozen at -80°C in marine broth 2216 (MB) (Difco, Becton Dickinson and Company, MD USA) or tryptic soy broth (TSB) (BBL, Becton Dickinson and Company, MD USA) + 1.5 % NaCl containing 80%

(v/v) glycerol while being investigated.

Bacterial identification and grouping

The isolates were identified to the generic levels following standard biochemical tests as follows: Gram reaction was conducted 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) and cell shape and cultural characteristics as they developed onto MA. Motility test, endospore staining (Schaeffer and Fulton, 1933) and metabolism of glucose in oxidation/fermentation basal medium (Merck, Germany) (Hugh and Leifson, 1953) were carried out for selected strains. Species level identification was performed using API kit (API, BioMerieux, France) and BBL Crystal identification system (Becton Dickinson and Company, MD USA). Purity of the selected isolates were regularly checked and maintained throughout the study. Grouping of the isolates was carried out based on the phenotypic traits observed as described above. The isolated species were finally classified according to Bergey's Manual of Systematic Bacteriology.

Preliminary screening by using cross streak method

A total of 123 isolates were collectively screened for their antagonistic activity by using a perpendicular cross streak method as described by Alippi and Reynaldi (2006) with minor modifications. A range of known fish pathogens (that is, *Vibrio alginolyticus* ATCC33839, *V. parahaemolyticus* ATCC43996, *V. harveyi* ATCC35084 and *Aeromonas hydrophila* ATCC35654) were used as target strains. Briefly, the isolates to be tested and the target strains were grown overnight in 10 ml of TSB+ 1.5% NaCl. A streak of 25 mm of 10^8 cfu/ml isolates was made at the centre of a plate of TSA+ 1.5% NaCl. The plates were seeded with 10^8 cfu/ml target strains by a single streak across the original streak of isolates following the incubation at 25°C for 3 days. Plates were further incubated for two days and the inhibition zone was observed. Isolates with antagonistic activity against all four target strains were short listed as probiotics candidates (probionts). The experiment was performed in triplicate.

Haemolytic activity assay

The shortlisted probionts were further subjected to a haemolytic assay by inoculating them onto blood agar base (Scharlau Chemie, S.A., Spain) supplemented with 0.5% defibrinated horse blood. The plates were incubated and the haemolytic zones were observed. The probionts were subsequently classified as alpha, beta or gamma haemolysis.

Deoxyribonucleic acid (DNA) extraction, 16S rRNA gene sequencing and phylogenetic analysis

Those probionts which exhibit gamma haemolysis were selected for 16S rRNA gene sequence-based identification. Bacterial cells for DNA extraction were collected with a sterile cotton swab and inoculated into MB. Overnight culture of 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 primers 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 mM MgCl, 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, extension at 72°C for 1 min and final extension step at 72°C for 10 min. The PCR product was visualized on 1% agarose gel elctrophoresis. The amplified products were then purified with GeneJET PCR purification kit (Fermentas, Europe) and subsequently sent for sequencing (1st BASE, Malaysia). 16S rRNA gene sequences of probionts were used as query to search for homologous sequences in the GenBank, RDP and DDBJ database. Sequences were aligned by using the ClustalW program and MEGA 4.0 software for phylogenic inference.

Antibiotics susceptibility assay

Kirby-Bauer method as proposed by Lalitha (2004) was used to determine the antibiotic susceptibility of all the selected strains. The susceptibility against the following antibiotics; chloramphenicol (30 µg), tetracycline (30 µg), streptomycin (10 µg), kanamycin (30 µg), gentamicin (10 µg) and ampicilin (10 µg) were determined on the surface of Mueller-Hinton agar (Difco, Becton Dickinson and Company, MD USA) plates with Bio-discs (BioMerieux, France).

Bacteriocin-Like inhibitory substances (BLIS) assay

An advanced antagonistic activity assay was performed on selected probionts by means of BLIS or cross-streaking method as described by Hill et al. (2009). Selection of three inhibitory isolates for BLIS assay was done based on the results of previous experiments. Screening process started from preliminary characterization on culture media followed by preliminary test on antagonistic activity against four fish pathogens, haemolytic nature of isolates and 16S rRNA gene sequence-based identification.

Modification of the original method was made to assess the effect of the initial densities of isolates administered and different pre-incubation period of isolates prior to the antagonism test 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 and 10^9 cfu/ml. 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 (probionts) at four different cell densities. The first set of plates, denoted as 0 h pre-incubation period was cross- streaked with the 10^8 cfu/ml active growing target strains (perpendicularly across the vertical streak). The plates were then incubated at 25°C for 24 and 48 h followed by observation of the inhibition zones. The width of inhibition zones were measured to the nearest millimeters (mm) and recorded. Significant inhibition zones were considered for those greater than 10 mm. A set of plates for each strain was cross-streaked for every 24 h. The experiment was performed in triplicate for 72 h and the experimental design used was illustrated in Figure 2.

Results were scored and presented as the levels of inhibition which were defined as 0 (no inhibition), 1 (1 – 5 mm), 2 (6 – 10 mm), 3 (11 – 15 mm), 4 (16 – 20 mm), 5 (21 – 25 mm), 6 (26 – 30 mm), 7 (31 – 35 mm) and 8 (36 – 40 mm).

RESULTS

Bacterial isolation, identification and grouping

A total of 123 intestinal aerobes and facultative anaerobes

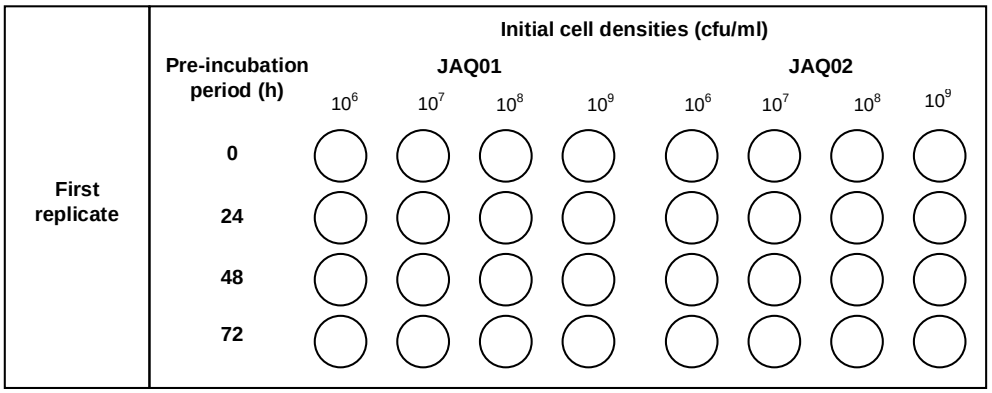

Figure 2. Illustration Shows the First Replicate of BLIS Assay. Each circle represents a combination treatment of initial cell densities (cfu/ml) and pre-incubation period (h). Each row signifies the term 'set' in explanatory text.

were successfully isolated from 51 healthy juveniles *E. fuscoguttatus* cultured at the three farms in Malaysia. Phenotypic analysis of grouper intestinal microflora has discerned 20 genera out of the 101 isolates. Based on Bergey's Manual of Systematic Bacteriology classification (Table 1), 22 isolates remained unidentified, which included 10 Gram negative and 12 Gram positive isolates.

Preliminary screening on the antagonistic activity of isolates and haemolytic assay

Out of the 123 isolates, only 40 (32.5%) displayed strong antagonistic activity, mostly (n=32; 80%) Gram-positive bacteria. Of these 40 isolates, nine (22.5%) showed gamma haemolysis and they were preliminarily coded with a JAQ number (Table 2). Among those, six isolates originated from Farm A, two were isolated from fishes of Farm B and one isolates from Farm C.

16S rRNA gene sequence analysis

Following sequencing of the 16S rRNA gene, nine probionts were classified into respective genus (Figure 3). Approximately, 750 nucleotides of 16S rRNA gene sequence of the probionts were revealed to their closest relative with >98% similarity. Phylogenetic analysis clearly revealed their relation between one another (Figure 3).

The phylogenetic analysis showed that 55.6% (n = 5) of the probionts were in agreement with the results of their respective biochemical characterization. The remaining four showed negative correlation between phenotypic and genotypic characters (Table 2). At the end of the experiment, five probiotics candidates namely *Vibrio harveyi* JAQ01, *V. harveyi* JAQ02, *Bacillus cereus* JAQ04 and *Micrococcus luteus* JAQ06 and JAQ07 were selected for further assessments.

Antibiotics susceptibility

All five promising probionts investigated showed susceptibility to tetracycline and two of the aminoglycosides, the gentamicin and kanamycin. Both JAQ04 and JAQ06 were susceptible to all antibiotics tested. Resistance response on ampicilin was recorded for JAQ01 and JAQ02. Both JAQ02 and JAQ07 seem to be resistant to streptomycin while JAQ01 showed an intermediate response.

Bacteriocin-like inhibitory substances (BLIS) assays

Evaluating the results from the antibiotics susceptibility assay, three candidates (*B. cereus* JAQ04 and *M. luteus* JAQ06 and JAQ07) were chosen. Figure 4 illustrates the level of inhibition of target strains when tested against these three selected probionts at different pre-incubation periods. The growth inhibition of target strains was greatly affected by pre-incubation periods and initial densities of the test strains. For all target strains tested, the strongest inhibitory activity has always corresponded with the JAQ04 at 48 and/or 72 h pre-incubation time and 10^8 and/or 10^9 cfu/ml initial cell densities. However, the same levels of inhibitions were recorded for *V. alginolyticus* when tested against all three probionts. For *A. hydrophila*, the strongest inhibition was also contributed by the initial addition of *M. luteus* JAQ06 at 10^8 cfu/ml with 72 h pre-incubation period. In contrast, the weakest antimicrobial activity was contributed by JAQ06 against *V. harveyi* and *V. parahaemolyticus* as shown in Figure 4. Since the analysis was made on the individual target strain, no comparative data on the most susceptible target strains can be generated. For all target strains, relatively high differences were observed among

Table 1. The isolated species classified according to Bergey's Manual of Systematic Bacteriology (1984).

Phylum	Family	Genus	Species
Proteobacteria	Alteromonadaceae	*Shewanella*	*S. putrefaciens*
	Vibrionaceae	*Photobacterium*	*P. damsela*
		Vibrio	*V. alginolyticus*
			V. vulnificus
			V. fluvialis
			V. mimicus
	Aeromonadaceae	*Aeromonas*	*A. salmonicida spp salmonicida*
			A. hydrophila group 1
			A. hydrophila group 2
	Enterobacteriaceae	*Enterobacter*	*E. sakazaki*
		Shigella	*Shigella sp.*
	Pasteurellacea	*Pasteurella*	*P. pneumotropica haemolytica*
	Burkholderiaceae	*Burkholderia*	*B. pseudomallei*
	Neisseriaceae	*Chromobacterium*	*C. violaceum*
Firmicutes	Bacillaceae	*Bacillus*	*B. licheniformis*
			B. cereus
			B. megaterium
			Bacillus sp.
	Paenibacillaceae	*Brevibacillus*	*B. brevis*
	Staphylococcaceae	*Staphylococcus*	*S. cohnii spp cohnii*
			S. equorum
			S. sciuri
	Carnobacteriaceae	*Alloiococcus*	*A. otitidis*
	Streptococcaceae	*Streptococcus*	*S. intermedius*
	Incertae Sedis	*Helcococcus*	*H. kunzii*
Actinobacteria	Micrococcaceae	*Micrococcus*	*M. luteus*
	Microbacteriaceae	*Leifsonia*	*L. aquaticum*
	Bifidobacteriaceae	*Gardnerella*	*G. vaginalis*
	Dermacoccaceae	*Kytococcus*	*K. sedentarius*
	Corynebacteriaceae	*Corynebacterium*	*Corynebacterium sp.*
			C. jeikeium

the pre-incubation periods with 72 h as the optimum pre-incubation period needed prior to introduction of target strains. No inhibition of target strains was observed at 0 h pre-incubation period of the test strains.

DISCUSSION

The primary steps involved in the selection of a bacterial probiotics to be used in an aquaculture system are collection of background information and acquisition of potential probiont (Gomez-Gil et al., 2000). Based on the preliminary grouping strategy, we found that the phenotypic characterization of fish intestinal isolates herein has resulted in a wide collection of bacterial species. This collection is an advantage to our study as hundreds (or thousands) of bacteria need to be isolated and tested before eventually nominated as potential probiotic bacteria (Gram and Hjelm, 2002). Sugita et al. (2002) have claimed that, in most studies, only 1 to 10% of intestinal bacteria of fish possess an inhibitory activity against fish pathogenic bacteria.

In search of the best suited bacterial probiotics for grouper larviculture, a series of consecutive screening process was performed to cover both functional and safety aspects of the probiotics for aquaculture. These *in vitro* tests are commonly being used to eliminate the less potential microorganisms from available large pool of candidate probionts to a more acceptable number for further testing (Vine et al., 2006). In many probiotic studies (El-Rhman et al., 2009; Goldschmidt-Clermont et al., 2008; Riquelme et al., 1997), assessment on the

Table 2. A comparative data on the basic generic characteristics of probionts and genus or species as identified using kits with respective confidence percentages and 16S rRNA gene sequence analysis.

Characteristics	Probionts								
	JAQ01	JAQ02	JAQ03	JAQ04	JAQ05	JAQ06	JAQ07	JAQ08	JAQ09
Gram staining	-	-	+	+	+	+	+	+	+
Morphology	SR	LR	C	LR	C	C	C	C	C
Catalase	+	-	+	+	+	+	+	+	+
Oxidase	+	+	-	-	-	-	-	-	-
Indole	+	-	-	-	-	-	-	-	-
Swarming on TSA (+ 1.5 % NaCl)	+	-	-	+	-	-	-	-	-
Growth on TCBS agar	Y	G	nd	nd	nd	nd	nd	nd	nd
Identification based on kits	*Pasteurella multocida* (51.4%)	*Photobacterium damsela* (62.7%)	*Micrococcus* sp. (74.3%)	*Brevibacillus brevis* (78.8%)	*Kytococcus sedentarius* (98.3%)	*Micrococcus luteus* (96.7%)	*M. luteus* (97.3%)	*M. luteus* (95.6%)	*M. luteus* (97.4%)
Identification based on 16S rRNA gene sequence	*Vibrio harveyi*	*V. harveyi*	*Staphylococcus pasteuri*	*Bacillus cereus*	*Kytococcus sedentarius*	*M. luteus*	*M. luteus*	*M. luteus*	*M. luteus*
Origin	Farm C	Farm A	Farm B	Farm A	Farm A	Farm A	Farm B	Farm A	Farm A

Note: [a] TCBS, thiosulfate citrate bile salt sucrose agar; +, positive; -, negative; Y, yellow colonies formed; G, green colonies formed; SR, short rods; LR, long rods; C, cocci; nd, not determined.

antagonistic activity of the isolates were set as the main screening process. Similarly, in this study, the functional requirement of the intestinal isolates to be use as probiotics was assessed by measuring their inhibitory spectrum towards fish pathogens. The ability of a bacterial strain to inhibit the growth of pathogenic bacteria has been proposed to be the first criteria used for the selection of probiotics (Vine et al., 2006). The selection of *Vibrio alginolyticus* and *V. parahaemolyticus* as target strains reflected the most significant causative agents of vibriosis in cultured *E. fuscoguttatus* (Ali et al., 2008). *Aeromonas hydrophila* and *V. harveyi* on the other hand are the common opportunistic bacteria associated with diseased freshwater and marine organisms, respectively (Thayumanavan et al., 2003). Potential probionts displaying a broad antagonistic activity were chosen, since the principal *in vivo* effects are not known. Thus, only isolates with antagonistic activity against all four target strains were selected at present and categorized as strong antagonists. Of 123 isolates, only 40 displayed strong antagonistic activity while the remaining was categorized as either weak (growth inhibition of only one target strain), medium (growth inhibition of two or three target strains) or no antagonistic activity (no growth inhibition of any target strains). This preliminary screening has resulted in an elimination of 67.5% (n =83) of the total isolates from current study. Successful growth inhibition of all four pathogenic bacteria by 40 other isolates suggesting their potential to control the detrimental effect offered by a broad spectrum of pathogens in *E. fuscoguttatus* culture.

To further reducing the number of candidates for *in vivo* study, the haemolytic nature of 40 selected probionts were evaluated. Nine isolates showing gamma haemolysis indicating no breakdown of red blood cells are finally classified as potential probiotics and being used for further characterization. Such safety consideration is very important when considering a new strain to be use as probiotics. Haemolytic activity of a bacterial strain regularly signifies pathogenicity. Previously, a study on *Mycobacterium avium* by Maslow et al. (1999) has concluded that haemolysin expression may play a role in the pathogenesis of invasive disease. Alternatively, probiotics strains that showed gamma haemolysis herein were suggested harmless to tiger grouper as they were isolated from the intestine of healthy individuals with a good growth. Therefore, the ability of these

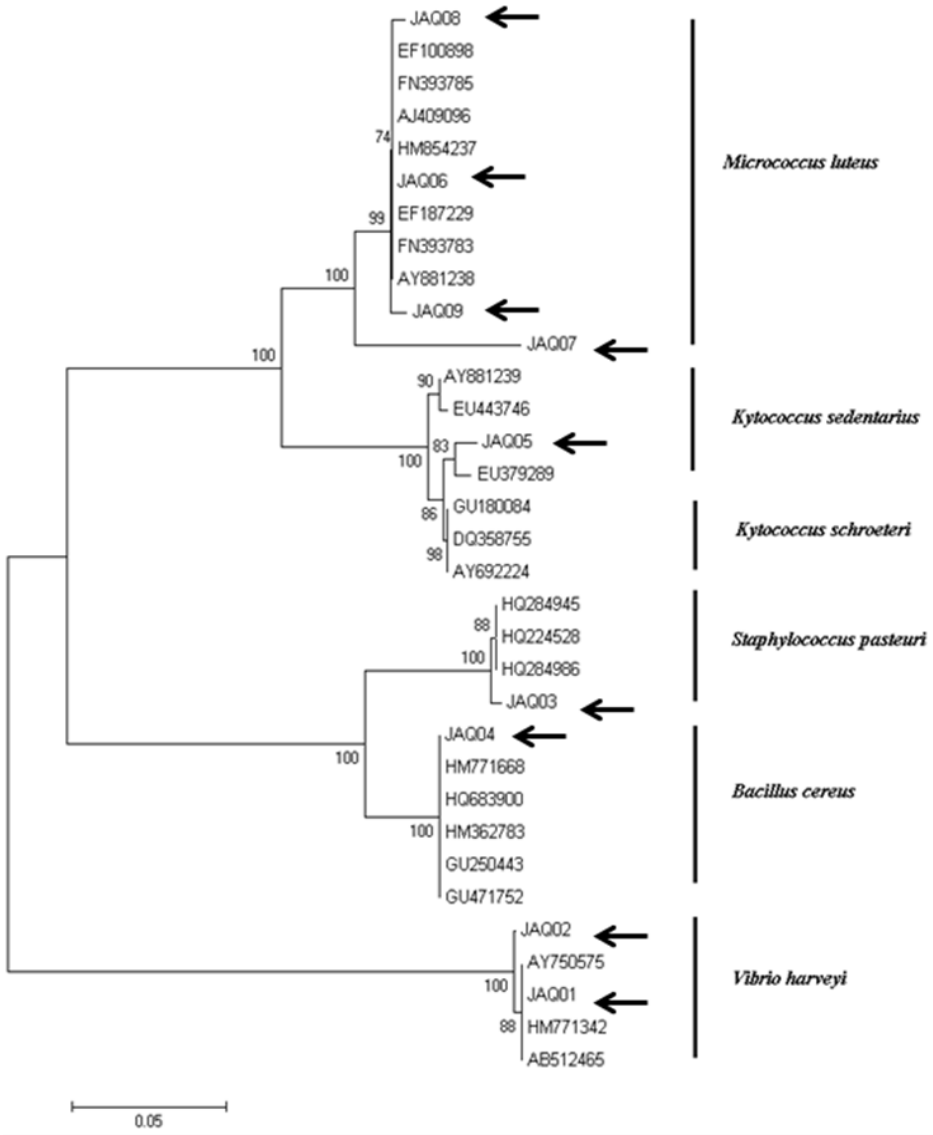

Figure 3. Evolutionary distances of 16S rRNA gene of nine probionts computed using Kimura-2 parameter method with 1000 bootstrap test. Reference sequences are labeled with their respective GenBank, DDBJ and RDP accession number.

nine strains to act as biocontrol agents in aquaculture is worth an extensive research.

Depending too much on commercial kit for phenotypic characters tend to be a problem and in this study four probionts; JAQ01, JAQ02, JAQ03 and JAQ04 were wrongly identified. Similarly, Gatesoupe (1997) reported the presence of a *Vibrio* isolate that showed phenotypic characteristics that were different than those of known *Vibrio* species. Phenotypic variability among strains belonging to the same species may result in misidentification of the bacterial isolates. Error in interpretation of the test results may partly contribute to the failures in such conventional identification. Alternatively, 16S rRNA gene sequence analysis was proposed to be used and successfully identified the probionts down to the species level. A study by Drancourt et al. (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 750 bp of 16S rRNA gene generated by a pair of universal primer was successfully grouped the probionts into five genera (Figure 3). At the end of the experiment, two potential human pathogens namely *Staphylococcus pasteuri* JAQ03 and *Kytococcus sedentarius* JAQ05 were eliminated from this study. Considering their phenotypic characters and sequence similarity as observed in 16S rRNA gene sequences analysis, we postulated that *M. luteus* JAQ06, JAQ08 and JAQ09 are actually the same bacterial species. Thus, only

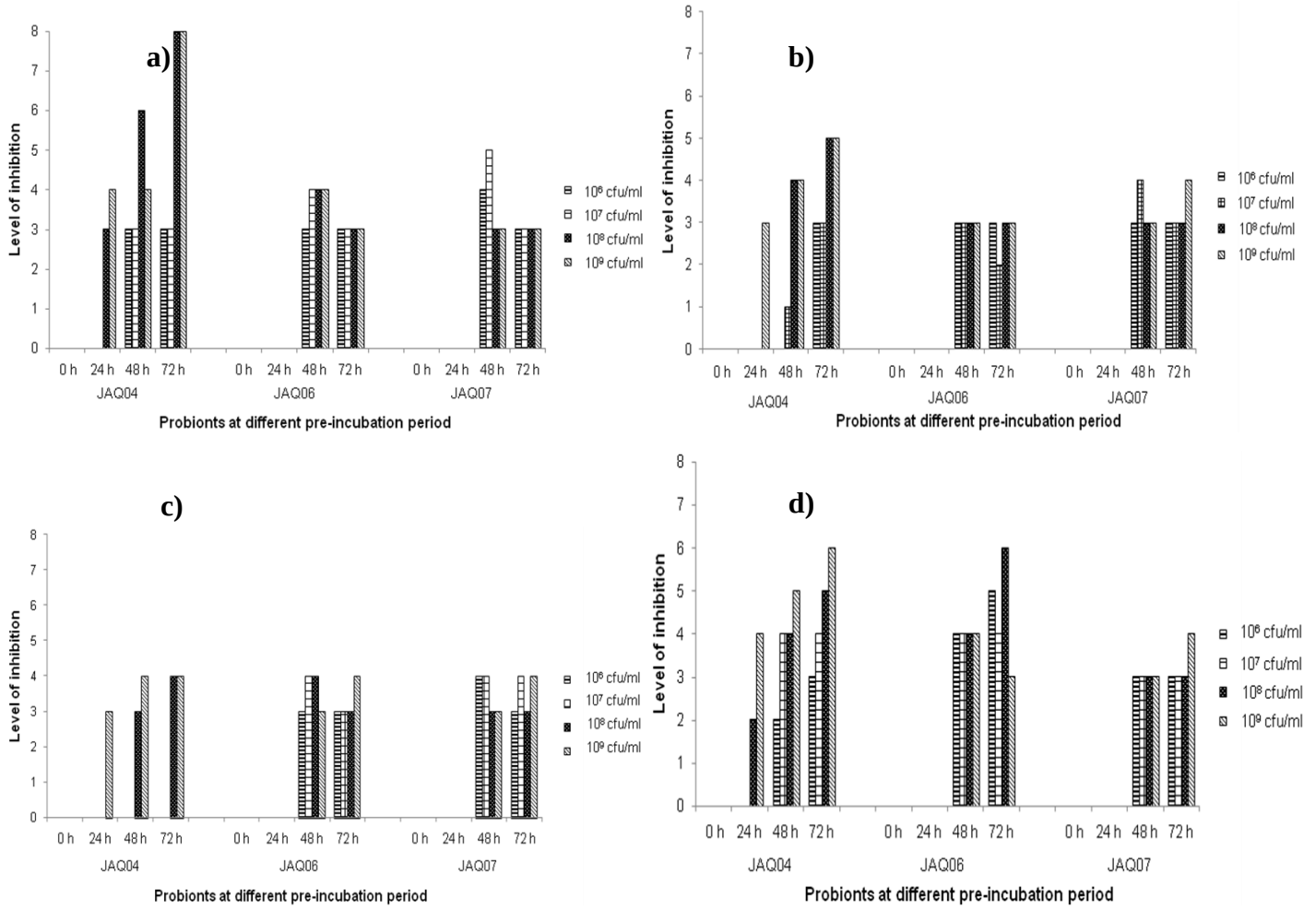

Figure 4. The level of inhibition for each probionts when tested against a) *Vibrio harveyi*; b) *V. parahaemolyticus*; c) *V. alginolyticus* and d) *Aeromonas hydrophila* at different pre-incubation periods of probionts prior antagonism test. The probionts were administered at the initial densities of 10^6, 10^7, 10^8 and 10^9 cfu/ml.

JAQ06 was chosen as representative for further study.

Safety consideration was further being investigated by an antibiotic susceptibility assay against common clinical and farmed-used antibiotics. In the present study, the pattern of ampicillin and streptomycin resistance showed by strains JAQ01 and JAQ02 are in accordance to studies done on *Vibrio* spp. (Thakur et al., 2002; Manjusha et al., 2005; Vaseeharan et al., 2005). Similar resistance activity on streptomycin was also recorded for JAQ07. 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 those three probionts are intrinsic.

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. In addition, the presence of some *V. harveyi* was associated with mortalities in cultured grouper in Kuwait (Saeed, 1995) and thus vibrios are categorized as opportunistic bacteria which support our suggestion to exclude both *V. harveyi* JAQ01 and JAQ02 from this study. Thus, only three isolates were finally selected for advance study.

A modified BLIS or cross-streaking method was further employed to validate the first screening process as proposed by Hai et al. (2007). It is apparent that the inhibition of pathogens by all potential probiotics tested is largely dependent on the initial concentration introduced and time to grow prior encountering the target strains. According to Hill et al. (2009), such results suggesting the importance of good water quality and maintenance of a healthy gut microflora to prevent pathogens from being able to establish.

At present, BLIS assay has successfully revealed the potential use of *Bacillus* and *Micrococcus* spp. as probiotics

for grouper culture. Similarly, the application of *Bacillus* sp. as probiotics in aquaculture has been documented in many research articles (Guo et al., 2009; Jamilah et al., 2009; Wang and Gu, 2010; Powedchagun

et al., 2011; Nayak and Mukherjee, 2011) and mostly were focused on their application on shrimp culture. Considering their capacity to inhibit the pathogenic bacteria in this assay, *B. cereus* JAQ04 seems to be the most potent candidate over the other two isolates. This result was in agreement with a study by Guo et al. (2009) as the *B. cereus biovar toyoi* tested has also exhibited antagonistic activity against selected target organisms. On the other hand, the prospect of *M. luteus* as probiotics was demonstrated delightfully by El-Rhman et al. (2009). In their study, the organism tested was able to inhibit growth of *A. hydrophila in vitro*. Further *in vivo* study recommended the use of *M. luteus* as probiotic for promoting growth performance and health of Nile tilapia, *Oreochromis niloticus*.

Collectively, three potential probiotics associated with farmed tiger groupers investigated herein may play a role in disease control. In our study, the *B. cereus* JAQ04 was nominated as the most competent candidate. However, the host-origin and non-haemolytic nature of *M. luteus* JAQ06 and JAQ07 in addition to their ability to inhibit pathogens and their sensitive and intermediate susceptibility to all the antibiotics tested cannot be neglected. Therefore, thorough studies on all three bacteria should be taken into consideration including an extensive assessment on their effect *in vivo*.

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