FIRST ISOLATION OF *VIBRIO PROBIOTICUS* FROM THE GUT OF *PORTUNUS PELAGICUS* (LINNAEUS, 1758) AND ITS PROBIOTIC COMPETENCY AGAINST SHELLFISH PATHOGENS

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Abstract

In this study, the *Vibrio probioticus* was isolated from the gut of female *Portunus pleagicus*, identified through 16S rDNA gene sequencing, and was evaluated for its probiotic features. The probiont was tested in co-culture assay at various concentrations against three target shellfish pathogens including *V. harveyi*, *V. parahaemolyticus* and *Pseudalteromonas piscicida*. In a co-culture assay it was apparent that the *V. probioticus* had shown to outcompete the growth of tested pathogenic bacteria due to its antibacteriocin properties. Different ratios produced different effect on tested pathogens, however, the highest ratio (pathogen:probiiont) was more effective in suppressing the pathogen. A maximum inhibition growth was recorded for *V. harveyi* and *P. piscicida*. The probionts could further be tested *in vivo* culture environment to understand the effectiveness in microbial control of *P. pelagicus* larviculture and other aquaculture species.

Keywords: *Vibrio probioticus*; 16S rDNA sequencing; Antibacteriocin; Probiiont; Larviculture

Introduction

The intensive rearing conditions for marine fish larvae provide an environment favourable for pathogenic bacteria [1] that may cause microbial problems and high larval mortality. To overcome of the microbial problems encountered in the intensive rearing of marine larvae, controlled colonization of the bacterial flora can be achieved by replacing with probiotic bacteria [2]. Most important bacterial pathogens of fish include the gram-negative species, which cause the infections and result high mortality in fish hatcheries. At present, owing to disease and high mortalities in hatchery operations, microbial infections are generally cured either through chemotherapeutic treatment of antibiotics or disinfectants. However, frequent use of antibiotics has led to drug-resistant bacteria that are difficult to control and exterminate [3, 4, 5, 6]. Probiotics can be the alternative, which may reduce the frequency of microbial proliferation or diminish the sternness of disease eruptions. Probiotics are defined as “live microorganisms, which when administered in sufficient quantity bestow a health benefit to the host” [7]. When selecting probiotic microorganisms with beneficial health effects for the host, many criteria have to be met. For example, probiotic species selection requires cautious testing of potential species benefits both *in vitro* and *in vivo*. The mechanisms used include the production of inhibitory substances against pathogens, a benefit effect to the host; moreover, species of bacteria require careful screening and selection before incorporation as a probiotic.

The aim of the current study was to characterize the probiotic properties of *V. probioticus* isolated from the gut of blue swimmer crab, *P. pelagicus* by examining antibacterial properties against three shellfish pathogens (previously isolated from *P. pelagicus*).

Materials and Methods

Study site and sampling site

The present study was conducted in the laboratory of the Institute of Tropical Aquaculture, Universiti Malaysia Terengganu (UMT), Malaysia. Crab sample was collected from Strait of Tebrau, Johor, Malaysia, (1° 22' N and 103° 38' E) with 188.4 gm of body weight (BW), 13.4 cm carapace width (CW) and 6.3 cm carapace length. The collected sample was transferred to 40 litres round polythene tanks filled with sea water from sampling site and equipped with aeration along with other crab samples. Salinity at site was measured between 31 - 33 ppt with Refracto-meter (Reichert Tech). To avoid contamination, the sample was kept in same sea water until it dissected for microbes study.

Removal of the gut and bacteria culture

Prior to segregation of the gut, crab specimen was bathed in 10% formalin for 20 minutes. Subsequently, it was again washed with fresh tap water for 5 minutes and finally washed with sterilised de-mineralised water in order to get rid of surface micro flora if attached to it. Sterilised dissecting materials were used for dissecting purpose.

Aseptically crab specimen was dissected, the whole gut (foregut and hindgut) was removed and washed two times with sterilised de-mineralised water then pulverised with pestle and mortar vigilantly and mixed with sterilised sea water to prepare the inoculums.

Bacteria isolation were carried out by 10 fold serial dilution up 10^{-5} . A volume 0.1 ml of inoculums was plated in selective culture media Thiosulphate Citrate Bile Salts (TCBS, Difco, USA) for the bacterial growth. Culture media was prepared in sterilised sea water (31 ± 2 ppt). Inoculated plates were incubated at 37°C for 24 hours. A pure culture was obtained by repeated streaking method.

Total genomic DNA extraction

Total genomic DNA of sample was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instruction. Briefly, 1 ml of overnight culture was centrifuged for 2 minutes at 13,000 rpm. The cells were then suspended in 480 μl of 50mM EDTA and 120 μl of lytic enzyme before incubation at 37°C for 60 minutes. The mixtures were then centrifuged for 2 minutes at 13,000 rpm and the supernatant was removed. Nuclei Lysis Solution 600 μl was added to the pellet and mixed gently by pipetting. The mixture was then incubated for 5 minutes at 80°C and left over to cool down at room temperature. RNase Solution 3 μl was added to the mixture, and incubated at 37°C for 30 minutes and subsequently cooled at room temperature. Afterwards 200 μl of Protein Precipitation Solution added to mixture, vortexed and incubated on ice for 5 minutes, followed by centrifugation at 13,000 rpm for 3 minutes and supernatant was transferred to a clean tube containing 600 μl of room temperature isopropanol and mixed properly. The mixture was centrifuged for 2 minutes at 13,000 rpm, and the supernatant decanted, and then 600 μl of 70 % ethanol (room temperature) was added, mixed, and centrifuged for 2 minutes at 13,000 rpm. The ethanol subsequently aspirated and the pellet air-dried for 10–15 minutes. Following the rehydration of DNA pellet in 100 μl of Rehydration Solution for 1 hour at 65°C or overnight at 4°C .

Polymerase Chain Reaction (PCR) amplification of 16S rDNA

The 16S ribosomal DNA was amplified by PCR using bacterial universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R-(5'-GGTTACCTTGTTACGACTT-3'). Briefly, the PCR reaction was performed in a Bio Thermal cycler (Bio-Rad, USA), with an initial denaturing step at 95°C

for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C for 30 sec, and 72°C for 1 minute, and ended with a final extension step of 72°C for 15 minutes. The PCR product was electrophoresed in 1% molecular grade agarose gel (Aventis)) stained with 1 $\mu\text{g}/\text{ml}$ of ethidium bromide and was then visualised using Alpha Imager gel documentation system (Alpha Innotech, UK). Sequences obtained were analysed and compared with sequences from GenBank, using BLAST NCBI citation (<http://blast.ncbi.nlm.nih.gov>).

Morphological, physiological and biochemical characteristics of isolate

Morphological, physiological and biochemical features were carried out using standard procedures and BBL Crystal Kit (Becton, Dickinson and company, USA), was also used.

Target pathogen

Three shellfish pathogens isolated from the gut of female *P. pelagicus* and were identified as *V. harveyi*, *V. parahaemolyticus* and *P. piscicida* [8] were used as the target pathogens.

Co-culture antagonistic assay

The co-culture method was performed according to Rahman et al. [9] to observe the antagonistic potential of the probiont growing with the target pathogens including *V. harveyi*, *V. parahaemolyticus* and *P. piscicida*. Bacterial population of 10^6 cells measured to optical density ($\text{OD}_{630\text{nm}}$) was used as standard ratio for the experiment. The culture broth of the *V. probioticus* and the target organisms were inoculated separately into 50 ml marine broth (Difco) prepared in natural sea water. The different combinations of bacterial population at ratios 1:1, 1:2, 1:3 and 1:4 were obtained by mixing the culture of target pathogens and the *V. probioticus* bacteria respectively. The co-cultures was prepared in duplicates and incubated at 37°C , samples were serially diluted up to 10^{-5} and 0.1 ml from each co-culture was plated daily in TCBS agar and marine agar up to four days for vibrios and *P. piscicida* enumeration respectively.

Statistical analysis of data

Comparison followed by ANOVA ($p < 0.05$), treatment and inoculum concentration being the factors. Post hoc comparisons were compared using LSD test and Tukey's test. All statistics were performed using SPSS version 16 for Microsoft Windows.

Results

Segregated foregut and hindgut of *P. pelagicus* is shown in Figure. 1 respectively.

Figure1. Images show the foregut (a) and hindgut (b) of blue swimming crab, *P. pelagicus*.

Morphological, physiological and biochemical features of *V. probioticus*

Cells of *V. probioticus* strain appeared bright yellow, smooth-rounded colonies of >6 mm diameter on TCBS agar at 37°C after 48 hours. Physiological and biochemical characteristics of *V. probioticus* are illustrated in Table 1.

Identification of *V. probioticus*

Sequences obtained through 16S rDNA were analyzed and compared with sequences from data base GenBank using BLAST (megablast) National Center for Biotechnology Information (NCBI, USA) online data bank citation (<http://blast.ncbi.nlm.nih.gov>). Result obtained from GenBank isolate as *V. probioticus* (Accession No. AJ345063.1).

Co- culture antagonism assay

The *V. probioticus* isolate which demonstrated maximum reduction of pathogens count following each day, preferably at a lower ratio were considered to be the most potential candidate. As a result, the *V. probioticus* isolate demonstrated the antagonistic activity against all pathogens. A maximum inhibition growth was recorded for *V. harveyi* and *P. piscicida* (Fig. 2 and 3), whereas the isolate showed moderate activity against the target *V. parahaemolyticus* (Fig. 4).

Figure 2. Co-culture experiments using *V. harveyi* as a target pathogen and probiotic bacteria at different population ratios (Pathogen: Probiot). The result is expressed as a mean of duplicate observations in terms of *Vibrio* count on TCBS agar for four-day long antagonistic activity of the probiont. Means with different letters on bars were not significantly different from control ($p>0.05$).

On the day four, only *V. harveyi* pathogens was eliminated by tested *V. probioticus*. Different ratios had shown different effect on target pathogens and higher concentrations of probiont showed better result compared to lower concentration. The concentration ratio at 1:4 totally eliminated *V. harveyi* after 96 hours (Fig 2). In general all pathogens were affected by the antibacterial activity of the tested *V. probioticus*.

Figure 3. Co-culture experiments using *P. piscicida* as a target pathogen and probiotic bacteria at different population ratios (Pathogen: Probiot). The result is expressed as a mean of duplicate observations in terms of *p. piscicida* count on marine agar for four-day long antagonistic activity of the probiont. Means with different letters on bars were not significantly different from control ($p>0.05$).

Figure 4. Co-culture experiments using *V. parahaemolyticus* as a target pathogen and probiotic bacteria at different population ratios (Pathogen: Probiot). The result is expressed as a mean of duplicate observations in terms of *V. parahaemolyticus* count on TCBS agar for four-day long antagonistic activity of the probiont. Means with different letters on bars were not significantly different from control ($p>0.05$).

Table 1: Physiological and biochemical characteristics of *V. probioticus*.

Characteristics	Results	Characteristics	Results
Growth on TCBS	Yellow	Arginine dihydrolase	-
Growth in NaCl		Amylase production	+
0%	+	D-serine	-
2%	+	2-aminoethanol	-
3%	+	Fermentation	+
7%	+	Phenylalanine Deaminase	-
10%	+	Fermentation to acid	
12%	-	Glucose	+
Growth in sea water	+	Sucrose	+
Growth at °C		Lactose	+
4°C	-	Amygdalin	+
25°C	+	Utilization of	
35°C	+	Citrate	-
42 °C	+	Glucose	+
55°C	-	Lactose	-
DL-carnitine	-	Sucrose	+
Oxidase	+	Casein	+
Catalase	+	Lipid	+
Motility	+	Glycine	+
Indole	+	L-Arginine	-
H ₂ S formation	-	L-Tyrosine	+
Urease	-	L-Serine	+
Inositol	-	Acetate	-
Rhamnose	-	Gelatin liquefaction	-
Melibiose	-	Glycogen	-
L-threonine	-	N-acetyl-D-galactosamine	-
Tween 40	-	D-glucosaminic acid	-
Xylitol	-	NO ₃ reduction	+

Note: Abbreviations; - negative, + positive

Discussion

With the development of commercial-scale aquaculture, application of probiotics in aquaculture is increasing [4, 10]. During the present study, the growth of pathogenic microbes was controlled by non-pathogenic *V. probioticus*. In co-culture experiments result showed that the inhibitory activity of the *V. probioticus* increased with increasing density of the bacteria. A relatively higher concentration of antagonist (probiotic) was required to inhibit the targeted pathogens in the co-culture experiments. The present study demonstrated that the probiotics at higher levels than the pathogens, the degree of inhibition increased with the level of probiotic. Therefore, a potential probiotic co-culture must either be supplied on a regular basis or be able to colonise and multiply on or in the host. Hence, it can be suggested that the isolate of *V. probioticus* species, as discovered from the gut of female *P. pelagicus* crab are very considerable in terms of their capability to produce anti-bacterial component, which suppressed or eliminated pathogens.

antibacterial capabilities due to bacteriocin production of gut *Vibrio* species bacteria isolated from juvenile and larvae of Japanese flounder (*Paralichthys olivaceus*) inhibited *Pasteurella piscicida* growth [11]. In addition, five isolates from the gut of common clownfish (*Amphiprion percula*) were found to be inhibitory against a wide variety of pathogens, such as *Aeromonas hydrophila*, *A. salmonicida*, *V. harveyi*, *V. anguillarum*, *V. damsela*, *V. alginolyticus* and *Carnobacterium piscicola* [12]. In another study, *Carnobacterium* bacteria inhibited the *V. anguillarum* in the turbot (*Scophthalmus maximus*) and author concluded the use of intestinal bacteria with antagonistic activity may be used to reduce the load of *Vibrio* species in turbot hatcheries [13].

As above mentioned, the target pathogens were influenced by the production of inhibitory compounds by the tested probiotic. However, the results of co-culture assay suggest that pathogens were killed or their growth was inhibited as a result of production of antimicrobial substances by the tested probiotic. The results of present study was in match with the previously mentioned studies [12, 13] that probiotic are able to inhibit the growth of pathogens. In cited literature *V. probioticus* also

Antibacterial activity between microorganisms in nature occurs frequently. The

known as *V. hepatarius* and can be found in aquatic animals and the marine environment (14). *V. probioticus* isolate/strain when administrated showed high levels of colonisation in the hepatopancreas of *Litopenaeus vannamei* shrimp, outcompeted, excluded the shrimp pathogen *V. harveyi*, and enhanced the health and weight of shrimps (14). Relating to *P. pelagicus* literature cited do not offer any information on this probiont, therefore, this is first report for isolation and characterisation of this bacteria as probiont from the gut of female *P. pelagicus*.

Probiotic usages offer a very promising alternative to the use of antibiotics in aquaculture. Further study is needed to elucidate the exact mode of action of the observed beneficial effects and to understand the possibilities and limitations of microbial control in aquaculture particularly in hatchery operations.

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