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Isolation and Characterization of Probiotic Bacteria from the Gastrointestinal Tract of Pond-cultured *Litopenaeus vannamei* in Tuaran, Sabah

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Abstract: Sabah has the highest annual white leg shrimp (*Litopenaeus vannamei*) aquaculture production in Malaysia. However, disease infection is one of the big problems to white leg shrimp aquaculture. One of the promising control strategies is the using of probiotics. Currently, the study on probiotic in Sabah is very limited. Therefore, this study aimed to isolate, characterize and identify potential probiotic bacteria from the gut of pond-cultured white leg shrimp in Tuaran, Sabah. Eight out of a total of twenty one isolates from TSA, Rogosa and MRS agar were screened out and designated as GS4, GS11, GS12, GS14, GS15, WS1, WS3 and WS5. However, four isolates (GS11, GS12, GS15 and WS5) had probiotic potential for shrimp culture. They are identified as *Shewanella* sp. (WS5), *Bacillus thuringiensis* (GS11), *Lactobacillus plantarum* (GS12), and *B. cereus* (GS15). Among the four selected isolates, GS15 has the highest probiotic potential due to positive antagonistic activity against pathogenic *V. harveyi*. Further research such as *in vivo* assay still needs to be conducted to test the probiotic potential of the four selected isolates from the gut of collected *L. vannamei* samples. Besides being used to support disease management of *L. vannamei* aquaculture in Sabah, all four isolates might help aquaculture of other penaeid shrimp or non-penaeid shrimp species.

Keywords: Probiotic Potential, *Bacillus*, Shrimp Industry, Probiotic Screening, Antagonistic Test

Introduction

Global demand and consumption for shrimp can be fulfilled, due to the change of wild caught shrimp increased in relation to aquaculture produced shrimp. Aquaculture of shrimp enables higher production that lowers the price down. According to FAO (2016), the annual per capita availability of shrimp has increased from 0.4 kg in 1961 to 1.8 kg in 2013. The sharp decrease of price can be seen with 15-20% drop in price from the first half of 2014 to the first half of 2015. In Sabah, the production of white leg shrimps (*Litopenaeus vannamei*) has reported a hike of 38% from 2013 to 2014, with RM321.08 million in 2014 (Borneo Post Online, 2015). Although the production of white leg shrimp is high in Sabah, instead of price falling, the price continues to soar from 2.91 USD/kg in 2012 to 4.12 USD/kg in 2014 (Annual Fisheries Statistics, 2000-2016). However, shrimp production encountered with a decrease in 2015 when the industry was hit by disease problem (FAO, 2016). The production of white leg shrimp decreased from around 14,000 metric tonnes in 2015 to around 10,000 metric tonnes in 2016 (Annual Fisheries Statistics, 2000-2016). This is bad news, given that some private companies had invested RM1.12 billion in shrimp farms establishment in Sabah, particularly in Pitas, Kota Belud, Tawau and Kudat (Borneo Post Online, 2015).

The diseases that impacted white leg shrimp can be classified into infectious and non-infectious diseases. Among these two categories, infectious diseases that include viral, bacterial, fungal and parasitic diseases are the most common and troublesome (Lavilla-Pitogo *et al*., 2000; Leaño, 2001; Briggs *et al*., 2004; Manual of Diagnostic Tests for Aquatic Animals, 2016). Since disease is such a big problem to the white leg shrimp aquaculture, control strategies must be implemented to prevent the disease outbreak. The control strategies can be the use of specific pathogen free (SPF) postlarvae (PL), disease management, effluent management, phage therapy, chemical and antimicro-bials treatments, vaccination and probiotic (Lakshmi *et al*., 2013). Some of these practices are effective but intensive and superintensive culture of shrimp become more common until these practices cannot follow up with the culture size (Lakshmi *et al*., 2013). The use of antibiotic has resulted in antibiotic resistant bacteria in shrimp that can be harmful to human (Angulo *et al*., 2004; Le *et al*., 2005). Although shrimp's immune system can be induced with DNA vaccination or viral envelope protein, however the effectiveness of vaccine decreases over time (Witteveldt *et al*., 2004;

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Rout *et al.*, 2007).

Therefore, probiotic is chosen in this study for its advantages. Probiotic can secrete antimicrobial or diffusible and volatile compounds that can inhibit the growth of pathogens, stimulate the immune system of shrimp by up-regulating immune-related genes, increase environmental stress tolerance to salinity and temperature changes, enables higher survival rate for cultured shrimp, improve water quality parameters such as pH and ammonia level and stimulates the growth of shrimp (Gullian *et al.*, 2004; Chaurasia *et al.*, 2005; Das *et al.*, 2006; Balcázar and Rojas-Luna, 2007; Chiu *et al.*, 2007; Castex *et al.*, 2010; Liu *et al.*, 2010; Vieira *et al.*, 2010). Probiotic has been tremendous economic profitability, with the current price of probiotic ranges from USD 1 to USD 100 per kilogram. It was projected to have market value of USD 5.07 billion by 2022, with North American countries were expected to be primary markets and Asia-Pacific region were expected to improve the probiotic market (Markets Insider, 2017).

Many similar studies have been conducted to isolate the probiotic strain from the gut of *L. vannamei*. There are studies by Widarnarni *et al.* (2015) in Indonesia, Liu *et al.* (2014) in China, Leyva-Madrigal *et al.* (2011) in Mexico and Zokaei Far *et al.* (2013) in Malaysia. However, the report is incomplete since some of the information on the identity of the isolate is unknown and probiotic screening assay is insufficient. Moreover, there is no similar study has been conducted in Sabah.

Therefore, the bacterial strains were isolated, characterized and identified from the gastrointestinal tract of *L. vannamei* for potential probiotic bacteria. The objective of this study was to examine the characteristics and identify the potential probiotic bacteria from the gastrointestinal tract of *L. vannamei* in Sabah.

Materials and Methods

Bacterial Isolation

Pond-cultured adult shrimps were obtained from Borneo Venture Farm Sdn. Bhd. and packed into plastic bag containing half-filled oxygenated pond water. The plastic bags were tied with rubber band and put in a polyfoam box with ice pack. This is to maintain low temperature in the box while transporting the shrimp to laboratory (Goodrick and Paterson, 1992). The shrimp was then killed humanely by thermal shock through immersion in ice bath at 4°C before processed (Lucas and Southgate, 2012).

The shrimp was washed with 70% ethanol before the sampling of the gastrointestinal tract (GIT) (Liu *et al.*, 2014). The GIT was used for the isolation of probiotic since according to Shakibazadeh *et al.* (2009), the digestive system of *P. monodon* has the highest bacteria content, with varieties of bacteria species, compared to body surface and muscles. The isolation of probiotic bacteria was done according to Sánchez-Ortiz *et al.* (2015). The GIT was removed aseptically with sterile tweezers from the shrimp after dissection. About 1 g of GIT and a loopful of pond water sample were then streaked onto the surface of non-selective media agar such as Tryptone Soya Agar (TSA) (HiMedia, Mumbai, India) supplemented with 2% sodium chloride (Merck, Darmstadt, Germany) and selective media such as MRS (Merck, Darmstadt, Germany) and Rogosa (Oxoid, Basingstoke, United Kingdom) which were prepared following manufacturer's instructions. The GIT and water sample were inoculated in each medium in duplicate. The sample in TSA was then incubated at 37°C, whereas the sample in MRS or Rogosa was incubated at 30ºC. TSA, MRS and Rogosa agar were observed at 24, 48 and 72 h and after 10 days of incubation. Each bacterium was cross-streaked in the same medium to obtain pure cultures.

Preparation of Bacterial Culture, Bacterial Suspension and Bacterial Glycerol Stock

Single colony from pure culture was used to inoculate 150 mL of Tryptone Soya Broth (TSB) (HiMedia, Mumbai, India) or MRS broth (Merck, Darmstadt, Germany) according to the media used to isolate the respective bacteria. The media containing bacterial isolates were then incubated at 37°C for 24 h for TSB and 30°C for 48 h for MRS broth. About 25 mL of the bacteria culture was then centrifuged at 4,400 rpm for 10 minutes. The growth medium was decanted (gradually poured out without disturbing the sediment) and the pellet was double washed with respective growth medium. The pellet was resuspended in 2% sterile saline solution with adjusted pH of 6.5 ± 0.2 with 1 M NaOH to avoid false positive, in order to obtain a final bacterial suspension. Bacterial suspension was adjusted to an optical density of 0.5 ± 0.005 in a DiluPhotometer (Implen, Munich, Germany) at 600 nm (Saini, 2010 and Sánchez-Ortiz *et al.*, 2015).

Bacterial glycerol stock was prepared for preserving bacterial isolates for future probiotic bacteria selection assays, biochemical test and molecular identification. Bacterial glycerol stock was

prepared by mixing 250 μL glycerol (Virra-Vista, Kuala Lumpur, Malaysia) and 750 μL bacterial culture in 1.5 mL vial. The mixture was mixed thoroughly and stored in -80°C freezer (New Brunswick, New Jersey, United States).

Probiotic Bacteria Selection Assays

The selection for probiotic bacteria is conducted by assessing the characteristic of bacterial isolates using eight different types of assays. They are microbial adhesion test to hydrophobicity, aggregation assay, tolerance to ammonia, pH tolerance test, salinity tolerance test, extracellular enzymatic tests and antagonistic test against pathogenic bacteria.

Microbial Adhesion Test to Hydrophobicity (MATH)

Microbial Adhesion Test to Hydrophobicity (MATH) is conducted to study the adhesion of bacteria to hydrocarbon as a representation of adhesion to wall of intestines in shrimp. MATH was conducted as a screening assay according to the method by (Saini, 2010; Sánchez-Ortiz *et al.*, 2015). About 800 μL bacterial suspension was added into a 1.5 mL vial, followed by addition of 200 μL P-xylene (Bendosen, Masai, Johor Bahru, Malaysia). Next, the tube was vortexed vigorously for two minutes. The suspension was allowed to rest at room temperature for 15 minutes to allow hydrocarbon-aqueous phase separation. A portion of the aqueous phase was pipetted out from the base of the tube and transferred to a quartz cuvette for absorbance measurement at 600 nm and recorded as A_f. Triplicates were done between each sample. Between the triplicates from the same sample, cuvette was washed with distilled water, whereas ethanol and distilled water was used to wash the cuvette in between different samples. A control containing only the bacterial suspension and without the hydrocarbon was measured for its absorbance and recorded as A_0. The cell surface hydrophobicity in unit percentage was calculated using the formula $100 \times A_0\text{-}A_f/A_0$. The bacterial isolate with cell surface hydrophobicity value was considered low if it was less than 30%, the value was medium if it was 30% - 60% and was high with value of more than 60%. Bacterial isolates with low adhesion of less than 30% were not considered as potential probiotic bacteria and were not selected for next assay.

Aggregation Assay

Aggregation assay is conducted to determine the effectiveness of colonizing the intestinal tract. The aggregation assay is conducted following method described by (Del Re *et al.*, 2000). 150 mL of TSB or MRS broth, according to selected bacterial isolates, was inoculated with single bacterial colony from pure culture. The media were then incubated at 37°C for 24 h for TSB and 30°C for 48 h for MRS broth. 25 mL of the bacteria culture was then centrifuged at 4400 rpm for 10 minutes and suspended in 2% sterile saline solution with pH 6.5±0.2 to form bacterial suspension. Bacterial suspension was adjusted to optical density of 0.5±0.005 at 600 nm. 1.5 mL of bacterial suspension was transferred to 1.5 mL vial and centrifuged at 10000 g for 10 minutes. The bacterial pellet was then re-suspended with their respective culture medium. After incubation at 30 - 37°C for 2 h, 0.75 mL of the upper suspension was transferred for absorbance measurement. Aggregation was expressed as 1 - (O.D. upper suspension/O.D. total bacterial suspension)×100. The test was conducted in triplicates. Bacterial isolates with high auto-aggregation percentage of more than 80% aggregated immediately, forming a precipitate and clear solution. Bacterial isolates with auto-aggregation percentage of 20 - 80% that showed both a precipitate and constant turbidity, were considered having medium auto-aggregation percentage. Bacterial isolates with low auto-aggregation percentage of less than 20% were unable to auto-aggregate and produce constant turbidity. Bacterial isolates with low percentage were not considered as potential probiotic bacteria.

Tolerance to Total Ammonia Nitrogen

Tolerance against total ammonia nitrogen (TAN) is done because in intensive culture of shrimp, the high protein content of 20-25% in feed can release high amount of ammonium and organic nitrogen into the water. Probiotic can convert ammonia to nitrite and nitrate to reduce accumulation of toxic ammonia (Sánchez-Ortiz *et al.*, 2015).

A stock solution of ammonia of 9430 mg/L was prepared by dissolving 9.43 g of dehydrated ammonium sulfate $(NH_4)_2SO_4$ (R&M Chemicals, Essex, United Kingdom) in 1000 mL of distilled water. 9.43 g of dehydrated ammonium sulfate was dissolved in 100 mL of distilled water in beaker, and transferred to volumetric flask of 1000 mL volume. The beaker was washed with distilled water, followed by transferring the water into the volumetric flask. The volumetric flask was filled with more distilled water until it reached the indicator line. Different levels of TAN of 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 15.0, 20.0, and 25.0

mg/L were prepared, each up to 200 mL. Then, each TAN level of 200 mL was mixed with TSB and 1.5% NaCl. 5 mL of each TAN level was transferred into test tube and autoclaved before used (Devaraja *et al.*, 2013).

Bacterial culture of selected bacterial isolate was prepared by inoculating 20 μL of bacterial isolates from glycerol stock in TSB and incubated at 37°C for 24 h. Then, 100 μL bacterial culture were inoculated into each test tube containing different TAN levels, and incubated at 30°C for 7 days. The test was duplicated with control containing 5 mL of the mixture of 200 mL distilled water with TSB and 1.5% NaCl without addition of bacteria, and repeated for each selected bacterial isolates. Bacterial growth was observed after 7 days (Devaraja *et al.*, 2013).

Salinity Tolerance

Salinity tolerance determination is important as well because in the delivery pathway to the host species, the probiotic bacteria may need to go through environment with different range of salinity. Test tubes each with 5 mL TSB each was supplemented with 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12% NaCl. Bacterial culture was prepared by inoculating 20 μl of bacterial glycerol stock in TSB and incubated at 37°C for 24 h. Each tube with different salinity was then inoculated with 100 μL of bacterial culture and incubated at 37°C for 7 days. The experiment was conducted in duplicates and repeated for each selected isolates. Bacterial growth was observed after 7 days of incubation (Sánchez-Ortiz *et al.*, 2015).

pH Tolerance

pH tolerance test is done to observe the growth of bacteria in different pH and to study the resistance against gastric acid. Test tubes each with 5 mL TSB were adjusted to pH of 4, 5, 6, 7, 8, 9, and 10 with addition of 1M HCl and NaOH. Bacterial culture of selected isolate was prepared by inoculating 20 μl bacterial glycerol stock in TSB and incubated for 24 h. Each tube was then inoculated with 100 μL of bacterial culture and incubated at 37°C for 7 days. The experiment was conducted in duplicates and repeated for selected bacterial isolates. Bacterial growth was observed after 7 days of incubation (Sánchez-Ortiz *et al.*, 2015).

Extracellular Enzymatic Activity (Proteolytic and Lipolytic Properties Test)

The determination of enzymatic activity is done by using proteolytic and lipolytic tests. Ability of bacterial isolates to produce enzymes such as protease and lipase can increase the digestibility by the host species (Widarnarni *et al.*, 2015).

Skim milk agar with TSA and 2% skim milk (Sigma-Aldrich, St. Louis, Missouri, United States) was prepared for proteolytic test. Bacteria from broth culture was streaked in TSA with 2% skim milk, and incubated at 37°C for 24 h in proteolytic test. The test was conducted in duplicates and repeated for each selected bacterial isolates. The positive result in proteolytic test was recorded if the clear zone present at or around the bacteria colony (Colwell and Grigorova, 1987 and Widarnarni *et al.*, 2015). In lipolytic test, TSA with 1% Tween 80 (Merck, Darmstadt, Germany) was prepared and autoclaved. The bacterial culture was streaked onto the agar and incubated at 37°C for 24 h. The test was conducted in duplicates and repeated for selected bacterial isolates. After incubation, positive results were recorded when bright green color was observed around the colony (Colwell and Grigorova, 1987; Widarnarni *et al.*, 2015). Finally, bacteria isolates with at least one digestive enzyme property were considered as potential probiotic (Colwell and Grigorova, 1987; Widarnarni *et al.*, 2015).

Test of Antagonistic Activity

Antagonistic tests are conducted to select bacterial strains that are suitable to be probiotics. The test is conducted using cross-streak method by Zokaei Far *et al.* (2013). The bacteria isolates that show inhibitory effect against tested pathogen are considered as probiotics. Pathogen used was *V. harveyi* VHJR7 (Ransangan and Mustafa, 2009). The plate was incubated at 37°C for 8 h. Inhibition of *V. harveyi* growth towards the bacterial isolates was recorded as positive result for antagonistic activity against *V. harveyi*.

Biochemical Test

The selected bacterial isolates with probiotic potential from screening assay were used on subsequent biochemical test. Biochemical test using RapID NF Plus Panels (Remel, San Diego, California, United States) was carried out according to manufacturer's instructions. The inoculated panel was then incubated for 4 h at 35 - 37°C. After 4 h, colour changes were observed at all the ten reaction cavities and recorded in the report form, with comparison of colour with the provided interpretation guide from the kit. ERIC

Database was assessed online for identification of bacteria species based on the results recorded on report form (Remel, 2017; Song and Leff, 2005).

Molecular Identification of Selected Strains

DNA was extracted from each selected bacterial isolate using Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin, United States) with slight modifications. After bacteria inoculation in TSB, and overnight incubation at 37°C, about 1.5 mL of bacterial culture was pelleted at 13,000 rpm for 2 minutes. The supernatant was discarded, followed by suspension of pelleted cells in 480 µl of 50 mM ethylenediaminetetraacetic acid (EDTA). 120 µL of lysozyme was added to the cells suspension and incubated at 37°C for 45 minutes. After incubation, centrifugation was done at 13,000 rpm for 2 minutes. The supernatant was discarded, followed by addition of 600 µL Nuclei Lysis Solution (Promega, Madison, Wisconsin, United States) to lyse cells. Pipette was used to gently mix the Nuclei Lysis Solution with the pellet by repeatedly pipetting in and out of the solution. Incubation was done at 80°C for 5 minutes, then was cooled to room temperature. 3µL of RNase Solution (Promega, Madison, Wisconsin, United States) was added and pipetted gently to mix. Another incubation was done at 37°C for 45 minutes, then was cooled to room temperature. After the cells were completely lysed, the protein was precipitated by adding 200 µL of Protein Precipitation Solution (Promega, Madison, Wisconsin, United States) and vortexed. Incubation was performed close to ice for 5 minutes. Then, centrifugation was done at 13,000 rpm for 3 minutes. Supernatant from the centrifuged product was transferred to another clean 1.5 mL tube, added with 600 µL of room temperature isopropanol for DNA precipitation. The mixture was pipetted gently to mix. The new tube was centrifuged at 13,000 rpm for 2 minutes, and the supernatant was decanted after centrifugation. 600 µl of room temperature 70% ethanol was added to the pellet and pipetted gently to mix. Another centrifugation at 13,000 rpm was done for 2 minutes. The supernatant ethanol was decanted and aspirated for 15 minutes for complete removal of ethanol from the DNA pellet. Lastly, DNA pellet was rehydrated with 100 µL Rehydration Solution (Promega, Madison, Wisconsin, United States) for 1 hour at 65°C or overnight at 4°C. After rehydration, the extracted DNA was kept at -20°C for future use (Promega, 2010).

The quality of DNA was checked by electrophoresis with 1.5% 1x TAE agarose gel stained with 5 µL RedSafe (iNtRON Biotechnology, Seongnam, South Korea) for visualization. Amplification of 16S ribosomal DNA was performed. This was done by 31.1 µL autoclaved distilled water, 10 µL 5 × Colorless Go Taq® Flexi Buffer (Promega), 3.4 µL $MgCl_2$ (25 mM, Promega), 1.0 µL dNTPs (10 mM, Promega), 1.0 µL of each primer 33F (27 nmol, N-Gene Research Laboratories, New York, United States) and 1449R (26.8 nmol, N-Gene Research Laboratories), 0.5 µL Go Taq® Flexi DNA polymerase (5 U/µL, Promega) and 2.0 µL template DNA in the reaction mixture totaling to 50 µL. The primers used were 33F 5'-GAA CGC TGG CGG CAG GCC TAA-3' and 1449R 5'-ACT CCC ATG GTG TGA CGG CGG-3' with a final concentration of 10 mM. The reaction mixture was then subjected to conditions of pre-heating at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, an annealing step at 56°C for 30 s and an extension at 72°C for 30 s, followed by a final extension at 72°C for 5 minutes. After amplification, the amplified products were kept at -20°C for future use (Leyva-Madrigal *et al.*, 2011). The amplified products were analyzed with electrophoresis as well in a 1.5% agarose gel. Purification was done with Invisorb® Fragment CleanUp (Stratec Molecular, Berlin, Germany). A GeneQuant™ pro RNA/DNA calculator (Biochrom, Cambridge, United Kingdom) was used to measure the concentration of DNA. The purified PCR product was sent for sequencing at Biotechnology Research Institute, Universiti Malaysia Sabah. The sequencing result was analyzed using BLAST analysis to find closes homology for the identity of the isolates (Leyva-Madrigal *et al.*, 2011).

Results

Bacterial Isolation

A total of twenty one bacterial isolates were found from both the gastrointestinal tract of *L. vannamei* and the pond water sample. Table 1 shows the number of bacterial isolates isolated from the gastrointestinal tract of shrimps and water samples in different agar media. From TSA, ten of the bacterial isolates from the shrimps' gastrointestinal tract were obtained, whereas the other six bacterial isolates were isolated from the water sample of the pond where the shrimps were cultured. Two bacterial isolates were isolated from the shrimps' gastrointestinal tract using Rogosa agar, a type of selective medium for isolation of lactic acid bacteria. No bacterial isolates were isolated from

the water sample using Rogosa agar. In MRS agar, which was a type of selective medium for isolation of lactic acid bacteria, three bacterial isolates from the shrimps' gastrointestinal tract were isolated. No bacterial isolates were isolated from the water sample using MRS agar. A total of 15 isolates from gastrointestinal tract were labeled as "GS" which stands for gut sample, whereas 6 isolates from water sample were labeled as "WS" which stands for water sample.

Tab. 1: Number of bacterial isolates on different non-selective and selective media from gastrointestinal tract of pond-cultured *Litopenaeus vannamei* and pond water sample.

Media	SGT	Water Sample
Tryptone Soy Agar (TSA)	10	6
Rogosa Agar	2	0
MRS Agar	3	0
Total	15	6

SGT: Shrimps' Gastrointestinal Tract

Microbial Adhesion Test to Hydrophobicity (MATH)

The MATH showed eight isolates GS4, GS11, GS12, GS14, GS15, WS1, WS3 and WS5 had medium hydrophobicity of between 30% to 60% and were selected for further probiotic selection assays, biochemical test and molecular identification. All results are shown in Table 2. GS4, GS11, GS12, GS14, GS15, WS1, WS3 and WS5 has medium ability in adhering to the inner wall of the gastrointestinal tract and occupying space to reduce pathogens adhesion in shrimp.

Aggregation Assay

The eight selected isolates except GS4 had medium aggregation percentage ranging in between 20% and 80%. GS4 had low aggregation percentage of less than 20%. Table 2 shows the aggregation ability of the eight selected isolates. The eight isolates except GS4 produce both a precipitate and constant turbidity. GS4 with low aggregation percentage were unable to aggregate and produce constant turbidity.

Tolerance to Total Ammonia Nitrogen

All the selected eight isolates could tolerate ammonia nitrogen concentration up to 25 mg/L. All results of tolerance to different TAN levels are shown in Table 2. This means the eight isolates can survive in aquatic environment with ammonia nitrogen concentration up to 25 mg/L.

pH Tolerance

All eight selected isolates survived in pH ranging between 4 to 10, except GS15 and WS3. GS15 had no tolerance to pH 4, whereas WS3 had no tolerance to pH 4 and 5. All eight isolates can tolerate alkaline aquatic environment. However, GS15 and WS3 could not survive in acidic condition. The results of tolerance of eight selected isolates on different pH are shown in Table 2.

Salinity Tolerance

All eight isolates had tolerance up to 12% salinity except GS4 and WS5. GS4 had no tolerance at 11% and 12% salinity, whereas WS5 had no tolerance from 7% to 12%. The results for salinity tolerance of all eight isolates are shown in Table 2. All eight isolates although with various salinity tolerance for some isolates, have high salinity tolerance, with the least reaching 6%.

Extracellular Enzymatic Activity

The extracellular enzymatic activity test showed GS11, GS15, WS3 and WS5 had proteolytic activity whereas no lipolytic activity was observed from all the 8 isolates. The results for extracellular enzymatic activity, expressed as proteolytic activity and lipolytic activity are shown in Table 2. GS11, GS15, WS3 and WS5 have protease, but all the eight isolates have no lipase.

Test of Antagonistic Activity to Pathogen

Among the eight selected isolates, only GS15 showed antagonistic activity to pathogen, *V. harveyi* through the inhibition of pathogen's growth away from the marked spot and away from GS15. The other isolates showed no inhibition to pathogen's growth. Therefore, pathogen continued to grow past the marked spot, and towards the isolates. The results for test of antagonistic activity by selected isolates to pathogen, *V. harveyi* are shown in Table 2.

Biochemical Test

The biochemical tests showed variable results (Tab 3). WS3 was successfully identified as *A. hydrophila*, the other isolates were not successfully identified.

Molecular Identification

The Basic Local Alignment Search Tool (BLAST) results showed that GS4 was *Vibrio* sp., GS11 was *B. thuringiensis*, GS12 was *Lac. plantarum*, GS14 was

Tab. 2: Results of probiotic selection assays of the bacterial isolates. NT: not tested, "-": negative result, "+": positive result.

		MATH	Aggregation	GS1	GS2	GS3	GS4	GS5	GS6	GS7	GS8	GS9	GS10	GS11	GS12	GS13	GS14	GS15	WS1	WS2	WS3	WS4	WS5	WS6
TAN (mg/L)	0.05	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	0.1	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	0.5	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	1.0	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	5.0	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	10.0	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	15.0	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	20.0	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	25.0	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
Tolerance to pH	4	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	-	+	NT	-	NT	+	NT
	5	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	-	NT	+	NT
	6	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	7	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	8	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	9	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	10	Low	NT	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT

Tab. 2: Continued

	GS1	GS2	GS3	GS4	GS5	GS6	GS7	GS8	GS9	GS10	GS11	GS12	GS13	GS14	GS15	WS1	WS2	WS3	WS4	WS5	WS6
Tolerance to Salinity (%) 0.5	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
1	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
2	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
3	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
4	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
5	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
6	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
7	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	-	NT
8	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	-	NT
9	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	-	NT
10	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	-	NT
11	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	-	NT
12	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	+	-	NT	-	+	-	NT	+	NT	+	NT
Proteolytic Activity	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	-	NT	-	NT
Lipolytic Activity	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	-	NT	-	NT
Antagonistic Test against *V. harveyi*	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	+	-	NT	-	NT	-	NT

Tab. 3: Results of catalase test, oxidase test and biochemical tests of the bacterial isolates. NT: not tested, NA: not available, "-": negative result, "+": positive result.

		GS1	GS2	GS3	GS4	GS5	GS6	GS7	GS8	GS9	GS10	GS11	GS12	GS13	GS14	GS15	WS1	WS2	WS3	WS4	WS5	WS6
	Catalase Test	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
	Oxidase Test	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	+	NT	-	NT
Biochemical Tests	ADH	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	-	NT	-	NT
	TRD	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	+	+	+	NT	+	NT	-	NT
	EST	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	-	NT	-	-	-	NT	+	NT	-	NT
	PHS	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	+	NT	+	+	-	NT	+	NT	+	NT
	NAG	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	+	-	NT	+	NT	+	NT
	αGLU	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	+	+	NT	-	+	-	NT	-	NT	-	NT
	βGLU	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	-	+	NT	-	+	+	NT	-	NT	-	NT
	ONPG	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	+	NT	-	NT
	URE	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	-	NT	+	-	-	NT	-	NT	-	NT
	GLU	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	+	-	-	NT	+	NT	-	NT
	PRO	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	+	NT	+	NT
	PYR	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	-	-	NT	-	+	+	NT	-	NT	+	NT
	GGT	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	-	NT	+	NT
	TRY	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	+	NT	-	-	-	NT	-	NT	-	NT
	BANA	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	-	NT	+	NT
	IND	NT	NT	NT	-	NT	NT	NT	NT	NT	NT	-	-	NT	-	-	-	NT	-	NT	-	NT
	NO_3	NT	NT	NT	+	NT	NT	NT	NT	NT	NT	+	+	NT	+	+	+	NT	+	NT	+	NT
Identification		NT	NT	NT	NA	NT	NT	NT	NT	NT	NT	NA	NA	NT	NA	NA	NA	NT	*	NT	NA	NT

* *A. hydrophila*

Staphylococcus sp., GS15 was *B. cereus*, WS1 was *A. hydrophila*, WS3 was *Vibrio* sp. and WS5 was *Shewanella* sp. Table 4 shows the BLAST results for all the eight selected isolates.

Tab. 4: BLAST results for the eight selected isolates (16S ribosomal RNA gene, partial sequence).

Isolates	Homology Sequence	E value	Homology (%)	Species
GS4	WAB2255	0.0	100	*Vibrio* sp.
GS11	WAB2159	0.0	100	*B. thuringiensis*
GS12	DL7X	0.0	99	*Lac. plantarum*
GS14	O_li_AC4	0.0	100	*Staphylococcus* sp.
GS15	KK2	4e-20	100	*B. cereus*
WS1	TPF-2	0.0	100	*A. hydrophila*
WS3	WAB2135	9e-99	100	*V. alginolyticus*
WS5	X17XC10	0.0	100	*Shewanella* sp.

Discussion

Eight isolates were screened out as potential candidate for probiotic from a total of twenty one isolates from gastrointestinal tract of white leg shrimp, and water sample from the cultured pond of white leg shrimp. The eight isolates were GS4, GS11, GS12, GS14, GS15, WS1, WS3 and WS5. According to results in biochemical identification tests, only WS3 was identified as *A. hydrophila*, whereas the GS4, GS11, GS12, GS14, GS15, WS1 and WS5 were not identified. Based on the results of molecular identification, GS4 were *Vibrio* sp., GS11 was *B. thuringiensis*, GS12 was *Lac. plantarum*, GS14 was *Staphylococcus* sp. and GS15 was *B. cereus*, WS1 was identified as *A. hydrophila*, WS3 was *Vibrio* sp. and WS5 was *Shewanella* sp. According to Moraes *et al.* (2013), biochemical tests are usually used for identifying small number of isolates because the biochemical tests database normally consists of only clinically important bacteria. Therefore, most of the selected isolates are not identified through biochemical tests. Comparing the results of biochemical tests and molecular identification, the results from molecular identification is preferred over the biochemical tests identification. This is supported by Moraes *et al.* (2013), in the study to compare phenotypic tests such as Biolog and API50CHL and molecular test such as 16S rDNA sequencing, in lactic acid bacteria identification. According to this study, molecular identification was more reliable and should be used to identify difficult isolates, whereas phenotypic tests had poor reproducibility, different results according to bacterial growth, difficulties for mass application, poor discriminatory power for biologically related species and inability of bacterial isolates to express genes simultaneously in different environmental conditions (Moraes *et al.*, 2013). Therefore, the selected isolates are identified as according to molecular identification.

The number of isolates obtained and species identified were not the same compared to previous studies. For example, in a study by Leyva-Madrigal *et al.* (2011) in Mexico, seven isolates of Gram positive cocci were selected and identified as *Pediococcus pentosaceus* and *S. haemolyticus*. In another study by Widanarni *et al.* (2015) in Karawang, West Java, Indonesia, four isolates were selected. In study by Liu *et al.* (2014) in Zuhai, China, five isolates were selected with one of the isolate was identified as *B. subtilis*. Zokaei Far *et al.* (2013) in Hatchery complex, Department of Aquaculture, Faculty of Agriculture of Universiti Putra Malaysia, six isolates selected and identified as *Pseudomonas* sp.. In the last study by Gullian *et al.* (2004), two isolates were selected. The number of isolates selected as potential candidate for probiotic is not the same in all the studies. The location of sampling might affect the number and distribution of microfauna living on white leg shrimp. In study by Shakibazadeh *et al.* (2009), the external or internal body parts of shrimp selected for isolation of bacteria is another factor affecting the difference in number of bacteria and bacteria species isolated.

In MATH, all eight selected isolates showed medium hydrophobicity in percentage range between 30% to 60%. The eight isolates can adhere to the gastrointestinal wall of the host and avoid elimination by peristalsis (Verschuere *et al.*, 2000). Therefore, constant supply of probiotic to the cultured shrimp is not needed, which further decreases the cost of production. Adhesion to the gastrointestinal wall by probiotic in the host also prevents the colonization of pathogens (Verschuere *et al.*, 2000). Probiotic with good hydrophobicity not only applies to adhesion to gastrointestinal wall of the host, but also in bioremediation. Probiotic with good hydrophobicity

has better access to soluble materials and organic matter attached to the tank surface or surface of uneaten feed (Sánchez-Ortiz *et al.,* 2015).

Seven of the eight selected isolates from MATH showed medium aggregation ability in aggregation assay. The seven isolates with medium aggregation ability can colonize the gastrointestinal tract of the host by forming a barrier to prevent the adhesion of pathogens to the intestinal wall. Formation of barrier is done once probiotics are adhered to the intestinal wall (Kos *et al.*, 2003). Therefore, aggregation is usually related with probiotic's adhesiveness. The strong relationship between aggregation and adhesiveness of probiotic is due to the presence of S layer surface protein on bacteria that enables high hydrophobicity, which is essential for aggregation and adhesion of probiotic on intestinal wall (Kos *et al*., 2003).

The eight selected isolates showed tolerance to ammonia up to 25 mg/L. Therefore, the eight selected isolates can tolerate aquatic environment with high ammonia level until 25 mg/L, due to release of ammonium and organic nitrogen from uneaten feed. Comparing to isolates from the study by Devaraja *et al.* (2013), the highest ammonia tolerance level was only 20 mg/L. However, there were isolates that could survive in ammonia level up to 200 mg/L (Sánchez-Ortiz *et al.*, 2015).

The eight selected isolates had high tolerance to alkaline condition, but with exception in acidic condition for some of the eight selected isolates. GS15 showed intolerance to pH 4, whereas WS3 showed intolerance to pH 4 and pH 5. Tolerance to wide range of pH is necessary. Probiotic needs to tolerate low pH to withstand acidic gastric acid in the host's stomach whereas tolerance to high pH enables probiotic to withstand alkaline bile salt from the intestine (Widanarni *et al.*, 2015). According to Waterman (2012), pH of digestive juice in shrimp is in the range of around 5 to 7. Among the two isolates, GS15 and WS3 that showed intolerance to lower pH, GS15's intolerance to pH 4 is still acceptable.

Among the eight selected isolates, GS4 showed intolerance to salinity levels at 11% and 12 % and WS5 showed intolerance to salinity levels from 7% to 12%. The remaining selected isolates showed tolerance to all the tested salinity from 0.5% to 12%. Comparing to study by Powedchagun *et al.* (2011), its isolate was able to grow at 8% salinity, whereas the isolates from study by Sánchez-Ortiz *et al.* (2015), was able to grow at 0.5% to 9% salinity. The salinity tolerance shown by most of the eight selected isolates is considered higher than the previous studies. Higher salinity tolerance among most of the nine selected isolates might be due to the isolation of bacteria from the white leg shrimp cultured in brackish water pond environment. This can be supported by the same finding by Sánchez-Ortiz *et al.* (2015), where the probiotic isolated from a mangrove bivalve species showed generally higher salinity tolerance. Extreme salinity tolerance by probiotic is necessary as it enables the probiotic to travel through environment with extreme salinity before reaching the location for exertion of probiotic effect (Sánchez-Ortiz *et al.,* 2015).

The information on the tolerance of the potential probiotic bacteria to various range of ammonia concentration, pH and salinity is necessary for future application of the isolates. However, in this study the physico-chemical water parameters of the pond water have not been collected, therefore, it is difficult to ensure the tolerance of the isolated probiotic bacteria to environmental condition of the pond. Thus, further study on the *in vivo* tolerance of the selected probiotic isolates need to be conducted.

Among the eight selected isolates, WS3, WS5, GS11 and GS15 had protease enzyme. However, none of the eight selected isolates contained lipase. Enzymatic activities such as proteolytic and lipolytic activity are essential characteristics for probiotic. Probiotic with enzymatic activities may produce microbial enzymes such as amylase, protease and lipase to break down bigger and complex molecules to improve digestibility and feed absorption, thus improving growth of the host (Tuan *et al.*, 2013). Moreover, probiotic itself can act as a supplementary source of fatty acids, vitamins and essential amino acids (Tuan *et al.*, 2013).

Isolate GS15 showed antagonistic or antibacterial property to pathogen *V. harveyi*. GS15, *B. cereus* can limit the growth of pathogen *V. harveyi* through competition for space or attachment sites and nutrients (Avendaño-Herrera *et al.*, 2005). *V. harveyi* is responsible for causing luminous disease in hatchery and grow-out conditions (Lavilla-Pitogo *et al.*, 2000). Luminous disease in hatchery condition causes larvae or postlarvae to become weak and opaque white (Lavilla-Pitogo *et al.*, 2000). When larvae or postlarvae is in total darkness, greenish luminescence is observed (Lavilla-Pitogo *et al.*, 2000). In grow-out condition, luminous diseases cause shrimp's hepat-opancreas to degenerate and inflame and develop-ment of brownish tissues in hepatopancreas (Lavilla-

Pitogo *et al.*, 2000). The affected shrimps in grow-out condition have slow growth, swimming behaviour of heads near the water surface and mass mortality within 45 days (Lavilla-Pitogo *et al.*, 2000). Many of the bacteria tested in previous studies such as *B. subtilis*, *V. alginolyticus*, *B. cerues* and *B. thuringiensis* showed antagonistic activity towards *V. harveyi* (Balcázar and Rojas-Luna, 2007; Gullian *et al.*, 2004 and Masitoh *et al.*, 2016).

GS15, *B. cereus* is among the eight selected isolates most likely be potential probiotic. This is due to antagonistic activity only shown by *B. cereus* against pathogen *V. harveyi*. In comparison with previous study by Masitoh *et al.* (2016), *B. cereus* reported has antagonistic activity to pathogen *V. harveyi* through *in vitro* and *in vivo* assays. Although other selected isolates such as *Shewanella* sp., *B. thuringiensis* and *L. plantarum* did not show antagonistic activity against pathogen *V. harveyi* in this study, but previous studies showed that these bacteria had antibacterial property to pathogens or have already been used as probiotic. In study by Interaminense *et al.* (2018), *Shewanella* sp. could inhibit *Vibrio* pathogen in the rearing of *L. vannamei*. *B. thuringiensis* showed antagonistic activity to pathogen *V. harveyi* through *in vitro* and *in vivo* assay (Masitoh *et al.*, 2016). *Lac. plantarum* had been used in *L. vannamei* postlarvae culture as probiotic and was observed to lower cumulative mortalities in comparison to control (Chiu *et al.*, 2007).

The remaining selected isolates such as *A. hydrophila*, *Vibrio* sp. and *Staphylococcus* sp. are pathogens. *A. hydrophila* according to Hernández Serrano (2005) is one of the sickness causing pathogen in fish farming. Most of the *Vibrio* sp. are pathogen except *V. alginolyticus* has antibacterial effect against *V. harveyi* (Gullian *et al.*, 2004). *V. alginolyticus* also has higher immune index in *L. vannamei* treated with *V. alginolyticus* as probiotic (Gullian *et al.*, 2004). However, no *V. alginolyticus* was identified in this study. In study by Sánchez-Ortiz *et al.* (2015), *Staphylococcus* strains with positive catalase test result, next characterized by aniline blue formation on MRS agar were removed in the isolation process. This has showed that *Staphylococcus* strains might not be selected as probiotic.

In conclusion, four isolates may be considered as potential probiotic bacteria (*Shewanella* sp., *B. thuringiensis*, *Lactobacillus plantarum*, and *B. cereus*) from the gastrointestinal tract of *L. vannamei* for shrimp culture. Among the four selected isolates, *B. cereus* is a potential probiotic bacteria because it has antagonistic activity against pathogenic *V. harveyi*. Further research such as *in vivo* assay still needs to be conducted to test the probiotic potential of the four selected isolates from the gut of collected *L. vannamei* samples. From this research, these four potential probiotic isolates might be used to support disease management of *L. vannamei* aquaculture in Sabah.

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