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In vitro Assessment of Bacterial Strains Associated with Microalgae as Potential Probiotics

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ABSTRACT

Bacteria and microalgae are essential elements in the aquatic ecosystem, co-existing and having constant interactions with each other which help microalgae to exert its beneficial effect as probiotics in aquaculture. This research aims to isolate and identify potential probiotics from different species of microalgae and to evaluate their antimicrobial activity against pathogenic *Vibrio* spp. via series of *in vitro* assays; disc diffusion, well diffusion, and co-culture assays. A total of 18 bacterial strains were isolated from five species of microalgae; *Chlorella* sp., *Nannochloropsis* sp., *Amphora* sp., *Chaetoceros* sp., and *Spirulina* sp.. The isolated strains were tested in *in vitro* antagonistic assay against four *Vibrio* spp. (*Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus*). Seventeen strains demonstrated antimicrobial activity with the highest inhibition was observed by strain SPS11 against *V. parahaemolyticus* (12.6 ± 0.36 mm) in disc diffusion assay and strain NAS32 showed 13.2 ± 0.45 mm clear zone against *V. vulnificus* in well diffusion assay. In co-culture assay, both the SPS11 and NAS32 were able to reduce the growth of *V. parahaemolyticus* and *V. harveyi* at concentration of 10^6 and 10^8 CFU mL^{-1}, respectively. Strains SPS11 and NAS32 were characterized as gram positive bacteria with rod shape and further identified as *Lysinibacillus fusiformis* (SPS11) and *Lysinibacillus sphaericus* (NAS32) using 16s rRNA. These two strains should be further studied in *in vivo* challenged experiments in fish and shellfish to explore their probiotic effects.

Keywords: *Lysinibacillus fusiformis*, *L. sphaericus*, microalgae, probiotics, *Vibrio* spp.

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INTRODUCTION

Microalgae are microscopic algae, usually found in fresh and marine water that varies in size depending on species. In aquaculture, microalgae are often used as supplements for additional nutritional value, serve as immunostimulants, improve defense mechanisms as well as enhance disease resistance towards pathogenic bacteria (Shah et al., 2018). Microalgae have great potential as antiviral agents, antifungal, antibacterial, enzyme inhibiting, immunostimulant, and antiplasmodial due to their ability to synthesize active substances (Ghasemi et al., 2004). The most common species of microalgae used in aquaculture are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Nannochloropsis*, *Pavlova*, *Chaetoceros*, *Skeletonema*, and *Thalassiosira* (Charoonnart et al., 2018). There are positive interactions between microalgae and bacteria in which bacteria can enhance the growth of microalgae by producing growth-promoting factors such as vitamins and regeneration of inorganic nutrients (Fuentes et al., 2016). In return, microalgae synthesize exudates that can be a source of fixed carbon to be absorbed by the bacteria (Yao et al., 2019). Microalgae are able to produce antibacterial compounds that inhibit bacterial growth and *vice versa* (Amin et al., 2012), which is species-specific and can be influenced by culture conditions (Grossart et al., 2006).

Disease outbreaks caused by infectious diseases have become the major limiting factor causing significant economic losses in aquaculture farms. This infectious disease can be caused by pathogenic bacteria, viruses and fungi that lead to severe damage in hatcheries and grow-out ponds. Pathogenic bacteria are the foremost significant pathogens in aquaculture which can cause heavy mortalities. The most common disease in aquaculture appears to be caused by *Vibrio* spp. and their virulence may be resistant to antibiotic treatments (Abraham, 2016). *Vibrio* spp. already exist as normal microbiota in marine and estuarine environments that associated with fish and other aquatic animals and act as primary or secondary opportunistic pathogens which can increase its populations in cultured pond water systems (Priyadarsani & Abraham, 2013). For instance, *V. parahemolyticus* has been identified as the causative agent for acute hepatopancreatic necrosis disease (AHPND) in shrimp including *Penaeus vannamei*, *Penaeus monodon*, and *Penaeus chinensis* (Food and Agriculture Organization [FAO], 2013).

Antibiotics remain as one of the preferred choices in dealing with the outbreaks. However, uncontrollable usage of antibiotics in aquaculture may result in disease resistance of bacteria, and negative impacts on the environment. It is estimated that 75 % of the antibiotic used for feeding are excreted back into the water (Burridge et al., 2010), as fish do not effectively metabolize the antibiotic. On top of that, the residue of antibiotics may accumulate in the flesh of cultured animals which can be transferred to humans through food handling and consumption that poses a high risk to human health. In the long term, antibiotic usage will result in the emergence of antibiotic resistant pathogens. It is

reported that many pathogenic *Vibrio* have been determined to be resistant to almost all available antibiotics (Baker-Austin et al., 2008; Sarter et al., 2007). Abraham (2016) also reported on resistance of *Vibrio* spp. that affected finfish, crustaceans and mollusc production, reduced larval growth as well as inhibited the defence mechanisms of fish larvae. Due to the negative impacts of antibiotic usage towards the environment and human, antibiotic used in aquaculture has been banned in some countries.

Uses of probiotics are known to be harmless as microorganisms obtained from the probiotic are endemic to their purpose, which avoids the introduction of other bacteria into the system (Abraham, 2016). Thus, probiotics have been widely used as an alternative method as a replacement of antibiotics as disease prevention measures in aquaculture. The range of probiotics in aquaculture varies from both Gram-negative and Gram-positive bacteria, bacteriophages, yeasts, as well as microalgae (Irianto & Austin, 2002). Microalgae play a major role as primary live food for fish and shellfish hatcheries and at the same time involves in the overall health management and disease prevention. Each microalgae species has its potential probiotic organism which allows them to strive in their natural environment. Probiotics are not only recognized to prevent common diseases (Sharifah & Eguchi, 2011), but also able to improve the overall growth of microalgae culture.

Thus, the purpose of this study was to identify potential probiotics isolated from different species of microalgae consists of *Chlorella* sp., *Chaetoceros* sp., *Spirulina* sp., *Amphora* sp., and *Nannochloropsis* sp. and its ability to inhibit pathogenic *Vibrio* spp. through series of *in vitro* assays.

MATERIALS AND METHODS

Phytoplankton Collection

Live microalgae culture of *Chlorella* sp., *Nannochloropsis* sp., *Spirulina* sp., *Amphora* sp., and *Chaetoceros* sp. used in this experiment were obtained from Aquatic Bioproduct Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia. Live microalgae were harvested during the late log phase (cell density reached 10^8 cells/mL).

Isolation of Potential Probiotic Candidates

The selected microalgae species were centrifuged at 4,000 ×*g* for 15 minutes. Both supernatant and pellet were placed in tubes following ten-fold serial dilution up to 10^3. A 50 µL from each diluted microalgae sample was pipetted on top of Trypticase Soy Agar (TSA, Difco™, USA) supplemented with 1.5% sodium chloride (NaCl) in triplicates and was spread evenly using a sterile glass stick. All plates were incubated overnight at 30ºC. Pure cultures of individual probiont candidates were preserved at -80°C in 20% sterile glycerol solution for future used.

Elimination of Pathogenic *Vibrio* Strains

Pure cultures of each isolate were streaked onto Thiosulfate Citrate Bile Salt Sucrose (TCBS, Difco™, USA) agar and incubated at

30°C for 24 hours to eliminate any possible pathogen species among the isolates. Isolated bacterial strains with negative growth on TCBS agar were selected for screening in *in vitro* assay.

Pathogenic Bacterial Strains

Four strains of marine pathogenic *Vibrio* sp., *V. harveyi* (NBRC 15634), *V. alginolyticus* (NBRC 15630), *V. vulnificus* (CMCP6), and *V. parahaemolyticus* were obtained from Fish Health Laboratory, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia and cultured in TCBS agar. The cultures were incubated at 30°C for 24 hours prior used.

Screening of Probiotic Candidates

The potential probionts underwent three assays in *in vitro* screening; disc diffusion, well diffusion, and co-culture.

Disc Diffusion Assay. A pure culture of each potential probiont was inoculated aseptically into Tryptic Soy Broth (TSB, Difco™, USA) with 1.5% NaCl and incubated at the same time and temperature as the pathogen culture. Inoculum densities of pathogens were adjusted to 10^5 CFU mL^{-1} (Jasmin et al., 2016) using saline seawater. Pathogens were then swabbed onto TSA + 1.5% NaCl agar plates using sterile polyester-tipped swabs. Sterile paper discs were dipped into each of these overnight cultures of potential probiont suspensions (10^9 CFU mL^{-1}) while negative control was done by dipping a sterile disk into a 0.22 mm filtered sterile saline seawater and placed onto the agar surface that already swabbed evenly with the pathogen. All agar plates were then incubated at 30°C for overnight. Plates were examined and the diameter of inhibition zone was measured and recorded.

Well Diffusion Assay. The potential probionts were further confirmed for their antagonistic activity in a well-diffusion agar against the target strains. The *Vibrio* strains were grown in TSB + 1.5% NaCl for overnight at 30ºC. On the next day, the cell density was adjusted to 10^5 CFU mL^{-1}. The *Vibrio* strains were swab onto TSA + 1.5% NaCl agar plate. Then, a hole with a diameter of 3 mm was punched aseptically using a sterile cork borer and filled with 2 µL of potential probiont overnight culture with cell density of 10^8 CFU mL^{-1}. The plates were then incubated at 30ºC for 24 hours and inhibition zones were recorded.

Co-culture Assay. Selected potential probionts with the highest inhibition zone towards the virulent *Vibrio* strains from previous screening were further tested in co-culture assay. Overnight culture of *V. harveyi* and *V. parahaemolyticus* was inoculated into TSB + 1.5% NaCl at an initial cell density of 10^5 CFU mL^{-1}, whereas the initial cell density of probiont candidates were 10^4, 10^6, and 10^8 CFU mL^{-1}. Each pathogen and potential probionts were co-cultured in 10 mL of TSB + 1.5% NaCl and incubated at 30 ºC with shaking. Samples were taken at time interval of 0, 6, 12, 24, 48, and 96 hour(s) of incubation for the determination of *Vibrio* densities by spreading the treatment samples

onto TCBS plate and incubated at 30 ºC for 24 hours. The number of colonies for each inoculum was counted and recorded as CFU mL^{-1} using the formula:

$$\text{CFU mL}^{-1} = \frac{(\text{No.of colonies} \times \text{dilution factor})}{\text{Volume of culture plate}}$$

Identification of Potential Probionts

Potential probionts obtained through the screening assays were preliminary identified through Gram staining (Bartholomew & Mittwer, 1952) for morphological characteristics observation and further identified using molecular identification method 16S rRNA sequence analysis (Labreuche et al., 2012; Walling et al., 2010).

Gram Staining. Gram staining was performed by using a loopful of a single bacterial colony and smeared onto a glass slide and heat fixed. The smear was stained with crystal violet for one minute and then washed with gentle water. Iodine reagent was added for one minute and decolorized using acetone for 3 to 5 seconds. The smear was then counterstained with safranin for 45 seconds, washed with water and air-dried. The slide was then observed for its shape and color under an oil immersion lens using a microscope.

Molecular Identification through 16s rRNA Sequence Analysis. A total genomic DNA of the potential probiotics was extracted using Genaeid™ Genomic DNA Mini Kit (Genaied Biotech, Taiwan). The universal primer used to amplify the 16s rRNA gene sequence from each DNA template extracted were; forward primer (5′ AGAGTTTGATCCTGGCTCAG 3′) and reverse primer (5′ ACGGCTACCTTGTTACGACTT 3′) (Amin et al., 2012). The PCR protocol involved the initial denaturation at 95ºC for 4-5 minutes, and then 40 amplification cycles of denaturation at 95ºC for 1 minute, annealing at 55ºC for 1 minute and extension at 72ºC for 2 minutes and a final extension at 72ºC for 5 minutes. Amplified PCR products were detected on agarose gel (1%) electrophoresis in 1x TAE buffer and visualized under UV light. The PCR products were sent for sequencing (First Base Laboratories Sdn. Bhd, Malaysia). The sequence identity of the concatenated 16S rRNA sequence was Blast using NCBI blast online tool.

Statistical Analysis

All the data collected were analyzed using one-way analysis of variance (ANOVA). Multiple comparison tests (Tukey's test) was used (IBM SPSS Statistic 2.0 software). Results were expressed as the mean ± standard error and the differences were considered significant at $p < 0.05$.

RESULTS

Isolation of Potential Probiotic Candidates

A total of 18 bacterial strains were successfully isolated from different species of microalgae as potential probionts (Table

1). Results showed 34% strains were isolated *Chlorella* sp., 22% from *Nannochloropsis* sp., 22% from *Amphora* sp., 11% from *Chaetoceros* sp., and 11 % from *Spirulina* sp..

Screening of Probiont Candidates

Elimination of Pathogenic Vibrio Strains. All 18 potential probiotics showed no growth on TCBS agar, hence it is confirmed that these potential probiotics did not belong to *Vibrio* spp..

Disc and Well Diffusion Assays. Positive inhibitory activities against four *Vibrio* strains were observed on each potential probionts in both assays except for strain CAS11 (Table 2). The highest inhibition was

Table 1
Potential probionts isolated from five different species of microalgae

Microalgae	Samples isolated	Label
Chlorella sp.	6	CP11, CP12, CP31, CS21, CS22, CS31
Nannochloropsis sp.	4	NAP11, NAS21, NAS31, NAS32
Chaetoceros sp.	2	CAP11, CAP31
Amphora sp.	4	AMP11, AMP31, AMS21, AMS31
Spirulina sp.	2	SPS11, SPS31

Table 2
Diameter of inhibition zone (mm, mean ± SE) by potential probionts (10^9 CFU mL^{-1}) against Vibrio *spp. (10^5 CFU mL^{-1}) in disc and well diffusion assays*

Isolate	*Vibrio vulnificus*		*Vibrio parahaemolyticus*		*Vibrio harveyi*		*Vibrio alginolyticus*	
Assay	Disc	Well	Disc	Well	Disc	Well	Disc	Well
AMP11	8.5 ± 0.58	7.6 ± 0.58	8.3 ± 0.15	9.0 ± 0.15	-	7.0 ± 0.06	-	10.6 ± 0.06
AMP31	8.4 ± 0.53	8.4 ± 0.53	8.6 ± 0.12	8.0 ± 0.06	-	8.4 ± 0.06	-	11.2 ± 0.12
AMS21	6.0 ± 0.58	7.8 ± 0.42	7.0 ± 0.15	10 ± 0.25	-	7.0 ± 0.06	-	8.6 ± 0.06
AMS31	7.2 ± 0.32	9.0 ± 0.58	2.0 ± 0.26	9.0 ± 0.06	-	8.8 ± 0.10	-	10.2 ± 0.10
SPS11	8.0 ± 0.32	8.8 ± 0.35	12.6 ± 0.36	8.0 ± 0.17	-	3.2 ± 0.06	-	10.0 ± 0.15
SPS31	5.7 ± 0.26	8.4 ± 0.52	8.8 ± 0.06	5.8 ± 0.15	-	5.8 ± 0.06	-	11.2 ± 0.06
CAP31	9.6 ± 0.25	9.0 ± 0.26	9.0 ± 0.06	9.0 ± 0.06	-	4.2 ± 0.12	-	4.2 ± 0.12
CS21	7.0 ± 0.12	7.0 ± 0.58	7.0 ± 0.06	7.0 ± 0.06	-	7.2 ± 0.12	-	11.2 ± 0.10
CS31	7.6 ± 0.31	9.0 ± 0.58	9.0 ± 0.06	9.0 ± 0.21	-	7.4 ± 0.06	-	9.6 ± 0.06
CS22	8.4 ± 0.32	9.0 ± 0.15	9.0 ± 0.06	9.0 ± 0.06	-	6.0 ± 0.15	-	11.0 ± 0.17
CP11	-	-	-	-	-	6.0 ± 0.12	-	-
CP31	4.6 ± 0.32	8.0 ± 0.38	10.8 ± 0.10	8.0 ± 0.15	-	7.2 ± 0.10	-	8.4 ± 0.10
CP12	5.4 ± 0.17	8.6 ± 0.20	9.5 ± 0.10	7.6 ± 0.06	-	6.4 ± 0.06	-	14.2 ± 0.17
NAS31	5.8 ± 0.06	8.2 ± 0.29	8.3 ± 0.21	7.8 ± 0.10	-	7.0 ± 0.06	-	8.0 ± 0.06
NAS21	7.6 ± 0.20	7.6 ± 0.11	8.0 ± 0.12	8.6 ± 0.15	-	4.0 ± 0.12	-	9.2 ± 0.15
NAP11	6.0 ± 0.10	8.0 ± 0.17	8.5 ± 0.15	10.0 ± 0.06	-	9.6 ± 0.10	-	8.4 ± 0.06
NAS32	13.2 ± 0.45	9.0 ± 0.10	9.6 ± 0.06	10.6 ± 0.10	-	7.5 ± 0.06	-	12.0 ± 0.21

observed on NAS32 against *V. vulnificus* with a diameter of 13.2 ± 0.45 mm in disc diffusion and *V. alginolyticus* with a diameter of 12.0 ± 0.21 mm in well diffusion assay. Meanwhile, SPS11 demonstrated highest inhibition zone against *V. parahaemolyticus* with a diameter of 12.6 ± 0.36 mm in disc diffusion assay. NAS32 and SPS11 showed high inhibition zones against all *Vibrio* strains tested with a range of inhibition between 7.5 to 13.0 mm. Strain CP12 had the highest inhibition against *V. alginolyticus* at 14.2 ± 0.17 mm, however, it was later identified to be as the same species as NAS32. Thus, only NAS32 and SPS11 were selected for co-culture assay.

Co-Culture Assay. Potential probionts SPS11 and NAS32 which had the highest inhibition towards *Vibrio* strains in previous screening assays were tested in co-culture assay to identify the optimum concentration of potential probionts that could inhibit the growth of *V. harveyi* and *V. parahaemolyticus*.

In this assay, the growth of pathogenic *V. parahaemolyticus* was inhibited by isolate SPS11 at three different initial concentrations of 10^4, 10^6, and 10^8 CFU mL^{-1} (Figure 1). A higher concentration of SPS11 (10^8 CFU mL^{-1}) reduced the growth of *V. parahaemolyticus* after 6 hours incubation period. Whereas, lower concentrations of SPS11 (10^4 and 10^6 CFU mL^{-1}) managed to reduce *V. parahaemolyticus* effectively at 12 hours co-incubation until 96 hours.

Potential probiont NAS32 able to inhibit *V. parahaemolyticus* at all concentrations (10^4, 10^6, and 10^8 CFU mL^{-1}) from 6 to 24 hours (Figure 2). NAS32 with the highest concentration (10^8 CFU mL^{-1}) inhibited *V. parahaemolyticus* better compared to the other concentrations from 48 hours onwards. Higher concentrations of NAS32 (10^6 and

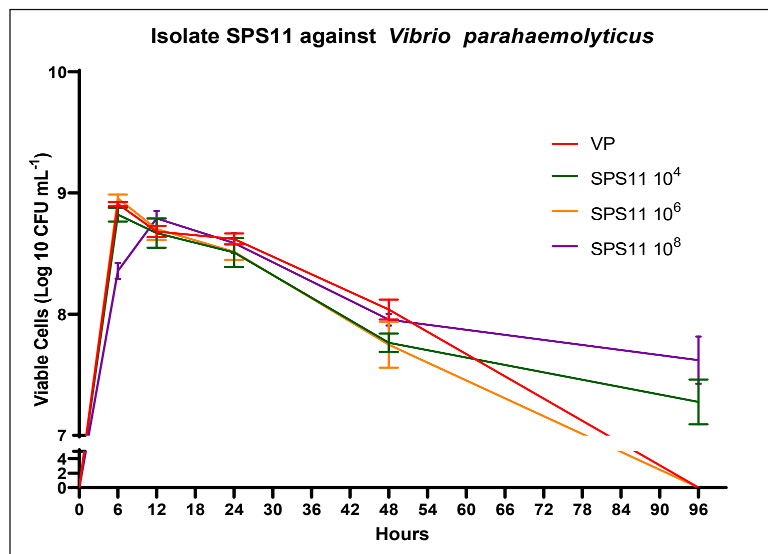

Figure 1. Growth pattern of *Vibrio parahaemolyticus* (VP) CFU mL^{-1} incubated with different concentrations of potential probiont SPS11 (10^4, 10^6, and 10^8 CFU mL^{-1}) against time. Error bars indicate standard error (SE)

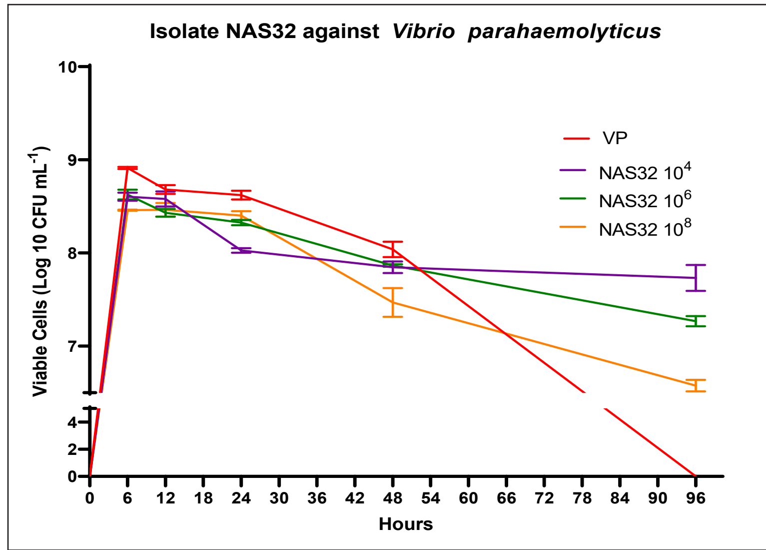

Figure 2. Growth pattern of *Vibrio parahaemolyticus* (VP) CFU mL^{-1} incubated with different concentrations of potential probiont NAS32 (10^4, 10^6, and 10^8 CFU mL^{-1}) against time. Error bars indicate standard error (SE)

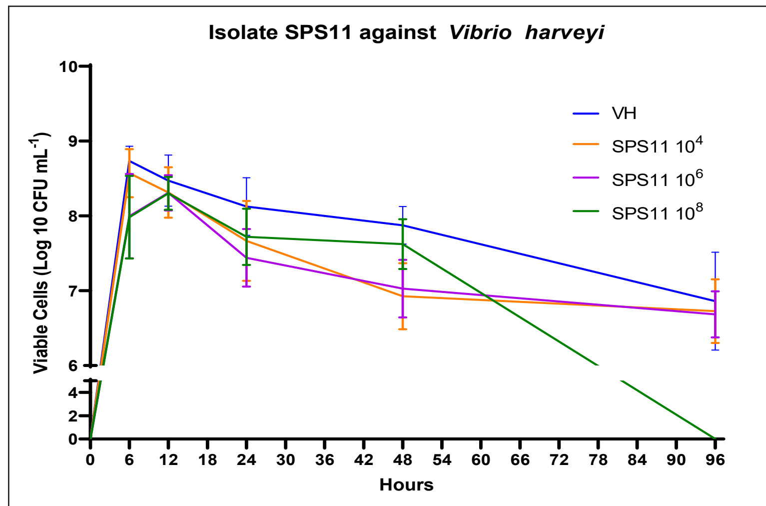

Figure 3. Growth pattern of *Vibrio harveyi* (VH) CFU mL-1 incubated with different concentrations of potential probiont SPS11 (10^4, 10^6, and 10^8 CFU mL-1) against time. Error bars indicate standard error (SE)

10^8 CFU mL^{-1}) able to reduce the total viable counts compared to the lowest concentration of NAS32 (10^4 mL^{-1}).

SPS11 at concentrations of 10^4 and 10^6 CFU mL^{-1} had significant inhibition against *V. harveyi* compared with the growth of *V. harveyi* with no probiont added (Figure 3). However, only slight inhibition of *V. harveyi* occurred at 10^8 CFU mL^{-1} of SPS11 from 24 to 96 hours. SPS11 strain at 10^6 CFU mL^{-1} was the most effective concentration in reducing the growth of *V. harveyi*.

Strain NAS32 at 10^8 CFU mL^{-1} showed significant inhibition towards *V. harveyi* from 6 to 48 hours compared to the lower concentrations. However, the other two NAS32 concentrations; 10^4 and 10^6 CFU mL^{-1}, did not show any viable growth (probionts and vibrios) until the end of the experimental period at 96 hours, hence the results for these two concentrations were omitted (Figure 4).

Identification of Potential Probiotics

Gram Staining. Preliminary identification of potential probionts, SPS11 and NAS32 using Gram staining revealed that these two strains were characterized as Gram positive with rod in shape (Figure 5).

Molecular Identification. Both strains were identified using 16S rRNA sequence analysis (Labreuche et al., 2012; Walling et al., 2010).

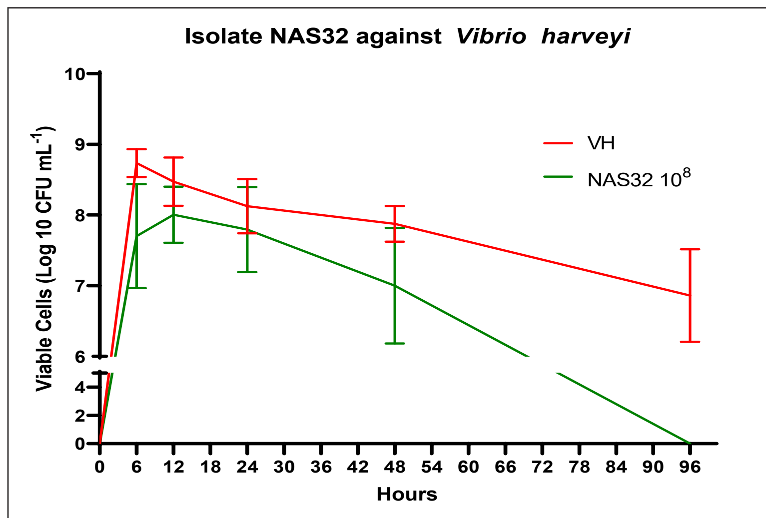

Figure 4. Growth pattern of *Vibrio harveyi* (VH) CFU mL^{-1} incubated with different concentrations of potential probiont NAS32 (10^8 CFU mL^{-1}) against time. Lower concentration of NAS32 (10^4 and 10^6 did not show a valid data). Error bars indicate standard error (SE)

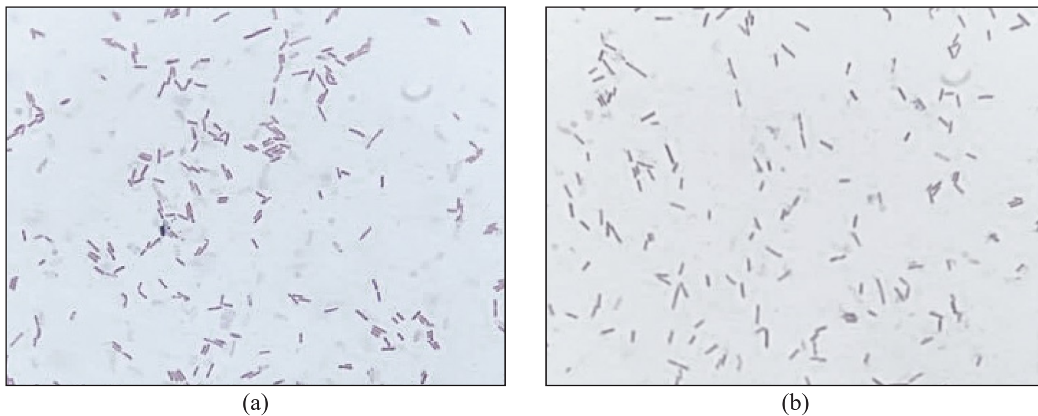

Figure 5. Gram staining of potential probionts (a) SPS11 and (b) NAS32 shows blue staining and bacillus in shape

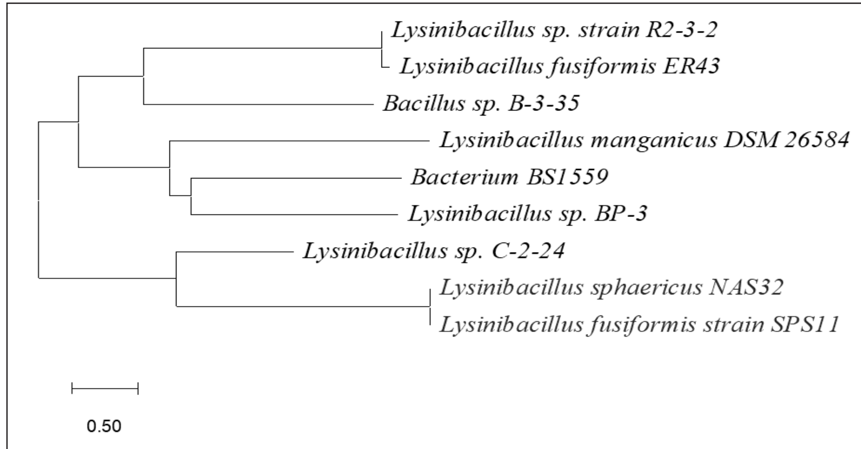

Figure 6. Phylogenetic tree of sequence analysis of SPS11 identified as *Lysinibacillus fusiformis* and NAS32 as *Lysinibacillus sphaericu*s. Samples were determined by comparing the homology with the existing GenBank database

The PCR products were sequenced and the results were blast using National Centre for Biotechnology Information (NCBI). The phylogenetic tree was determined by comparing the homology with the existing GenBank database as shown in Figure 6. The comparative analysis revealed their closest neighbors. Results showed that SPS11 was identified as *Lysinibacillus fusiformis* with 98% similarity while NAS32 was identified as *Lysinibacillus sphaericus* with 99% similarity.

DISCUSSION

Probiotic strains isolated from various hosts and sources have been proven to be beneficial in enhancing diseases resistance as well as growth promoters in aquaculture. In this study, 90% of bacterial strains isolated from five species of microalgae showed potential as probiotics. The two most effective strains were SPS11 which was isolated from *Spirulina* sp. and NAS32 isolated from *Nannochloropsis* sp.. These potential probionts demonstrated the highest inhibition activities against *V. parahaemolyticus* and *V. vulnificus* among other isolated strains and were identified as *Lysinibacillus fusiformis* for SPS11 and *Lysinibacillus sphaericus* for NAS32.

The present study successfully isolated a total of 18 potential probiotics from different species of microalgae; *Amphora* sp., *Chaetoceros* sp., *Chlorella* sp., *Spirulina* sp., and *Nannochloropsis* sp.. Most of the isolates were obtained from *Chlorella* sp., which had been found to have a selection of symbionts, which included bacteria that may have potential as probiotics (Ferro et al., 2019; Myers, 2016; Watanabe et al., 2005).

Microalgae have been used in aquaculture especially for shrimp and fish larvae cultures as a growth promoter as well as to increase the antimicrobial activity of the cultured species. *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*,

Nannochloropsis, *Skeletonema*, and *Thalassiosira* are among the most frequently used microalgae species in aquaculture. The synergistic relationship between microalgae and bacteria may increase the density of both in which will improve the growth performance of microalgae (Han et al., 2016). Bacteria are able to successfully uptake the dissolved oxygen and organic materials produced by microalgae for their benefits (Ethier et al., 2011).

A previous study by Kokou et al. (2012) on microalgae strains of *Chlorella minutissima*, *Tetraselmis chuii*, *Nannochloropsi*s sp., and *Isochrysis* sp. showed antibacterial activity against *Vibrio alginolyticus*, *Vibrio lentus*, *Vibrio splendidus*, *Vibrio scophthalmi*, *Vibrio parahaemolyticus*, and *Vibrio anguillarum* when co-cultivated with the microalgae. The antibacterial activity showed by these microalgae against the pathogenic bacteria might be due to the active compounds with antibacterial properties synthesized by the microalgae which were able to inhibit bacterial growth. These active compounds include fatty acids (Desbois et al., 2009), terpenoids, carbohydrates (Duff & Bruce, 1966) peptides, polysaccharides, and alkaloids (Borowitzka, 1995).

In this study, 17 potential probionts exhibited antibacterial activity on both disc and well diffusion assay against four strains of pathogenic *Vibrio* spp.. The highest inhibition was observed by isolates SPS11 and NAS32 against *V. harveyi* and *V. parahaemolyticus*. This may be due to the production of antibacterial compounds that was diffused through the media inhibiting the growth of vibrios as observed in previous studies (Karim & Hasan, 2019; Ravi et al., 2007; Vaseeharan & Ramasamy, 2003). SPS11 and NAS32 were further tested in co-culture assay to test their ability in inhibiting the growth of *V. harveyi* and *V. parahaemolyticus* in motile conditions.

In the co-culture assay, each potential probiotic was able to inhibit the growth of *V. harveyi* and *V. parahaemolyticus* with different patterns and time intervals. SPS11 at lower concentrations (10^4 and 10^6 CFU mL^{-1}) were able to inhibit *V. parahaemolyticus* and *V. harveyi* while NAS32 performed better with a higher concentration (10^8 CFU mL^{-1}) against both pathogens. These findings are correlated with a recent study by Jasmin et al. (2016) which stated that increasing the amount of probiotic at a specific time might improve the effectiveness in inhibiting pathogens. The inhibition of pathogens by the potential probionts might be due to the production of bacteriocin-like compounds, competition for attachment sites, competition for nutrients (particularly iron in marine microbes), alteration of enzymatic activity of pathogens and immunostimulatory functions (Kesarcodi-Watson et al., 2008). This could explain the decrease of *V. harveyi* and *V. parahaemolyticus* population when co-cultured with potential probionts in the study. Based on the period of incubation interval, early incubation periods from 0 to 12 hour(s) exhibit the rapid increase in the growth of *V. harveyi*, *V. parahaemolyticus*, and potential probiotics (Chen et al., 2019; Stalin & Srinivasan, 2017; Wang et al., 2017).

Several probiotic strains have been reported to be able to reduce and inhibit the growth of pathogens in aquaculture and at the same time improve resistance against disease infection. The most common probiotic strains; *Bacillus* sp., are effective in inhibiting vibriosis (Doroteo et al., 2018; Tepaamorndech et al., 2019). Giri et al. (2013) reported Lactobacillus *plantarum* VSG3 was beneficial towards *Labeo rohita* by improving the growth performance, immunity, and disease resistance. *Leuconostoc mesenteroides* was found to inhibit pathogenic bacteria in Nile tilapia (Zapata & Lara-Flores, 2013). Meanwhile, *Lactobacillus rhamnosus* and *Lactobacillus sporogenes* were able to improve the disease resistance of common carp (Harikrishnan et al., 2010). The ability of probiotics in inhibiting pathogenic bacteria may be due to the competitive action of the probiotics against the pathogenic bacteria for adhesion sites. In order for the pathogenic bacteria to initiate the development of a disease, it requires abundance of attachment at the mucosal layer of the host gastrointestinal tract (Adams, 2010). Besides, probiotic itself needs to have bactericidal effects on other microbial populations by synthesizing active compounds such as bacteriocins, hydrogen peroxide, siderophores, lysozymes, proteases (Panigrahi & Azad, 2007), organic acid, and volatile fatty acids that can reduce pH in gastrointestinal tract, which can prevent opportunistic pathogenic bacteria to grow (Tinh et al., 2007).

Both isolates SPS11 and NAS32 were observed to be Gram positive bacteria with rod shape. Through 16s rRNA molecular identification, SPS11 was identified as *Lysinibacillus fusiformis* while NAS32 was identified as *Lysinibacillus sphaericus*. *Lysinibacillus fusiformis* and *L. sphaericus* were previously known as *Bacillus fusiformis* and *Bacillus sphaericus*, respectively. Reclassification from genus *Bacillu*s to *Lysinibacillus* is based on the fact that the *Lysinibacillus* genus contains peptidoglycan with lysine, aspartic acid, alanine, and glutamic acid, which bacteria form genus *Bacillus* do not have. Several reports on *Lysinibacillus* sp. as probiotics have been reported. *Lysinibacillus fusiformis* isolated from Nile tilapia reportedly exhibits antagonistic characteristics towards *Aeromonas* sp. (Reda et al., 2018). Similar findings were reported on *L. fusiformis* isolated from rainbow trout against pathogen *Flavobacterium psychrophilum* (Burbank et al., 2011, 2012). Compared to *L. fusiformis*, there are limited researches on the probiotic activity of *L. sphaericus* in aquaculture. Recently, *L. sphaericus* had been found isolated from Catla, *Catla catla* (Seelam et al., 2017). In India, *L. sphaericus* was isolated from the gut of Asian catfish, *Clarias batrachus*, and showed positive antagonistic activity towards common *Vibrio* pathogens; *V. harveyi*, *V. vulnificus*, and *V. parahaemolyticus* (Ganguly et al., 2019).

These current findings are the key in developing potential probiotics that can benefit both microalgae and marine aquaculture since not many studies have been done on the application of *L. fusiformis* and *L. sphaericus* as probiotics in the marine aquaculture systems.

CONCLUSION

In conclusion, two potential probiotics; *Lysinibacillus fusiformis* which was isolated from *Spirulina* sp. and *Lysinibacillus sphaericus* from *Nannochloropsis* sp. displayed inhibitory effects against *V. harveyi* and *V. parahaemolyticus*, in *in vitro* assay. These bacteria may have positive inhibition towards pathogenic *Vibrio* spp. in laboratory condition yet the efficiency of these potential probiotics in *in vivo* studies is still unclear. Thus, further study in *in vivo* is very much needed to determine the efficiency and to understand better the mechanism of the two potential probiotics. Although there are still more researches that need to be done before it can be considered to be used in the culture systems, the potential of the two potential probiotics, *L. fusiformis* SPS11 and *L. sphaericus* NAS32 are promising and deserve to be further evaluated.

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