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Evaluation of Bacterial Isolates from Seaweed *Sargassum oligocystum* Montagne as Probiotic Supplements in Commercial Fish Feeds

Jenifer E. Ealdama[a]

[a]*Igbaras National High School-Special Program in Science, Technology and Engineering, Escala St., Igbaras, Iloilo 5029, Philippines*

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A B S T R A C T

This study focused on the isolation, characterization, and screening of bacterial isolates from *Sargassum oligocystum* Montagne against *Vibrio harveyi*. The most effective isolate was identified and inoculated in commercial fish feeds as a probiotic supplement.Six bacterial isolates were isolated from the seaweed sample and screened against *Vibrio harveyi.* Two of the most effective among the six isolates were chosen and further screened against *Vibrio harveyi.* Bacterial isolate 1 identified as *Bacillus cereus* complex was incorporated in commercial fish feeds with and without seaweeds. Results showed that *B. cereus* complex with *S. oligocystum* had a viability of 12 days. The final population count was also higher in *Bacillus cereus* complex with *S. oligocystum* (M=9.83 log CFU/g) showing a significant difference in the treatment with *B. cereus* complex only. Thus, *B. cereus* complex grows better in fish feed supplemented with powdered *S. oligocystum.*

Keywords: Bacterial isolates; seaweed *Sargassum oligocystum*; probiotic supplements; commercial fish feeds

1. Introduction

Seaweeds or macroalgae are an extensive group of macroscopic marine organisms that comprise of a few thousand species (Kim *et al.*, 2008; Kumari *et al*., 2010). Seaweeds are mainly involved in global primary production and providing food and shelter for a variety of organisms; for instance, providing protection and nutrient rich conditions for bacterial growth. Seaweeds have a rich diversity of associated microorganisms compared with other multicellular organisms. These microorganisms may be beneficial or harmful to the seaweeds. Epiphytic bacterial communities have been reported as vital for morphological development of seaweeds, and bacteria with antibacterial properties are thought to protect the seaweeds from pathogens and the other competition organisms. Some bacterial species show host specificity and bactericidal activity against specific pathogens. This specificity engage complex biochemical interactions between seaweed and bacteria (Susilowati *et al.*, 2015).

Seaweeds can also be considered as a source of biologically active compounds for animals and represent a potential feed alternative. For this reason they are recommended as dietary supplements and have an economic potential in animal nutrition, since they may compensate nutritional deficiencies of diets (Taboada *et al*., 2010).

Probiotics are usually defined as live microbial feed supplements that are administered in such a way as to enter the gastro-intestinal tract which beneficially affects the host animal by improving its intestinal microbial balance and in turn its health (Gatesoupe, 1999; Ghosh *et al.,* 2003). The benefits of such supplements include improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, anti-mutagenic and anti-carcinogenic activity, increased immune response and increase in the growth and survival of fish species (Pandiyan, Balaraman, Thirunavukkarasu, George, & Mannikam, 2013).

On the other hand, seaweed meal is used as a feed ingredient to reduce cost. In aquaculture, seaweed with a high protein level is used in the production of food for fish (Azad & Xiang, 2012). At present, seaweed with elevated protein content and production rate is receiving increasing attention as a novel food with potential benefits (Buschmann *et al*., 2001) and as a possible ingredient in fish diets (Davies *et al*., 1997).

* *Corresponding author*
E-mail address: elumbajenifer@gmail.com

Sargassum is a genus of brown macroalgae (seaweed) in the order *Fucales*, Class *Phaeophyceae*. *Sargassum* has the highest protein level of 14% dry weight of all seaweeds (lDW) (Azad & Xiang, 2012). The protein apparent digestibility coefficient (ADC) content was higher in *Sargassum* (65.1%) than other seaweed. Commercial utilization of *Sargassum sp*. is limited. These might be potential resources for aquaculture (Pereira *et al*., 2012).

Hence, this study focused on the isolation and identification of bacterial isolates from seaweed *Sargassum oligocystum* and its viability in commercial fish feeds.

2. Methods

The materials used in the study were petri dishes, inoculating loop, alcohol lamp, Erlenmeyer flasks, beakers, sterile sample bottles, sterile vials, culture tubes, test tubes, test tube rack, micropipette tips, aluminum foil, and autoclavable plastics.The chemicals and reagents needed were MRS agar, MRS broth, Thiosulfate Citrate Bile Salt Sucrose (TCBS), Tryptic Soy Broth (TSB), Nutrient Agar (NA), liquid molasses, normal saline solution (NSS), rock salt, and Gram Staining chemicals (iodine, crystal violet, alcohol, and safranin). The equipment needed were biosafety cabinet, refractometer (salinity), blender, glass jars (anaerobic incubator), 0.1ml, 1ml and 10ml micropipettors, magnetic stirrer, digital weighing scale, microwave oven, hot plate, autoclave, colony counter, and refrigerator.

*Collection of Sargassum oligocystum.*Species of brown seaweeds *Sargassum oligocystum* was collected during low tide at Barangay Maninila, Miag-ao, Iloilo, Philippines with geographical coordinates of 10. 65 ˚N and 122.26˚ E. *Sargassum oligocystum* was cut or uprooted from rocks and put into ziplock plastic bags and place in a cool box.

Authentication of Sargassum oligocystum Montagne.Authentication of *Sargassumoligocystum* was done at SEAFDEC Aquaculture Department, Tigbauan, Iloilo, Philippines by Dr. Ma. Rovilla J. Luhan, Associate Scientist a specialist in seaweeds. The identification of *Sargassum oligocystum* was done also through qualitative observations and based on the descriptions presented in the book (Seaweeds of Panay) by Hurtado *et al*. (2006). The *Sargassum* sp. sample evaluated in the study was a species of *Sargassum oligocystum*. A voucher specimen was prepared and deposited at the Phycology Collection of SEAFDEC AQD in Tigbauan, Iloilo, Philippines.

*Determination of Salinity used as Diluents.*The samples were rinsed with sterile seawater, weighed (100 grams) and cut into smaller pieces using a sterile knife aseptically. The cut samples were macerated and placed in a blender and 100 ml sterile seawater was added. A drop of the sample solution was placed on top of the prism assembly. The daylight plate was closed in order for the solution to spread across the entire surface of the prism without any bubbles or dry spots. The sample solution was allowed to sit on the prism for approximately 30 seconds in order to adjust to the ambient temperature of the refractometer. Afterwards, the refractometer was held in the direction of a natural light source and observed through the eyepiece and took the reading of the salinity of the algal sample.

Preparation of Diluents based in Salinity of Algal Sample.In every 100 ml of distilled water, 3 grams of table salt was mixed to make a 3% solution. The salt was completely dissolved. This was autoclaved and used as diluents for bacterial isolates from algae and test bacteria.

Isolation of Bacterial Isolates from Brown Algae (*Sargassum oligocystum* Montagne).About 100 grams of algal sample was homogenized in 90 ml 3% salt solution previously prepared and sterilized. About 1 ml of the solution was serially diluted up to the 10^{th} dilution. About 0.1 ml of all even dilutions was evenly plated in MRS agar plates. After inoculating the plates, they were incubated in a plastic box using improvised candle jar method for 48 to 72 hours tightly sealed at room temperature. After incubation, visible distinct well isolated colonies were picked and characterized according to form, elevation, margin, appearance, optical property, pigmentation and texture. All colonies picked were further purified three times and screened against *Vibrio harveyi*.

2.1 Experiment proper

Gram-staining and Screening of Six (6) Bacterial Isolates against Vibrio harveyi.The six bacterial isolates were gram stained (Appendix Q) and were tested against fish pathogen, *Vibrio harveyi* from cultured samples obtained from SEAFDEC-AQD laboratory. The six bacterial isolates were screened for probiotic activities against *Vibrio harveyi*. The six probiotic strains were inoculated in the MRS broth and incubated in an anaerobic condition for 24-48 hours. The pathogenic strain of *Vibrio harveyi* was also inoculated in TSB (Tryptic Soy Broth) and incubated at room temperature.

After the incubation of six bacterial isolates, each probiotic isolate was co-cultured in an Erlenmeyer flask, serially diluted following 3% salt solution as diluents and plated in MRSA plates and TCBS plates using spread plate method in an anaerobic condition for 48-72 hours. The culture of each probiotic isolate with *Vibrio harveyi* was stored at room temperature. The bacterial count was monitored every 48 hours after incubation for 7 days. This was done in three trials with two replicates each.

2.2 Production of bacterial isolate inoculum

Preparation of Substrate.The prepared fresh algae was weighed 30 grams and homogenized in a blender. The homogenized algal sample was transferred in 1 liter Erlenmeyer flask. The homogenized sample was diluted with 3% salt solution in 1:5 ratio (Chellapandi, 2007), 300 g algae to 1000 ml distilled water.

Addition of Molasses as Carbon source. The molasses was utilized because it is an industrial by-products or wastes have been evaluated as substrate

with the aim of decreasing the cost of the process, such as sugarcane (Calabia & Tokiwa, 2007), molasses (Dumbrepatil *et al.,* 2008) as carbon source. The cane molasses was obtained from a local poultry supply in Iloilo City as an additive for animal feeds. Upon acquisition of the sample, the substrate was hydrolyzed by adding 1ml of 20% $H_2SO_4$to 100ml of molasses solution. The acidified molasses solution was heated in a boiling water bath for 20 minutes. The pH of the medium was adjusted to 6.0 with 4.0 M KOH prior to sterilization. The sugar cane molasses contained 100% reducing sugar (Coelho *et al.,* 2011).

The molasses solution was mixed in the solution of homogenized algal sample. A volume of 120 ml of molasses per liter of homogenized algal sample solution was mixed. The solution was autoclaved at 121° C for 15 minutes 15psi and cooled down for further use.

Inoculation of Starter Culture. Microbial culture of bacterial isolates and *Vibrio harveyi* in broth solutions were compared to 0.5 McFarland Standard. About 10% (v/v) of starter culture (Wardani *et al.,* 2017) for each set up was added to the sterile solution per 100 ml of the solution. According to Wardani *et al.* (2017), 10% inoculum resulted in the significantly increase population of the cultured bacteria during fermentation.

The following set ups with starter culture (24 hour old culture of test bacteria) were prepared:

- Set up A: Algal solution with bacterial isolate only;
- Set up B: Algal solution with *Vibrio harveyi* only; and
- Set up C: Algal solution with bacterial isolate+ *Vibrio harveyi*

Microbial count was monitored after days 1, 3, 5 and 7. The set ups were done in three trials with two replicates each.

2.3 Formulation of commercial fish feeds with bacterial isolate and evaluation of viability

Preparation of Bacterial Culture and Harvesting.About 10 ml of MRS broth solution was prepared for the bacterial culture. One loopful of bacterial isolate was inoculated to the broth solution and incubated for 48-72 hours. After incubation, it was compared to 0.5 McFarland Standard solution and the probiotic standard is $1x10^8$. The bacterial pellets were harvested by centrifuging bacterial cultures at 4000 g for 15 min at 4˚C, the pellets were washed twice in normal saline solution and suspended in same buffer saline. The bacterial culture was stored for further use.

*Preparation of Commercial Fish Feeds Inoculated with Bacillus cereus complex Isolate.*The procedure by Sreeja (2014) was followed with modification. The commercial fish feeds product of Santeh Feeds Corporation were obtained at Iloilo supermarket with ISO 9001:2008 certified. The fish feed was manufactured on August 17, 2018 and with an expiration date of December 17, 2018. About 100 g of commercial fish feed pellet was autoclaved for 15 min at 121˚C and cooled at room temperature. About 1 g *Bacillus cereus* complex isolate produced from centrifuged broth per 100 g granules was mixed with 15 g powder algae, and 3 g tapioca as natural binder and culture was mixed with 1 ml normal saline solution. It was further added with 100 ml water per 100 g mixture to uniformly distribute the mixture. The 100 g bacterial culture was mixed thoroughly on feeds at two different formulations (Londoño, Toro, & Bolivar, 2015).

The composition of the commercial fish feeds with *Bacilluscereus* complex isolate is shown in Table 1.

Table 1-Composition of Commercial Fish Feeds with *Bacillus cereus* complex Isolate.

Components	Composition
Commercial Fish Feed	100 g
Bacterial Isolate	1 g
Powderized Algae	15 g
Normal Saline Solution	100 ml

The prepared feeds were packed in airtight polyethylene bags, labelled and stored at room temperature, 26˚C with a relative humidity of 75%. This was done in three trials with three replicates each. The two fish feed formulations include the following:

1. Fish Feed Formulation A: Fish Feed + *Bacillus cereus* complex + *Sargassum oligocystum*; and
2. Fish Feed Formulation B: Fish Feed + *Bacillus cereus* complex.

*Evaluation of Duration of Viability of Bacillus cereus complex Isolate in Commercial Fish Feeds.*The duration of viability of *Bacillus cereus* complex isolates in commercial fish feed was determined for 24 days at a 3-day interval with a population count between 10^6 CFU/ml to 10^9 CFU/ml per sampling as the accepted viable count. This was supported by the studies conducted on halibut, *H. hippoglosus* (Ottensen & Olafsen, 2000), crab, *P. pelagicus* larvae and *Lactobacillus* based probiotics in teleost models (Uma *et al*., 1999; Dash *et al.,* 2014; Londoño, Toro & Bolivar, 2015).

Ten milligrams of feed pellet was taken and mixed with 1 ml phosphate buffered saline under aseptic condition and then transferred to 9 ml PBS. From this, it was serially diluted to 10^{-10} dilutions wherein all even dilutions were spread –plated in MRS agar plates and incubated using candle jar method for 48-72 hours at room temperature.

2.4 Characterization and Identification of Most Effective Isolate

Preparation of Agar Slant.About 100 ml Nutrient Agar (NA) with 3% sodium chloride (NaCl) was prepared and autoclaved at 121˚C for 15 minutes.

Afterwards, 10 ml NA slant was poured and cooled down in a 15 ml culture tube.

Inoculation and Incubation.The most effective bacterial isolate was streaked in a 10 ml NA slant. The two prepared slants were incubated in anaerobic condition with 10% carbon dioxide for 48-72 hours at room temperature.

Characterization and Identification of Isolate.The most effective isolate was purified three times through gram-staining and the prepared smear was viewed using binocular microscope under oil immersion objective. The purified isolate was further identified using Biomerieux Vitek 2 Identification System, BCL Card. The identification of unknown bacterial isolate was done at Philippine National Collection of Microorganisms (PNCM), National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB).

Waste Disposal. The bacterial isolates in culture plates and culture tubes were placed in autoclavable plastics (Ulysses PP) and autoclaved for 15 minutes at 121˚C, 15 psi before being discarded. The decontaminated glass plates and culture tubes were washed with soap detergent and disposable culture plates were placed and disposed in a garbage bag.

2.5Data analysis

Descriptive Data Analysis. For descriptive data, frequency of bacterial isolates, colony and cell characterization of bacterial isolates, and mean and standard deviation were used for the determination of the effective bacterial isolate from brown algae and screened against *V. harveyi*.

Colony counting was done in the range of 25-250 CFUs (Zipkes, Gilchrist, & Peeler, 1981) based on Bacteriological Analytical Manual (BAM) Standards. Dilutions were recorded and total number of colonies counted. When the number of CFU per plate exceeds 250 for all dilutions, they were recorded as TNTC. The CFU per milliliter or gram with replicates was computed using the following formula:

$$\text{CFU/ml or g} = \frac{\text{N (total no. of colonies)}}{\frac{\text{N}}{1}+\frac{\text{N}}{10}+\frac{\text{N}}{100}\ldots} \times \text{1st countable dilution}$$

Inferential Data Analysis.The CFU/ml or g was converted to log CFU/ml or g to minimize disparities among the values obtained and used for inferential data analyses. The paired sample t-test was used to determine any significant differences between the six bacterial isolates from *Sargassum oligocystum* and *Vibrio harveyi* co-cultured while repeated measures ANOVA was used to determine any significant differences between *Bacillus cereus* complex only and *Bacillus cereus*complex with *Vibrio harveyi* and *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex population count over a 21-day sampling period. In addition, ANOVA with repeated measures was used to determine any significant difference in the viability (CFU/ml) of *B.cereus* complex in two fish feed formulations over a 15-day sampling period. The t-test for independent samples was used to determine any significant difference in the duration of viability (days) of *B. cereus* complex in the two types of fish feed formulations. The level of significance was set at 5 percent alpha level. All the statistical tests were processed via the Statistical Package for Social Sciences (SPSS) software, version 11.5.

3. Results

4.1 Descriptive data analysis

Characterization of Isolates. Table 2 shows the frequency, colonial morphology and cell characteristics of bacterial isolates from *Sargassum oligocystum*. There were six bacterial isolates obtained from *S. oligocystum*. These were characterized based on form, elevation, margin, appearance, optical property, pigmentation, texture, gram reaction and cell shape and arrangement.

Table 2-Frequency, Colonial Morphology and Cell Characteristics of Bacterial Isolates from *Sargassum oligocystum.*

Isolate No.	Form	Elevation	Margin	Appearance	Optical Property	Pigmentation	Texture	Gram Reaction	Cell shape and arrangement
1	puncti-form	flat	entire	dull	translucent	Non-pigmented	smooth	positive	Bacilli; clusters/ individual
2	rhizoid	flat	filamen-tous	shiny	translucent	Non-pigmented	smooth	positive	cocci; individual
3	circular	flat	entire	shiny	opaque	Non-pigmented	smooth	positive	Bacilli; clusters
4	circular	flat	entire	shiny	opaque	pigmented	smooth	positive	cocci; clusters/ individual
5	irregu- lar	flat	undu-late	dull	opaque	Non-pigmented	smooth	positive	Bacilli; individual
6	irregu-lar	flat	lobate	dull	opaque	Non-pigmented	smooth	positive	Bacilli; chain

Viability of Test Isolates. Figure 1shows the population counts of *Bacillus cereus* complex isolate in the two fish feed formulations. The *B. cereus* complex isolate had the same growth pattern observed in the two fish feed formulations, with and without seaweed *S. oligocystum*. The bacterial isolate, *B. cereus* complex achieved its peak growth after 12 days (M= 9.83 log CFU/g; SD= 2.96) in fish feed formulation with *S. oligocystum* and declined after 15 days. However, *B. cereus* complex in commercial fish feed without *S. oligocystum* achieved its peak growth after 9 days (M= 7.88 log CFU/g; SD= 1.15) and declined after 12 days.

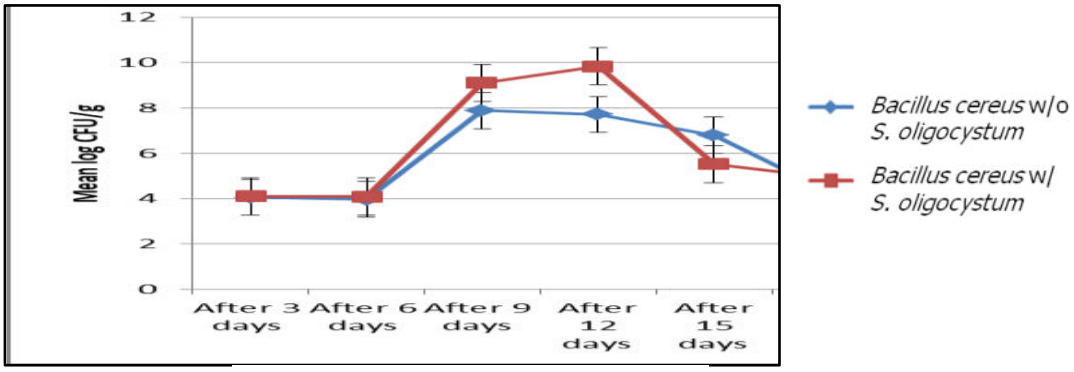

Fig. 1-Population counts of *Bacillus cereus* complex isolate in the two fish feed formulations.

Viability of Isolate in Fish Feed Formulations. Figure 2 shows the mean viability of *Bacillus cereus* complex in two types of fish feed formulations. The bacterial isolate, *B. cereus* complex in fish feed with *S. oligocystum* had a higher viability count (M=14 days; SD= 2.92) while *B. cereus* complex in fish feed without *S. oligocystum* had a lower viability (M=13 days; SD= 1.32).

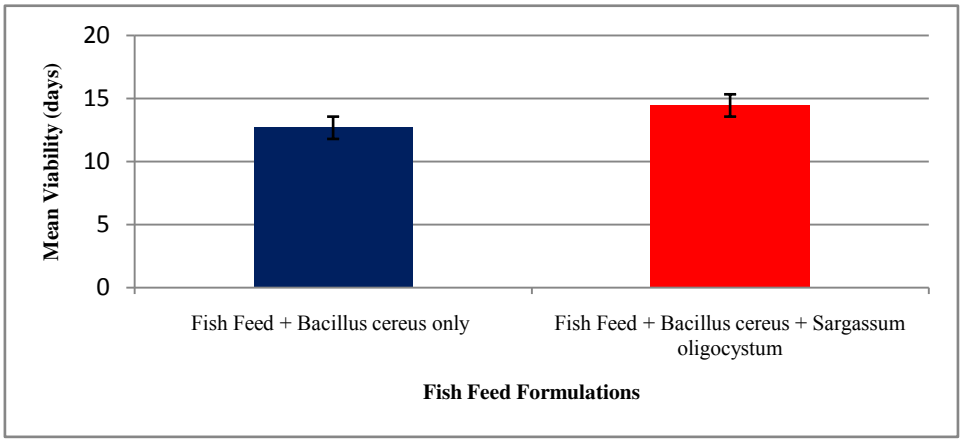

Fig. 2-Mean Viability of Bacillus cereus complex in Two Types of Fish Feed Formulations.

4.2 Inferential data analysis

Paired Sample t-test on the Population Count between Bacterial Isolates from *Sargassum oligocystum* and *Vibrio harveyi*. Figure 3shows the paired sample t-test on the population count between the bacterial isolates from *Sargassum oligocystum* and *V. harveyi*. It shows that in pair 1, bacterial isolate 1 and *Vibrio harveyi* has the only significant difference in the population count, t (df=3) =16.712, p (0.000) <0.05. This means that bacterial isolate 1 have competitively excluded the population of *V. harveyi*. The rest of the paired isolates and *V. harveyi* did not show any significant differences, p>0.05. This means that the *V. harveyi* with bacterial isolates have comparable population counts. The partial eta squared value of all the treatments has a large effect size (eta squared value > 0.14). This means that the effect is due to the treatment used in the experiment.

Group		Mean	N	Std. Deviation	Mean Difference	t	df	Sig. (2-tailed	Partial Eta Squared Value
Pair 1	B1	6.8725	4	1.177	2.778	16.712*	3	0.000	0.989
	V1	4.0950	4	1.441					
Pair 2	B2	6.7675	4	2.693	2.768	1.704	3	0.187	0.491
	V2	4.0000	4	1.462					
Pair 3	B3	4.8450	4	0.836	0.970	1.729	3	0.182	0.499
	V3	3.8750	4	0.302					
Pair 4	B4	4.3175	4	0.084	0.300	1.476	3	0.236	0.420
	V4	4.0175	4	0.485					
Pair 5	B5	5.1300	4	1.553	1.385	1.946	3	0.147	0.557
	V5	3.7450	4	0.234					
Pair 6	B6	6.4725	4	2.739	2.330	1.300	3	0.285	0.360
	V6	4.1425	4	0.951					

Note: *p<0.05 is significant; partial eta squared=0.01 is small; 0.06 is medium; and 0.14 is large.

Fig. 3- Paired Sample t-test on the population count between bacterial isolates from *Sargassum oligocystum* and *Vibrio harveyi*.

Analysis of Variance with repeated measures on Tests of Within-Subjects Contrasts on the population count between *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi.* Table 3 shows the ANOVA with repeated measures tests of within-subjects contrasts on the population count between *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi*. It shows a significant difference on the population count in relation to time, F (1,4)=239.509, p (0.00)<0.05. This means that *Bacillus cereus* complex significantly decreased the population of *V. harveyi*. The effect size (partial eta squared value=0.984) is large. This means that the effect is due to the treatment used. However, there is no significant interaction between time and group on the population count of *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi*, F (1,4)=239.509; p (0.253)>0.05. This means that the population count of *Bacillus cereus* complex with *Vibrio harveyi* did not significantly vary with the population of *Bacillus cereus* complex only. The effect size (partial eta squared value= 0.308) is also large. This means that the effect size is due to the treatment used.

Table 3 - ANOVA with repeated measures Tests of Within-Subjects Contrasts on the population count between *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi*.

Source	Time	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
TIME	Linear	8.096	1	8.096	239.509*	.000	.984
TIME* GROUP	Linear	.060	1	.060	1.784	.253	.308
Error (TIME)	Linear	.135	4	.034			

Note: *p<0.05 is significant; partial eta squared=0.01 is small; 0.06 is medium; and 0.14 is large.

Based on pairwise comparison using Least Significant Difference as indicated in Appendix V in relation to time, there is a significant difference between day 1 and day 7, p(0.000), day 3 and day 7, p(0.013), day 5 and day 7, p(0.001), p<0.05. This means that time is a factor that influences the change in the population count of *B. cereus complex*.

Analysis of Variance with repeated measures result on Tests of Between- Subjects Effects on the population count between *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi*. Table 4 shows the ANOVA with repeated measures tests of between-subjects effects on the population count of *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi*. It shows no significant difference between the populations of *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi*, F (1,4)=1.202, p (0.335) >0.05 level of significance. This means that the population count of *Bacillus cereus* complex with *Vibrio harveyi* is comparable with *Bacillus cereus* complex only. The effect size is large (partial eta squared value =0.231). This means that the effect is due to the treatment used.

Table 4 - ANOVA with repeated measures on Tests of Between-Subjects Effects on the population count between *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi*.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	754.545	1	754.545	3294.897*	.000	.999
GROUP	.275	1	.275	1.202	.335	.231
Error	.916	4	.229			

Note: *p<0.05 is significant; partial eta squared=0.01 is small; 0.06 is medium; and 0.14 is large.

Based on pairwise comparison using Least Significant Difference as indicated in Appendix W, there is no significant difference between the population count of *Bacillus cereus* complex only and *Bacillus cereus* complex with *Vibrio harveyi*, p(0.335) >0.05. This means that the two treatments are comparable.

Analysis of Variance with repeated measures results on Tests of Within-Subjects Contrasts on the population count between *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex. Table 5 shows the ANOVA with repeated measures tests of within-subjects contrasts on the population count between *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex. There is a significant difference on the population count of *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex in relation to time F(1,4)=19.839, p(0.011)<0.05. This means that the population count of *Vibrio harveyi* with *Bacillus cereus* complex has significantly decreased upon the treatment of *Bacillus cereus* complex. The effect size (partial eta squared value=0.832) is large. This means that the effect is due to the treatment used. There is also a significant interaction between time and group on the population count of *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex, F (1,4)= 167.586, p (0.000) <0.05. This means that the population count of *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex significantly differ with time and treatment used. The effect size (partial eta squared value= 0.977) is also large. This means that the effect is due to the treatment used.

Table 5 - ANOVA with repeated measures on Tests of Within-Subjects Contrasts on the population count between *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex.

Source	Time	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
TIME	Linear	2.437	1	2.437	19.839*	.011	.832
TIME* GROUP	Linear	20.584	1	20.584	167.586*	.000	.977
Error (TIME)	Linear	.491	4	.123			

Note: *p<0.05 is significant; partial eta squared=0.01 is small; 0.06 is medium; and 0.14 is large.

Based on pairwise comparison using Least Significant Difference as indicated in Appendix X, there is a significant difference on the population count of *V. harveyi* in relation to time, between day 1 and days 3, p(0.028), 5 p(0.003), and 7 p(0.030); day 3 and days 1 p(0.028) and day 5 p(0.038); day 5 and days 1 p(0.003), 3 p(0.038), 7 p(0.044); and day 7 and days 1 p(0.030) and 5 p(0.044), p<0.05. This means that the change in the population of *V. harveyi* is significantly reduced in the presence of *B. cereus* complex.

Analysis of Variance with repeated measures result on Tests of Between- Subjects Effects on the population count between *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex. Table 6 shows the ANOVA with repeated measures tests of between-subjects effects on the population count of *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex. There is a significant difference between the population count of *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex, F (1,4)=102.006, p (0.001) <0.05. This means that the population count of *Vibrio harveyi*with *Bacillus cereus* complex was significantly excluded by the presence of *Bacillus c*ereus complex while a normal growth pattern was observed in the *Vibrio har*veyi only setup. The effect size is large (partial eta squared value =0.962). This means that the effect is due to the treatment used.

Table 6 - ANOVA with repeated measures on Tests of Between-Subjects Effects on the population count between *Vibrio harveyi* only and *Vibrio harveyi* with *Bacillus cereus* complex.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	300.900	1	300.900	2027.742*	.000	.998
GROUP	15.137	1	15.137	102.006*	.001	.962
Error	.594	4	.148			

Note: *p<0.05 is significant; partial eta squared=0.01 is small; 0.06 is medium; and 0.14 is large.

Based on pairwise comparison using Least Significant Difference as indicated in Appendix Y, there is a significant difference in the population count between *V. harveyi* only and *V. harveyi* with *B. cereus* complex, p(0.001)<0.05. This means that there is a significant alteration in the growth pattern of *V. harveyi* with *B. cereus* complex.

Test of Within-Subjects Contrasts on the Population Count of *Bacillus cereus* complex Isolate in the Two Fish Feed Formulations.Table 7 shows the Analysis of Variance with repeated measures on tests of within-subjects contrasts on the population counts of *Bacillus cereus* complex isolate in the two fish feed formulations. There is a significant difference on the population count of *B. cereus* complex in the different sampling periods, F(1,4)=26.563, p(0.007) < 0.05. This means that the population counts vary in time significantly. As shown in Figure 5, it follows a normal growth pattern with its peak after days 9 to 12 of monitoring. The effect size (partial eta squared value = 0.869) is large. This means that the sampling is due to the effect of the treatment used. There is also a significant interaction between time and the types of feed formulations with *B. cereus* complex, F(1,4)=8.197, p(0.046) < 0.05. This means that the growth of *B. cereus* complex was favored upon the addition of *S. oligocystum* pulverized and incorporated in one of the fish feed samples. The effect size (partial eta squared value = 0.672) is large. This means that the growth rate of *B. cereus* complex is due to the effect of the treatment used.

Table 7 - Analysis of Variance with Repeated Measures on Tests of Within-Subjects Contrasts on the Population Counts of *Bacillus cereus* complex Isolate in the Two Fish Feed Formulations.

Source	TIME	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
TIME	Linear	13.296	1	13.296	26.563*	.007	.869
TIME * GROUP	Linear	4.103	1	4.103	8.197*	.046	.672
Error(TIME)	Linear	2.002	4	.501			

*p < 0.05 is significant; partial eta squared=0.01 is small; 0.06 is medium; and 0.14 is large.

Furthermore, based on pair-wise comparison using least significant difference as indicated in Appendix L, there is significant difference in the population count of *B. cereus* complex in varying time intervals, p< 0.05. This may be explained by the fact that there is large discrepancy in the population count observed in this time. This may also imply that the addition of the substrate may enhance the growth and stability of *B. cereus* complex on the ninth to twelveth day of monitoring.

Tests of Between-Subjects Effects on the Population Count of *Bacillus cereus* complex Isolate in the Two Fish Feed Formulations.Table 8 shows the Analysis of Variance with repeated measures on tests of between-subjects effects on the population counts of *Bacillus cereus* complex isolate in the two fish feed formulations. There is no significant difference on the population count of *B. cereus* complexin the two fish feed formulation, with and without *S. oligocystum* mixed in the fish feed, F(1,4)=26.563, p(0.093) > 0.05. This means that *B. cereus* complex can survive in the fish feed with and without powderized *S. oligocystum* as additional substrate. As shown in Figure 5, both formulations show the same pattern of growth from the initial day of monitoring until the end. However, better growth rate was observed in fish feed with *S. oligocystum* than without it. The effect size (partial eta squared value = 0.546) is large. This means that the growth rate is due to the effect of the treatment used.

Table 8 - Analysis of Variance with Repeated Measures on Tests of Between-Subjects Effects on the Population Counts of *Bacillus cereus* complex Isolate in the Two Fish Feed Formulations.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Intercept	1420.166	1	1420.166	502.707	.000	.992
GROUP	13.579	1	13.579	4.807	.093	.546
Error	11.300	4	2.825			

P> 0.05 is not significant; partial eta squared=0.01 is small; 0.06 is medium; and 0.14 is large.

Furthermore, based on pair-wise comparison using least significant difference as indicated in Appendix M, there is no significant difference between two fish feed formulations with and without pulverized *S. oligocystum* as substrate for *B. cereus* complex. This means that *B. cereus* complex can survive in fish feeds with its peak after nine to twelve days of monitoring period.

t-Test on the Viability of *Bacillus cereus* complex on Fish Feed Formulations with and without *Sargassum oligocystum*. Table 9 shows the t-Test for independent samples on the viability of *Bacillus cereus* complex on fish feed formulations with and without *Sargassum oligocystum*. There is no significant difference on the viability of *B. cereus* complex in the two fish feed formulations, t (df=16)=-1.562, p(0.138) > 0.05.This may be due to the narrow mean values obtained in the duration of viability. In addition, the feeds with and without *Sargassum oligocystum* as supplement to *Bacillus cereus* complex are the same. Also, *Bacillus cereus* complex is able to survive due to its endospores that may aid in survival in various conditions. *Bacillus cereus* complex may not have or come from seaweed but from soil since no significant difference in population count in 2 feed formulations (with or without *Sargassum*). The effect size (partial eta squared value = 0.13) is moderate. This may imply that the viability is affected partly by the substrate inoculated in one of the fish feed formulated and due to other factors that may not be controlled during the experimentation.

Table 9 - t-Test for Independent Samples on the Viability of *Bacillus cereus* complex on Fish Feed Formulations with and without *Sargassum oligocystum*.

		t-test for Equality of Means			
		t	df	Sig. (2-tailed)	Partial Eta Squared Value
Days Viable	Equal variances assumed	-1.562	16	.138	0.1323

p> 0.05 is not significant; partial eta squared=0.01 is small; 0.06 is medium; and 0.14 is large.

Identification of the Bacterial Isolate. The bacterial culture was identified using the Biomerieux Vitek 2 Identification System, BCL Card. The isolate was reported to be *Bacillus cereus/Bacillus thuringiensis/Bacillus mycoides* with acceptable identification confidence. This is shown in Table 10.

Table 10 - Identity of the Bacterial Isolate used in the study

Isolate code	Identification	Confidence	Percentage Probability	Vitek 2 Bionumber
Bacterial culture isolated from brown algae (*Sargassum oligocystum*)	*Bacillus cereus/ thuringiensis/ mycoides*	Excellent Identification	96%	0327100144446621

Microorganisms belonging to the *Bacillus cereus* group, which includes *Bacillus thuringiensis* and *Bacillus mycoides* are not notable for their phenotypic relatedness. *Bacillus mycoides* can be distinguished from the rest of the group by its rhizoidal colonies. Since the culture does not form rhizoidal colonies, it is unlikely to be *Bacillus mycoides*. Hence, the isolate may be either *Bacillus thuringiensis* or *Bacillus cereus*. The members of *Bacillus thuringiensis* may be distinguished from *Bacillus cereus* by: (1) pathogenicity to *Lepidoptera* larvae (2) production of a protein parasporal crystal (Claus & Berkeley, 1986), (3) sequence analysis of the 16S rRNA sequence and *cry* gene (Chen & Tsen, 2002).

The bacterial culture isolated from brown algae (*Sargassum oligocystum*) had the morphological characteristics characterized in terms of cultural and cellular characteristics. In terms of cultural characteristics: off-white, circular, opaque, dull, convex colonies with smooth surface, entire margin and soft consistency; 1.0-2.0 mm colony diameter. For cellular characteristics: Gram-positive straight to slightly curved rods, arranged in singles, pairs, and short chains with central ellipsoidal spores that occupy nearly all of the sporangium; 2.0-4.0 µm length, 0.8 µm width. Table 10 shows the biochemical characteristics of the most effective isolate.

The result for the identification of the bacterial isolate is consistent with the work of Sharon and Rani (2017) based on the comparative 16srRNA gene sequence analysis. The isolate belongs to the genus *Bacillus cereus* group and it is closely related to *Bacillus cereus* (97.99%), *Bacillus anthracis* (97.99%), *Bacillus thuringiensis* (97.81%), *Bacillus toyonensis* (97.81%), *Bacillus mycoides* (97.45%), *Bacillus weihenstephanensis* (97.45%). The phylogenetic tree showed that isolate *Bacillus cereus* is closely related to *B. mycoides* as indicated in Figure 5. According to Susilowati, Sabdonob, and Widowati (2015), the result from BLAST homology of the bacterial strain is affiliated to the genus *Bacillus* which comes from *Sargassum polycystum*.

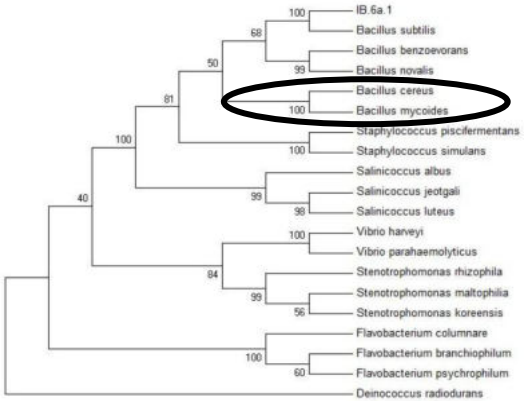

Fig. 4 - Neighbor-joining phylogenetic tree based on comparative 16S rRNA gene sequence analysis of the genus Bacillus showing the phylogenetic affiliation of strain IB.6a.1. Selected sequences Deinococcus radiodurans was used as an outer group.

4.Discussions

Bacterial Isolates and Their Antibacterial Properties from Brown Algae. There were six (6) bacterial isolates obtained from *S. oligocystum*. The presence of these bacterial isolates may be due to an endophytic interaction of marine algae and microorganisms which has also been observed in some seaweeds which provides an interesting biotic environment for these bacterial communities (Se-Kwon Kim, 1807). The seaweed surface provides a suitable substratum for the settlement of microorganisms and also secretes various organic substance that function as nutrients for multiplication of bacteria and the formation of microbial biofilms (Steinberg *et al*., 2002; Staufenberger *et al*., 2008; Singh, 2013). In addition, algal polysaccharides are a potential source of carbon and energy for numerous marine bacteria (Hehemann *et al*., 2012) that produce specific molecules, which in turn facilitate seaweed–bacterial associations (Steinberg *et al*., 2002; Lachnit *et al*., 2013).

It is also suggested on previous research, that bacteria associated had been isolated from 66 strains bacteria isolated from brown algae *Undaria pinnatifida* (Lee, Hyun, & Hong, 2006). These bacteria could acquire the necessary nutrition such as vitamin, polysaccharide a fatty acid from their animal or plant hosts; while on the other, they could excrete products such as amino acid, antibiotic and toxin propitious for the development and metabolism of the hosts, or to improve the chemical defense capability of the hosts (Armstrong, Yan, Boyd, Wright, & Burgess, 2001).

The bacterial isolates from brown algae were effective against *V. harveyi*.

According to Susilowati, Sabdonob, and Widowati (2015), previous research showed that bacteria associated from brown algae *Sargassum* spp. had antibacterial activity with the inhibition zone between 0,5-4,5 mm against pathogenic bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis*,

Serratia marcescens. *Salmonella typhi*, *Klebsiella pneumoniae*, and *Salmonella enteritidis* (Kanagasabhapathy, Sasaki, Soumya, & Yamasaki, 2006). In addition, Susilowati *et al.* (2015) studied the isolation and characterization of bacteria associated with brown algae *Sargassum* spp. from Panjang Island and their antibacterial activities. Bacteria associated with brown algae represent a rich source of bioactive metabolites. Twenty-three marine bacterial strains associated with three species of brown algae Sargassum (*S. polycystum, S. duplicatum* and *S. echinocarphum)* were isolated using ZoBell 2216E from Panjang island, Jepara, North Java.

All bacterial isolates proved to have antibacterial effect against *V. harveyi*. They are able to exhibit competitive exclusion of pathogenic bacteria via producing inhibitory compounds, enhancing the immune response of the host, improving water quality and producing supplemental digestive enzymes that improve nutrition of the host species (Thompson *et al*., 1999; Verchuere *et al*., 2000; Qi *et al*., 2009; Sun *et al*., 2010). The effectiveness of the isolates could be related to the action of competitive exclusion, by which probiotics may create a hostile environment for pathogen colonization (Zokaeifar, 2012). Moreover, according to Verschuere *et al.* (2000), *Bacillus* spp. are able to compete with pathogenic bacteria for nutrients and space, increasing their proportion in the intestinal microbiota of shrimps. A number of studies have demonstrated that *Vibrio harveyi* in shrimp is suppressed by various *Bacillus* spore formers (Rengpipat, Phianphak, Piyatiratitivorakul, & Menasveta, 1998; Vaseeharan & Ramasamy, 2003). Manipulation of microbiota using probiotics have been reported as a worthy practice for aquaculture in order to control or inhibit the pathogen bacteria (Balcazar *et al.,* 2006; Verschuere, Sorgeloos, & Verstraete, 2000; Perez *et al.,* 2010).

According to Vidal *et al*. (2018), probiotic bacteria were more aggressive in competing for space and nutrients when compared to *Vibrio* spp. and promoted a positive substitution of harmful bacteria to the animals, such as *V. parahaemolyticus*, by beneficial bacteria such as *B. cereus.*

Bacterial Isolate, *Bacillus cereus* group. The *Bacillus cereus* group, also known as *B. cereus sensu lato*, consists of Gram-positive, rod-shaped, aerobic bacteria that are wide spread in natural environments (Sharon & Rani, 2017). The bacteria of the *B. cereus* group produce various valuable enzymes and metabolites, (Nilegaonkar *et al*., 2007) degrade different types of pollutants and promote growth of both animals and plants when used as probiotic (Guinebretiere *et al*., 2013; Hong, Duc, & Cutting, 2005). In light of the significance of the *B. cereus* group, the identification and taxonomy of the isolates within the group are of fundamental importance, and therefore have been extensively studied using various typing methods from phenotype to genotype (Sharon & Rani, 2017). In the past, the bacteria of this group were classified into different species according to 16S rRNA gene sequences and characteristics such as the presence or absence of virulence plasmids (*B. anthracis* and *B. thuringiensis*), colonial morphology (*B. mycoides* and *B. pseudomycoides*), psychrophilic or thermotolerant ability (*B. weihenstephanensis* and *B. cytotoxicus*) (Yang Liu, 2015).

A short phylogenetic marker previously used in the reconstruction of the Order *Bacillales* and the genus *Bacillus* was assessed here at a lower taxa level: species in the *Bacillus* cereus group: *B. anthracis, B. cereus, B. thuringiensis* and *B. weihenstephanensis.* This maker is 220 bp in length. It is a combination of 150 bp at the 3' end of the 16S rDNA and 70 bp at the 5' end of the 16S-23S ITS sequence. Three additional *Bacillus* species, *B. halodurans*, B. *licheniformis* and *B. subtilis*, and *Clostridium tetani* were included for comparison purposes. A total of eight bacterial species and 12 strains were analyzed. A boot strapped neighbor joining tree was inferred from comparative analyses of all allelic sequences of the bacterial species and strains under study. Based on its topology, four major Groups were revealed at the 90% nucleotide sequence identities, Group I to IV. Group I contains all alleles of the *Bacillus* cereus group. Group II contains all alleles of *B. halodurans*. Group III contains all alleles of *B. licheniformis* and *B. subtilis*. Group IV contains all alleles of *Clostridium tetani.* The 220 bp phylogenetic marker used here could resolve different species from different genera. At the genus level, distant species could be distinguished. Very closely related species, however, were undistinguishable. Species in the *B. cereus* group, most notably *B. cereus*, *B. anthracis* and *B. thuringiensis*, could not be distinguished. After successfully inferring the phylogenies of the Order *Bacillales* and the genus *Bacillus*, we have met the resolving limit of this short phylogenetic marker: *B. cereus, B. anthracis* and *B. thuringiensis* (Yakoubou& Côté, 2010).

Survival rate and Viability of *Bacillus cereus* in Fish Feed Formulations. The survival rate and viability of *B. cereus* in fish feed formulations may be due to the strong adaptability to diverse conditions and that several species produce highly resistant spores. One of the advantages of using *Bacillus* spp. as probiotics in aquaculture is that they are unlikely to use genes from Gram negative bacteria (e.g., *Vibrio*) that may confer antibiotic resistance (Murillo & Villamil, 2011).Studies have proved that several Gram-positive spore forming *Bacillus* species have the potential to serve as probiotics as well as biocontrol agents in aquaculture (Hong *et al.,* 2005).

Bacillus cereus has been known to be used as probiotics in farmed animals such as swine, calves, poultry and rabbits as well as for human use (Hong *et al*., 2005). There are strains that are deemed safe as health food supplements or novel foods (Hong *et al*., 2005; Mietke *et al*., 2010).

Products containing endospores of members of the genus *Bacillus* (in single doses of up to 10^9 spores/g or 10^9 spores/ml) are used commercially as probiotics, and they offer some advantages over the more common *Lactobacillus* products in that they can be stored indefinitely in a desiccated form (Mazza, 1994). This was observed in *B. cereus* in fish feed with *S. oligocystum*.

The viability can be due to formation of spores. Thus, they are heat stable and can be stored at room temperature as dehydrated products without any deleterious effect affecting their viability. Navin Chandran *et al.* (2014) evaluated the effect of *B. cereus* as probiotic on post-larvae of *Pennaeus monodon* and found a high survival rate in groups treated with feed supplemented with probiotic compared to the control group.

The result of the experiment showed that there were six (6) bacteria isolated from seaweeds *Sargassum oligocystum*. The bacterial isolate 1 showed the highest potential to be a probiotic.Bacterial isolate 1, *B. cereus* complex had higher population count (M=5.33 log CFU/ml) compared to bacterial isolate 6 (M=5.00 log CFU/ml). *Bacillus cereus* complex achieved its peak growth after 12 days in fish feed formulation with *S. oligocystum* and had a higher viability count (M=5.97 CFU/g).There is a significant difference in terms of population count through time but no significant difference on the viability of *Bacillus cereus* complex isolate in the two fish feed formulations and *Bacillus cereus*complex showed better growth rate in terms of population count and viability in fish feed formulation with *Sargassum oligocystum* as additional substrate. Thus, the said bacterium can be utilized by fish feed manufacturers to incorporate probiotic bacteria in commercial fish feed products.

Since bacteria isolated from the seaweed sample were of *Bacillus cereus* complex, it may be implied that seaweed supports the growth of such

bacteria. Bacterial isolate 1 had the highest potential to be a probiotic, it may be implied that this bacterial isolate contain metabolites which can control the population of *Vibrio harveyi*.*Bacillus cereus*complex was able to survive and is viable in fish feeds. This may imply that the viability and survival rate may be due to its spore form *i.e.* heat stable. Products containing endospores belong to the genus *Bacillus* and can be used commercially as probiotics which can be stored in desiccated form.

It is recommended that a study can be conducted using other species of *Sargassum* available in the Philippines since, it is possible that they can isolate more bacteria with probiotic capability.The isolated bacteria, *Bacillus cereus*complex can be used as probiotic in other fish feed formulations.Other preparations of *B. cereus*complexin fish feeds such as lyophilized form or freeze-dried form can be done. Also, consider the recovery rate of the said bacteria in the different fish feed formulations.Screening of probiotic bacteria *Bacillus cereus*complex to other fish pathogens can be done. It can be tried to fish as in vivo studies.Further studies be conducted to identify other marine bacteria from seaweed *Sargassum oligocystum* which are beneficial for industrial use.

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