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Effect of Red Seaweed *Kappaphycus alvarezii* on Growth, Survival, and Disease Resistance of Pacific White Shrimp *Litopenaeus vannamei* Against *Vibrio harveyi* in the Nursery Phase

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Abstract

In this study, the effect of red seaweed *Kappaphycus alvarezii* by-product meal on growth, survival, and disease resistance of white shrimp *Litopenaeus vannamei* against *Vibrio harveyi* was evaluated in the nursery phase. Shrimp was fed for 30 days with four different diets: control (0 g kg^{-1}), 5 g kg^{-1}, 10 g kg^{-1} and 15 g kg^{-1} seaweed supplemented feed. The seaweed supplementation at the higher concentrations (10 and 15 g kg^{-1}) was found to increase the survival of the shrimp, even though not significantly. The highest total biomass obtained in the shrimp group fed with 15 g kg^{-1} seaweed diet ($P>0.05$). Following feeding test, bacterial challenge test using pathogenic *V. harveyi* was done on the shrimp group previously fed with 15 g kg^{-1} seaweed diet. The findings showed that seaweed-supplementation significantly increased up to 10% higher shrimp survival after *Vibrio* challenge. Based on the results of histopathological analysis, hepatopancreas from seaweed-supplemented shrimp showed decreasing tubular epithelial cell lesion by *Vibrio* infection, suggesting that compounds contained in *K. alvarezii* by-product meal could have protected shrimp hepatopancreas from distructive effect of *Vibrio* infection. In general, red seaweed *K. alvarezii* enrichment on shrimp diet provides protection against *V. harveyi* infection during the shrimp nursery phase.

Keywords: Enrichment; Seaweed; Immunostimulant; Shrimp; *Vibrio*

Introduction

Aquaculture sector plays an important role towards food security and nutrition. According to the latest available statistics collected globally by FAO, world aquaculture production of fish and plants combined reached 101.1 million tonnes (MT) in live weight in 2014, for an estimated total farm gate value of US$165.8 billion, with farmed aquatic plants, mostly seaweed, contributing 27.3 MT (US$5.6 billion) as per FAO in 2016. Indonesia is currently the major contributor (36.9%) to the growth in aquatic plant production in the world, with its farming of tropical seaweed species *Kappaphycus alvarezii* and *Eucheuma* spp. as per FAO in 2016. After being the most-traded product for decades, shrimp now ranks second in value terms. In 2014, farmed crustaceans accounted for 8.2% (8.3 MT) of aquaculture production by volume but up to 21.7% (US$35 billion) by value. Asia still accounts for 90% of global shrimp aquaculture, with Indonesia as the 2nd top producer following China as per FAO in 2016 followed by India. The proportional share of Pacific white shrimp (*Litopenaeus vannamei*) in global shrimp production continues to increase, especially after the decline in giant black tiger prawn (*Penaeus monodon*) production due to the white spot syndrome disease outbreaks world-wide. In recent years, although global farmed shrimp production has increased, major producing countries has experienced a decline in output because of shrimp disease. This is mainly as a result of disease-related problems. *Vibrio* spp., especially the luminous *V. harveyi*, has been implicated as the main bacterial pathogens of shrimps [1]. Antibiotics have been used in attempts to control these bacteria, but their efficacy is now, in general, very poor. Antibiotic-resistant strains of pathogens evolve rapidly, and there are also risks of the transfer of resistance to human pathogens and gut. Therefore, development of alternatives to antibiotic treatments, e.g. immunostimulants, for a more sustainable aquaculture is of major concern in disease management in aquaculture production. In recent years, several seaweed species have been studied for their biocompounds/bioactive molecules that can act as immunostimulant. It has been suggested that bioactivity of diverse compounds extracted from seaweeds plays an imperative role in preventing diverse diseases and has antioxidant, antiviral and antimicrobial properties [2]. The antibacterial activity of *Kappaphycus alvarezii* was reported against animal borne bacterial pathogens [3]. It has been suggested by several studies that seaweed or macroalgae provide a great variety of metabolites and natural bioactive compounds with antimicrobial activity, such as polyunsaturated fatty acids, polysaccharides, phlorotannins and other phenolic compounds, and carotenoids [4-16]. Recently, Sakthivel et al. [17] reported that the application of red seaweed by-product paste through enrichment on live food *Artemia* nauplii can enhance the shrimp growth and resistance against salinity stress and *Vibrio* infection in white shrimp culture during the hatchery phase. This current study aims to explore the potential of red seaweed by-product meal as an alternative of growth modulator and anti-infective strategy for white shrimp *L. vannamei* production. The effect of seaweed meal enrichment in the shrimp diet, on the survival, growth and disease resistance of white shrimp culture against pathogenic *V. harveyi* was assessed in the nursery phase.

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Material and Methods

Experimental set-up

This study used Pacific white shrimp *L. vannamei* post larvae for animal test and performed in compliance with guidelines outlined in the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (NRC 2011). Pacific white shrimp 10-days old post larvae (PL10) were collected from shrimp hatchery PT. Suri Tani Pemuka, Indramayu, West Java and acclimated for five days to seawater of 30 ± 1 ppt at 28 ± 1°C in a 220-L (1.1 × 0.8 × 0.25 m) polyethylene tank connected to a biological filter. Before stocking, raw natural seawater was treated to reach 5-10 ppm chlorine concentration 30 min post treatment. The PL were fed live feed *Artemia* nauplii to satisfaction. Following acclimation period, shrimp (PL15) with the average individual body weight of 1.62 ± 0.57 mg were distributed to four treatment groups in a total of 12 100 L-rectangular replicate tanks (200 PL per tank) supplied with dechlorinated heated seawater (30 ± 1 ppt). Water temperature and photoperiod was maintained at 28 ± 1°C and 12L:12D (light:dark). A 10% water renewal was done weekly to maintain optimal water quality parameters. Water physicochemical parameters including pH, temperature, dissolved oxygen (DO), and salinity level was measured two times a week. The measurement of NH_4^+, NO_2^- and NO_3^- level was analyzed using Nessler, Diazotation, and Nitrate HCl method [4].

Seaweed characterizations

Red seaweed by-product meal used in this study contained 44% total carbohydrate (as carrageenan), 7% crude protein and 0.25% crude fat (Table 1). The dried seaweed meal were grounded well in laboratory pulverizer, sieved through a 0.5 mm mesh and used as raw seaweed powder for ingredient of experimental diets/micropellets.

Feeding regime

The experimental seaweed diets were prepared by addition of seaweed powder into commercial shrimp diet (containing total protein and lipid of 30% and 5% dry weight, respectively) at the concentration of 5 g kg^{-1} diet, 10 g kg^{-1} diet and 15 g kg^{-1} diet. The moist mixture was extruded through a 3-mm diameter meat grinder (Hobart Corp., Troy, Idaho, USA). The resulting moist pellets were air-dried at room temperature to a moisture content of about 100 g kg^{-1}. Pellets were ground into small pieces, sieved to obtain appropriate sizes (± 800 µm). For 30 days of feeding experiment, shrimp of each treatment group were fed with either control (commercial) diet or one of the three different seaweed experimental diets at the level of 10% body weight (BW) per day. Total daily food was given in four equal meals at 09:00, 12:00, 15:00 and 18:00. Each diet was tested in four replicates. Due to weekly shrimp sampling with no shrimp replacement, feeding rate was adjusted weekly based on total number of shrimp samples. At the end of the study, proximate analyses were done at at PT. Saraswanti Indo Genetech, Bogor, Indonesia, to determine the total energy, energy from fat, water and ash content, total lipid, total protein, and total carbohydrate, as well as protein (amino acid) and lipid (fatty acid) profiles were performed on all four diets. Water content measured using drying method. The ash content was measured using heating method at temperature 600°C then weighed until constant. Protein content was measured using Kjeldhal method while the lipid content was measured using Soxhlet method. Fatty acid analysis was conducted by using gas chromatography, while carbohydrate content (including nitrogen free extract and crude fiber) was calculated following the equation: Carbohydrate level (%) = 100% - (moisture content + fat content + ash content + protein content). All analyses were performed following the Official Methods of Analysis (AOAC 1999).

Compounds	Percentage (%)
Moisture (by weight)	8
Total carbohydrate (as carrageenan)	44
Crude protein	7
Crude fibre	5
Crude fat (ether extract)	0.25
Salt (water soluble)	16
Total sulfur	3.5
Ash	32

Table 1: Nutritional characteristics of red seaweed *K. alvarezii* by-product meal.

Shrimp survival

Shrimp survival for individual treatments was determined as the number of surviving shrimp at the end of feeding experiment (day 30) relative to the number of shrimp at the beginning of the feeding experiment (day 0). The average weight gain of the shrimp in a tank over the 30 days is calculated by subtracting the weight of the shrimp sampled on the final day of the experiment with the average weight of the shrimp as measured at the beginning of the experiment, and then taking the average. The specific growth rate (SGR) is calculated as follows:

$$SGR\,(\%\,body\ weight\ gain\ per\ day) = \left[\frac{\ln W - \ln W_0}{t}\right] * 100$$

where W is the average body weight after 30 days, W_0 is the average initial body weight, and t is experimental period (30 days). The same approach is used to calculate the feed conversion ratio (FCR), expressed as the feed consumption over the total gained shrimp biomass per treatment, including the biomass of shrimp samples that were taken weekly for microbial community profile evaluation and histology analysis.

Microbial community (MC) profile evaluation

Three shrimp PL from each replicate tank were randomly collected weekly to monitor the shrimp gut microbial community (MC) profile. Shrimp were pooled, rinsed and homogenized in pestle and mortar with 100 µL of 9 g L^{-1} NaCl sterile saline solution. Subsequently, 50 µL of the homogenate was plated on Marine Agar plates. 50 µL of the homogenate was also plated on Thiosulphate Citrate Bile Salts Agar (TCBS) agar plates to count the total number of *Vibrio*. In addition to the shrimp samples, culture water was also sampled weekly from each replicate tank to monitor the microbial community (MC) profile in the culture water. 50 µL of the culture water was plated on Marine Agar plates. 50 µL of the culture water was also plated on TCBS agar plates to count the total number of *Vibrio*. All of the inoculated plates were incubated at 27 ± 1°C for 24 h and the total number of bacteria was counted to determine the total bacteria count (TBC) and *Vibrio* count. Identification of bacteria was carried out using molecular approach. Bacterial DNA extraction (using commercial Qiagen DNeasy blood and tissue kit), polymerase chain reaction/PCR amplification (using 27F/1492R primer) and bacterial rRNA 16S gene sequencing (using 785F/907R primer) was done at the Macrogen Inc., Korea. Sequence homology search for the test sequences was done using Nucleotide Blast versus Genebank and RDP (Ribosomal Database Project) data. Results were validated with a phylogenetic analysis using the Winclada program and the Ratchet method (Island Hopper).

Bacterial challenge procedure

Following the feeding experiment, bacterial challenge test using

pathogenic *Vibrio harveyi* was done on the shrimp from the 15 g kg^{-1} seaweed diet treatment group (that reached the highest final total biomass following 30 d feeding experiment). The shrimp from 15 g kg^{-1} diet treatment group of the feeding test was divided into two groups: (1) kept on previous 15 g kg^{-1} seaweed diet, then challenged or not challenged; and (2) switched onto control (commercial) diet, then challenged or not challenged. Additionally, the control group from the feeding test were kept on the control diet and divided into two groups, challenged or not challenged. A total of six experimental groups were tested in the bacterial challenge procedure (Table 2). At the start of the challenge period, the shrimp culture density was adjusted to 35 PL per replicate tank. Shrimp PL50 were fed at feeding level of 10% BW per day. Total daily food was given in four equal meals a day. For the bacterial challenge, suspension of pathogenic *Vibrio harveyi* culture was harvested by centrifuging at 1000 × *g* for 10 min and washed twice in its culture medium followed by 1 time washing using shrimp culture water before added to the respective replicate tanks. The density of the bacterial suspension prior to addition was determined based on the McFarland standard (BioMérieux, Marcy L'Etoile, France) by measuring the turbidity with a spectrophotometer (Genesys 20, Thermospectronic) at 550 nm. For each replicate tank, bacteria inoculum was added at a pathogenic density level of 10^6 CFU mL^{-1} [17].

Shrimp survival and growth evaluation

Following 16 days of challenge period, shrimp body weight gain, SGR, and survival for each of the treatment groups is calculated as mentioned in section 2.4.

Shrimp gut MC profile evaluation

Three shrimp PL from each replicate tank were randomly collected every five days during the bacterial challenge period to monitor the dynamics of shrimp gut MC profile. Shrimp were pooled, rinsed and homogenized in pestle and mortar with 100 µL of 9 g L^{-1} NaCl sterile saline solution. Subsequently, 50 µL of the homogenate was plated on Marine Agar plates. The inoculated plates were incubated at 27 ± 1°C for 24 h and the total number of bacteria was counted. 50 µL of the homogenate was also plated on TCBS agar plates to count the total number of *Vibrio*. Identification of grown bacteria culture was carried out using molecular approach as mentioned in section 2.5.

Treatment	Previous diet during feeding experiment	Diet during challenge period	Vibrio challenge
1	15 g kg^{-1} seaweed diet	Kept on 15 g kg^{-1} seaweed diet	Not challenged
2	15 g kg^{-1} seaweed diet	Switched onto control diet	
3	Control diet	Kept on control diet	
4	15 g kg^{-1} seaweed diet	Kept on 15 g kg^{-1} seaweed diet	Challenged
5	15 g kg^{-1} seaweed diet	Switched onto control diet	
6	Control diet	Kept on control diet	

Table 2: Experimental set up of bacterial challenge.

Parameters	Unit	Control	5 g kg^{-1}	10 g kg^{-1}	15 g kg^{-1}
Total energy	kkal / 100 g	329.25	341.34	343.13	344.45
Energy from fat	kkal / 100 g	41.85	56.34	55.53	55.17
Water content	%	11.11	11.8	10.52	10.16
Ash content	%	12.39	10.69	11.41	11.39
Total Lipid	%	4.65	6.26	6.17	6.13
Total Protein	%	30.93	31.28	31.46	31.59
Total carbohydrate	%	40.92	39.97	40.44	40.73

Table 3: Proximate analysis of different experimental diets.

Histology analysis

Three shrimp PL from each replicate tank were randomly collected at the beginning and end of the feeding experiment as well as the bacterial challenge for histology analysis of the shrimp hepatopancreas. Following collection, shrimp PL was fixed in Bouin's solution for at least 72 hours. After that, samples were dehydrated through a series of alcohol solution as follows: 70% alcohol for 1 h continued by 80% alcohol, 90% alcohol, and 100% alcohol (30 min each). The samples were then cleared according to processing procedure for animal tissues by soaking the dehydrated samples into xylene I, xylene II, xylene III (30 min each) for infiltration. Finally, samples were embedded into melted paraffin for at least 3 hours. After that, the samples were cut and sectioned with 6-7 µm thickness using microtome, resulting floating tissue ribbons. Sections were placed on slides. Shrimp PL were then stained using hematoxylin and eosin procedure according to the procedure as followed: xylene I, continued by xylene II, 100% alcohol I, 100% alcohol II (each for 5 min), 95% alcohol for 2 min, 70% alcohol for 2 min, running tap water for 2 min, hematoxylin for 10 min, running tap water for 2 min, acetone acid for 3 min, running tap water for 2 min, 2% potassium acetate for 3 min, running tap water for 2 min, eosin, 95% alcohol, 95% alcohol, xylene I, xylene II (each for 5 min) and finished with mounting. The slide samples were labeled and analyzed using inverted microscope Nikon.

Statistical analysis

Statistical analysis was performed with SPSS Version 11.0 statistic software package. Normalization of the distribution of the survival percentage data was done using arcsin transformation. Comparison of the shrimp survival, final body weight, SGR, and FCR were done using one-way analysis of variance (ANOVA) analysis. Data were expressed as means ± Standard Deviation (SD). Grouping of treatments based on significant differences in mean values from treatment replicates was done according to Duncan test (0.05 level of confidence).

Results

Nutritional properties of experimental diets

Based on the results of the proximate analyses, all seaweed-supplemented diets (5, 10, and 15 g kg^{-1} diet) were found to contain higher dietary energy level than the control (commercial) diet, with the differences of 12.1 to 15.2 kkal per 100 g diet (Table 3). A higher level of total lipid, as well as total protein, was observed in all three seaweed-supplemented diets when compared to the control diet (Table 3). As the consequences, lower levels of ash, water and carbohydrate content were found in the seaweed-supplemented diets compared to the control diet. No statistical analyses were performed on these data as single data were produced from the proximate analyses of each experimental diet. Related to the higher total protein content that was found in all seaweed-supplemented diets (5 g, 10 g, and 15 g seaweed powder kg^{-1} diet), amino acids analyses confirmed that the seaweed-supplemented diets contained higher level of essentials amino acids compared to the control diet (Table 4). Furthermore, related to the higher total lipid content that was found in all seaweed-supplemented diets (5, 10, and 15 g seaweed powder kg^{-1} diet), fatty acids analyses confirmed that the seaweed-supplemented diets contained higher level of fatty acids (unsaturated lipids, i.e. mono-unsaturated fatty acids/MUFA), poly-unsaturated fatty acids/PUFA, saturated lipids, arachidonic acid/AA), docosahexaenoic acid/DHA), eicosapentaenoic acid/EPA) compared to the control diet (Table 5).

Shrimp survival and growth following 30 d feeding period

Following 30 days of feeding experiment, the groups fed with seaweed-supplemented diets showed lower specific growth rate when compared to the control group (P<0.05) (Table 6). The food conversion rate (FCR) of the 5 g kg^{-1} seaweed diet treatment group was found to be significantly lower compared to the control group. However, no significant differences were found in the FCR between the other seaweed diet treatment groups (10 g kg^{-1} or 15 g kg^{-1}) compared to the control diet (P>0.05). The shrimp survival was in a range of 75% to 86%, while the average of final total biomass of shrimp was in a range of 3.5 g to 4.7 g. There were no significant differences in survival nor final total biomass observed among different treatment groups (P>0.05) (Table 6).

Amino acid (g kg^{-1})	Control	5 g kg^{-1}	10 g kg^{-1}	15 g kg^{-1}
Histidine	8.52	15.61	16.4	15.59
Threonine	15.69	26.91	32.36	27.91
Valine	15.36	25.25	33.66	26.47
Methionine	6.91	11.99	13.66	12.24
Isoleucine	13.57	22.42	29.17	23.32
Leucine	25.98	43.63	55.96	45.27
Phenylalanine	19.03	36.47	36.53	36.73
Lysine	22.64	34.13	55.66	37.78
Tryptophan	1.87	21.85	22.46	23.71

Table 4: Amino acid profile of different experimental diets.

Fatty Acid (g kg^{-1})	Control	5 g kg^{-1}	10 g kg^{-1}	15 g kg^{-1}
Mono-unsaturated fatty acids (MUFA)	11.15	15.24	14.4	14.42
Poly-unsaturated fatty acids (PUFA)	18.87	24.63	24.27	24.02
Unsaturated lipids	30.02	39.86	38.66	38.44
Saturated lipids	16.48	22.74	23.04	22.86
Arachidonic acid (AA)	0.62	0.87	0.82	0.88
Docosahexaenoic acid (DHA)	3.7	4.73	4.53	4.54
Eicosapentaenoic acid (EPA)	3.35	4.32	4.15	4.12
Omega 3 total	8.15	10.55	10.2	10.15
Omega 6 total	10.45	13.72	13.74	13.54
Omega 9 total	7.29	10.03	9.18	9.18

Table 5: Lipid (fatty acid) profile of different experimental diets.

Treatment	Survival (%)	Final total biomass (g)	SGR (%BW day^{-1})	FCR
5 g kg^{-1} seaweed diet	74.86 ± 2.23 ^{a}	3.57 ± 0.04 ^{a}	9.34 ± 0.14 ^{ab}	0.89 ± 0.11 ^{a}
10 g kg^{-1} seaweed diet	86.59 ± 5.59 ^{a}	3.50 ± 0.50 ^{a}	8.77 ± 0.65 ^{b}	1.19 ± 0.01 ^{b}
15 g kg^{-1} seaweed diet	86.78 ± 10.74 ^{a}	3.65 ± 0.08 ^{a}	8.93 ± 0.45 ^{b}	1.24 ± 0.18 ^{b}
Control diet	83.05 ± 9.08 ^{a}	4.73 ± 0.54 ^{a}	9.92 ± 0.51 ^{a}	1.16 ± 0.03 ^{b}

Table 6: Shrimp survival and growth following 30 d feeding period with four different experimental diets at the nursery phase.

Treatment	Bacteria load (CFU mL^{-1})					
	TBC			*Vibrio* count		
	Initial (day 0)	Middle (day 14)	Final (day 30)	Initial (day 0)	Middle (day 14)	Final (day 30)
Control diet	1.53 × 10^{5}	9.10 × 10^{6}	2.70 × 10^{6}	2.68 × 10^{4}	1.14 × 10^{6}	3.00 × 10^{4}
5 g kg^{-1} seaweed diet	*Na*	1.00 × 10^{7}	5.10 × 10^{7}	*Na*	1.04 × 10^{6}	2.96 × 10^{4}
10 g kg^{-1} seaweed diet	*Na*	5.35 × 10^{6}	5.80 × 10^{7}	*Na*	1.01 × 10^{6}	1.39 × 10^{5}
15 g kg^{-1} seaweed diet	*Na*	7.95 × 10^{6}	8.65 × 10^{6}	*Na*	9.85 × 10^{5}	1.88 × 10^{5}

Table 7: Bacterial load in shrimp during 30 d feeding experiment with four different experimental diets at the nursery phase.

Treatment	Bacteria load (CFU mL^{-1})					
	TBC			Vibrio count		
	Initial (day 0)	Middle (day 14)	Final (day 30)	Initial (day 0)	Middle (day 14)	Final (day 30)
Control diet	1.00 × 10^{5}	7.65 × 10^{5}	9.80 × 10^{5}	5.95 × 10^{2}	2.48 × 10^{3}	9.60 × 10^{2}
5 g kg^{-1} seaweed diet	1.45 × 10^{5}	4.25 × 10^{5}	1.80 × 10^{5}	2.65 × 10^{2}	1.86 × 10^{3}	1.63 × 10^{3}
10 g kg^{-1} seaweed diet	1.50 × 10^{5}	2.45 × 10^{5}	9.85 × 10^{5}	3.75 × 10^{2}	6.55 × 10^{3}	1.32 × 10^{4}
15 g kg^{-1} seaweed diet	2.75 × 10^{5}	7.55 × 10^{5}	2.18 × 10^{5}	4.20 × 10^{2}	2.22 × 10^{3}	1.46 × 10^{4}

Table 8: Bacterial load in culture water during 30 d feeding experiment with four different experimental diets at the nursery phase.

Culture microbial community (MC) profile during 30 d feeding period

The *Vibrio* count in the shrimp and the culture water at the end of the feeding period was in a range of 3.00 × 10^{4} to 1.88 × 10^{5} CFU mL^{-1} and 9.60 × 10^{2} to 1.46 × 10^{4} CFU mL^{-1}, respectively. The total bacteria count (TBC) in the shrimp and the culture water at the end of the feeding period was in a range of 2.70 × 10^{6} to 5.80 × 10^{7} CFU mL^{-1} and 1.80 × 10^{5} to 9.80 × 10^{5} CFU mL^{-1}, respectively, with relatively similar bacterial loads among dietary treatments (Tables 7 and 8). Results of the 16S rDNA gene sequencing analysis showed that the *Vibrio* sp. group was the dominant component in both the shrimp and culture water microflora during the 30 days of feeding period. The rest of the bacteria groups existed at a low density, including *Pseudoalteromonas* sp. (closely related to *Pseudoalteromonas piscicida*) and *Alteromonas* sp. that were found in both the shrimp and culture water, *Pantoea* sp. (closely related to *Pantoea anthophila*) that were found only in the shrimp, and also *Kocuria* sp. that were found only in the culture water.

Shrimp survival and growth following 16 d challenge period with *V. harveyi*

The treatment group that was fed with the 15 g kg^{-1} seaweed diet during the feeding as well as challenge period resulted in about 10% significantly higher survival percentage (73.3 ± 1.6%) (P<0.05) after *Vibrio* challenge when compared to the survival percentage of the challenged control group at the end of the challenge period (63.8 ± 1.6%) (Table 9). In addition, protection was still observed in the group that was fed with the 15 g kg^{-1} seaweed diet during the feeding but then switched to the control diet during the challenge period, with about 4% higher survival percentage (67.6 ± 4.4%) (P>0.05) after challenge when compared to the challenged control group at the end of the challenge period (Table 9). There was no difference in either final body weight or specific growth rate of the shrimp among all different experimental groups due to the *Vibrio* challenge (Table 9).

Culture microbial community (MC) profile during challenge period

The *Vibrio* counts at the end of the feeding period were relatively

similar in all six treatment groups, at a range of 8.40×10^6 to 2.58×10^7 CFU mL^{-1} and 1.80×10^3 to 1.73×10^4 CFU mL^{-1} in the shrimp and the culture water, respectively. In contrast, the total bacteria count (TBC) in both the shrimp and culture water of the control groups –fed with control diet since the beginning– at the end of the challenge period were much higher than the treatment groups –once fed or continued fed with 15 g kg^{-1} seaweed diet. The TBC in the shrimp and culture water of the control groups was in a range of 2.98×10^{13} to 2.94×10^{14} CFU mL^{-1} and 5.00×10^9 to 8.35×10^{10} CFU mL^{-1}, respectively, while in the treatment groups it was only in range of 9.90×10^7 to 2.92×10^8 CFU mL^{-1} and 8.10×10^6 to 2.72×10^7 CFU mL^{-1}, respectively (Tables 10 and 11).

Histology of shrimp hepatopancreas following feeding experiment and *Vibrio* challenge

Histopathological section of the control hepatopancreas (Figure 1a) showed normal structure of hepatopancreas with large compact tubules consisting of single layer of vacuolated epithelial cells enclosed a lumen and surrounded by intertubular space and connective tissues. Moreover, histopathological observations of hepatopancreas of seaweed–diet groups showed less number of vacuolated epithelial cells that are supposed to form single layer of epithelial cells in hepatopancreas tubules (Figures 1b and 1c). In addition, the diameter of hepatopancreas tubules of seaweed-diet groups is relatively larger compared to the hepatopancreas tubules of control-diet group. The tubules of control-diet groups after challenged tend to disintegrated and several parts of hepatopancreas cortex exhibit necrotic foci. In shrimp hepatopancreas, the predominant cells that constitute the tubules are B- and R-cells whose characteristics are cylindrical in shape, containing subapical vacuoles, and nucleus positioned in the basal part of the cells (Figure 1).

No	Treatment			Survival	Final BW (g)	SGR
	Previous diet during feeding test	Diet during challenge test	*V. harveyi* challenge	(%)		(%BW day^{-1})
1	15 g kg^{-1} seaweed diet	15 g kg^{-1} seaweed diet	Not challenged	76.2 ± 4.4^{a}	0.093 ± 0.009^{a}	7.71 ± 0.61^{a}
2	15 g kg^{-1} seaweed diet	Control diet		69.5 ± 6.6^{abc}	0.090 ± 0.021^{a}	7.42 ± 1.60^{a}
3	Control diet	Control diet		73.3 ± 1.6^{ab}	0.097 ± 0.013^{a}	7.94 ± 0.86^{a}
4	15 g kg^{-1} seaweed diet	15 g kg^{-1} seaweed diet	Challenged	73.3 ± 1.6^{ab}	0.080 ± 0.026^{a}	6.56 ± 2.28^{a}
5	15 g kg^{-1} seaweed diet	Control diet		67.6 ± 4.4^{bc}	0.092 ± 0.014^{a}	7.60 ± 1.01^{a}
6	Control diet	Control diet		63.8 ± 1.6^{c}	0.078 ± 0.021^{a}	6.49 ± 1.84^{a}

Table 9: Shrimp survival and growth following 16 d challenge period with *V. harveyi*.

No	Treatment			Bacteria load (CFU mL^{-1})			
	Previous diet during feeding period	Diet during challenge period	*V. harveyi* challenge	TBC		*Vibrio* count	
				Initial (Day 0)	Final (Day 16)	Initial (Day 0)	Final (Day 16)
1	15 g kg^{-1} seaweed diet	15 g kg^{-1} seaweed diet	Not challenged	*Na*	1.65×10^8	*Na*	8.40×10^6
2	15 g kg^{-1} seaweed diet	Control diet		*Na*	2.92×10^8	*Na*	1.39×10^7
3	Control diet	Control diet		*Na*	2.98×10^{13}	*Na*	2.58×10^7
4	15 g kg^{-1} seaweed diet	15 g kg^{-1} seaweed diet	Challenged	9.67×10^4	9.90×10^7	2.30×10^5	1.05×10^7
5	15 g kg^{-1} seaweed diet	Control diet		*Na*	2.03×10^8	*Na*	1.22×10^7
6	Control diet	Control diet		5.90×10^5	2.94×10^{14}	1.05×10^5	7.30×10^6

Table 10: Bacterial load in shrimp during 16 d challenge period with *V. harveyi*.

No	Treatment			Bacteria load (CFU mL^{-1})			
	Previous diet during feeding period	Diet during challenge period	*V. harveyi* challenge	TBC		*Vibrio* count	
				Initial (Day 0)	Final (Day 16)	Initial (Day 0)	Final (Day 16)
1	15 g kg^{-1} seaweed diet	15 g kg^{-1} seaweed diet	Not challenged	1.40×10^5	1.05×10^7	8.45×10^3	7.75×10^3
2	15 g kg^{-1} seaweed diet	Control diet		2.86×10^5	8.10×10^6	1.52×10^4	1.80×10^3
3	Control diet	Control diet		2.10×10^5	5.00×10^9	1.04×10^4	2.80×10^3
4	15 g kg^{-1} seaweed diet	15 g kg^{-1} seaweed diet	Challenged	2.98×10^5	2.72×10^7	1.24×10^4	1.73×10^4
5	15 g kg^{-1} seaweed diet	Control diet		2.08×10^5	2.03×10^7	1.01×10^4	2.20×10^3
6	Control diet	Control diet		2.80×10^5	8.35×10^{10}	1.38×10^4	1.69×10^4

Table 11: Bacterial load in culture water during 16 d challenge period.

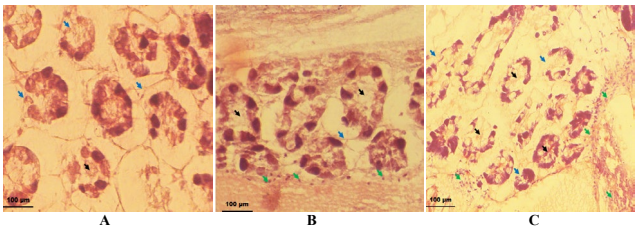

Figure 1: Hepatopancreatic organs of shrimps treated with seaweed or control diet and challenged with Vibrio. (A) Hepatopancreas section of shrimps fed with 15 g kg^{-1} seaweed diet during both feeding and challenge period then challenged. (B) Hepatopancreas section of shrimps fed with 15 g kg^{-1} seaweed diet during feeding period and switched to control diet during challenge period then challenged. Intact morphology of hepatopancreatic tissue with less damage on the hepatopancreatic tubule epithelium (blue arrowhead) and lumen (black arrowhead) (A and B). (C) Hepatopancreas section of shrimps fed with control diet during both feeding and challenge period then challenged. Hepatopancreatic defects showing massive sloughed of hepatopancreatic tubules, as well as phagocytes as signs of tissue necrosis (green arrowhead) (C). Scale bars 100 µm.

Discussion

Shrimp growth and survival following 30 d feeding period using different seaweed diets

In this study, the results of the proximate analysis showed that the seaweed diets contained higher energy levels (around 137 kkal per g higher than the control diet), which was found to be the extra energy from lipid. The lipid content of the seaweed diets was about 1.5% higher than the control diet. Dietary lipids are a highly digestible and

concentrated source of energy which supply 9 kcal/g, about double of that supplied by either carbohydrate or protein [10]. Dietary lipids are a source of essential fatty acids, phospholipids, sterols and carotenoids, required for growth, survival and normal metabolic function of all organisms. However, dietary lipids have a sparing effect on the utilization of dietary protein. It has been suggested that diet with excess energy content will inhibit/result in decreased food intake and also reduce the protein available for growth (causing depressed growth/reduced weight gain), which are likely a result of nutrient imbalances or deficiencies related to energy and the toxic products of lipid oxidation [10]. Even though the seaweed diets were found to contain of about 0.5% higher level of protein than control diet, this increase of dietary protein was not enough to contribute to a significantly higher growth. The excess energy in the diet may have limited the food consumption and thereby reducing the intake of protein, which might have been the reason for the depressed growth in the shrimp fed with seaweed-supplemented diets. Unlike the negative growth effect, the effect of dietary seaweed on the shrimp survival was neutral, as there was no significant difference in survival between groups fed with control and seaweed diets.

Shrimp growth and survival following 16 d *Vibrio* challenge period

The effect of dietary seaweed-supplementation on the disease (*vibrio*sis) resistance of the shrimp was positive as the results of the *Vibrio* challenge test showed that the seaweed-supplemented diet (15 g kg^{-1}) can provide protection to *Vibrio* infection. The use of 15 g kg^{-1} seaweed diet contributed to up to 10% higher survival after *Vibrio* challenge compared to the challenged control group, which may have been resulted from the higher level of fatty acids (especially the polyunsaturated fatty acids) in the seaweed diet. It has been suggested by several studies that seaweed or macroalgae provide a great variety of metabolites and natural bioactive compounds with antimicrobial activity, such as polyunsaturated fatty acids, polysaccharides, phlorotannins and other phenolic compounds, and carotenoids [12-16]. Similar results in protection of shrimp from *Vibrio* infection due to %utilization was also reported by Siegel and Weiser [18], where the seaweed extract (with the bioactive compounds including alcohols, phenols, alkenes, esters and ethers) was found to have antagonism effect against luminescence disease causing *Vibrio harveyi* during shrimp *Penaeus monodon* larviculture, possibly by reducing the exopolysaccharide and protease produced by *V. harveyi* that play a key role in developing infections among the shrimps.

Microbial community in shrimp and culture water following feeding experiment and *Vibrio* challenge

Vibrio sp. group was the dominant component in both the shrimp and culture water microflora following both feeding experiment and *Vibrio* challenge. Following the feeding experiment, the rest of the bacteria groups existed at a low density, including *Pseudoalteromonas* sp. (closely related to *Pseudoalteromonas piscicida*) and *Alteromonas* sp. that were found in both the shrimp and culture water, *Pantoea* sp. (closely related to *Pantoea anthophila*) that was found only in the shrimp, and *Kocuria* sp. that was found only in the culture water. Following *Vibrio* challenge, *Pantoea* sp. and *Kocuria* sp. were not observed anymore in the shrimp/culture water. At the end of the challenge period, the *Vibrio* counts (in both shrimp and culture water) in the groups fed with seaweed diet was similar to the control group. This suggested that the higher survival in the groups fed with seaweed diets was not necessarily due to a direct effect of the seaweed product on the *Vibrio* culture. However, the significantly lower total bacteria count after 16 days of *Vibrio* challenge in both the shrimp and culture water of the treatment group fed with seaweed diet could have contributed to the higher shrimp survival compared to the control groups.

Histology of shrimp hepatopancreas following *Vibrio* challenge test

Similar *Vibrio* counts in the shrimp and culture water between control and seaweed treatment groups indicated that the higher survival after *Vibrio* challenge obtained in the seaweed- treatment groups may thus not be related to the *Vibrio* bacterial load in the culture. This suggested that the protection against *Vibrio* infection obtained in the seaweed treatment groups could be due to direct physiological effects of seaweed supplementation to the shrimp. In this study, however, we found that in the hepatopancreas of control shrimp after challanged, the integrity of tubules was distrupted. This may be due to the inflitration of bacteria into the hepatopancreas and those bacteria infiltrating surrounding tissues disrupted the integrity of tubules through stimulation of necrosis of infected cells. Similarly, a study done by others shown that several *Vibrio* species were found in healthy hepatopancreas in *P. vannamei* [9]. Although, other researchers have found that there were no sign of bacteria in masssive sloughing of hepatopancreas because gastric barrier prevents bacteria to infiltrate the hepatopancreas [6]. We hypothesized that the barrier failed to prevent bacteria or their toxin for entering the gut and eventually infiltrate to hepatopancreas leading to massive sloughing of the tubules. One study has postulated that several compunds, such as protein from lectin family of *Gracilaria fischeri* may opsonize the bacterial surface causing decrease of colonization of bacteria; thus, reducing the production of bacterial toxins that can damage hepatopancreas [19-23]. Another study suggests that red seaweed %powder could prevent the infection by *Vibrio* by stimulating the immune system [14]. This may be achived by the immunostimulant ability of polysaccharides consisted in the seaweed product [2-14]. In addition, one study also argued that seaweed could defend shrimps from bacterial infection because it contains halogenated compounds [11]. In this study, there was no difference observed regarding the feeding administration pattern of seaweed, whether seaweed-supplemented diet was given before and after challenged with *Vibrio* or only before challenged with *Vibrio*, the hepatopancreas tissues were protected from bacterial infection.

Conclusion and Recommendations

In general, the growth enhancing effect of seaweed products could not be observed in this study, which was likely due to the rather unbalance nutritional property (excessive energy/dietary lipid level) of the seaweed-supplemented diets. Nevertheless, the results of this study suggest that seaweed supplementation can provide protection against *Vibrio harveyi* infection and hence increase the shrimp survival after the pathogen infection, although growth improvement of the shrimp culture when exposed to pathogenic *Vibrio* could not be observed. Further research must focus on the formulation of the seaweed-supplemented diets which fit the requirement of nutrient levels for shrimp culture. Fermentation of the seaweed products prior use can be done to increase the protein level of the formulated diet, which may enhance the growth of shrimp culture. Furthermore, detailed chemical characterization, identification and isolation of bioactive compounds through bioassay guided fractionation of the seaweed products needs to be performed in order to increase the effectiveness and efficiency of the seaweed products utilizations.

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