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Comparative study on length-weight relationship and *Vibrio* composition between normal and stunted growth in pond-cultured whiteleg shrimp, *Litopenaeus vannamei*

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ABSTRACT

The occurrence of stunted growth in *L. vannamei* culture causing large size variation of shrimps may lead to huge profit loss. Despite causing concerns to farmers, detailed information on the stunted growth in *L. vannamei* related to *Vibrio* loads and its composition is limited. Normal and stunted growth in shrimp was collected from shrimp ponds in Tuaran, Malaysia. Investigation of the length-weight relationship and *Vibrio* composition between normal and stunted grown shrimps were conducted. Statistical analysis showed that size variations were present in all age groups between normal shrimps and stunted shrimps. The regression coefficient (b value) for normal shrimp was 2.92, which is not significantly less than 3.00 indicating an isometric growth pattern. Meanwhile, a positive allometric growth pattern was observed in stunted grown shrimp with b value of 3.41. The coefficient correlation (r value) of normal shrimp and stunted shrimp was 0.95 and 0.94, respectively. No significant difference was found regarding the total bacteria count and the total *Vibrio* count between normal and stunted grown shrimps. However, the tests' results revealed that *Vibrio* isolates obtained from stunted shrimp showed a higher variation of phenotypic characteristics, compared to isolates from normal shrimp. The study revealed that normal shrimp had negative allometric growth, while stunted shrimp recorded positive allometric growth. The finding in this study indicates that stunted growth shrimp might have a more diverse and complex bacterial composition than normal shrimp. The present study managed to provide information on the size difference and length-weight relationship of normal and stunted grown *L. vannamei*, opening up the opportunity to conduct more research to find out the possible cause of stunted shrimp problems.

INTRODUCTION

Shrimp aquaculture industry is quickly expanding and now contributes 15% of products from the worldwide seafood production. One of the main species in shrimp aquaculture is the white legged shrimp, or scientifically known as *Litopenaeus vannamei*.

This species has been the choice for shrimp farmers since they have a fast-growing rate and good market value **(Liao & Chien, 2011)**. Although many studies have been conducted on *L. vannamei*, problems related to growth still occur especially under a normal culture environment.

In recent years, there have been increasing reports of smaller shrimp sizes in production, causing large size variation of shrimps in one crop. According to a survey conducted by the Global Aquaculture Alliance, the proportion of small-sized shrimp in harvest yield rose from 27% in 2010 to 48% in 2017. Although the percentage of small-sized shrimp counts declined to 37% in the most recent study, it remains higher than the percentages recorded prior to 2011. Growth retardation in shrimp development was regarded as an effect of pathogenic agents such as bacterial *Vibrio* causing acute hepatopancreatic necrosis disesase (AHPND); infections of *Enterocytozoon hepatopenaei* (EHP) parasites or viral pathogens **(Aranguren *et al.*, 2017**; **Fang *et al.*, 2019)**. However, affected farms reported very few mortality cases of shrimps with this condition, whereas slow growing shrimp survived until harvest. Despite causing concerns to shrimp farmers since it affects the production yield causing profit loss, there are currently few studies with detailed information on the features of the affected *L. vannamei*. The study on its growth or length and weight analysis in relation to bacterial loads should be gathered and processed in order to make an in-depth analysis.

The occurrence of stunted growth was recently linked to that affected by the *Enterocytozoon hepatopenaei* (EHP) parasite **(Tang *et al.*, 2015)**. Nevertheless, possible association of *Vibrio* and viruses was also thought to contribute to the occurrence of the stunted growth in shrimp. The *Vibrio* spp. are common bacteria found in shrimp and known to cause diseases for shrimp **(Souza Valente & Wan, 2021)**. It is well reported that the pathogen responsible for vibriosis is highly associated with acute hepatopancreatic necrosis disease (AHPND), a disease that can possibly cause slow growth in shrimp **(Hong *et al.*, 2016)**. In other cases, viral infections (e.g. IHHNV, LSNV, 320 MBV, HPV) were previously discovered leading to the slow or stunting growth of *Penaeus* species **(Flegel *et al.*, 1999; Flegel *et al.*, 2004; Rai *et al.*, 2009)**. Only until recently, the hypothesis for the mechanism of slow growth was supported by gene expression profiles, since many genes of shrimp, which participate in the infecting process of viruses were up-regulated in sick shrimp with stunting symptom. This may originate from the reason that the shrimp infected by viruses could not appropriately metabolize protein and sugar (starch and sucrose) **(Fang *et al.*, 2019)**. In the current case, stunted shrimp did not show diseased conditions related to viral infections. Therefore, the first information that should be retreived in this case is the bacterial composition of stunted growth, *L. Vannamei*, that needs to be further studied since literature is restricted.

This research aimed to establish a clear distinction between normal and stunted shrimps in terms of weight and length, as well as determine and compare the length-

weight relationship of normal and stunted shrimps. In addition, the study investigated the bacterial aspects, specifically the composition of *Vibrio* species in stunted grown shrimp compared to normal grown shrimp. The current results would contribute to our understanding of the characteristics of stunted growth in shrimp in *L. vannamei* culture.

MATERIALS AND METHODS

Sampling location

Samples were collected from a local farm in Tuaran, Sabah, Malaysia (6^o 13' 2.92'', 116^o 12' 35.61''). The local farm is located approximately at 2.5km from the nearest coastal area in the estuarine area of Sungai Tuaran, a river which flows to the South of the China Sea. The shrimp farm has five earthen ponds; one was used as a reservoir and the other four ponds were used for shrimp culture. The area of the ponds ranged from 4000-5000m^2, with a stocking density of 100 piece of postlarvae m^{-2}. The farm practices a closed water system, with an inlet and an outlet for water exchange purpose. Paddle wheel aerators were used in their pond water aeration system.

Shrimp samplings

Ten normal shrimps and ten stunted shrimps were collected in each sampling. Shrimp was classified as stunted if it showed slow growth, and the coefficient of size variation was greater than 35% **(Pratoomthai *et al.*, 2008)**. Live shrimp samples were collected from culture pond using mesh net and *in-situ* water parameter such as pH, temperature (°C), salinity (‰) while dissolved oxygen levels of culture pond were recorded using portable multi-parameter water quality meter (HANNA Instruments, USA).

Shrimp were grouped into normal and stunted shrimps. Shrimp samples were kept in an ice box for preservation and immediately transferred to Borneo Marine Research Institute in Universiti Malaysia Sabah. All samples were immediately processed in the disease laboratory. Total length was measured from the tip of shrimp rostrum to the end of telson using a ruler (±0.1 cm precision). Shrimp body weight was measured using analytical balance (±0.1 g precision). The pictures of shrimps were also taken.

Length – weight relationship analysis

The total length of shrimp was regressed with the body weight to find the length-weight relationship and the equation parameters 'a' and 'b'. Length-weight relationship is practical in biological study and stock assessments of aquatic species **(Aydin, 2018)**. Through length-weight relationship analysis, the estimation of weight can be determined from a given length, while the morphology between population of the same or different species can be compared.

Length-weight relationship of shrimp was expressed using the formula: $\mathbf{W = a\ L^b}$ **(Jones *et al.*, 1999)**; where, W=body weight (g); L= total length (cm); a=constant or coefficient of condition or length-weight factor, and b=constant or change rate of weight and length. The b value was used to determine the allometric growth pattern of shrimp. The growth pattern is isometric when the b value is 3 and considered allometric (positive: b>3 or negative: b<3) when it varies significantly from 3.

To estimate the a and b parameters, the equation was logarithmically transferred and expressed as: **Log W=Log a + b Log L (Khademzadeh & Haghi, 2017)**; where: Log W is the dependent variable (y); Log L is the independent variable (x); b is the regression coefficient or slope, and Log a is the y– intercept. Log a and the regression coefficient (b) were estimated by the usual method of least squares. Logarithmic transformation of the formula gives a straight-line relationship of the form.

The degree of correlation between total length and body weight was represented by the R^2 value, which is the coefficient of determination. Following the method by **Sripathip (2015)**, correlation coefficient (r) is the strength of the linear relationship between points of body weight and total length on a regression plot which can test the significance of the correlation coefficient using the following formula: $\boldsymbol{t = \sqrt{(n-2)R^2/(1-R^2)}}$.

The t_{Normal} and $t_{Stunted}$ then were compared to the t (70-2) from t-distribution table at 95% confidence level.

The formula: $\boldsymbol{t = (b-3)/(S.E.\ \times \sqrt{\sum(x-\bar{x})^2})}$ was used to determine the significance difference of b value obtained from the analysis to the theoretical value according to "cube law" (b=3, p<0.05) **(Asadi *et al.*, 2017)**. Paired t-test were used to find if there is any significance difference between the means of total length and body weight between normal and stunted shrimp.

Total bacteria and total *Vibrio* count analysis

After measurements, each sample was dissected. Hepatopancreas (HP) was extracted and processed for bacterial enumeration using the method of **Soto-Rodriguez *et al.* (2015)**. HP were weighed and homogenized in 1.0mL sterile saline solution with 1.5% pf sodium chloride (NaCl) before diluted using serial ten-fold dilution method. After dilution, 0.1mL was inoculated onto tryptic soy agar (TSA) with 2% of NaCl to enumerate heterotrophic bacteria, and another 0.1mL was inoculated onto TCBS agar to enumerate *Vibrio* spp. After incubation at 30^{o}C for 24 hours, the colonies growth in agar were counted, and results were presented in colony forming unit, CFU g^{-1}.

Identification of *Vibrio* species

Bacterial isolates were purified up to four times in TCBS agar to obtain pure colony. The plates were incubated at 30^{o}C for 24 hours and preserved at -80^{o}C in tryptic soy broth (TSB) with 15 % glycerol. The isolates were characterized phenotypically using Remel RapID NF Plus System (Thermo-Scientific, USA) according to the manufacturer's instructions. Through the biochemical test scores from Remel RapID NF Plus System, bacteria isolates sharing the same result pattern were grouped together. A few representatives from each group of isolates were selected for 16S rDNA sequencing.

Boiling method was used to extract DNA from bacteria isolates, following the methods of **Barbosa *et al*. (2016)**. About 50uL of each DNA samples were kept in -20^{o}C. PCR were done using the primers (16S32F: 5'TCAGRWYGAACGCTGGCGC and 16S1432R: 5'CGATTACTAGCGATTCCGRC) on all selected isolates **(Ang & Lal, 2019)**. The PCR mixture consisted of 5 X PCR buffer (Promega, USA), 3.4 mM MgCl2 (Promega, USA), 400µM of dNTPs (Promega, USA), 0.8µM of forward and reverse primers, 2U of Taq DNA polymerase (Promega, USA) and 100ng of DNA in 50µL reaction. The PCR were carried out under the succeeding conditions; three minutes of initial denaturation in 95^{o}C, followed by 30 cycles of alternating temperatures as follows: denaturation step at 95^{o}C for 30 seconds, annealing step at 56^{o}C for 30 seconds, and extension step at 72^{o}C for 30 seconds. Final extension step was for 5min at 72^{o}C. PCR reactions were performed in thermal cycler (Kyratec, Australia). PCR products were separated on 1.5% agarose gel electrophoresis, stained with blue/orange loading dye (Promega, USA), and visualized using Gel Documentation System (Alpha Innotech). The PCR products were purified using DNA purification kit (Promega), and both primers and PCR products were sent to Biotechnology Research Institute, Universiti Malaysia Sabah for sequencing. Sequence results were analyzed in BLAST online search engine (http://www.ncbi.nih.gov/cgi-bin/BLAST), compared to the strain sequences in the database. The composition of the *Vibrio* species from normal grown shrimp was compared to stunted grown shrimp.

Statistical analysis

The regression of length and weight were analyzed using Microsoft Excel 2013. Data on shrimp length, shrimp weight, total bacterial counts and total *Vibrio* counts were statistically analyzed using IBM SPSS Statistics version 22. Student t-test was used to compare different mean values between two different shrimp conditions at 95% significanc level.

RESULTS

1. Comparison between normal and stunted shrimps

The physico-chemical parameters in the farm were recorded. Ranges were recorded as follows: salinity 12.80 – 19.87 ‰; pH 6.12 – 7.57; temperature 27.0 - 29.1 °C and dissolve oxygen 4.7-7.4 mg L^{-1}. The stunted grown shrimp appeared shorter compared to normal shrimp (Fig. 1). The analysis showed that the mean weight and mean total length of normal shrimps were higher than stunted shrimps. This finding was detected in all age groups. The average weight and average total length between normal and growth- retarded shrimp according to the age group was analyzed using independent sample t-test. The results revealed that the body weight and total length between normal and stunted shrimp in all age groups varied significantly at 0.05 level as shown in Table (1).

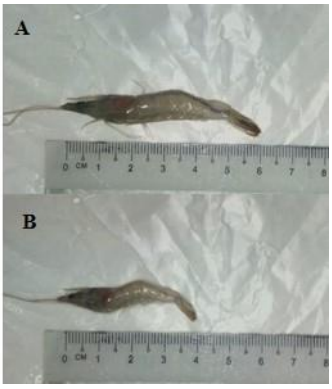

Fig. 1. Comparison of length between normal (A) and stunted growth (B) shrimp collected on the same day

Table 1. Summary of the comparison of means between normal shrimp and stunted shrimp; independent sample t-test used to test significant difference in means

Age (days after restocking)	Body Weight (g)			Total Length (cm)		
	Mean±SD		t	Mean±SD		t
	Normal	Stunted		Normal	Stunted	
33	2.50±0.39	0.84±0.18	12.28 *	7.75±0.28	5.87±0.29	14.63 *
45	3.84±0.92	2.34±0.68	4.16 *	7.75±0.63	6.72±0.52	3.99 *
47	6.21±0.91	1.84±0.41	13.92 *	9.95±0.43	6.68±0.48	16.08 *
51	6.08±0.92	3.29±0.84	9.39 *	9.88±0.67	7.31±0.61	9.07 *
63	8.56±2.77	4.73±0.96	4.13 *	11.07±0.89	8.54±0.94	6.16 *
79	9.75±1.5	5.02±1.15	6.60 *	11.37±0.60	9.12±0.87	6.72 *
94	14.13±1.98	7.90±1.74	7.47 *	12.79±0.57	10.34±0.83	7.67 *

* Significant at $P<0.05$; N= 10

The size variation of shrimps in each age group was determined from the coefficient of variation calculated based on the body weight. Results in Table (2) show the percentages of coefficient of variation of shrimps in each age group. The coefficient of variation in all age groups showed high values (more than 33%), indicating large size variation between shrimps within the same age. Shrimps at age 47 days after restocking showed the highest coefficient of variation at 58.23%. The second highest coefficient of variation was observed on shrimps aged 33 days after restocking (53.95%), followed by shrimps aged 63 days after restocking (42.39%), 51 days after restocking (40.45%), 79 days after restocking (39.05%), 45 days after restocking (35.57%) and the least on shrimps aged 94 days after restocking (33.36%).

Table 2. The coefficient of variation regarding shrimps in each age group

Age (Day after restocking)	Standard deviation	Mean	Coefficient of variation (%)
33	0.90	1.67	53.95
45	1.10	3.09	35.57
47	2.34	4.03	58.23
51	2.08	5.14	40.45
63	2.82	6.65	42.39
79	2.88	7.39	39.05
94	3.67	11.02	33.36

2. Length – weight relationship

Regression analysis on total length and weight of normal and stunted shrimp samples were performed to find the b value and correlation coefficient (r) value. The b value for the stunted shrimp was 3.41, while it was 2.92 for normal shrimp. The r value of the stunted shrimp and normal shrimp was 0.94 and 0.95, respectively. Both groups showed high correlation between body weight and total length. However, normal shrimps recorded greater r value compared to the stunted shrimps (Table 3 & Fig. 2).

The b value for normal shrimp was 2.92, and it was 3.41 for the stunted shrimp. In order to find if the b value was significantly different from the cube law, the value of t_{Normal} and $t_{Stunted}$ were calculated by using formula (5). The result revealed that the t_{Normal} was 0.68, which was less than t-distribution $t_{0.05}(70\text{-}2)$. This indicates that the b value of normal shrimp was not significantly different from the isometric growth *(P*>0.05). Meanwhile, the b value of the stunted shrimp varied significantly from the isometric growth because for the stunted shrimp, it was 2.61, which was greater than t-distribution $t_{0.05}(70\text{-}2)$ (*P*<0.05). The result showed that b value of the stunted shrimp was

significantly different from the cube law. The b value more than three (b>3) indicates positive allometric growth.

Table 3. Regression analysis results for length-weight relationship of normal shrimp and stunted shrimp

Parameter	Normal	Stunted
Number of shrimp sample	70	70
Range of total length (cm)	7.75-12.79	5.87-10.34
Range of body weight (g)	2.5-14.13	0.84-7.90
b- value	2.92^{ns}	3.41
Coefficient correlation (r)	0.95*	0.94*
Regression equation	y = 2.924x – 4.853	y = 3.407x – 5.855
T-test	0.68^{ns}	2.61*
	$T_{count} < T_{distribution}$	$T_{count} > T_{distribution}$
Growth allometry	Isometric	Positive allometric

* Significant at 0.05 level
x = Log Total Length (cm)
y = Log Body Weight (g)
ns = Not significant at 0.05 level

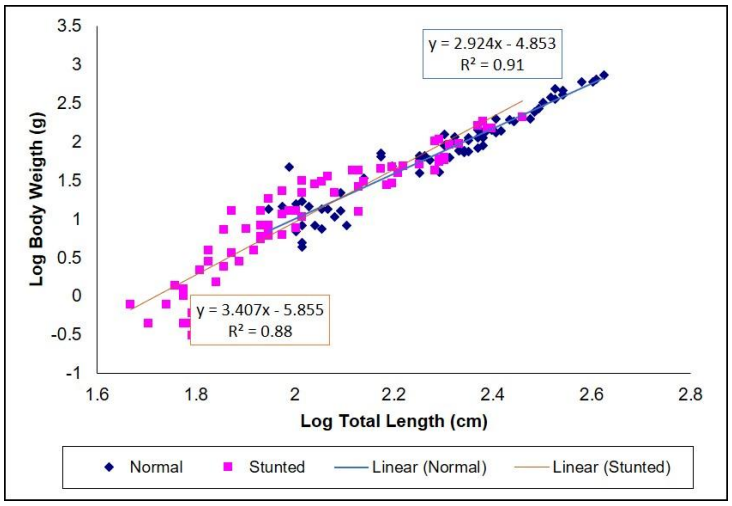

Fig. 2. Regression of body weight on total length of normal and stunted grown shrimp

3. Bacterial enumeration of normal and stunted grown shrimps

The total bacteria and *Vibrio* count from the tissue of pond-cultured normal shrimp and stunted grown shrimp are shown in Table (4)**.** The independent t-test were used to compare the overall means of total bacteria count and total *Vibrio* counts of normal and stunted shrimps**.** The result showed that there is no significant difference of

the total bacteria count (*P*=0.129) and total *Vibrio* count (*P*=0.523) between normal and stunted shrimps**.**

Table 4. Average of total bacteria counts and total *Vibrio* counts isolated from normal and stunted growth of whiteleg shrimp

Sampling Date	**Total bacteria count (CFU g^{-1})**		**Total *Vibrio* count (CFU g^{-1})**	
	Normal	**Stunted**	**Normal**	**Stunted**
01/08/2017	3.77×10^{7}	2.61×10^{9}	1.40×10^{6}	1.22×10^{6}
15/08/2017	2.23×10^{9}	9.13×10^{8}	2.75×10^{8}	3.04×10^{8}
22/08/2017	1.28×10^{9}	1.97×10^{9}	1.11×10^{5}	2.18×10^{4}
08/09/2017	1.98×10^{9}	3.85×10^{8}	6.39×10^{8}	1.25×10^{9}
26/09/2017	9.90×10^{8}	5.19×10^{7}	3.79×10^{6}	7.53×10^{5}
12/10/017	4.60×10^{7}	8.23×10^{7}	1.83×10^{4}	1.68×10^{6}
27/10/2017	7.44×10^{7}	1.49×10^{8}	1.83×10^{6}	2.31×10^{6}

4. Identification of *Vibrio* species from the normal and stunted grown shrimps

A total of 34 *Vibrio* isolates were recovered from shrimp samples**.** About 15 isolates were recovered from normal grown shrimps and 17 isolates were recovered from stunted shrimps**.** Biochemical test was performed on all 15 and 17 isolates from normal and stunted grown shrimps, respectively**.** The isolates were grouped according to their same phenotypic characteristics**.** From the normal shrimps, nine different phenotypes were detected (Table 5). From the stunted grown shrimps, 17 different phenotypes were recorded, based on the differences in biochemical tests; the characteristics of each isolate are shown in Table (6)**.**

Table 5. Phenotypic characteristics of *Vibrio* isolates from normal shrimp

Vibrio isolates	Biochemical characteristics													Phenotype
	Negative	TRD	EST	PHS	NAG	βGLU	ONPG	GLU	PRO	PYR	BANA	IND	NO_3	OXI	
NS01	Arginine hydrolysis, ρ-Nitrophenyl-α,D-glucoside hydrolysis, Urea hydrolysis, γ-Glutamyl β-napthylamidehydrolysis, Tryptophane β-napthylamidehydrolysis	-	-	+	+	+	-	-	+	-	+	-	-	-	1
NS02		-	+	+	+	+	-	-	+	-	+	-	-	-	2
NS03		-	+	+	+	-	+	-	+	-	+	-	+	-	3
NS04		-	+	+	+	+	-	-	+	-	+	-	-	-	2
NS05		-	-	+	+	+	-	-	+	-	+	-	-	-	1
NS06		-	+	+	+	-	+	-	+	-	+	-	-	-	4
NS07		-	+	+	+	+	-	-	+	-	+	-	-	-	2
NS08		-	-	+	+	+	-	-	+	-	+	-	-	-	1
NS09		-	+	+	+	+	-	-	+	-	+	-	+	+	5
NS10		-	-	+	+	+	-	-	+	-	+	-	-	-	1
NS11		+	+	+	-	-	-	-	-	-	-	+	+	+	6
NS12		-	+	+	+	+	-	-	+	-	+	-	-	-	7
NS13		-	+	+	+	-	+	-	+	-	+	-	+	-	3
NS14		-	+	+	+	-	+	-	+	-	+	-	+	+	8
NS15		-	+	+	+	-	+	+	+	+	+	-	+	-	9

*TRD = Aliphatic thiol utilization; EST = Triglyceride hydrolysis; PHS = ρ-Nitrophenyl-phosphoester; NAG = ρ-Nitrophenyl-N-acetyl-β,D-glucosaminide hydrolysis; βGLU = ρ-Nitrophenyl-β,D-glucoside hydrolysis; ONPG = ρ-Nitrophenyl-β,D-galactoside hydrolysis; GLU =Glucose utilization ; PRO = Proline-β-napthylamide hydrolysis; PYR = Prryolidine-β-napthylamide hydrolysis; BANA = N-Benzyl-arginine-β-napthylamide hydrolysis; IND = Trypthophane utilization; NO_3 = Nitrate reduction; OXI = Cytochrome c oxidase production; + = positive; – = negative

Table 6. Phenotypic characteristics of *Vibrio* isolates from stunted growth shrimp

Vibrio isolates	Biochemical characteristics																Pheno type
	Negative	ADH	TRD	EST	PHS	NAG	αGLU	βGLU	ONPG	URE	GLU	PRO	PYR	TRY	BANA	NO_3	OXI	
SS01	γ-Glutamyl β-napthylamide hydrolysis, Trypthophane utilization	-	-	+	+	+	-	-	+	-	-	+	-	-	-	-	+	1
SS02		-	-	+	+	+	-	-	+	-	-	+	-	-	-	-	-	2
SS03		-	-	-	+	+	-	-	-	-	-	+	-	-	+	+	-	3
SS04		-	-	+	+	-	-	-	-	-	-	+	-	-	+	-	+	4
SS05		-	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-	5
SS06		-	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-	5
SS07		-	-	+	+	+	-	-	-	-	-	+	-	-	+	-	-	6
SS08		-	-	+	+	+	-	+	-	-	-	+	-	-	+	-	-	7
SS09		-	-	+	+	+	-	+	+	-	-	+	-	-	+	-	+	8
SS10		-	-	+	+	+	-	+	-	-	-	+	-	-	+	+	+	9
SS11		-	-	+	+	+	-	+	-	-	-	+	-	-	+	+	+	9
SS12		-	-	+	+	+	-	-	-	-	-	+	-	-	+	+	+	10
SS13		-	+	-	+	+	-	+	+	-	+	+	-	-	+	+	+	11
SS14		-	-	+	+	+	-	+	+	-	+	+	-	-	+	+	+	12
SS15		-	-	+	+	+	-	+	+	-	-	+	-	-	+	+	+	13
SS16		-	+	-	+	+	+	-	-	+	-	-	-	-	-	+	-	14
SS17		-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	15
SS18		+	-	-	+	+	-	+	-	-	+	+	+	-	+	+	-	16
SS19		-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	17

*ADH = Arginine hydrolysis; TRD = Aliphatic thiol utilization; EST = Triglyceride hydrolysis; PHS = ρ-Nitrophenyl-phosphoester; NAG = ρ-Nitrophenyl-N-acetyl-β,D-glucosaminide hydrolysis; αGLU = ρ-Nitrophenyl-α,D-glucoside hydrolysis, βGLU = ρ-Nitrophenyl-β,D-glucoside hydrolysis; ONPG = ρ-Nitrophenyl-β,D-galactoside hydrolysis; URE = Urea hydrolysis; GLU =Glucose utilization ; PRO = Proline-β-napthylamide hydrolysis; PYR = Prryolidine-β-napthylamide hydrolysis; TRY = Tryptophane β-napthylamidehydrolysis; BANA = N-Benzyl-arginine-β-napthylamide hydrolysis; IND = Trypthophane utilization; NO_3 = Nitrate reduction; OXI = Cytochrome c oxidase production; + = positive; – = negative

5. 16S rRNA Gene Analysis of *Vibrio* Isolates from Normal and Stunted Shrimp

Representatives of nine *Vibrio* isolates with different phenotypic characteristics were selected from 15 isolates for 16S rRNA gene sequencing**.** Selected isolates were NS02 **(**Phenotype 2**)**, NS05 **(**Phenotype 1**)**, NS06 **(**Phenotype 4**)**, NS09 **(**Phenotype 5**)** NS11 **(**Phenotype 6**)**, NS12 **(**Phenotype 7**)**, NS13 **((**Phenotype 3**)**, NS14 **(**Phenotype 8**)** and NS15 **(**Phenotype 9**)**. Through Basic Local Alignment Search Tool **(**BLAST**)**, the identity of each isolate is shown in Table (7)**.** NS02, NS05, NS09, NS12, NS13, NS14, NS15 were revealed as *Vibrio parahaemolyticus*, while NS06 and NS11 were identified as *V. azureus* and *V. diabolicus*, respectively**.**

Representatives of 17 *Vibrio* isolates with different phenotypic characteristics were selected from 19 isolates for 16S rRNA gene sequencing**.** Selected isolates were SS01 **(**Phenotype 1**)**, SS02 **(**Phenotype 2**)**, SS03 **(**Phenotype 3**)**, SS04 **(**Phenotype 4**)**, SS05 **(**Phenotype 5**)**, SS07 **(**Phenotype 6**)**, SS08 **(**Phenotype 7**)**, SS09 **(**Phenotype 8**)**,SS10 **(**Phenotype 9**)**, SS12 **(**Phenotype 10**)**, SS13 **((**Phenotype 11**)**, SS14 **(**Phenotype 12**)**, SS15 **(**Phenotype 13**)**, SS16 **(**Phenotype 14**)**, SS17 **(**Phenotype 15**)**, SS18 **(**Phenotype 16**)**, and SS19 **(**Phenotype 17**)**. Through BLAST, the identity of each isolate is shown in Table (8)**.** SS01, SS02, SS03, SS04, SS05, SS07, SS09, SS10, SS13 and SS17 were identified as *Vibrio parahaemolyticus***.** Meanwhile, SS12 and SS19 were identified as *V. diabolicus***.** SS08 was identified as *V. azureus***.** Interestingly, SS16 and SS18 were identified as *Photobacterium damselae* subsp**.** *damselae* and *Photobacterium damselae* subsp**.** *piscicida*, respectively**.** Lastly, two isolates, SS14 and SS15 were identified as *Vibrio* sp**.** only**.**

Table 7. Highest sequence homology from BLAST results of nine selected isolates

Vibrio Isolates	Homolog	E value	Homology (%)	Species name
NS02	*Vibrio parahaemolyticus* strain MVP1 chromosome 1, complete sequence	0.0	98.88	*Vibrio parahaemolyticus*
NS05	*Vibrio parahaemolyticus* strain BC36 16S ribosomal RNA gene, partial sequence	0.0	97.52	*Vibrio parahaemolyticus*
NS06	*Vibrio azureus* strain AN11 16S ribosomal RNA gene, partial sequence	0.0	97.91	*Vibrio azureus*
NS09	*Vibrio parahaemolyticus* strain AC7 16S ribosomal RNA gene, partial sequence	0.0	97.33	*Vibrio parahaemolyticus*
NS11	*Vibrio diabolicus* strain LV 34 16S ribosomal RNA gene, partial sequence	0.0	98.92	*Vibrio diabolicus*
NS12	*Vibrio parahaemolyticus* strain HY3 16S ribosomal RNA gene, partial sequence	0.0	98.56	*Vibrio parahaemolyticus*
NS13	*Vibrio parahaemolyticus* strain BC36 16S ribosomal RNA gene, partial sequence	0.0	97.15	*Vibrio parahaemolyticus*
NS14	*Vibrio parahaemolyticus* strain HY3 16S ribosomal RNA gene, partial sequence	0.0	99.14	*Vibrio parahaemolyticus*
NS15	*Vibrio parahaemolyticus* strain HY3 16S ribosomal RNA gene, partial sequence	0.0	94.45	*Vibrio parahaemolyticus*

Table 8. Highest sequence homology from BLAST results of 17 selected isolates

Isolates	Homolog	E value	Homology (%)	Species name
SS01	*Vibrio parahaemolyticus* strain HNPH13 16S ribosomal RNA gene, partial sequence	0.0	99.24	*Vibrio parahaemolyticus*
SS02	*Vibrio parahaemolyticus* strain MAI-4 16S ribosomal RNA gene, partial sequence	0.0	97.83	*Vibrio parahaemolyticus*
SS03	*Vibrio parahaemolyticus* strain BC36 16S ribosomal RNA gene, partial sequence	0.0	98.37	*Vibrio parahaemolyticus*
SS04	*Vibrio parahaemolyticus* strain HY3 16S ribosomal RNA gene, partial sequence	0.0	99.62	*Vibrio parahaemolyticus*
SS05	*Vibrio parahaemolyticus* strain HY3 16S ribosomal RNA gene, partial sequence	0.0	98.92	*Vibrio parahaemolyticus*
SS07	*Vibrio parahaemolyticus* strain HY3 16S ribosomal RNA gene, partial sequence	0.0	98.51	*Vibrio parahaemolyticus*
SS08	*Vibrio azureus* strain AN11 16S ribosomal RNA gene, partial sequence	0.0	96.51	*Vibrio azureus*
SS09	*Vibrio parahaemolyticus* strain HNPH13 16S ribosomal RNA gene, partial sequence	0.0	97.52	*Vibrio parahaemolyticus*
SS10	*Vibrio parahaemolyticus* strain SEM52 16S ribosomal RNA gene, partial sequence	0.0	98.76	*Vibrio parahaemolyticus*
SS12	*Vibrio diabolicus* strain LV 34 16S ribosomal RNA gene, partial sequence	0.0	96.90	*Vibrio diabolicus*
SS13	*Vibrio parahaemolyticus* strain HY3 16S ribosomal RNA gene, partial sequence	0.0	98.89	*Vibrio parahaemolyticus*
SS14	*Vibrio sp*. strain BC76 16S ribosomal RNA gene, partial sequence	0.0	93.17	*Vibrio sp*
SS15	*Vibrio sp*. strain ECSMB92 16S ribosomal RNA gene, partial sequence	0.0	98.89	*Vibrio sp*
SS16	*Photobacterium damselae* subsp. damselae strain KC-Na-NB1 chromosome 1, complete sequence	0.0	95.98	*Photobacterium damselae*
SS17	*Vibrio parahaemolyticus* strain HNPH13 16S ribosomal RNA gene, partial sequence	0.0	98.34	*Vibrio parahaemolyticus*
SS18	*Photobacterium damselae* subsp. piscicida strain L09110601O 16S ribosomal RNA gene, partial sequence	0.0	99.23	*Photobacterium damselae*
SS19	*Vibrio diabolicus* strain LV 34 16S ribosomal RNA gene, partial sequence	0.0	99.46	*Vibrio diabolicus*

6. Comparison of the Presence of *Vibrio species* in normal and stunted- grown shrimp

The sequencing result revealed that three species of *Vibrio* **(***V. parahaemolyticus, V. azureus, V. diabolicus***)** were present in the normal shrimp**.** Meanwhile, there were five species of identified bacterial species presented in the stunted- growth shrimp**.** They were *V. parahaemolyticus, V. azureus, V. diabolicus, P. damselae* subsp**.** *damselae* and *P. damselae* subsp *piscicida***.** In the stunted- growth shrimp, unidentified *Vibrio* species were also present**.** Based on Fig. (3), *V. parahaemolyticus* was dominant in both normal shrimp (78%) and stunted shrimp **(**59%). Nevertheless, the number of *V. parahaemolyticus* strains in stunted- growth shrimp was higher compared to normal- growth shrimp**.**

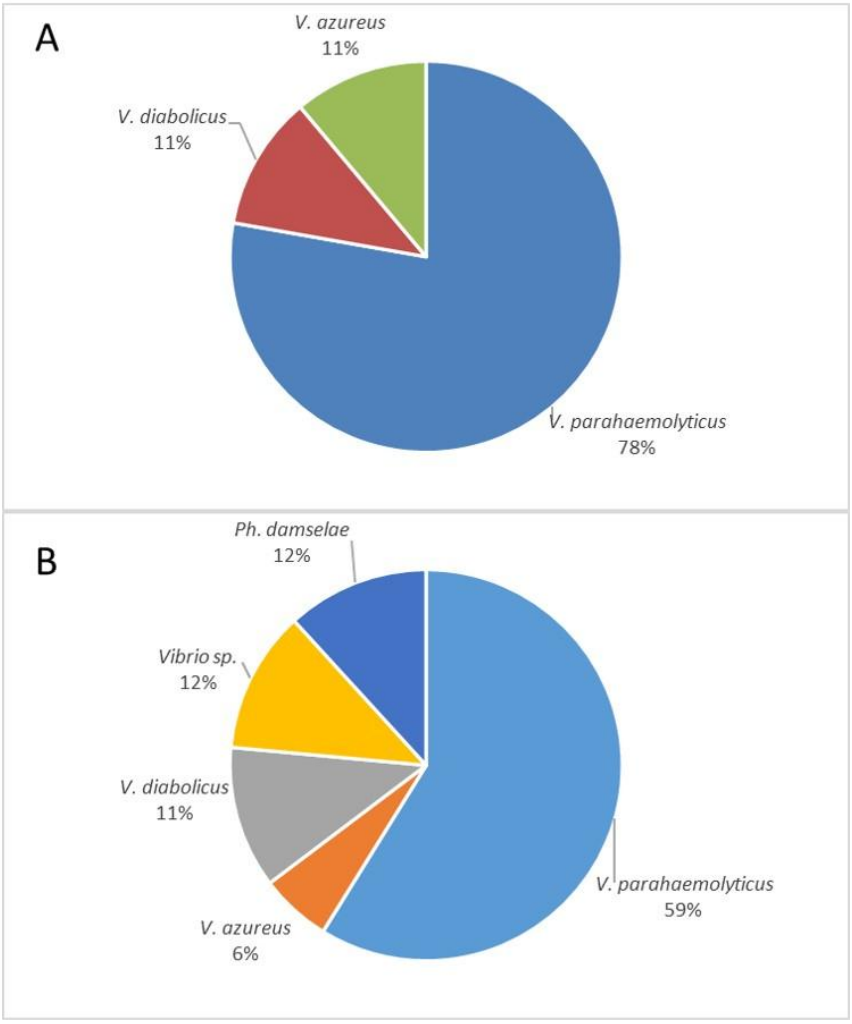

Fig. 3. Composition of *Vibrio* species identified from normal- growth shrimp **(**A**)** and stunted- growth shrimp **(**B**)**

DISCUSSION

In this present study, the mean body weight and mean total length of normal shrimp differ significantly from the stunted shrimp. This trend was reported in all age groups. The result indicates that there is an apparent size difference in terms of length and weight between normal shrimp and stunted- growth shrimp. They are consistent with the findings of **Santhoshkumar *et al.* (2017)** who reported that, at particular age, the size of stunted *L. vannamei* can be nearly half the size of the normal ones, and added that the stunted shrimp does not necessarily show mortality. The coefficient of variation in each age group was high (more than 35%) in all age groups, except age 94 days after restocking (33.36%). This result is comparable to the finding of **Pratoomthai *et al.* (2008)** on Monodon slow-growth syndrome (MSGS), where *P. monodon* culture pond with slow growth had coefficient of variation more than 35%. At the age 33 and 47 days after restocking, the coefficient of variation was very high at 53.95% and 58.23%, respectively. These results revealed that, within these two age groups, the size between normal and stunted shrimp differed more than 50%, averagely. Overall, it can be deduced that there was a large size variation of shrimp in each age group **(Peacor *et al.*, 2007)**.

Length-weight regression analysis on normal shrimp and stunted shrimp revealed that the b values of them were 2.92 and 3.41, respectively. According to the cube law, it can be indicated that normal shrimp has negative allometric growth, while stunted shrimp showed positive allometric growth. However, through paired t-test, the b value of normal shrimp was not significantly different from the cube law (b=3). This indicates that normal shrimp has an isometric growth pattern, where body weight changes proportionally with the total length. Thus, the normal shrimp would appear to have a uniform body shape. The isometric growth pattern for normal shrimp might be due to the farm stocking density. Growth pattern following the cube law (b=3) can be considered as one of the indicators of shrimp with good growth performance **(Valenzuela-Madrigal *et al.*, 2017)**. Most *L. vannamei* culture farms in Malaysia applied intensive culture system, with minimum stocking density of 80 postlarvae m^{-2} on average **(Ghee-Thean *et al.*, 2016)**. In the present study, the stocking density is 100 postlarvae m^{-2}. **Arambul-Munoz *et al.* (2019)** reported that *L. vannamei* cultured in grow-out pond with 100 shrimp m^{-2} showed the best growth performance.

In contrast, it was found that the b value of stunted shrimp in this study deviates significantly from the cube law. This indicates that the stunted shrimp does not grow isometrically, but have a positive allometric growth pattern (b=3.41, t=2.61, $P>0.05$). **Valenzuela-Madrigal *et al.* (2017)** stated that, the deviation from isometric growth (b=3) might indicate changes in the physical well-being of the shrimp under study. This result revealed that, as compared to normal shrimps, stunted shrimp generally would appear to have a short but more rounded body shape, as the gain rate of weight is higher than the

increase rate of the total body length. According to **Chow and Sandifer (1991)**, shrimp with positive allometric growth pattern has a thicker body compared to normal shrimp. Interestingly, **Araneda *et al.* (2008)** stated that, the shrimp with positive growth allometry is the more preferable in aquaculture since it would require a smaller number of individuals to reach a kilogram of product, assuming the shrimp were sold in whole (no parts discarded) at the market. However, to agree with this point, the shrimp must be large and have similar length to the shrimp with isometric growth. For that reason, it can be deduced that even though stunted- growth shrimp had positive allometric growth pattern, they do not fit to the condition stated by **Araneda *et al.* (2008)** since the average total length of stunted shrimps was significantly lower than the normal shrimp in all age groups. The growth allometry of stunted shrimp in the present study is in contrast with the findings reported in previous studies on length-weight relationship of diseased penaeid shrimp. In 2015, a study on *L. vannamei* with loose shell syndrome revealed that, the affected shrimp showed negative allometry growth **(Raja *et al.*, 2015)**. Diseased *P. monodon* with loose shell syndrome was also reported to have negative allometric growth pattern **(Gopalakrishnan *et al.*, 2014)**.

The coefficient of determination (R^2) of normal shrimp was 0.91 and 0.88 for stunted growth shrimp. Although R^2 values of shrimp in both conditions were relatively high, indicating strong length-weight relationship, analysis using paired t-test revealed that the R^2 value between normal shrimp and stunted shrimp was significantly different. **Mane *et al.* (2019)** stated that there is a possibility of shrimp with same species to have different morphological characteristics if they are bred in different areas due to differences in food availability, temperature, etc. However, in this study normal shrimp and stunted growth shrimp were taken from same culture pond in each age group thus suggesting that there might be a possibility of variation of water quality in the culture pond that affected the shrimps' growth **(Ariadi *et al.*, 2019)**. Thermal and dissolved oxygen stratification are the common causes of variation of water quality in pond culture, as temperature of water highly influenced the distribution of dissolved oxygen in different region of a pond **(Oberle *et al.*, 2019)**.

The length – weight relationship analysis in this study to this point has proved that there were morphological differences between normal shrimp and stunted shrimp. Due to the fact that the shrimp was collected from the same pond and at the same time, the other factor such as water parameter was not a main reason for the difference between the shrimp. According to **Xiong *et al.* (2017)**, bacterial composition can affect the growth of shrimp and in some cases might be the causative factor of retardation or overgrowth of cultured shrimp. To further investigate the distinction between normal shrimp and stunted shrimp, the bacterial composition, primarily *Vibrio* species presence in normal and stunted shrimp were examined.

The total bacteria count and total *Vibrio* count in normal shrimp and stunted shrimp were determined in this study. The average total bacteria population in normal shrimp was between 3.77 x 10^7 – 2.23 x10^9 CFU g^{-1}. Meanwhile, the average total bacteria population in stunted growth shrimp was between 5.19 x 10^7 – 2.61 x 10^9 CFU g^{-1}. Statistical analysis revealed that there was no significant difference in the total bacteria count between normal and stunted shrimp. Both normal and stunted shrimp samples contained high total bacteria count ranged from 10^7-10^9 CFU g^{-1}. This result was comparable to the findings reported by **Samia *et al.* (2014)**, which recorded that shrimp samples from collected from local market around Dhaka city exhibited high bacterial loads ranged 1.5 x 10^4 CFU g^{-1} to 3.3 x 10^8 CFU g^{-1}. Total viable bacteria isolated from the shrimp's head, which is the location of hepatopancreas, was the highest within the range of 2.3 x 10^7 – 3.3 x 10^8 CFU g^{-1} **(Samia *et al.*, 2014)**. This range was higher than the standard set by the International Commission on Microbiological Specifications for Foods (ICMSF), where shrimp was considered not fresh if the total count of bacteria reaches 10^6 CFU per gram or millilitre **(Yousuf *et al.*, 2008)**. The total bacteria count of *L. vannamei* samples from several farms in Ratnagiri, India was between 0.57 x 10^6 – 1.03 x 10^6 CFU g^{-1} **(Tawade *et al.*, 2019)**. The total bacteria count in this present study was recorded high as compared with **Tawade *et al.* (2019)**.

The average total *Vibrio* count in normal shrimp ranged from 1.83 x 10^4 CFU g^{-1} to 6.39 x 10^8 CFU g^{-1}, and 2.18 x 10^4 CFU g^{-1} to 1.25 x 10^9 CFU g^{-1} in stunted shrimp. There was no significant difference of the total *Vibrio* count between normal and stunted shrimp in this study. A study on *Vibrio* species in hepatopancreas of healthy juvenile *L. vannamei* revealed the average numbers of *Vibrio spp*. found in the hepatopancreas was 4.30 x 10^4 CFU g^{-1} **(Gomez-Gil *et al.*, 1998)**. Prior to any treatments, the total plate count of *Vibrio sp*. from pond-culture *L. vannamei* in a farm in Surabaya, Indonesia was 3.0 x 10^3 CFU g^{-1} **(Marwiyah *et al.*, 2019)**. The total *Vibrio* count of normal and stunted shrimp in this study are relatively higher than the amount reported by **Gomez-Gil *et al.* (1998)** and **Marwiyah *et al.* (2019)**. In this present study, the total bacteria and total *Vibrio* count in normal and stunted shrimp showed fluctuation and were on the higher range. However, it can be observed that the total *Vibrio* count in both normal and stunted shrimp generally did not exceed the total bacteria count, which is a favourable condition since **Widiyanto *et al.* (2020)** stated that if *Vibrio* population is higher than other bacteria, there might be a decline in development and survival rate of shrimp.

Vibrio isolates were extracted from normal and stunted shrimp hepatopancreas and selected isolates were run with a series of biochemical tests to observe the phenotypic characteristics. The tests result revealed that *Vibrio* isolates obtained from stunted shrimp showed higher variation of phenotypic characteristics than isolates from normal shrimp. Based on 16S rRNA gene sequence on selected *Vibrio* isolates from normal shrimp and stunted shrimp, *Vibrio parahaemolyticus* was the most abundant *Vibrio* species detected in both normal and stunted shrimp hepatopancreas. This bacterium along with different

types of *Vibrio* species such as *V. alginolyticus, V. harveyi* or *V. cholerae* were often found in shrimp or other crustaceans as they occur naturally in all marine environments **(Takemura *et al*., 2014)**. In normal shrimp, *Vibrio parahaemolyticus* made up 78% of the *Vibrio* isolates, followed by 11% *Vibrio diabolicus* and 11% *Vibrio azureus*. Meanwhile, more variety of bacteria species were detected from stunted shrimp. 59% of the isolates were detected to be *Vibrio parahaemolyticus*, followed by 12% *Vibrio diabolicus*, 12% *Photobacterium damselae* and 6% *Vibrio azureus*. Another 11% were unidentified species of *Vibrio*. The finding in this study indicates that the different composition of *Vibrio* in the shrimp hepatopancreas might contribute to the occurrence of stunted shrimp in this present study. This finding also suggested that the hepatopancreas of a stunted shrimp might have a more diverse and complex bacterial composition than a normal shrimp. However, comprehensive representation of *Vibrio* species composition in normal and stunted shrimp were not able to be obtained in this present study as there was lacking data on total bacteria and *Vibrio* population in the other organs of the shrimp. This is subject to future study where the extensive research on bacterial and *Vibrio* community in all important parts of the shrimp's digestive tract (stomach, intestine, hepatopancreas) should be conducted in future research.

CONCLUSION

The occurrence of stunted shrimp is an ongoing problem in shrimp farming. The apparent size differences in length and weight of stunted shrimp compared to their normal counterparts proved to pose a serious issue for shrimp farmers as this can lead to low production biomass. This present study has managed to provide information on the size difference between normal and stunted *L. vannamei*, as well as the length-weight relationship of normal and stunted *L. vannamei*. Based on the findings, it can be deduced that stunted *L. vannamei* would appear in smaller size compared to the normal ones and possibly with body shape slightly more rotund (short and round). Through regression of length and weight, it is proved that normal shrimp has isometric growth pattern, obeying the cube law. Meanwhile, stunted shrimp in this study has positive allometry growth pattern, which deviates from the cube law. The information on the *Vibrio* species composition in normal and stunted *L. vannamei* were also obtained through this study. The findings revealed that *Vibrio parahaemolyticus* make up the majority of *Vibrio* species present in hepatopancreas of both normal and stunted *L. vannamei*. However, more types of different bacteria species were detected in stunted shrimp compared to the normal ones. The bacteria species detected in stunted *L. vannamei* also showed greater variety of phenotypic characteristic than normal shrimp. Thus, it can be concluded that stunted *L. vannamei* has more a more complex and diverse *Vibrio* composition in its hepatopancreas compared to normal *L. vannamei*. The bacteria detected however, does not have any significant association with the occurrence of stunted shrimp. Therefore, this opens up the opportunity to conduct more research to find out the possible cause

stunted shrimp problem and with that coming into light, research can be further progressed to find the solution for the cause.

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