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Response of Indigenous *Bacillus megaterium* Supplementation on the Growth of *Litopenaeus vannamei* (Boone), a New Target Species for Shrimp Culture in East Java of Indonesia

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ABSTRACT

Screening and characterization of indigenous bacteria isolated from digestive tract of health *Litopenaeus vannamei* resulted in four species which were identified as *Bacillus megaterium* BM1, *Bacillus firmus* BM2, *Actinobacillus* sp BM3 and *Pseudomonas stutzeri* BM4. In this study *B. megaterium* BM1 was chosen as a probiotic candidate in promoting growth on *L. vannamei* for several reasons, viz. it has high antimicrobial activity, complete extracellular enzymes, the shortest value of generation time and the highest value of constant growth rate. Safety and viability assay were also carried out to ensure the application of *B. megaterium* BM1 as a probiont. Results of in-vivo study suggested that *B. megaterium* BM1 supplemented in shrimp diet at concentration of 10^6 cells g^{-1} diet could be beneficial for the growth and feed utilization of *L. vannamei.*
KEYWORDS: Indigenous bacteria, probiotics, digestive enzyme, growth.

INTRODUCTION

Indonesia is a world producer of penaeid shrimp with the total production of 410.000 mt in 2008 [1]. Since 2006, the dominant shrimp species cultured in Indonesia have shifted from *Penaeus monodon* to *Litopenaeus vannamei*. Under the shrimp revitalization program, 140.000 ha of extensive and 8.000 ha of intensive monodon shrimp ponds have been changed to *L. Vannamei* ponds. The main shrimp aquaculture producing areas in Indonesia are Nanggroe Aceh Darussalam, North Sumatra, South Sumatra, Lampung, West Java, East Java, East Kalimantan and South Sulawesi. In East Java, the production of cultured *L .vannamei* reached 12,039.6 mt in 2008 [2].

In the culture of penaeid shrimp species, practical feeds are important nutrient. Feed cost is considered to be the highest expenditure in aquaculture. It comprises about 30-60% of total production cost. Therefore, any reduction in feed expenditures either through diet development or other direct or indirect improved husbandry is essential for the growth of the shrimp industry. As an alternative method for improving feed efficiency, the application of probiotic in fish and shrimp culture has been increasing rapidly. Probiotic-supplemented feed could contain single or mixed cultures of micro-organisms capable of improving the health of the host [3]. A probiotic is defined as a living microbial supplements that: (a) positively affects hosts by modifying the host-associated microbial community, (b) improves food degradation enhancing its nutritional value, and (c) improves the quality of the environmental parameters [4].

Most of the probiotic experiments in aquaculture were conducted for disease control purposes. A few studies were carried out to evaluate probiotics in promoting growth of aquatic organisms [5][6]. The main strategy in the use of probiotics is to isolate intestinal bacteria with favorable properties from mature animals and include large numbers of these bacteria in the feed of immature animals of the same species [5]. However, only a few of study conducted considered the bacterial origin to guarantee the success of probiotic application. Therefore, this study was designed to characterize the indigenous bacteria isolated from *L. vannamei* digestive tract and evaluate their potency as probiont in increasing growth rate and feed utilization of *L. vannamei*.

MATERIALS AND METHODS

Sampling, bacterial isolation and identification

Bacteria were isolated from the gastrointestinal of *L. vannamei* and cultured in duplicate in the general media (nutrient agar with 1.5% w/v NaCl) for 18-24 hours at a temperature of 30^{o}C. Pure isolates were taken after subculture on Tryptic Soya Agar (TSA) Identification of bacteria was performed using biochemical tests and Microbact Identification Kits GNB 12A/B/E.

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In-vitro-Antimicrobial assay

Isolated bacteria were assessed for the production of antimicrobial substances against *Vibrio harveyi*. Each isolate was grown in 10 ml Tryptic Soya Broth (TSB, with 2% NaCl) at 30^{o}C for 48 hours. After centrifugation (2000 rpm), the culture supernatant was filtered with 0.45 µm-pore-size filter. *V. harveyi* was grown in 1 ml of TSB (with 2% of NaCl) at 30^{o}C for 8 h. The broth was centrifuged (2000 rpm), washed and re-suspended with PBS (pH 7.2). This bacterial suspension was transferred on TSA plate (with 2% NaCl). After solidified, 3 mm well were punched and 10 µl of supernatant from four candidates were added. The plates were incubated at 30^{o}C, and zones of inhibition around the wells were measured after 24–48 h. Antibacterial activity was defined as the diameter in millimeters of the clear inhibitory zone. All experiments were carried out in triplicate to ensure feasibility and reproducibility.

Production of extracellular enzymes

Production of extracellular enzymes (protease, amylase and lipase) of isolated bacteria was evaluated using in-vitro assay [7]. The isolated bacteria were plated onto skim milk agar plates and were incubated at 37ºC for 24h. Protease was detected by the presence of a clear zone. For screening of amylase, isolates were streaked on starch (1%) supplemented nutrient agar plates and incubated at 37ºC for 48 h. After appearance of the colonies, the culture plates were flooded with 1% Lugol's iodine solution. Bacteria were considered amylolytic when they were able to grow in the presence of starch as the major carbon source and formed a degradation zone around the colony. The lipase production was detected using agar plates with olive oil 1% (w/v) and a drop of neutral red solution.

Identification of Bacterial Growth

To further reduce the number of candidate probionts for in vivo studies, those that have no ability to inhibit pathogen and have incomplete extracellular enzymes were excluded. The growth profiles of the bacteria were then determined growing the bacteria in 1000 ml erlenmeyer flask with 100 ml of broth at 30^{o}C. Samples were taken every two hours from the growth medium and quantified spectrophotometrically at 600 nm. The value of generation time and constant growth rate were calculated [8]. Isolate which has the shortest generation time and the highest constant growth rate will be used for the next assay.

Safety of *B. megaterium* BM1 as probiotics

B. megaterium BM1 was assayed on *L.vannamei* culture to evaluate the safety as probiotic bacteria. A range of *B. megaterium* BM1 applied was 10^4-10^7 cells ml^{-1}. All groups of shrimp were kept under observation for 7 days. If there were mortalities, shrimp were subjected to laboratory examination.

Preparation of feed with *B. megaterium* BM1 supplementation

Preparation of feed with was started by culturing *B. megaterium* BM1 in TSB (with 2% NaCl) for 48 h at 30^{o}C. They were centrifuged for 30 min at 2000 rpm and washed twice with PBS. The bacterial cell resuspended in 5 ml of PBS with the final concentration of 10^9 cells ml^{-1}and mixed with 5 ml fish oil. The bacterial suspension was added to commercial pellet to give 10^8cells g^{-1} for the viability experiment. The same procedure then applied to make the dosage of *B. megaterium* BM1 in the diet (10^4 - 10^7 cells g^{-1}) for feeding experiment.

Viability of *B. megaterium* BM1 in feed

The viability of *B. megaterium* BM1 in the diets was assessed based on [9] every week for 3 weeks following storage. One g of diet was homogenized in 9.0 ml of sterile saline and 9-fold serial dilutions of this suspension prepared in saline and 0.1 ml of each dilution was spread onto triplicate plates of TSA and the colony count determined after 24 h incubation at 30 °C.

Feeding Experiment

The commercial diet (SGH, Indonesia) was used as a control and as a basal diet for the *B. megaterium* BM1 supplementation (10^4 - 10^7 cells g^{-1}). During 28 days feeding trial, shrimp were fed three times daily at 5% BW d^{-1} with 10% of water change. The amount of food was adjusted in relation of body weight which was determined weekly. Specific growth rate (SGR; % BW day^{-1}), feed conversion ratio (FCR), protein and energy retention (RP and RE) and protein efficiency ratio (PER) were calculated using standard methods [10].

Assay of digestive enzyme activities

Assay of digestive enzyme activities of *L. vannamei* were conducted based on [11]. Gastrointestinal and midgut gland of representative shrimp (± 2 g) were homogenized in cooled 10 mmol L^{-1} Tris-HCl buffers, pH 7.5. The extracts were centrifuged at 4^{0}C for 30 min (10.000 rpm). The supernatant was assayed for the protease, amylase and lipase activity. Total soluble protein was calculated with the Bradford methods with

bovine serum albumin as a standard. Total protease activity was measured using substrate of casein and Folin reagent. Total amylase activity was calculated using dinitrosalicylic acid method based on the estimation of reducing sugars with maltose as the standard. Activity of total lipase was assayed using emulsion of olive oil and 2% polyvinyl alcohol solution as the substrate. Enzyme activities were measured as the absorbance of the reactions using spectrophotometer. One amylase unit was calculated as the amount of enzyme per milliliter culture that released one microgram reducing sugar per minute. One unit of lipase was measured as the amount of enzyme liberated one micromole of free fatty acid per minute (1 ml of 0.02 N NaOH ≈100 micromoles of free fatty acids).

Statistical analysis

Statistical analysis was performed using one-way analyses of variance (ANOVA) and Duncan's Multiple Range Test (1995) to determine differences between treatments. Levels of significance are expressed as $P<0.05$.

RESULTS

Bacterial isolation and Identification

Biochemical characters of isolated bacteria were illustrated in Table 1. Based on the descriptions in Bergeys Manual of Sytematic Bacteriology, the isolated strains were identified as *Bacillus megaterium BM1*, *Bacillus firmus BM2*, *Actinobacillus* sp *BM3* and *Pseudomonas stutzeri BM4*.

Antimicrobial assay and the production of extracellular enzyme

The antimicrobial assay of four isolated bacteria showed that all isolate were exhibited antimicrobial activity against *V. harveyi* (Table 2). A high activity of antimicrobial was only seen in isolate BM4 (>20.0 mm). The antagonistic effect of isolated bacteria can be attributed to the bioactive compounds produced by them to inhibit the pathogens. The production of extracellular enzyme by isolated bacteria was assayed qualitatively (Table 2.). Isolate BM1 and BM2 (strain of *Bacillus*) showed their capacity to produce all the three studied enzymes (protease, amylase and lipase). Isolate BM3, (*Actinobacillus* sp) was found to be amilolytic species. Meanwhile isolate BM4 was an amilolytic and lipolytic species.

Table 1 Phenotypic and biochemical characters of isolates

Character	*BM1*	*BM2*	*BM3*	*BM4*	Character	*BM1*	*BM2*	*BM3*	*BM4*
Gram staining	+ve	+ve	-ve	-ve	Citrate utilization	+	+	-	-
Shape	Rod	Rod	Rod	Rod	Gelatin liquefaction	+	+	-	-
Arrangement	Single	Single	Single	Single	Malonate utilization	-	NO	-	-
Spore	+	+	-	-	Acid from - Inositol	-	-	-	-
Motil	+	+	-	+	- Sorbitol	+	-	-	-
Oxidase	-	-	+	+	- Rhamnose	-	-	-	-
Catalase	+	+	-	+	- Sucrose	+	+	-	-
Lysine decarboxylase	-	-	+	+	- Lactose	-	-	-	-
Ornithinine decarboxylase	-	+	-	-	- Arabinose	+	-	-	-
H_2S production	-	-	+	-	- Adonitol	-	-	-	-
Acid from glucose	+	-	+	-	- Raffinose	-	-	-	-
Acid from manitol	+	+	+	-	- Salicin	-	+	-	-
Acid from xylose	+	-	+	+	Arginine dihydrolase	+	+	-	-
β-galactosidae	-	-	+	-	Growth at 5°C	-	-	-	-
Indole production	-	-	-	-	Growth at 45°C	+	+	+	+
Urea hydrolysis	-	-	+	-	Growth on 7% NaCl	+	+	+	+
VP reaction	-	+	-	-	**Beta-hemolysis**	+	+	NO	NO

+ Positive, - negative, NO not observed

Table 2 Antimicrobial ability and extracellular enzyme production of isolated bacteria

Isolates	Inhibitory area (mm)	Extracellular enzyme		
		Protease	Amilase	Lipase
BM1	18.32 ± 0.04	+	+	+
BM2	16.27 ± 0.16	+	+	+
BM3	11.29 ± 0.09	-	+	-
BM4	**20.36 ± 0.06**	-	+	+

Category: + (positive), - (not detected)

Identification of Bacterial Growth

Based on the results of antimicrobial assay and the production of extracellular enzyme, only isolate *BM1* and *BM2* were used for the identification of bacterial growth. The value of generation time and constant growth rate were 19.60 minutes and 2.12 h^{-1} for *B. megaterium BM1* and 19.89 minutes and 2.09 h^{-1} for *B. firmus BM2* respectively. As *B. megaterium BM1* has a shorter value of generation time and a higher constant growth rate, this isolate then used for the next assays.

Safety assays

In order to be safe for application in shrimp culture, the microbial strain has to be evaluated for safety to the host. Based on of the safety assay, *B. megaterium BM1* did not cause any harmful effects to *L. vannamei* upon challenge even at a dose of 10^7 cells ml^{-1} introduced by immersion (data are not shown). Therefore this isolate considered safe to be used for the shrimp.

Determination of the viability of *B. megaterium BM1* in feed

The viability of putative probiotics *B. megaterium BM 1* in shrimp diet varied at different storage temperatures (4 and 25 °C) over the three week storage period (Table 3). There was a sharp decline in bacterial count in the first week of storage at both temperatures (4 and 25 °C). Decreasing of bacterial number still occurred until the third week of storage.

Table 3 Viability of putative probiotics *B. megaterium BM1* in feed

Temperature	Day	Bacterial count (cells g^{-1})
4^0C	0	$1.20 \times 10^8 \pm 1.41 \times 10^7$ d
	7	$2.94 \times 10^7 \pm 3.54 \times 10^5$ c
	14	$2.19 \times 10^6 \pm 8.49 \times 10^4$ b
	21	$1.30 \times 10^4 \pm 1.41 \times 10^3$ a
25^0C	0	$1.05 \times 10^8 \pm 7.07 \times 10^6$ c
	7	$1.39 \times 10^4 \pm 1.20 \times 10^3$ b
	14	$1.21 \times 10^4 \pm 1.06 \times 10^3$ b
	21	**$4.60 \times 10^2 \pm 4.24 \times 10^2$ a**

Mean ± S.E. in the same row (temperature) having the same letter are not significantly different at P<0.05.

Digestive enzymes activity

The hepatopancreas-intestinal protease, amylase and lipase activities of *vannamei* were recorded at the beginning and at the end of feeding trial (Table 4). The activities of those enzymes increased from the initial values in all dietary treatments. The intestinal protease, amylase and lipase activities reached the highest value on the diet supplemented with 10^6 cells g^{-1} of *B. megaterium BM1*. The decline of digestive enzyme activities in the shrimp fed with 10^7 cells g^{-1} probiotic diet confirmed that there was a natural limit of extrinsic bacteria to induce digestive enzyme activities.

Table 4 Activity of intestinal protease, amylase and lipase of Litopenaeus vannamei fed with varied levels of *B. megaterium BM1* for 28 days

Bacillus Concentration (cells g^{-1})	Enzyme activity (U/ml)					
	Protease		Amylase		Lipase	
	Initial	Final	Initial	Final	Initial	Final
0	22.94 ± 2.29a	32.66 ± 1.17a	1.17 ± 0.13a	1.83 ± 0.02a	0.006 ± 0.0006a	0.0074 ± 0.0002a
10^4	22.94 ± 2.29a	38.44 ± 1.17b	1.17 ± 0.13a	3.08 ± 0.14c	0.006 ± 0.0006a	0.014 ± 0.0011b
10^5	22.39 ± 1.01a	39.06 ± 0. 67b	1.16 ± 0.05a	3.18 ± 0.16c	0.005 ± 0.0007a	0.015 ± 00002bc
10^6	24.61 ± 2.87a	41.22 ± 0.51c	1.13 ± 0.11a	3.67 ± 0.21d	0.006 ± 0.0006a	0.016 ± 0.0004d
10^7	**22,50 ± 0,83a**	**38,94 ± 0,86b**	**1,12 ± 0,08a**	**2,77 ± 0,19b**	**0,006 ± 0,0007a**	**0,015 ± 0,0001cd**

Mean ± S.E. in the same column having the same letter are not significantly different at P<0.05.

Growth performance and feed utilization

Data pertaining to shrimp survival and performance are presented in Table 5. There was no evidence of disease in any of the shrimp receiving the *B. megaterium BM1* during the 28 days of feeding trial. The survival rate was not significantly different among the groups fed with diet containing *B. megaterium BM1* bacteria and control group. *L. vannamei* fed with *B. megaterium BM1* supplemented diet exhibited better growth performance compared to the *L. vannamei* fed with control diet. Diet supplemented with 10^6 cells g-1 of *B.*

megaterium BM1 gave a significantly higher specific growth rate (SGR), lower feed conversion ratio (FCR), higher protein and energy retention (RP, RE) and higher protein efficiency ratio compared to other diets.

Table 5 Growth performance and feed utilization of vannamei fed with diet supplemented with *B. megaterium BM1*.

Parameters	Concentration of *B. megaterium BM1* (cells g^{-1})				
	0	10^4	10^5	10^6	10^7
SR	78.67 ± 2.31a	80.00 ± 4.00a	80.00 ± 4.00a	81.33 ± 4.62a	80.00 ± 4.00a
SGR (% BW day^{-1})	1.55 ± 0.04b	2.00 ± 0.052b	2.06 ± 0.03b	2.16 ± 0.04c	2.06 ± 0.04b
FCR	3.11 ± 0.09a	2.41 ± 0.072b	2.33 ± 0.02b	2.21 ± 0.04c	2.31 ± 0.05bc
RP (%)	14.00 ± 1.97a	14.33 ± 0.83a	15.88± 0.91ab	17.23 ± 0.67b	16.74 ± 0.28b
RE (%)	10.10 ± 1.11a	9.77 ± 0.57a	10.89± 0.93ab	12.39 ± 0.61b	11.62 ± 0.16b
PER (gram/gram)	**0.98 ± 0.03a**	**1.29 ± 0.01b**	**1.31 ± 0.01bc**	**1.38 ± 0.02d**	**1.32 ± 0.03c**

Mean ± S.E. having the same letter are not significantly different at P<0.05.

DISCUSSION

There were a number of studies observing the use of probiotics in shrimp culture for controlling diseases, enhancing the growth rate and improving water quality. As the source of probionts is an important factor to be a successful probiotics, this study was conducted to evaluate the characteristics of L. *vannamei* indigenous bacteria and their potency as probiotic to enhance the growth performance of *L. vannamei.* Even though the decline production of *Penaeus monodon* have been reported since 1993 [12], *L. vannamei* definitely overtook the position of *P. monodon* to be a dominant shrimp cultured in East Java in the year of 2006.

Several assays (antagonism to pathogen, production of extracellular enzymes, and identification of growth rate) were conducted to characterize the candidate of probiotic bacteria. Bacterium which had antimicrobial ability, complete production of extracellular enzymes (protease, amylase and lipase) and high growth rate then was used for in-vivo assay. The viability of putative bacteria on shrimp diet under storage and their safety assays were also carried out to guarantee their application on shrimp culture system.

Bacillus isolated from digestive tract of *L. vannamei* in this study likewise were reported and isolated from channel catfish [13], goldfish [14] and Atlantic cod [15]. *Pseudomonas* was also recorded in *Macrobrachium rosenbergii* hatchery system [16]. *Actinobacillus* was the first time reported as microbial organism in the shrimp or fish digestive tract. The Pseudomonas in this study was confirmed on the description of identification key [17].

All four isolated bacteria were able to inhibit the growth of *V. harveyi* in vitro. Generally, the antimicrobial activity of bacteria is as a result of any of the following factors (separately or in combination) production of antibiotics, siderophores, bacteriocins, lysozymes and proteases, and alteration of pH values by organic acids produced [14]. However, the inhibitory mechanism of the interaction was not characterized in this study. Previous studies in *Bacillus* have suggested that the inhibitory effects might be caused by either alteration of pH in the growth medium, utilization of essential nutrients, production of volatile compounds or production polypeptide antibiotics [18] [19] [20]. A heat-labile siderophore with a molecular mass of less than 5 kDa was an antibacterial substance produced by *Bacillus* sp. strain NM 12 [14]. Meanwhile, some studies have found that siderophores were antibacterial substances produced by *Pseudomonas* species [21] [22].

The next criteria used to screen probiotic bacteria was the production of beneficial enzymes for the host. Results of qualitative extracellular enzyme assay showed that only *B. megaterium* BM1 and *B. firmus* BM2 produced complete extracellular enzymes studied, viz., protease, amylase and lipase. While *Actinobacillus* sp BM3 exhibited only amilolytic activities, *P. stutzeri* BM 4 were found to produce amilolytic dan lipolytic activities. Several studies found that bacteria within fish gut were capable in producing various extracellular enzymes including amylase, protease, cellulose and lipase [23][24][25]. Bacteria in the surrounding environment and feeding habit may have influence on the composition of the gastrointestinal organism in fish and potentially could have a significant role in digestion [25]. As *L. vannamei* is omnivorous species, it would benefit most from microbial with complete producing enzyme capacity.

Assessment of bacterial lifetime was conducted to figure out the constant growth rate and the generation time of the bacteria. Probiotic bacteria will be most competitive in vivo if it has a fast growth rate. As *B. megaterium BM1* had a shorter value of generation time and a higher constant growth rate compared to *B. firmus* BM2, this species then was chosen for the in vivo assays. Many researchers used *Bacillus* as a probiont in their studies [26] [27] [28] [29], yet this is the first report of study using single *B. megaterium* as a probiont.

Probiotic microorganisms have to be nonpathogenic and nontoxic in order to avoid unwanted side-effects when applied to aquatic organisms. The safety assay was conducted by immersion of *L. vannamei* in 10^4-10^7 cells ml^{-1} of *B. megaterium BM1*. Insignificant differences of survival rate were recorded among treatments and control. The administration of *B. megaterium BM1* via immersion did not give any signs of diseases.

Mortalities recorded during assay were mainly due to molting process. *Bacillus* species were not related with diseases in aquatic organisms. Therefore this isolate considered safe to be used for the shrimp.

To maintain confidence in probiotic application in shrimp culture system, it is important to demonstrate good survival of the bacteria in products during their shelf life. Even though there was a decline number of bacterial count over the period of storage, *B. megaterium BM 1* incorporated in diet was still viable after 3 weeks at 4°C and 25 °C. The number of probiotic bacteria declined in activity when included into diets over several week of storage. Diet containing *Bacillus firmus* and *Citrobacter freundii* were no longer viable after 2 and 1 weeks at 25^{o}C storage [9]. However, *Bacillus pumilus* could still be counted at 25^{o}C after 5 weeks storage. *Bacillus* can be kept in the spore form and therefore stored indefinitely on the shelf [30].

In this study, *B. megaterium BM1* suplemented in the diet resulted in an increase in the specific activity of protease, amylase and lipase in the shrimp's digestive tract. Some bacteria may contribute in the digestion processes by producing complement of enzymes (such as proteases, lipases) and providing necessary growth factors [31]. *Bacillus* also produced vitamin K and B12 and secret a wide range of exo-enzymes [32] to promote better growth. After transition through stomach, probiotics germinate in the intestine, use a number of carbohydrates and produce a range of digestive enzymes such as amylase, protease and lipase [33]. In this study, shrimps fed the probiotic bacteria can facilitate digestion of all protein constituents. Furthermore, the enzymes produced by the bacteria can complement the activity of shrimp and then increase the nutrient digestibility. The main modes of action of probiotic in aquaculture organism is nutrition improvement of host by the production of supplemental digestive enzymes and higher growth and feed efficiency, prevention of intestinal disorders and pre-digestion of anti-nutritional factors present in the ingredients [34].

The intestinal protease, amylase and lipase activities were highest with diet supplemented with 10^6 cells g^{-1} of *B. megaterium BM1*. The decline of digestive enzyme activities in the shrimp fed with 10^7 cells g^{-1} probiotic diet confirmed that there was a natural limit of inducing digestive enzyme activities by extrinsic bacteria. The specific enzyme activities were recorded as accumulation of enzymes synthesized by the shrimp and the *B. megaterium BM1* in the shrimp digestive tract. The exogenous enzyme produced by extrinsic bacteria would give only a small contribution to total digestive enzyme activity or the presence of the probiotic bacteria might stimulate the production of endogenous enzyme by shrimp [6] [35].

Shrimp fed with *B. megaterium BM1* supplemented diet exhibited better growth performance compared to the *L. vannamei* fed with control diet. This revealed that *L. vannamei* could utilize the dietary nutrient efficiently when feed is supplemented with probiotic bacteria. Similar improvements of weight gain were also reported in Indian carp (*Labeo rohita*) [26] and the Indian white shrimp *Fenneropenaeus indicus* [6]. There were several ways of probiotics enhance nutrition: (1) by synthesis of essential nutrients (vitamins and short chain fatty acids) and enzymes, (2) by detoxifying the potentially harmful compounds in feed and (3) by denaturing the potentially indigestible components in the diet [35].

In this study, various concentration of *B. megaterium BM1* was considered to identify the effective probiotic dosage administrated to shrimp. Diet supplemented with 10^6 cells g-1 *B. megaterium BM1* gave a significantly higher specific growth rate (SGR), lower feed conversion ratio (FCR), higher protein and energy retention (RP, RE) and higher protein efficiency ratio compared to other diets. The inclusion of higher concentration of *B. megaterium BM1* than 10^6 cells g^{-1} in diet did not lead to better growth performance. A probiotic concentration of 10^6 to 10^8 cells g^{-1} was sufficient for improved survival and growth performance [36]. Even though the improvement of growth was recorded in this study, we found that survival rate of *L. vannamei* were insignificantly different between treatment and control. In line with that results, the treatment of a commercial *Bacillus* probiotic did not significantly increase *P. monodon* survival in the poorly prepared ponds [37]. On the other hand, a probiotic study in *P. monodon* showed that probiotic was able to colonize both of the culture water and shrimp digestive tract, thereby it increased the black tiger shrimp survival [38]. However, it was difficult the evaluate the effectiveness of probiotic as it was determined by many factors such as species composition, application level, frequency of application and environmental conditions [35].

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