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RESEARCH PAPER

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Determining the Dose of Dietary Probiotic (*Bacillus licheniformis*) for the Nursing of Blue Swimming Crablets (*Portunus pelagicus*, L., 1758)

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Abstract

Various efforts have been made to use probiotics instead of antibiotics in aquaculture to solve the problem of low survival and slow growth of hatchlings. Selection of probiotics strain and their right dose have been the major challenges. Limited research has been done in blue swimming crab (*Portunus pelagicus* L., 1758). Therefore, present study was carried out to determine the effects of varying doses of probiotic *Bacillus licheniformis* 10^7 colony forming unit (CFU)/mL i.e. 0.0, 1.5, 3.0, and 6.0 mL/kg on the survival, growth and moulting of blue swimming crablets nursed for 45 days and determine the right dose. The four treatments were replicated thrice in 500 L plastic tanks. Regression analysis revealed that the probiotic dose had significant ($P<0.05$) positive relationships with carapace length gain, weight gain, and moulting percentage; but negative relationship with food conversion ratio. *Vibrio* colony growth was significantly ($P<0.05$) inhibited at the dose of 3.3 mL/kg feed or higher. Results showed that increased doses of *Bacillus licheniformis* enhanced the growth and moulting of blue swimming crablets. The highest moulting percentage of 89 % was found at an extrapolated dose of 6.9 mL/kg feed.

Introduction

Blue swimming crab *Portunus pelagicus* (Linnaeus, 1758) is one of the most commercially important among the species. Wild stock is the main source of supply, but it is declining along with other fishery resources. The crab farming has been promoted in many countries. As a result, a global production of blue swimming crab has increased by about 45% from 184,249 tons in 2010 to 420,158 tons in 2017 (FAO, 2020). However, due to low survival in the early stages of its life and the cannibalism problem, the expansion of its farming is not taking off. Low survival of crablets during early stages, which drops down quite often to 3% is the bottleneck for the growth of crab culture (Kedmuean *et al*., 2004). Feed supplementation approach is one of the ways to improve survival as well as growth. The mortality in the larval stage is due mainly to cannibalism. Our recent study showed that avoiding cannibalism by nursing crablet individually in cups increased survival significantly (Boonyapakdee & Bhujel, 2019).

Young crablets are normally weak and vulnerable as their digestive and immune systems are not fully developed. Supplementation of some nutrients or feed additives are helpful in improving digestion of external food, reducing stress and boosting the immune systems, thereby improving the survival and growth. More importantly, the gravid female crabs have sometimes highly pathogenic bacteria in their guts which may transmit to larvae and crablets via eggs and cause slow growth and even heavy mortality (Talpur *et al*., 2011). Use of antibiotics may help but the antimicrobial resistance (AMR) and residues have been major concerns nowadays. Instead, a number of probiotics are

being tested as alternatives to antibiotics. During the last decade, several studies have reported beneficial effects of probiotics in aquaculture (Bhujel *et al*., 2020; Wu *et al*., 2014; Wang and Gu, 2010). As a result, many probiotic bacteria have been developed, produced commercially and marketed.

In Thailand, various taxonomic divisions of microbial organisms have been used as probiotics or tested as potential probiotics including *Bacillus* considering its positive impacts in various species of aquaculture. For example, supplementation of *B. subtilis* in tilapia (*Oreochromis niloticus*), crucian carp (*Carassius auratus* var. Pengze), eel (*Anguilla japonica*) and whiteleg shrimp (*Litopenaeus vannamei*) resulted in increased growth, immune response and disease resistance (Galagarza *et al*., 2018; Cao *et al*., 2019; Lee *et al*., 2018; Chai *et al*., 2016). In addition, the supplementation of *B. licheniformis,* one of the commonly available probiotics, has been studied in some crustaceans i.e. freshwater prawn (*Macrobrachium rosenbergii*), whiteleg shrimp (*Litopenaeus vannamei*) and *Penaeus japonicas.* It has been found to boost the growth, microflora bacteria in the gastro-intestinal tract, immune systems and reduce gut pathogenic bacteria (Ranjit Kumar, 2012; Madani *et al.*, 2018; Zhang *et al*., 2011). The efficacy of probiotic doses differs with the life stages of culture species. However, normally manufacturers of probiotics recommend a common dose 1-2 mL/kg feed (Jha *et al*., 2014) which may not be effective in all the species and for all stages. Therefore, more research is needed to determine the dose specific to crabs. Bhujel *et al*., (2020) reported that commercial probiotics of *Bacillus* was found to be effective at the dose of 1.5 and 1.7 g probiotics/kg diet in Rohu hatchlings. The same dose might not be effective in crabs as it has not been determined for blue swimming crabs especially for nursing stage. Therefore, present study was carried out to determine the optimal dose of *B. licheniformis* supplementation on the survival, moulting, and other growth performance of blue swimming crablets and ability to inhibit some pathogenic bacterial growth such as *Vibrio sp.*, as an indicator, during nursing period especially in the hatcheries.

Materials and Methods

Crablets – Experimental Animals

A total of 1,000 blue swimming crablets were acquired from Thung Tala Forest Royal Development Study and Crab Conservation Centre Krabi Province Thailand in April 2018. The weight of crablets, 12 days metamorphosed from megalopa, ranged from 0.1–0.15g with carapace width of about 1 cm. The crablets were transported at a density of 15 crablets/litre of seawater with sea pine leaves used as shelter by a vehicle to the Silpakorn Aquaculture Research Station at Petchaburi province Thailand and then were held in a 500 L capacity tank (109 cm × 61.5cm × 53 cm) with salinity 30 ppt.

Experimental Design and Culture Method

The crablets were acclimatized for a week before stocking into 12 experimental plastic tanks for four treatments with three replicates, at a density of 42 crablets per tank. Thirty crablets were sampled for initial microbial test. *B. licheniformis* (BL) kmp-9 strain from K.M.P. biotech Co., Ltd. with 10^7 CFU/mL was used at the doses of 0.0, 1.5, 3.0 and 6.0 mL/kg diet, and diluted with water then sprayed onto the feed coated with 5% squid oil for those treatments as per the recommendation by Kewcharoen & Srisapoome, (2019) and Bunnoy *et al*., (2019). A temperature-controlled room (air temp. at 30°C) was used where water temperature remained within the range of 26-30°C in all the treatment/replicate tanks. A continuous water recirculation system was used which was able to recirculate all the water four times a day via a flow-through filtration tank (50 L). Air-stones were provided in all the replicate tanks for oxygen supply during the whole period of experiment and the crablets were fed with the pellet produced or the shrimp post-larva (Kung Best Co., Ltd.). The trial ran for 45 days. On the find day, 10 crablets from each replicate tank were randomly selected, weighed, and measured by photograph using imageJ (software name). All the crablets were nursed using Cup-method which resulted in a high survival (Boonyapakdee & Bhujel, 2019). A plastic cup (500 mL) was used for each individual crablet with a bottom diameter of 5.5 cm and height 14.5 cm. Each cup had several holes (1 mm diameter) made by drilling for the purpose of good water exchange. The cups were floated mounting on styrofoam raft. The tanks were filled with 150 L of seawater. Pellet feed No.2 (Kung Best Co., Ltd.) with the size of 1 mm was used for the experiment which had 43% crude protein, 4% crude lipid, 4% crude fibre, 3% vitamin mix and 12% moisture. Feeding was done once a day at 3 pm at the rate of 30% of the biomass per day. Uneaten food and wastes were siphoned out from the nursing tanks daily. Water quality indicators such as dissolved oxygen (DO), alkalinity, salinity, pH, ammonia, nitrite and nitrate were monitored weekly and 80% water was exchanged every 3-days intervals.

Microbial Testing

For the microbial testing, seven crablets were sampled on the initial day of the experiment and 3-5 crablets per treatment at the end of the experiment. The whole body of crablets was included to make samples, excluding appendages. The samples were dipped in 70% ethanol and crushed/ground using sterilized homogenizer. The liquid extract was drawn with a syringe and then poured into the tube containing 0.1% normal saline buffer at the ratio of 1:9 (crablet fluid: buffer) then bacteria were enumerated by plate count

technique. For *Vibrio* count, all samples in triplicate were prepared in Thiosulfate Citrate Bile Salts (TCBS). Liquid extract samples were used to identify *Vibrio* sp. in terms of green and yellow colours. While for *Bacillus*, seven samples before trial and nine samples from each treatment at the end of the experiment were randomly prepared. The crablet fluid samples were dipped in 80°C in water for 10 minutes before it was made ready in *Bacillus* medium M1383.

Survival and Growth Measurements

The growth parameters were calculated following the method described by Bagenal (1978) as follows:

Average daily weight gain (ADG) = Final weight - Initial weight / Experiment period

Weight gain (WG) = Final weight - Initial weight

Carapace length gain (CLG) = Final carapace length - Initial carapace length

Specific growth rate (SGR) = (L_n Final weight - L_n Initial weight) x 100 /Experiment period

Food conversion ratio (FCR) = Total dry feed intake (g) / Total wet weight gain (g)

Survival (%) = (Number of crablets which survived / initial number of crablets) ×100

Moulting percentage (MP %) = (Number of crablets moulted / number of crablets) ×100

Water Quality Parameters

Temperature and dissolved oxygen (DO) were measured directly with DO meter (YSI 550A, Japan), pH by (Cyber Scan pH11, Japan), salinity by (Prima tech Salinity meter, China). The total ammonia nitrogen (TAN), nitrite (NO_2-N), nitrate (NO_3-N) and alkalinity were analysed following the guidelines in APHA *et al.,* (2005). All the parameters were measured/analysed before changing water scheduled weekly at15:00 h.

Statistical Analysis

One-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was performed to confirm the effects of probiotic dose significant at P<0.05. Regression analysis was used to analyse the relationship between the growth parameters and the probiotic dose. The association of water quality parameters and growth parameters were analysed using correlation to see any association. SPSS ver. 18 for Windows was used considering the differences are significant at P<0.05.

Results

Growth and Survival

The testing parameters with Duncan post hoc comparison (± SD) (Table 1) showed that probiotic dose, during 45 days of nursing, had significant effects (P<0.05) on the growth parameters i.e. carapace length gain (CLG), weight gain (WG), average daily gain (ADG), and moulting percentage (MP). The highest dose used i.e. 6.0 mL/kg diet resulted in significantly higher average values than other treatments in all of these growth parameters. With the dose of probiotic 3.0 mL/kg feed also significantly (P<0.05) increased CLG and MP as compared to the control. The lowest dose i.e. 1.5 mL/kg feed increased (P<0.05) CLG but did not increase the ADG. At the same time, 6.0 mL/kg feed decreased the FCR significantly as compared to all the treatments whereas other lower doses did not show any difference against the control.

At the same time, regression analysis revealed that the probiotic dose had a significant relationship (P<0.05) with CLG, WG, ADG, MP and FCR. Survival percentage remained high ranging from 95-100%. Its relationship with probiotic dose was not be decisive to determine the best dose of the probiotic as the rate of change was not significant. When considered the CLG, it was found

Table 1. The testing parameters with Duncan post hoc comparison (± SD)

Testing parameters	Initial day	Dose of probiotic *Bacillus licheniformis* (mL/kg feed) After 45 days of nursing			
		0.0	1.5	3.0	6.0
ADG		$0.022±0.004^{a}$	$0.021±0.004^{a}$	$0.024± 0.005^{a}$	$0.032±0.003^{b}$
FCR		$2.55±0.31^{a}$	$2.25±0.35^{ab}$	$2.36±0.24^{a}$	$1.77±0.12^{b}$
CLG		$0.87±0.72^{a}$	$1.0±0.04^{b}$	$1.04±0.05^{b}$	$1.23±0.04^{c}$
WG		$0.57±0.064^{a}$	$0.60±0.085^{a}$	$0.56±0.06^{a}$	$0.74±0.05^{b}$
Molting %		$75.4±3.64^{a}$	$81.7±3.64^{ab}$	$84.1±3.65^{b}$	$88.9±3.64^{bc}$
BL (CFU/mL)		0.0^{a}	$3.0±0.6 \times 10^{4b}$	$3.2±1.4 \times 10^{4b}$	$3.0±0.3 \times 10^{4b}$
Vibrio CFU/mL (green)	$1.4±0.8 \times 10^{4ab}$	$1.9±0.6 \times 10^{4a}$	$7.9±6.4 \times 10^{3bc}$	$3.0±1.8 \times 10^{3c}$	$3.3±1.7 \times 10^{3c}$
Vibrio CFU/mL (yellow)	$1.8±1.5 \times 10^{4a}$	$2.0±1.0 \times 10^{4a}$	$3.9±2.2 \times 10^{3b}$	$1.8±0.1 \times 10^{3b}$	$1.2±0.1 \times 10^{3b}$

Note: Values in the same row with different superscript are significant different at P<0.05

that increase in probiotic dose increases in CLG linearly (y=0.0575x+0.883, R^2=0.8915, n=12, P<0.01) (Figure 1). Based on the results, it reveals that every mL/kg diet of the probiotic dose increases 0.058 cm off CLG. Probiotic dose had a quadratic relation with WG (y=0.0087x^2-0.0274x+0.5835, R^2=0.5879, n=12, P<0.05) and ADG (y=0.0004x2-0.0006x+0.0215 R^2=0.623, n=12, P<0.05); however, it was also not possible to determine the best dose because when considered the supplementation dose up to 3.0 mL/kg diet found that there was no effect on WG and ADG when further increasing to 6.0 mL/kg diet of supplementation the progressive growth can be found (Figure 2 & 3).Thus, these indicated that higher growth is possible by using further increase in the probiotic dose higher than the one applied in this experiment i.e. 6.0 mL/kg diet. However, economic analysis needs to be done. Fortunately, the relationship of the probiotic dose with MP was found to be significant and quadratic (y=-0.2806x2+3.86x+75.722, R^2=0.7182, n=12, P<0.01, Figure 4), which helped to determine the optimum dose. Based on the equation, maximum (89%) extrapolated MP is likely to be achieved at the dose of 6.9 mL/kg diet, which is almost the same level when the supplementation level was 6.0 mL/kg

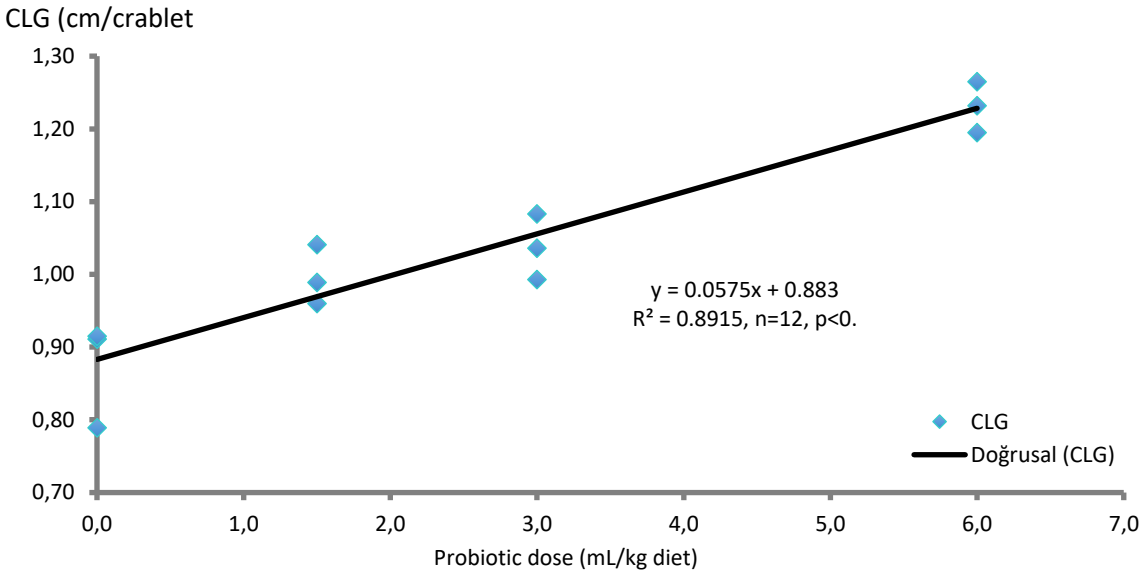

Figure 1. Relationship between carapace length gain and probiotic dose (mL/kg diet).

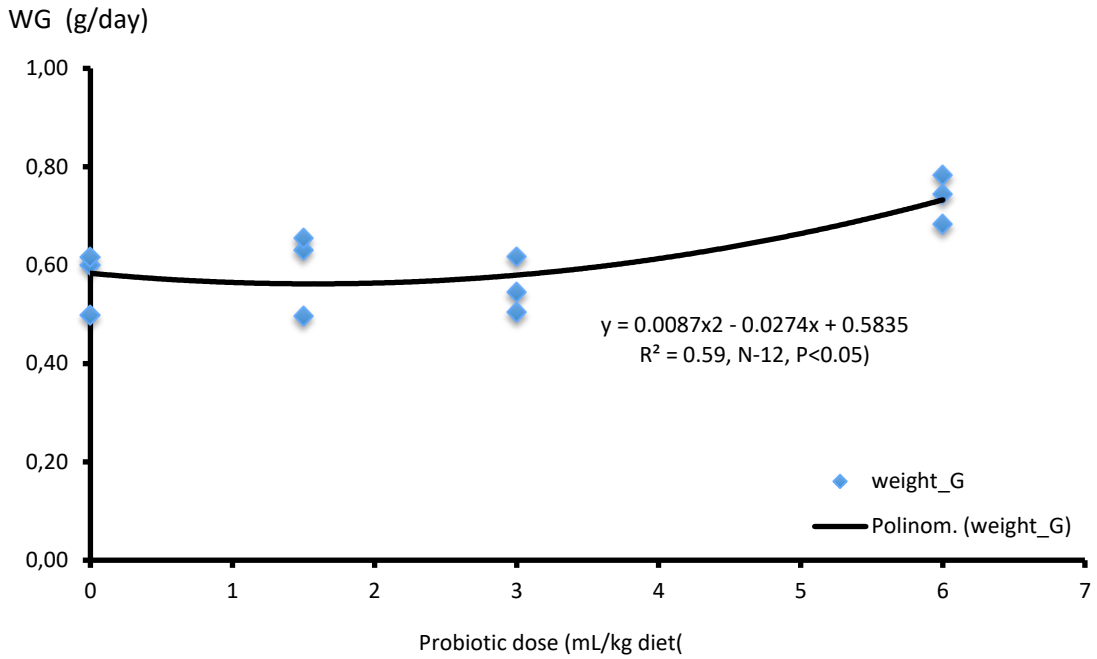

Figure 2. Relationship between weight gain and probiotic dose (mL/kg diet(.

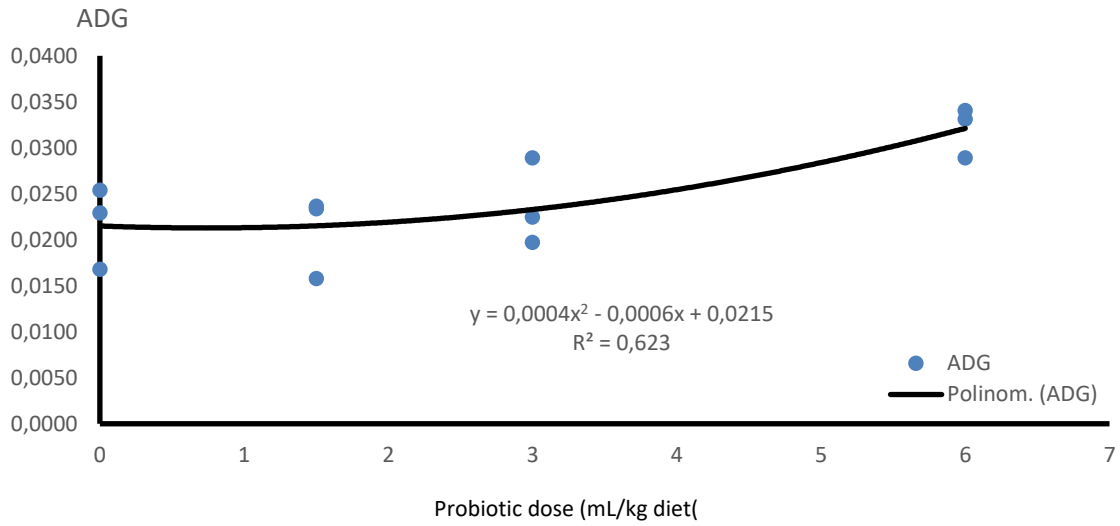

Figure 3. Relationship between ADG and probiotic dose (mL/kg diet(.

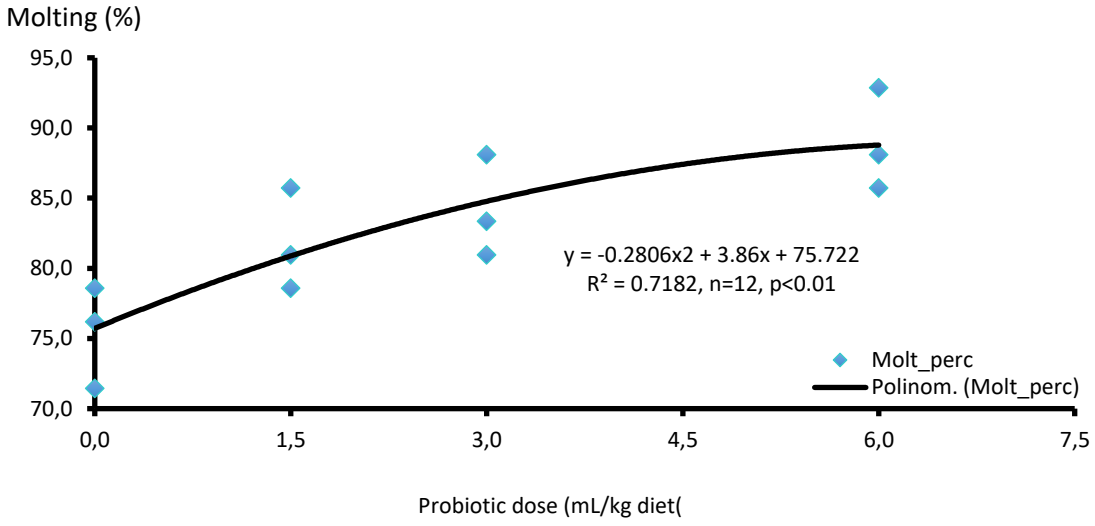

Figure 4. Relationship between moulting percentage (MP) and probiotic dose (mL/kg diet(.

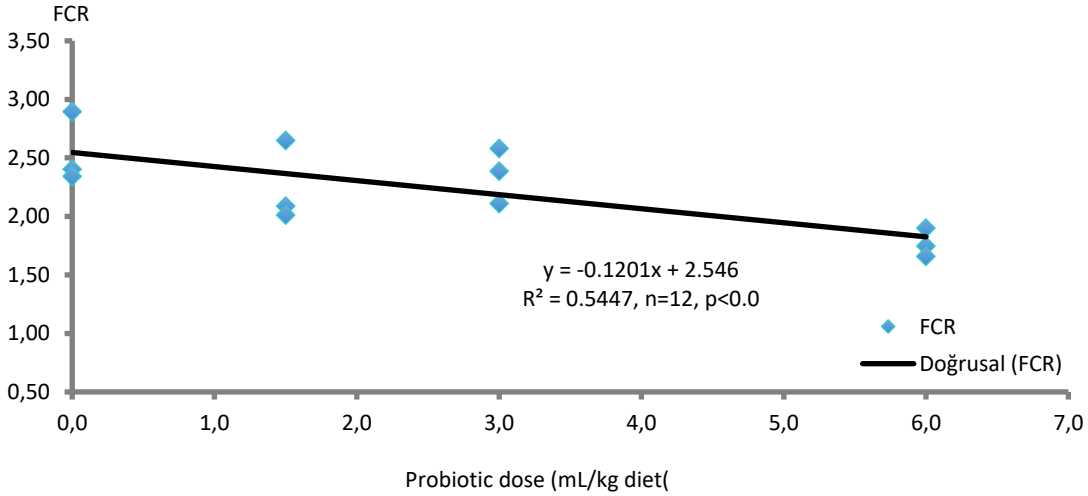

Figure 5. Relationship between FCR and probiotic dose (mL/kg diet(.

diet (Table 1, Figure 4). Similarly, an increase in probiotic dose decreases FCR linearly i.e. inverse relationship (y=- 0.1201x+2.546, R^2=0.5447, n=12, P<0.01) (Figure 5). It shows every mL/kg diet of the probiotic dose decreased FCR by 0.12.

***Bacillus licheniformis* Test**

Seven samples before the experiment (0 day: TR0) that were randomly tested by plate count technique found no BL colony on any of those plates. After 45 days of nursing, when nine samples from the treatment TR1 to TR4, the average of BL colonies were found to be 0, 3.0±0.6 × 10^4, 3.2±1.4 × 10^4 and 3.0±0.3 × 10^4 CFU/mL respectively. ANOVA showed that there were significant differences (P<0.05) among the treatments. Likewise, the regression analysis showed that CFU/mL of BL had a quadratic relationship (P<0.05) with concentration of supplementation (y=-23.709x^2+186.18x–46.255, R^2=0.6734, n=135) which revealed that the highest CFU/mL of BL would be at the supplementation dose of 3.9 mL/kg diet with 3.2 × 10^4 CFU/mL

***Vibrio* Test**

Before the experiment (0 day: TR0) when analysed taking seven samples from each treatment tested by plate count technique, average green (g) and yellow (y) colonies were found at 1.4±0.8 × 10^4 and 1.8±1.5 × 10^4 CFU/mL After 45 days of nursing the number of crablets sample tests for TR1 to TR4 were 3, 4, 4 and 5 respectively and the findings of the average of green and yellow of *Vibrio* colonies were (g) 1.9±0.6 × 10^4 (y) 2.0±1.0 × 10^4 CFU/mL, (g) 7.9±6.4 × 10^3 (y) 3.9±2.2 × 10^3 CFU/mL, (g) 3.0±1.8 × 10^3 (y) 1.8±0.1 × 10^3 CFU/mL and (g) 3.3±1.7 × 10^3 (y) 1.2±0.1 × 10^3 CFU/mL respectively. ANOVA showed that there were significant differences (P<0.05) in *Vibrio* colonies whether it be green or yellow (Table 1 and Figure 6-7). Additionally, regression analysis showed quadratic relations with the green and yellow colonies of *Vibrio* (y=39.685x^2 - 258.91x + 417.49, R^2=0.7681, N=16, P<0.01) and (y=29.341x^2 - 199.92x+363.49, R^2=0.7321, N=16, P<0.01) which also assisted to determine the dose of probiotics. Based on these quadratic equations, the lowest green and yellow colonies of *Vibrio* were found at the doses of 3.3 mL probiotic/kg.

Water Quality Analysis

Average temperature (27.4±0.39 °C), salinity (28.0±0.0 ‰), DO (5.95±0.29 mg/L), alkalinity (175.07±5.85 mg/L), pH (7.6±0.1), total ammonia nitrogen (0.01±0.01 mg/L), NO_2-N (0.93±0.79 mg/L) and NO_3-N (1.10±0.47mg/L) were within the suitable range for crablet nursing. In addition, one-way ANOVA revealed that there were no significant differences (P<0.05) in water quality parameters among all the treatments. Correlation analysis (Table 2) showed that NO_2-N had significant negative correlations (P<0.01: 2-tailed) with WG, CLG ADG, and MP, whereas total ammonical nitrogen showed significant positive correlations (P<0.05: 2-tailed) with survival.

Discussion

Crabs are healthy as well as high value commercial products traded worldwide but they are still being harvested mainly from the wild. As most of the fishery resources are declining efforts are also being made to develop the farming of crabs. It is becoming an emerging aquaculture sector with great potential. However, major obstacles are low survival of crablets and slow growth during nursing (Fielder, 2004; Waiho *et al*., 2018). Although, the low survival is considered mainly due to cannibalism among crablets themselves, but it is still not fully understood yet and remains as one of the major bottlenecks (Chen, *et al*., 2014). A number of factors

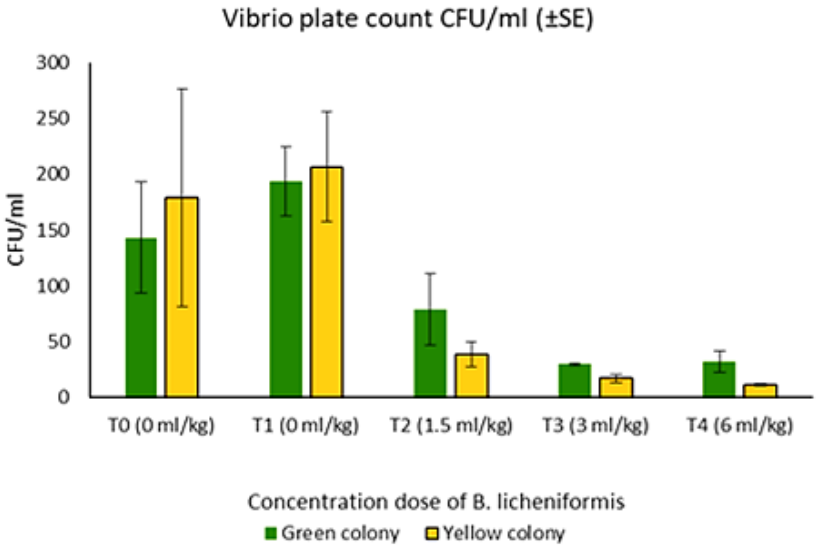

Figure 6. *Vibrio* plate count in crablets after rearing for 45 days at different doses of *B. licheniformis* treatments.

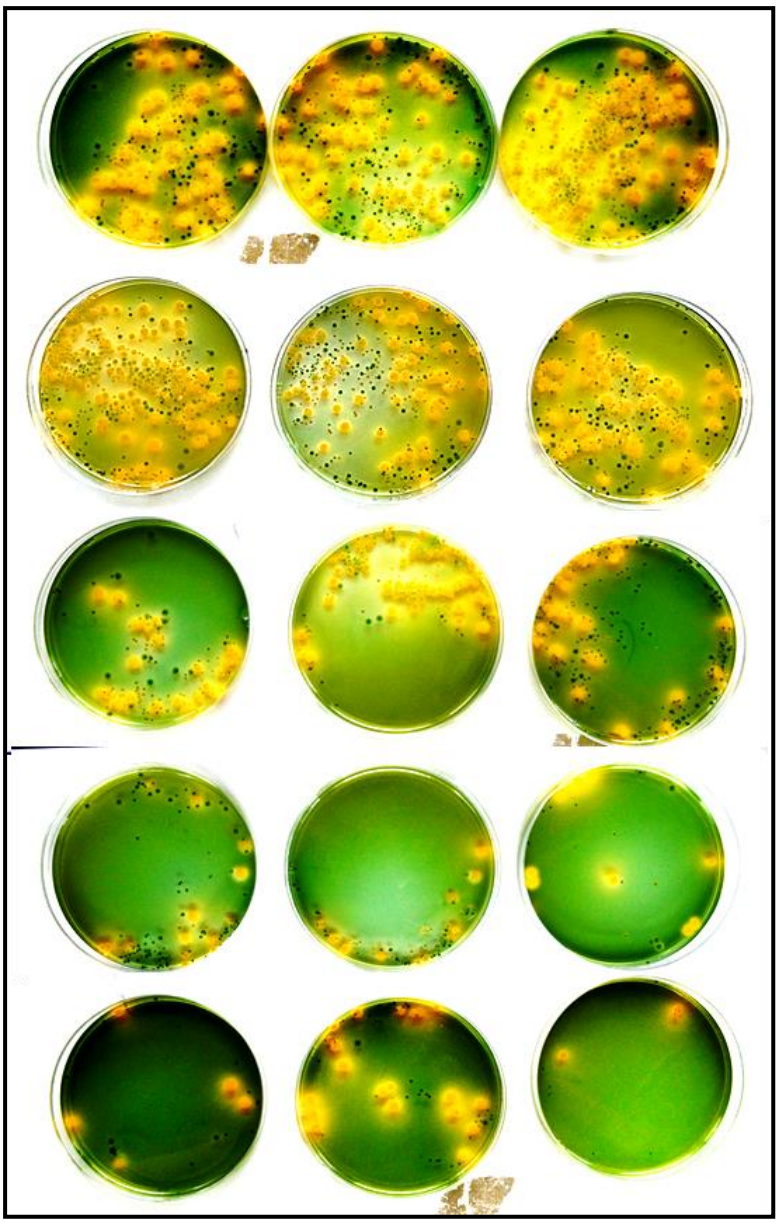

Figure 7. Plate count of *Vibrio* sp. with different concentration dose of BL (first row from top = before initiation; second row = 0.0 mL/kg diet (control); Third row=1.5 mL/kg diet; fourth row=3.0 mL/kg diet; fifth row=6.0 mL/kg diet; second – fifth row are after 45 days).

Table 2. The Pearson Correlation Sig. (2-tailed) of No_2-N and TAN (± SD)

Parameters	Mean		Std	Pearson Correlation No_2-N	Sig. (2-tail)	Pearson Correlation TAN	Sig. (2-tail)	N
No_2-N	1.33	±	0.83	1.0		-.591**	0.00	48
No_3-N	1.01	±	0.49	-.379**	0.01	0.106	0.47	48
TAN	0.00	±	0.00	-.591**	0.00	1.0		48
ADG	0.01	±	0.01	-.772**	0.00	0.188	0.27	36
FCR	2.73	±	0.62	.563**	0.00	-0.2	0.24	36
SGR	4.35	±	0.57	0.034	0.84	-0.15	0.38	36
Survival (%)	98.40	±	1.87	.567**	0.00	-.346*	0.02	48
WG	0.62	±	0.34	-.860**	0.00	.341*	0.04	36
CLG	0.32	±	0.23	-.838**	0.00	0.227	0.18	36
MOL (%)	72.97	±	11.28	-.864**	0.00	.421*	0.01	36

Note: significant asterisk (*/**) is significant different at $P<0.05$ and $P<0.01$

affect their survival, namely; geography, climate, culture species, feeding regimes and salinities (Dan & Hamasaki, 2010; Azra, & Mohammad, 2015). Tree leaves, grasses or other substrates can reduce cannibalism to improve survival. Raising in cups individually avoids cannibalism. Our previous study revealed that nursing of blue swimming crablets using individual Cup-method increases survival by three folds i.e. 21% to 63% (Boonyapakdee & Bhujel, 2019). However, it is cumber some and less productive and not very practical for commercial farming. In addition to survival, slow growth is another hurdle. Efforts were made to optimize these factors such as use of various substrates, different colours of tank, stocking density, use of antibiotics, water exchange, provision of different types of culture systems and feeding regimes. However, only some of them were found to effective such as use of dark tank colour, lowering of stocking density, use of different types of artificial foods with feed supplements (Shi *et al*., 2019; Heasman & Felder, 1983; Williams et al., 1999; Hamasaki, *et al*., 2002; Mann, *et al*., 2007).

The present study was one of the attempts to see whether the application of one of the common probiotics in aquaculture could further improve survival and the growth of crablets. As in the past study using the same Cup method, survival has been found to be very high that ranged from 95-100%, improved further from our own results of the previous trial (Boonyapakdee & Bhujel, 2019). Despite being cumbersome, cup-method showed promising in terms of survival. The high survival in the present study, including in the control (without probiotic treatment), was most probably due to the better management of water quality that kept relatively higher dissolved oxygen (DO). In this study, application of the probiotic demonstrated its positive effects on moulting percentage and the growth in terms of average daily gain and carapace length. Linear increase in average daily weight gain and carapace length gain with the increasing dose of the probiotics clearly indicated that optimal dose is beyond the highest dose i.e. 6 mL/kg used in this study. The predicted dose for highest moulting was 6.9 mL/kg feed based on the quadratic model/relationship between the dose and the moulting. However, the moulting percentage at 6 and 6.9 mL/kg feed are almost the same. Therefore, it can be confirmed that the optimal dose of probiotic should be 6 mL/kg feed.

It is clear that crablets are highly vulnerable due to cannibalism and adverse environmental conditions or water quality parameters and they need high level of nutritional and antibacterial inputs to ensure survival and support growth especially during moulting. Gravid females have sometimes highly pathogenic bacteria in their guts which may transmit to larvae and crablets via eggs and may cause slow growth and heavy mortality during nursing Talpur *et al.,* (2011). This indicates that the first moulting or earliest stages are very critical and considerably higher doses of probiotics are needed than the common dose of 1-2 mg or mL/kg feed for most species recommended by suppliers (Jha *et al*., 2014; Bhujel *et al.*, 2020). Prediction model from the present study clearly showed the highest moulting percentage i.e. 89 % can be achieved at an extrapolated probiotic dose of 6.9 mL/kg feed. Indeed, the highest dose used in this trial i.e. 6.0 mL/kg diet showed very close to the maximized moulting percentage by the predicted model. Similar high doses, 2-3 folds, showed further increment in the growth and survival of some species e.g. in rohu (Bhujel *et al*., 2020). More research, however, needs to be done for the higher doses to prove cost effective. Nevertheless, it is confirmed that crablets require considerably higher doses than the recommended dose of 1-2 mg or mL/kg feed for other aquaculture species as a general rule.

Improvement in the growth of crablets might be due to various reasons; namely, improved immune system, suppression of growth of other pathogens and improved digestion. In the present study, significant reduction in *Vibrio* (green and yellow colonies) was clearly observed when the dose was 3 mL/kg of diet or higher indicating that application of higher dose might have helped to suppress the growth of pathogens or enhance the immunity of crabs resulting in better growth. However, it was not clear whether the probiotic enhanced digestion. In terms of growth results are clearly in line with the studies of Talib *et al*., (2016) and Talpur *et al*., (2012) who reported significant improvement on the growth performances and survival due to probiotics supplementation. Supplementation of probiotic bacteria such as lactic acid bacteria (LAB) (*Enterococcus faecalis* Y17 and *Pediococcus pentosaceus* G11) stimulate immune responses in the mud crab and increased significantly serum enzyme activities of phenoloxidase, lysozyme, as well as superoxide dismutase (SOD) which were detected in the haemolymph of mud crab (Yang *et al*., 2019).Moreover, the in vivo assay conducted on blue crab juveniles, revealed that *Bacillus amyloliquefaciens* strain L11 at 10^6 CFU/mL showed significant improvement in survival (42±1%) compared with the group challenged with *V. harveyi* with no probiotics supplemented (12 ±1%) after five days of exposure. Strain L11 was also able to minimize the number of *Vibrios* and maximized the weight of the juveniles (Azrin *et al*., 2019). Even though the results of this study showed there was no significant difference on survival among treatments probably due to a short period of rearing and the use of individual cup rearing method which also resulted in a high survival. A number of researches reported that in the long periods of supplementation it provided a positive result on survival i.e. Nile tilapia (*Oreochromis niloticus*), mud crab (*Scylla serrata*), White-leg shrimp (*Litopenaeus vannamei*) (Opiyo *et al*., 2019; Wang *et al*., 2019; Yang *et al*., 2019). Therefore, nursing on a longer period with the probiotic supplementation would be an advantageous option for a good research study.

In the present study probiotic against green and yellow colonies of *Vibrio*, the probiotic significantly

reduced 4 - 16 times as found by Talpur *et al*., (2011) who reported that the gut of *Portunus pelagicus* larvae contains several highly pathogenic fish bacteria including *Vibrio harvei* and use of probiotics can control pathogenic bacteria, thereby protecting the crablets. However, more study is needed to see whether the probiotic could inhibit other types of pathogenic bacteria such as *Staphylococcus*, *Micrococcus* and others. Considering the enhancement in the growth of crablets and gut microbe inhibition, it can be confirmed that the probiotic BL is beneficial in crablets of blue swimming crab. Most of the suppliers of commercial probiotics recommend 1-2 g or mL per kg of diet as a general dose for an example carp fish studied, even though higher doses were found to further increase growth performance (Bhujel *et al*., 2020).

Based on the results of present study, the dose of around 3 mL/kg diet could inhibit *Vibrio*, and higher doses such as 6.0 mL/kg diet is required to achieve highest moulting and better growth. Different mechanisms have been described their beneficial effects, including competition with pathogens by probiotic bacteria for shelter, competition for nutrients, enzymatic improvement for digestion, improved water quality and enhanced host immune response (Sahu *et al.*, 2008). The dietary supplementation of BL in *Litopenaeus vannamei* showed that there were significantly higher levels ($P<0.01$) of lysozyme, total protein, normal microflora and immunoglobulin (Li *et al.*, 2007; Madani *et al*., 2018). Further research is needed to examine the activities of digestive enzymes like protease, amylase and lipase in the blue swimming crabs that have received diets containing BL or other probiotics (Gobi *et al*., 2018). Present study showed that supplementation of BL improved growth by 1.2-1.4 folds and immune against *Vibrio* sp. by 4 - 17 folds then, this must be considered to use for the commercial production of crablets.

Bacillus licheniformis showed improved growth performances in individual cup nursing system, more research is needed to see whether it could also improve survival and growth of crablets in mass rearing system with the shelter of tree leaves. In addition, more studies are needed to see how this probiotic would perform when the adverse environmental conditions as water quality parameters play a key role to maintain quality of crablets in the nursing system (Boonyapakdee & Bhujel, 2019). In the present study, nitrite-nitrogen (NO_2-N) was negatively correlated with DO and salinity. Total ammonical nitrogen (NH_3-N) had positively correlated with pH and temperature. There are some reports showing decrease in growth performance due to exposure to high nitrite in crustaceans and other gilled aquatic organisms which they have (Romano & Zeng, 2013; Boonyapakdee & Bhujel, 2019). Therefore, providing good water quality is important. On the other hand, exploring appropriate feeds, optimal feeding regime and space for each individual crablet could be some of the areas for future research with a view to achieving higher growth along with high survival which are essential for commercial production.

Conclusion

Present study showed that BL supplementation benefitted the blue swimming crablets in terms of growth and *Vibrio* inhibition. Increase in the dose of probiotic (*Bacillus licheniformis*) enhances the growth and moulting of blue swimming crablets. Regression model predicted the dose of 6.9 mL/kg feed to result in the highest moulting percentage of 89 %; however, present study can confirm 6.0 mL/kg feed is the best recommended dose for growth performance. More importantly, an economically optimum dose should be determined and recommended performing an economic analysis using the prices or the costs of other inputs and outputs in each local context.

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