

Original Research Paper

Effects of Extended-Rotifers Inclusion and Live Food-Enrichment with Probiotics on the Survival, Metamorphosis, Development Time and Growth of Mud Crab, *Scylla paramamosain* (Estampador) Larvae

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Abstract: The present larval nutrition study was conducted to evaluate the effects of feeding probiotic-enriched rotifers inclusion mixed with *Artemia* until zoeal stages 4 (Z4) and Z5 on the survival, metamorphosis, development time and growth of mud crabs, *Scylla paramamosain* larvae. The efficiency of the feeding regime was tested by two-factor analysis. A 4×2 factorial experimental design with eight combinations of enriched or un-enriched or a mixture of enriched and un-enriched live food (factor 1) in each of two feeding regimes (factor 2) in triplicate was setup. After 24 days of larval cultivation, there were no significant ($p>0.05$) interactive effects between the two selected factors on survival and metamorphosis rate of larvae. Larval survival in all treatments, under both factors, decreased gradually ($p<0.05$) during the cultivation period. From 15 Days After Hatching (DAH), the survival and metamorphosis rates of larvae fed both enriched rotifers and *Artemia* were higher ($p<0.05$) than those of the larvae without enriched live food. Similarly, from 18 DAH onwards, the extension of rotifers inclusion mixed with *Artemia* until Z5, irrespective of enrichment, showed higher ($p<0.05$) survival, metamorphosis and growth of megalopa than when the rotifers were included only until Z4 stage. Overall, an amalgamation of extending rotifer feeding and enriching them at the same time with *Artemia* can result in considerable improvements in survival and metamorphosis which can in turn, have beneficial impact on the technical feasibility of a commercial crab hatchery.

Keywords: Mud Crab, *Scylla paramamosain*, Hatchery, *Artemia*, Rotifers-Extension, Probiotics

Introduction

Mud crab, *Scylla paramamosain*, is a commercially important source of income for coastal fishers in the Asia-Pacific region (Keenan, 1999). In Vietnam, *S. paramamosain* is the most prevalent cultured species in coastal areas (Le Vay *et al.*, 2001; Lindner, 2005) and the second most common crustacean species after the shrimp (*Penaeus monodon* and *Litopenaeus vannamei*) (Nghia *et al.*, 2007). The pond-based crab culture system totally relies on wild-caught seed (Keenan, 1999), which has become limited by over-exploitation and diminishing

mangrove habitats (Le Vay *et al.*, 2001). Although mud crab seed production has been studied extensively (Brick, 1974; Heasman and Fielder, 1983; Marichamy, 1996; Quintio *et al.*, 1999; Mann *et al.*, 1999; Davis, 2003; Nghia *et al.*, 2007; Yi *et al.*, 2009; Noorbaiduri and Ikhwanuddin, 2015), the survival of hatchery-produced crabs is still unreliable and inconsistent (Baylon and Failaman, 1999; Hamasaki *et al.*, 2011), mainly due to mass mortality during the zoeal (Z) and megalopa stages, especially during metamorphosis from Z5 to megalopa (Hamasaki *et al.*, 2002). Mass mortalities are mainly linked to poor larval nutrition (Hamasaki *et al.*, 2002;

Suprayudi *et al.*, 2004) caused by selection of improper feeding regimes.

Nutritional deficiencies and inappropriate feeding regime, for example, feeding either rotifers alone (Nghia, 2004; Baylon, 2009), or exclusively *Artemia* (Ruscoe *et al.*, 2004; Zeng *et al.*, 2004), or excessive or starved *Artemia* (Suprayudi *et al.*, 2002; Dan *et al.*, 2016) to mud crab larvae resulted in low survival and prolonged larval development time. On the contrary, *Artemia* enriched with lecithin and cholesterol (Holme *et al.*, 2007) as well as feeding a mixture of rotifers and *Artemia* to crab larvae (Zeng and Li, 1999; Nghia, 2004; Ruscoe *et al.*, 2004) improves survival. Zeng and Li (1999), Ruscoe *et al.* (2004) and Nghia (2004) have recommended that the inclusion of rotifers should be extended until Z2 or Z3 stages to increase the survival of crab larvae. Similarly, our previous study (Quy *et al.*, 2018) showed that including rotifers until the late zoeal stages (Z3 to Z5), mixed with *Artemia*, increased in a higher metamorphosis of megalopa, thus, confirming that including the extension of rotifers in the feeding regime of mud crab larvae can contribute additional nutrients essential for moulting of most of the larval stages, as *Artemia* alone are incapable of providing these additional nutrients to the larvae (Baylon, 2009).

Similar to larval mal-nutrition, fungal, parasites and bacterial infections contribute to poor survival; however, enriching live feeds with probiotics increases survival rates (Dan and Hamasaki, 2015). Probiotics are defined as bio-friendly agents that can control and compete with pathogenic bacteria as well as stimulate the growth of aquatic animals without undesirable side effects (Farzanfar, 2006). The beneficial effects of dietary inclusion of probiotics are displayed through competition for habitat and nutrients to displace harmful bacteria (Moriarty, 1998), provision of nutrients and enhanced digestive enzyme activities (Ziaei-Nejad *et al.*, 2006; Wang, 2007; Zhou *et al.*, 2009; Gupta *et al.*, 2016), improvement of water quality (Nimrat *et al.*, 2012), enhancing immune responses (Rengpipat *et al.*, 2000; Tseng *et al.*, 2009; Kumar *et al.*, 2013; Ambas, 2015) and producing antiviral substances (Lakshmi *et al.*, 2013; Pandiyan *et al.*, 2013). Although the beneficial uses of probiotics, including bacteria of the genus *Bacillus*, have been made in larval, post-larval and juvenile stages of shrimp (Rengpipat *et al.*, 1998; Ziaei-Nejad *et al.*, 2006; Balcázar and Rojas-Luna, 2007; Yu *et al.*, 2008; Guo *et al.*, 2009; Liu *et al.*, 2010; Nimrat *et al.*, 2012; Vinoj *et al.*, 2013; Dong *et al.*, 2014; Jamali *et al.*, 2015) and in mud crab larvae and juvenile (Wu *et al.*, 2014; Dan and Hamasaki, 2015; Talib *et al.*, 2017), no information is available on the effects of supplementing probiotics and extending the inclusion of rotifers on the survival, metamorphosis, development time and growth of mud crab larvae. Thus, the aim of this study was to evaluate

the effects of enriching live feeds (*Artemia* and rotifers) with commercially available *Bacillus* spp. as probiotics and extending the inclusion of rotifers until late zoeal stages of *S. paramamosain*.

Materials and Methods

Water Source

Seawater was pumped from the river and left one night in a reservoir tank before being disinfected with calcium hypochlorite at the rate of 20 mg L⁻¹. After two day of chlorination, when no chlorine residue detected, the disinfected seawater was transferred into a storage tank, where the water was treated again with 2 mg L⁻¹ Vikor-S (Bayer, Germany) for further use in the experiment.

Live Food Culture

According to the method described by Lind (2014), L-strain rotifers (*Brachionus plicatilis*) originated from a marine fish hatchery were stocked into indoor 4,000-L square concrete tank. The tank was filled 60% of disinfected seawater (30‰) and provided gently aeration by air-stone. Rotifers were fed a mixture diet of commercially available rotifer feed (S. parkle, INVE Aquaculture, Thailand) and baker's yeast (50:50) at a rate of 0.4 g for one million rotifers as recommended by Lind (2014) at 6 am, 12 pm and 6 pm daily. When density of rotifers achieved approximately 500 individuals mL⁻¹, they were partially harvested to feed to mud crab larvae.

Artemia franciscana cysts were incubated by an incubation container. Before incubating, the cyst were disinfected with sodium hypochlorite solution at a concentration of 20 mg L⁻¹ for 10 min to avoid any infection of harmful bacteria and fungi and then washed with seawater. Salinity and pH of disinfected seawater using for *Artemia* incubation were 30‰ and 8.3, respectively. Strong aeration and light were also require. At the temperature of 29°C, the newly hatched *Artemia* nauplii appeared after 15 h of incubation and they were harvested to enrich or directly feed to mud crab larvae.

Method of Enriching the Live Foods

Bacillus spp. is known as an effective probiotic bacteria in shrimp culture (Ambas *et al.*, 2013), so a commercial probiotic (Lymnozyme FT-2B, Cisbay, USA) including *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. Pumilus* was used to enrich rotifers and *Artemia* in the current study. The rotifers, partially harvested from mass production in the 4,000-L square concrete tank, were washed with fresh seawater and stocked in a 10-L plastic bucket containing 50%

volume of disinfected seawater with gentle aeration. Probiotics and *S. parkle* at a rate of 1 mg g⁻¹ of rotifer feed were placed together into a blender (HR2195/00, Philips, China) and blended with 100 mL of deionized water. This blended mixture was then poured into the bucket containing rotifers. The feeding rate of this mixture was adjusted following the rotifer stocking densities in the rearing medium which was 0.4 g of rotifer commercial feed meant to feed one million rotifers (for rotifer density of 1,000-1,500 rotifers mL⁻¹). This was equivalently to 40 mL of the mixture of probiotics and rotifer commercial feed. This dose was based on the recommendation of feed company. After 6 hours of enrichment, rotifers were harvested and fed to crab larvae.

In contrast, newly hatched *Artemia* were only soaked in the commercial probiotic suspension and disinfected seawater at a rate of 1 mg L⁻¹, with strong aeration. This enrichment process lasted only for 1 h in order to avoid nutrient losses in *Artemia* due to their quick assimilation efficiencies. The enrichment protocol was strictly followed and the supplemented weight of the probiotic powder was measured by an analytical balance (PA 214-Ohaus, USA) with 0.0001 g precision. The newly hatched *Artemia* (instar 1) is not fully developed and thus are unable to ingest the food from water, though, they can absorb water containing *Bacillus*. As a result, the *Bacillus* could be resident of both gut and the external body of *Artemia*. To verify the final concentration of the probiotics, homogenised sample taken from whole rotifers or *Artemia* was used to determine total *Bacillus* spp. on Tryptic Soy Agar (TSA) plates. Total *Bacillus* spp. concentration in enriched rotifers and *Artemia* were counted periodically and also at the end of the trial in plates containing TSA medium, according to the method described by Tomasiewicz *et al.* (1980).

Mud Crab Brood Stock and Larval Source

Mature and berried females of *S. paramamosain* were fattened and managed as described by Quy *et al.* (2018). During the fattening period, the salinity and water temperature ranged from 28 to 30‰ and 28 to 29°C, respectively, while photoperiod was exposed to 12: 12 of light: Dark cycle following natural lights. One day before hatching, the berried female was

transferred and individually reared in a 200-L round tank for hatching.

Newly hatched Z1 larvae, from single spawners, were collected and bathed in 0.1 µL L⁻¹ iodine solution for 30 seconds and they were then temporarily reared into a 100-L plastic bucket. The larvae were fed purely with the rotifers without enrichment with probiotics at a rate of 40-50 individuals mL⁻¹. On the next day, the larvae were used for the experiment.

Experimental Design

Based on our previous research (Quy *et al.*, 2018), which aimed to extend the inclusion of rotifers into the feeding regime of mud crab larvae, two feeding regimes *viz.* extending rotifers inclusion mixed with *Artemia* until Z4 and Z5 stages, were selected. In the current study, rotifers and *Artemia* in these feeding regimes (extending rotifers until Z4 and Z5) were enriched with a commercial probiotic. The enriched/un-enriched live food was defined as a first factor and extension of rotifers inclusion as a second factor. A 4×2 factorial experimental design with eight various combinations of enriched or un-enriched or a mixture of enriched and un-enriched live food (factor 1) in each of two feeding regimes (factor 2) in triplicate was tested (Table 1). Enriched or un-enriched rotifers were fed from Z1 crab larvae and extended until the next day of the moulting to Z4 and Z5 stages, depending upon the selection of the extension of rotifers inclusion, whereas enriched or un-enriched *Artemia* nauplii were offered to these treatments from Z2 stage until the megalopa stage.

Twenty-four 1.5-L plastic beakers in which each beaker was filled with 1 L of disinfected seawater (30‰) and stocked with 30 mud crab Z1 collected from the 100-L plastic bucket. The beakers were moderately aerated to prevent settlement of live food and larvae. All the experimental beakers were maintained at a constant temperature (28±1°C) by immersing in a 5,000-L square concrete tank. To improve ability to catch the prey and possibly reduce any mortality by cannibalism or infection, the photoperiod was maintained constantly for 24 h by a 40-W fluorescent lamp. The pH of disinfected seawater was balanced approximately to 8.3±0.2 by adding CaCO₃ and/or NaHCO₃ before being distributed into every experimental beaker.

Table 1: Experimental feeding treatments (design) showing combinations of enriched/un-enriched live food and extension of rotifer inclusion for *S. paramamosain* larvae

Extension of rotifer inclusion	Enrichment			
	Un-enriched <i>Artemia</i> and rotifers	En-Rotifers only	En- <i>Artemia</i> only	Both <i>Artemia</i> and rotifers enriched
Until zoea 4 stage	R & A	En-R & A	R & En-A	En-R & En-A
Until zoea 5 stage	R & A	En-R & A	R & En-A	En-R & En-A

R: Un-enriched rotifers; A: Un-enriched *Artemia*; En-R: Enriched rotifers; En-A: Enriched *Artemia*

Mud crab larvae were fed only rotifers at a rate of 20 individuals (ind.) mL⁻¹ from Z1 to Z2 and then fed a mixture of rotifers and *Artemia* at a rate of 10 and 5 ind. mL⁻¹, respectively, until Z4 and Z5, depending upon the treatment in factor 2. After rotifers inclusion was stopped, the density of *Artemia* in the feeding regime was increased from 5 to 10 ind. mL⁻¹ until the end of the experiment. The desired densities of rotifers and *Artemia* nauplii in every beaker was prepared following the method described by Baylon (2009). Feeding time was applied only once a day after all crab larvae were transferred into a newly prepared beaker. The newly beakers were filled with disinfected seawater of the same environmental parameters of the replaced beakers and the larvae were carefully and quickly transferred with the help of a large bore pipette. At the same time, larval metamorphosis and mortality was recorded in each beaker. When megalopa stage appeared, they were immediately moved out to avoid cannibalism. Ten megalopa were collected and preserved in 10% formalin solution for further measurements of carapace widths, body lengths and wet weights. The experiment was ended at 24 days after hatching. The survival of the larvae, consisting of zoea and megalopa was computed using the following equation:

$$\text{Survival rate} = \frac{\text{Final larvae}}{30 \text{ zoea stocked}} \times 100$$

Megalopa carapace width and body length were measured using a scale inserted into the eyepiece of a microscope (Olympus CX21, USA), then they were dried on tissue paper for wet weight. The wet weight of megalopa was weighted by an analytical balance (PA 214-Ohaus, USA) with 0.0001g precision.

Statistical Analysis

All data in the treatments presented in percentages, such as survival of larvae and percentage of successfully metamorphosed megalopa, were arcsine-square root transformed (Zar, 2010) before analysis. Two-way ANOVA was used to test for significant differences among treatments. In the case of no significant interactive effects between enriched/un-enriched live food and extension of rotifers inclusion, two-way ANOVA was continuously employed to test for various enrichment/non-enrichment and two levels of extensions of rotifers inclusion. If there were significant interactive effects between enriched/un-enriched live food and extension of rotifers inclusion, one-way ANOVA was employed to test for various effects of enrichment/non-enrichment, extension of rotifers inclusion and interaction between enrichment/non-enrichment and extension of rotifers inclusion. Significant differences between the treatments were detected by Tukey's

multiple range tests at the 0.05 level of significance. Kruskal-Wallis non-parametric tests was used to compare megalopa carapace width, body length and wet weight because of unequal sample size (amount of megalopa of some replicate in treatments were not enough); significant differences between the treatments were detected by Mann-Whitney Test at the 0.05 level of significance. All statistical analyses were performed using IBM SPSS Statistics 24.0.

Results

Environmental Parameters and Concentration of Total *Bacillus* spp. of Enriched Live Food

During the experiment, the salinity and pH in all experimental beakers were stable at 30‰ and 8.6, respectively. Alkalinity was constant at 140 mg CaCO₃ L⁻¹. While water temperature was 27.9±0.5°C throughout the experimental period.

After enriching live food, the total *Bacillus* spp. counted in enriched rotifers and *Artemia* were 2.0±0.70 (×10⁶) cfu g⁻¹ and 3.3±0.07 (×10⁶) cfu g⁻¹, respectively.

Larval Survival

There were no significant ($p>0.05$) interactive effects between enriched/un-enriched live food with probiotics (factor 1) and extension of rotifers inclusion (factor 2) on mud crab larval survival; the main effect was examined independently for two factors (Table 2). Larval survival in all treatments (in both factors) decreased gradually ($p<0.05$) during the culture period. The survival of larvae fed both enriched rotifers and *Artemia* was higher ($p<0.05$) than that of larvae fed a diet without enrichment (R & A) from 15 Days After Hatching (DAH) onwards and a diet enriched with rotifers only from 15 to 18 DAH. However, the larval survival at the end of the feeding experiment was not altered ($p>0.05$) by enrichment when enrichment was carried out individually either to rotifers or *Artemia*. Similarly, extension of rotifers inclusion influenced ($p<0.05$) the survival of larvae from 18 DAH onwards, in which the larvae fed rotifers mixed with *Artemia* until Z5 showed significantly higher survival than those fed rotifers inclusion until Z4.

There was a negative correlation between larval survival and culture period (Table 3), with regression indices (R²) ranging from 0.96 to 0.99. These indices were, however, independent ($p>0.05$) of enrichments and extension of rotifers inclusion in diets with *Artemia*.

Rate of Metamorphosis and Development Time of Megalopa

Mud crab larvae fed both enriched rotifers and *Artemia* (En-R & En-A) showed a higher ($p<0.05$) rate of metamorphosis of megalopa (37.2%) than those fed a

diet without enrichment (R & A) (12.8%) and a diet enriched with rotifers only (En-R & A) (17.2%). Similarly, the larvae fed rotifers inclusion mixed with *Artemia* until Z5 showed a significantly higher ($p < 0.05$) metamorphosis rates (27.5%) than larvae fed rotifers inclusion until Z4 (15.8%) (Fig. 1). Morphological

variation was also less seen in the feeding treatments that had high successful metamorphosis of megalopa. However, the duration to metamorphose from Z1 to the megalopa stage (development time) did not completely depend ($p > 0.05$) on enrichments and feeding duration with rotifers (Fig. 2).

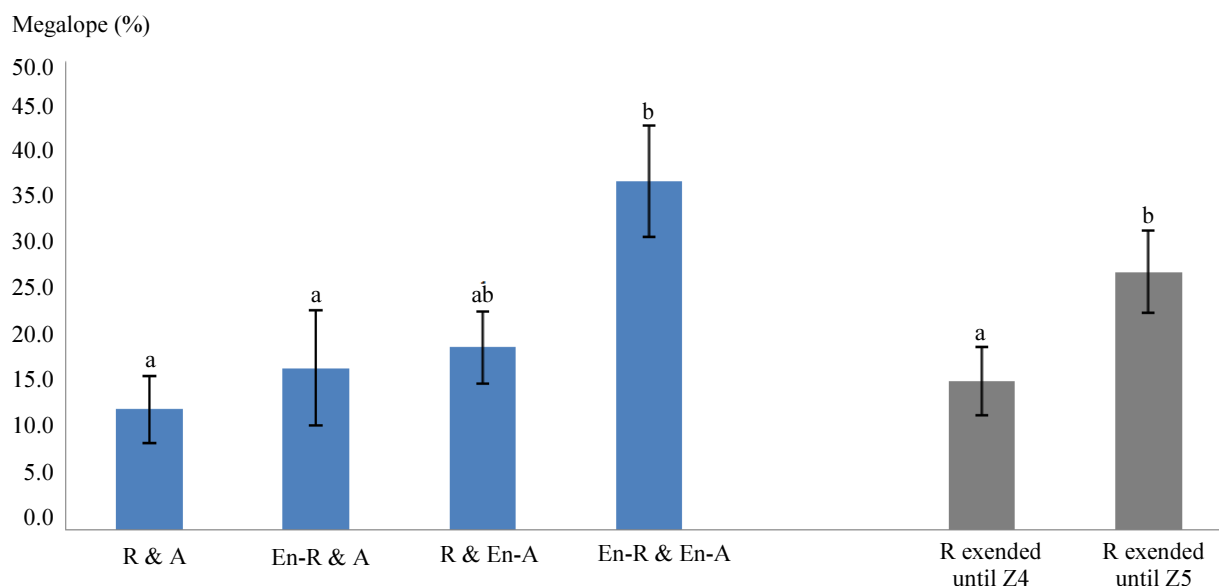


Fig. 1: Percentage of successful metamorphosis of megalopa fed according to different enrichments and extending rotifers inclusion mixed with *Artemia*. The bars, regardless to colours, present mean percentage of megalopa and error bars present \pm standard error ($n = 3$). R: Un-enriched rotifers; A: Un-enriched *Artemia*; En-R: Enriched rotifers; En-A: Enriched *Artemia*. Significant differences were found among all treatments with different superscript letters ($p < 0.05$) for bars with the same colour

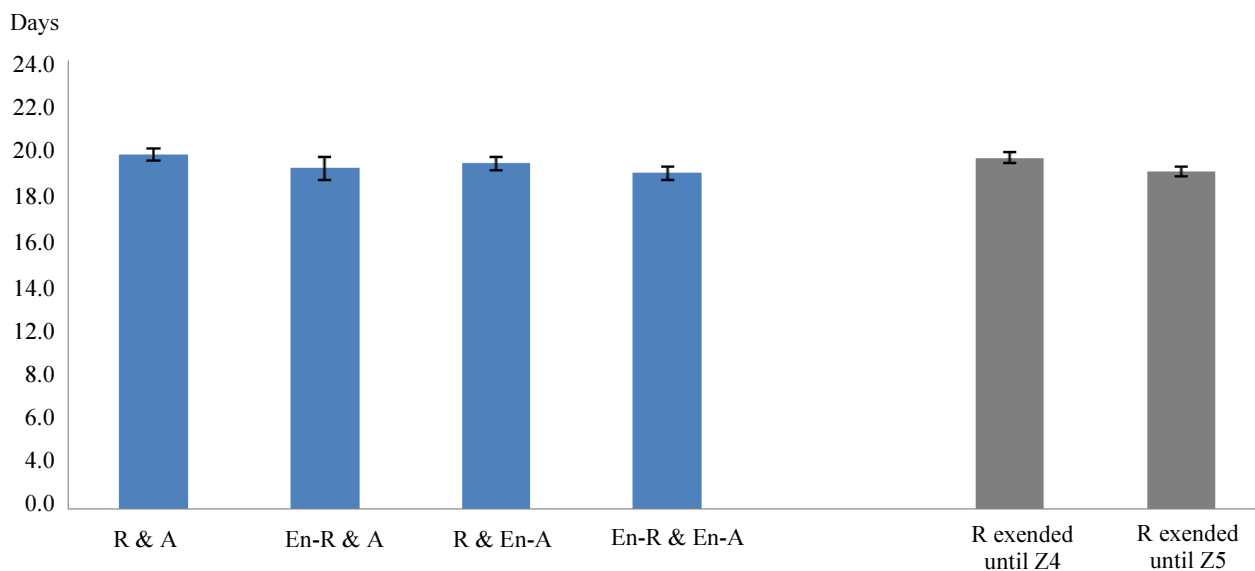


Fig. 2: Duration of rearing period needed for metamorphosis from zoea 1 to megalopa stage under various feeding regimes. The bars, regardless to colours, present mean development time of megalopa and error bars present \pm standard error ($n = 3$). R: Un-enriched rotifers; A: Un-enriched *Artemia*; En-R: Enriched rotifers; En-A: Enriched *Artemia*

Table 2: Mean survival ± Standard Error (SE) of crab larvae fed according to different enrichments and extending rotifer inclusion mixed with *Artemia* for 24 days of culture

Days	Factors					
	Enriched/un-enrichment live food				Extension of rotifers	
	R & A	En-R & A	R & En-A	En-R & En-A	Until Z4	Until Z5
3	¹ 95.0±1.67 ^a	¹ 93.3±2.11 ^a	¹ 96.7±1.49 ^a	¹ 97.2±0.56 ^a	¹ 96.9±0.76 ^a	¹ 94.2±1.31 ^a
6	² 72.2±2.94 ^a	¹² 78.3±4.01 ^a	² 79.4±2.91 ^a	¹² 86.1±2.78 ^a	² 79.7±2.61 ^a	² 78.3±2.61 ^a
9	²³ 57.2±7.95 ^a	²³ 69.4±3.98 ^a	²³ 70.0±4.39 ^a	²³ 76.7±1.92 ^a	²³ 65.8±4.48 ^a	²³ 70.8±2.76 ^a
12	²³⁴ 45.0±9.26 ^a	³⁴ 48.3±7.33 ^a	³⁴ 57.8±6.48 ^a	²³⁴ 71.1±3.51 ^a	³⁴ 51.1±6.03 ^a	³⁴ 60.0±4.91 ^a
15	³⁴⁵ 31.7±7.54 ^a	⁴⁵ 37.2±7.62 ^a	⁴ 47.8±4.99 ^{ab}	³⁴⁵ 61.7±4.77 ^b	⁴⁵ 38.9±5.32 ^a	⁴⁵ 50.3±5.15 ^a
18	⁴⁵ 23.3±5.84 ^a	⁴⁵ 29.4±6.96 ^a	⁴⁵ 37.2±4.16 ^{ab}	⁴⁵⁶ 54.4±5.62 ^b	⁵⁶ 30.0±4.85 ^a	⁴⁵⁶ 42.2±4.93 ^b
21	⁵ 16.1±4.67 ^a	⁴⁵ 22.2±6.19 ^{ab}	⁵ 24.2±3.06 ^{ab}	⁵⁶ 40.6±6.74 ^b	⁶ 20.0±3.74 ^a	⁵⁶ 31.7±4.56 ^b
24	⁵ 13.9±3.59 ^a	⁵ 20.0±5.84 ^{ab}	⁵ 21.7±3.19 ^{ab}	⁶ 38.9±6.70 ^b	⁶ 18.1±3.75 ^a	⁶ 29.2±4.38 ^b

R: Un-enriched rotifers; A: Un-enriched *Artemia*; En-R: Enriched rotifers; En-A: Enriched *Artemia*. Significant differences were found among all treatments with different superscript letters ($p < 0.05$) in the same row for each factor. Significant differences were also found throughout the cultivation period within each treatment with different superscript numbers ($p < 0.05$) in the same column

Table 3: Relationships between survival and day of culture of crab larvae fed with different enrichments and extension of rotifer inclusion mixed with *Artemia*

Treatment	Equation	Regression (R ²)
R & A	y = -3.91x+97.46	0.96±0.02
En-R & A	y = -3.69x+99.60	0.97±0.01
R & En-A	y = -3.54x+101.90	0.99±0.01
En-R & En-A	y = -2.74x+102.50	0.99±0.00
R extended until zoae 4	y = -3.81x+101.28	0.98±0.01
R extended until zoae 5	y = -3.13x+99.43	0.99±0.01

R: Un-enriched rotifers; A: Un-enriched *Artemia*; En-R: Enriched rotifers; En-A: Enriched *Artemia*. Y represents the survival of larvae and X the day of culture. Values are mean ± SE.

Table 4: Mean carapace width, body length and wet weight of one-day-old megalopa fed according to different enrichments and extension of rotifers inclusion mixed with *Artemia*

Parameters	Factors					
	Enriched/un-enriched live food				Extension of rotifers	
	R&A	En-R&A	R&En-A	En-R&En-A	Until Z4	Until Z5
CW (mm)	1.63±0.01 ^a	1.64±0.01 ^a	1.63±0.02 ^a	1.66±0.01 ^a	1.62±0.01 ^a	1.66±0.01 ^b
BL (mm)	4.91±0.05 ^a	4.97±0.03 ^a	4.96±0.01 ^a	4.98±0.03 ^a	4.93±0.03 ^a	4.98±0.02 ^a
WW (mg)	3.84±0.11 ^a	3.90±0.12 ^a	3.92±0.12 ^a	3.94±0.07 ^a	3.77±0.07 ^a	4.02±0.05 ^b

R: Un-enriched rotifers; A: Un-enriched *Artemia*; En-R: Enriched rotifers; En-A: Enriched *Artemia*; CW: Carapace width; BL: Body length; WW: Wet weight. Values represent mean ± SE. Significant differences were found among all treatments with different superscript letters ($p < 0.05$) in the same row for each factor.

Growth Performance of Megalopa

Enrichment of live food did not significantly ($p > 0.05$) influence carapace width, body length and wet weight of megalopa, but extension of rotifers inclusion mixed with *Artemia* until Z5 improved ($p < 0.05$) carapace width and wet weight of megalopa (1.66 mm and 4.02 mg, respectively) compared to the treatment in which rotifers were included until Z4 (1.62 mm and 3.77 mg, respectively; Table 4).

Discussion

Previous research has shown that *Bacillus* spp. can have significant effects on crustaceans (Table 5), mediated by improved water quality (Soundarapandian and Sankar,

2008; Nimrat *et al.*, 2012), enhanced digestive enzyme activities (Ziaei-Nejad *et al.*, 2006) and increased health status (Uddin *et al.*, 2013) of the host. Likewise, a feeding regime including rotifers and *Artemia* improved the survival of mud crab larvae (Baylon and Failaman, 1999; Zeng and Li, 1999; Nghia, 2004; Ruscoe *et al.*, 2004).

To our knowledge, this is the first study investigating the effects of extending rotifer feeding in an *Artemia*-based diet with enriched live foods, both *Artemia* and rotifers, with a commercial probiotic based on *Bacillus* spp. in mud crab (*S. paramamosain*) larvae. In the current study, a diet that included both enriched rotifers and *Artemia* with probiotics increased survival and metamorphosis rates of megalopa compared to a diet without enriched live food. The same results were obtained in *L. vannamei* fed both

enriched rotifer and *Artemia* (Jamali *et al.*, 2015). Total bacteria and probiotic *Bacillus* increased in the host (Jamali *et al.*, 2015) that resulted in increasing activity of digestive enzymes (Ziaei-Nejad *et al.*, 2005) and inhibiting the growth of pathogens (Decamp *et al.*, 2008), which in turn improved larval survival. In addition, Nogami *et al.* (1997) reported that the bacterial strain PM-4 (*Thalassobacterutilis*), which was added daily into rearing water increased the survival of swimming crab (*Portunus trituberculatus*) larvae by repressing the growth of harmful bacteria and fungi. Dan and Hamasaki (2015) also found that suppressing larval necrosis symptoms and improving survival of early larvae of *S. serrata* were done when probiotics was inoculated in the larval rearing water. Although the mode of probiotic administration differed between our study and the previously mentioned studies, the beneficial outcomes of probiotics were not significantly different (Ziaei-Nejad *et al.*, 2005). Similar to the factorial I outcome, the inclusion of rotifers mixed with *Artemia* until Z5 improved megalopa survival, metamorphosis rate and growth compared to the treatment where rotifers were included only until Z4.

Previous studies have also shown that a mixed diet of rotifers and *Artemia* until the late zoeal stages improved larval survival of *S serrata* (Baylon and Failaman, 1999), *P. Pelagicus* (Redzuari *et al.*, 2012), *Upogebia pusilla* (Faleiro and Narciso, 2009) and *Panopeus herbstii* (Harvey and Epifanio, 1997), but these studies failed to mention the benefits that achieved as extending of

rotifers inclusion. In the present study, morphological variation and formation of immature megalopa, resulting from accumulation of insufficient nutrients (Dan *et al.*, 2013) were less seen in the crab larvae fed rotifers inclusion mixed with *Artemia* until Z5 than in those fed rotifers inclusion only until Z4. This could be due to the starvation of *Artemia* that can be reduced to a certain extent by feeding them rotifer excrements (Dan *et al.*, 2016). Crab larvae can be exposed to less starved *Artemia* when rotifers are present until Z5 in a mixed diet with *Artemia*.

Additionally, morphological variation and formation of immature megalopa, which are caused by quantity of available food (Pestana and Ostrensky, 1995), may be less appearance as rotifers and *Artemia*, both are available in rearing water in our study. According to Genodepa *et al.* (2004), the feeding behaviour of mud crab larvae is raptorial. The late zoeal stages showed a strong preference for *Artemia*, which supplied the majority of energy intake required for moulting to the next stage (Harvey and Epifanio, 1997); however, after moulting, they preferred rotifers, which provided energy for survival (Baylon *et al.*, 2004). This indicates that extending rotifers inclusion, mixed with *Artemia*, until Z5 can provide additional benefits in reducing the morphological variation and the formation of immature megalopa, which would result in mass mortality of crab larvae (Dan *et al.*, 2013).

Table 5: Uses of probiotic *Bacillus* species in crustacean aquaculture

Probiotic	Target host species	Doses and administration duration	Response	References
<i>B. subtilis</i> UTM 126	<i>Litopenaeus vannamei</i>	10 ⁵ cfu g ⁻¹ feed for 28 days	Resistance of <i>Vibrio</i> species	Balcázar and Rojas-Luna (2007)
<i>B. subtilis</i>	<i>Litopenaeus vannamei</i>	10 ¹⁰ cfu g ⁻¹ feed for 30 days	Increased survival and yield and reduced <i>Vibrio</i>	Far <i>et al.</i> (2009)
<i>B. subtilis</i>	<i>Litopenaeus vannamei</i>	10 ¹⁰ cfu g ⁻¹ feed for 20 days	Against white spot syndrome virus	Fu <i>et al.</i> (2011)
<i>B. subtilis</i> - E20	<i>Litopenaeus vannamei</i>	10 ⁹ cfu L ⁻¹ for 14 days	Increased survival and immune response of larvae	Liu <i>et al.</i> (2010)
<i>B. subtilis</i> - E20	<i>Litopenaeus vannamei</i>	10 ⁸ cfu g ⁻¹ feed for 98 days	Increased survival and disease resistance	Tseng <i>et al.</i> (2009)
<i>B. subtilis</i>	<i>Penaeus monodon</i>	10 ¹⁰ cfu g ⁻¹ feed for 90 days	Disease protection	Rengpipat <i>et al.</i> (2000)
<i>B. S11</i>	<i>Penaeus monodon</i>	0.4 mg g ⁻¹ feed for 90 days	Increased growth and immunity	NavinChandran <i>et al.</i> (2014)
<i>B. cereus</i>	<i>Macrobrachium rosenbergii</i>	10 ⁹ cfu g ⁻¹ diet for 60 days	Increased survival, growth, feed use, digestive enzyme activity and innate immune response	Gupta <i>et al.</i> (2016)
<i>B. coagulans</i>	<i>Macrobrachium rosenbergii</i>	10 ⁹ cfu g ⁻¹ diet for 60 days	Increased growth, feed use and immune parameters	Kumar <i>et al.</i> (2013)
<i>B. licheniformis</i>	<i>Marsupanaeus japonicas</i>	10 ⁸ cfu g ⁻¹ diet for 60 days	Increased survival, growth and immune response and reduced <i>Vibrio</i>	Dong <i>et al.</i> (2014)
<i>Bacillus</i> spp.	<i>Litopenaeus vannamei</i>	10 ⁵ cfu mL ⁻¹	Increased survival and resistance of <i>Vibrio</i> species	Luis-Villaseñor <i>et al.</i> (2011)
<i>Bacillus</i> spp.	<i>Litopenaeus vannamei</i>	10 ⁹ cfu g ⁻¹ diet for 60 days	Improved growth and survival of larvae	Nimrat <i>et al.</i> (2012)
<i>Bacillus</i> spp.	<i>Fenneropenaeus indicus</i>	10 ⁶ cfu mL ⁻¹	Improved growth, survival and digestive enzyme activity of larvae	Ziaei-Nejad <i>et al.</i> (2006)

For cost-effective aspect, Ruscoe *et al.* (2004) and Baylon (2009) found that extending rotifers inclusion until late zoal stages with *Artemia* does not provide economic benefits due to the use of expensive resources in terms of floor space, microalgae and labour (Nghia, 2004; Ruscoe *et al.*, 2004). On the contrary, rotifers used in the present study produced according to the procedures described by Lind (2014) were of high daily productivity and low production cost. Furthermore, extending the period of rotifer inclusion in the feeding regime could save the usage of *Artemia* (Quy *et al.*, 2018). Therefore, extending rotifers inclusion mixed with *Artemia* until Z5 is possible to reduce cost production for mud crab hatchery.

Probiotics may rapidly alter the intestinal microbiota of the larvae. Gatesoupe (1999) showed that the intestinal microbiota of early larval stages was easily altered by the invasion of the microorganisms from water and food, even when their digestive tract was not fully developed and feeding had not yet commenced. In the present study, a mixture of *Bacillus* spp. was inoculated in live food at the beginning of the experiment; therefore, any harmful bacteria that could invade the cultured species through the food chain (Van Stappen, 1996, p.114) may have been reduced or eliminated. Enriched live food can also become a vector for bringing desirable microbes into the digestive system of the host (Lavilla-Pitogo *et al.*, 2002), thereby potentially improving the properties of the indigenous microflora of the larvae as well as interfering with the development of harmful bacteria. For example, when the probiotic *Bacillus* spp. was used, *Vibrio* numbers were reduced and no luminous *Vibrio* responsible for losses in shrimp hatcheries existed (Moriarty, 1998; 1999). Similarly, Rengpipat *et al.* (1998) and Far *et al.* (2009) have reported that *B. subtilis* proliferated and replaced *Vibrio* spp. in the digestive tract of shrimp (*P. monodon*) treated with probiotics that increased in survival and growth of the shrimp. In agreement with these results, our study showed that the survival and metamorphosis rates of the larvae fed both enriched rotifers and *Artemia* with probiotics were higher than those fed a diet without enrichment. The reduction in the number of probiotic bacteria (Dan and Hamasaki, 2015) or low concentrations of probiotics in the diet (Tseng *et al.*, 2009) induced individual differences among the replicated dietary treatments in any feeding trial. This may explain the fact that in the current study, enrichment of only one type of live feed could not significantly improve survival and rate of metamorphosis in crab larvae.

A study by Ruscoe *et al.* (2004) on *S. serrata*, as well as our earlier study (Quy *et al.*, 2018) on *S. parasamosain*, showed that feeding a mixture of un-enriched rotifers and *Artemia* until Z4 and Z5 stages obtained similar megalopa survival. On the contrary, in

the present study feeding a mixture of rotifers and *Artemia*, irrespective of enrichment, until Z5, rather than Z4, improved megalopa survival, metamorphosis rates and growth. It is apparent that enrichment can be considered as a factor contributing to these differences. However, there were no significant interactive effects between enrichment/non-enrichment with probiotics and extension of rotifers inclusion on mud crab larval survival and metamorphosis rates, probably because the beneficial effect of the two factors studied was more of an additive affect rather than a synergistic affect.

Conclusion

In conclusion, inclusion of enriched rotifers mixed with enriched *Artemia* until the stage Z5 improved survival, metamorphosis rate and growth of megalopa. Further, the enrichment of both rotifers and *Artemia* as a live feed is crucial to achieve beneficial outcomes in crab hatcheries. However, these results needs to be validated under commercial farming operations, wherein other natural and/or anthropogenic factors interplay. However, current study can be used as a baseline data on the use of appropriate feeding regimes to improve the survival of mud crab larvae. There is also a need to compare the bio economics between the use of exclusive formulated feed and the feeding regime used in the current study.

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Author's Contributions

Quy Moc Ong: Participated in built up, designed, carried out, analysed data and drafted manuscript.

Ravi Fotedar: Contributed to check all the work as well as correct the manuscript.

ThyThi Truong Ho: Contributed carrying out experiment and analysing samples (counted bacteria concentration...).

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