THE EFFECTS OF *BACILLUS* AND *LACTOBACILLUS* AS PROBIOTICS ON MUD CRAB *SCYLLA PARAMAMOSAIN* (ESTAMPADOR, 1949) ZOEA LARVAL CULTURE PERFORMANCE

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LIST OF SYMBOLS AND ABBREVIATIONS

Σ	Total
AA	Amino acids
ACP	Acid phosphatase
ANOVA	Analysis of Variance
BS11	*Bacillus subtilis* strain 11 or *Bacillus* S11
BIARC	Bribie Island Aquaculture Research Centre
BW	Body weight
cfu	Colony forming units
C1	Crablet 1 stage
CLPs	Cyclic lipopeptides
CP	Crude protein
DM	Dry matter
DNA	Deoxyribonucleic acid
DHA	Docosahexaenoic acid
DPH	Day(s) post hatching
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAO	Food and Agriculture Organization
FER	Feed efficiency ratio
FCR	Feed conversion ratio
g	Gram(s)

h	Hours
HUFA	Highly unsaturated fatty acid
ind.	Individuals
LAB	Lactic acid bacteria
LCFA	Long chain fatty acids
LSI	Larval Stage Index
LIM	Lysine Indole Motility
M	Megalopa(e)
MRS	Man-Rogosa-Sharpe
NA	Nutrient agar
OA	Organic acid
OTC	Oxytetracycline
P	Phosphorus
PCR	Polymerase chain reaction
PER	Protein efficiency ratio
PL	Post larvae
ppt.	Parts per thousand
PUFA	Poly unsaturated fatty acids
RCC	Reinforced Concrete
SEM	Scanning electron microscopy
Sp. or spp.	Species (for singular or plural term)
t	tonnes
TCBS	Thiosulphate citrate bile salts sucrose
TSA	Tryptic soy agar

TSB	Tryptic soy broth
TSI	Triple Sugar Iron
UFA	Unsaturated fatty acids
USD	United States dollar
VP	Voges-Proskauer
WGR	Weight gain ratio
Wt/ vol	Weight for volume
Z1 - Z5	Zoeal stages 1 – 5

KESAN *BACILLUS* DAN *LACTOBACILLUS* SEBAGAI PROBIOTIK DI ATAS PRESTASI KULTUR LARVA ZOEA KETAM NIPAH *SCYLLA PARAMAMOSAIN* (ESTAMPADOR, 1949)

ABSTRAK

Ketam nipah (genus *Scylla* spp.) merupakan sejenis krustasea yang penting dari segi pengkomersilan. Namun, salah satu halangan utama untuk perkembangan akuakultur ketam *Scylla* adalah kekurangan bekalan anak benih yang ditetaskan dalam pusat hatceri penetasan disebabkan isu-isu utama iaitu penyakit disebabkan bakteria dan kadar kematian yang tinggi. Pada masa kini, antibiotik digunakan secara meluas dalam penternakan larva ketam nipah untuk mempertingkatkan kelangsungan hidup dan bekalan yang berterusan. Walau bagaimanapun, penggunaan antibiotik untuk jangka panjang menimbulkan pelbagai risiko, termasuk pembangunan rintangan bakteria terhadap antibiotik, tahap kebisaan, dan pengesanan bahan kimia dihadkan dalam daging binatang yang diternak. Tujuan kajian ini adalah untuk menggunakan tiga jenis probiotik, iaitu *Bacillus* pelbagai jenis, *Lactobacillus casei* (Shirota), dan *Lactobacillus plantarum*, pada kepekatan yang berbeza untuk menentukan prestasi kultur di larva *Scylla paramamosain*. Terdapat kadar kemandirian larva yang lebih tinggi dalam rawatan dengan *Bacillus* pelbagai jenis ditambah berbanding dengan kawalan, terutamanya pada kepekatan 5.00×10^8 (12.33 - 18.33 ± 1.76 - 3.76 % berbanding 1.67 - 3.67 ± 1.67 - 1.76 %; $P < 0.05$) dan 1.00×10^3 cfu/ml. Kepekatan terbaik *Bacillus* pelbagai jenis untuk memberikan jumlah *Vibrio parahaemolyticus* yang lebih rendah dalam kajian ini adalah pada kepekatan 1.00×10^9 cfu/ml. Ini lebih rendah (siknifikan) dari kawalan (1.33 ± 1.33 cfu/ml berbanding

32.67 ± 11.05 cfu/ml) pada hari kultur (DPH) kemudian. *Vibrio parahaemolyticus* dikenali sebagai patogen untuk larva *Scylla*, dan nombor koloni yang lebih rendah itu adalah wajar dalam kultur. Rata-rata, tidak ada perbezaan yang ketara dalam kualiti air dan Larva Stage Indeks (LSIs) antara rawatan *Bacillus* pelbagai jenis dan kawalan, walaupun larva muncul lebih sihat dalam rawatan *Bacillus* pelbagai jenis. Peringkat perkembangan larva yang paling cepat dilihat dalam rawatan *Bacillus* pelbagai jenis pada 1.00×10^9 cfu/ml. Rawatan *Lactobacillus casei* pada kepekatan 1.88×10^8 dan 3.75×10^8 cfu/ml memberikan kadar kemandirian yang lebih tinggi (49.70 - 99.30 ± 11.90 - 29.20 and 70.70 ± 13.03 % berbanding 2.30 - 5.70 ± 1.20 - 5.70 %; P = 0.022 and 0.029) berbanding dengan kawalan semasa DPH awal hingga pertengahan. Terdapat kadar kemandirian yang lebih tinggi (74.33 - 90.67 ± 7.26 - 6.57 % berbanding 47.67 ± 2.19 %; P = 0.040 and 0.005) dalam rawatan *Lactobacillus plantarum* berbanding dengan kawalan semasa DPH awal, pada kepekatan 1.00×10^2 cfu/ml dan 5.00×10^2 cfu/ml di dalam air kultur. Dari segi kualiti air dan LSIs, tidak ada perbezaan yang ketara antara rawatan *Bacillus* pelbagai jenis dan kawalan, dan ini mungkin disebabkan oleh kepekatan yang rendah *Lactobacillus* ditambah. Berdasarkan kepekatan yang digunakan di dalam kajian ini, *Bacillus* pelbagai jenis adalah lebih berkesan dari *Lactobacillus* spesies tunggal untuk meningkatkan kadar kemandirian dan menurunkan nombor koloni *V. parahaemolyticus* di dalam kultur *S. paramamosain* larva. Oleh itu, kepekatan *Bacillus* pelbagai jenis terbaik untuk ditambah ke dalam kultur *S. paramamosain* larva ialah 5.00×10^8 dan 1.00×10^9 cfu/ml, manakala bagi *Lactobacillus* ia adalah 3.75×10^8 cfu/ml. Penggunaan *Bacillus* dan *Lactobacillus* sebagai probiotik adalah alternatif ideal untuk antibiotik dalam penetasan *Scylla* untuk menghasilkan pengeluaran anak benih yang berterusan dan boleh dilaksanakan secara komersial.

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ABSTRACT

Mud crabs (*Scylla* spp.) are commercially important crustaceans. One of the major obstacles to the growth of *Scylla* aquaculture is the low supply of hatchery produced seed due to bacterial diseases and high rates of mortalities. Currently, antibiotics are used extensively in *Scylla* larval culture to improve survival, but its use poses numerous risks including the development of resistance in bacteria to antibiotics, higher levels of virulence, and detection of restricted chemicals in the flesh of farmed animals. The aim of this study was to determine the culture performance of *Scylla paramamosain* larvae with the use of three different types of probiotics, namely a multispecies *Bacillus*, *Lactobacillus casei* (Shirota), and *Lactobacillus plantarum*, at various concentrations. There were higher average larval survivals in treatments with a multispecies *Bacillus* probiotics added compared with the control, especially at concentrations of 5.00×10^8 (12.33 - 18.33 ± 1.76 - 3.76 % compared to 1.67 - 3.67 ± 1.67 - 1.76 %; $P < 0.05$) and 1.00×10^9 cfu/ml. The best concentration of the multispecies *Bacillus* probiotic that gave lower *Vibrio parahaemolyticus* counts in this study was at a concentration of 1.00×10^9 cfu/ml. This was significantly lower than the control (1.33 ± 1.33 cfu/ml compared to 32.67 ± 11.05 cfu/ml) during the later days post hatching (DPH). *Vibrio parahaemolyticus* is known to be pathogenic to *Scylla* larvae, thus lower numbers are desirable in culture. On average, there were no obvious differences in water qualities and Larval Stage Indexes (LSIs)

between the multispecies *Bacillus* treatments and controls, although larvae appeared healthier in the multispecies *Bacillus* treatments. Water qualities were within tolerable ranges for *Scylla* larvae. The fastest larval developmental stage was seen in the multispecies *Bacillus* treatment at 1.00×10^9 cfu/ml. *Lactobacillus casei* treatments at concentrations of 1.88×10^8 and 3.75×10^8 cfu/ml gave significantly higher survival rates compared to the control during the early to mid DPHs (49.70 - 99.30 ± 11.90 - 29.20 and 70.70 ± 13.03 % compared to 2.30 - 5.70 ± 1.20 - 5.70 %, respectively; $P = 0.022$ and 0.029). There were significantly higher survival rates in the *Lactobacillus plantarum* treatments compared to the control during the early DPH (74.33 - 90.67 ± 7.26 - 6.57 % compared to 47.67 ± 2.19 %; $P = 0.040$ and 0.005), at concentrations of 1.00×10^2 and 5.00×10^2 cfu/ml in the culture water. In terms of water qualities and LSIs, there were no obvious differences between the *Lactobacillus* treatments and controls, and this could be due to the relatively low concentration of *Lactobacillus* added. Based on the concentrations used in this study, the multispecies *Bacillus* probiotic was more effective than single species *Lactobacillus* for improving the survival rate and lowering *V. parahaemolyticus* counts of *S. paramamosain* larvae. The recommended concentration of the multispecies *Bacillus* to be added into *S. paramamosain* larvae was therefore 5.00×10^8 and 1.00×10^9 cfu/ml, and for *Lactobacillus* it was 3.75×10^8 cfu/ml. The use of *Bacillus* and *Lactobacillus* as probiotics is an ideal alternative to antibiotics in *Scylla* hatcheries to provide a consistent, commercially feasible production of seedlings.

CHAPTER 1

INTRODUCTION

The term "probiotic" has been defined as "a mono- or mixed culture of live microorganisms that, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora" (Havenaar et al., 1992). In aquaculture, Moriarty (1999) suggested that the definition further applies to the adding of live, naturally occurring bacteria to tanks and ponds in which the animals live, because these bacteria modify the bacterial composition of the water and sediment. The health of the animals is thus improved by removal, or decrease in population density of pathogens and by improving water quality through the more rapid degradation of waste organic matter (Moriarty, 1999). Fuller (1989) gave the meaning of probiotics as a living bacterial food addition which provides advantages to the cultured organism through the enhancement of its gut bacterial equilibrium. Kesarcodi-Watson et al., (2008) defined probiotics as bacterial strains which can out-compete pathogens while benefiting and enhancing the growth of the host, with no detrimental side effects.

Nevertheless, different probiotic species and strains provide differing benefits (Senok et al., 2005). Probiotics can control the microflora and pathogenic bacteria, degrade unwanted substances, enhance the environment through lowering toxic gasses, increase the number of food organisms in the culture water, improve the host nutrition, and enhance their immunity to diseases (Kesarcodi-Watson et al., 2008). Improved water quality has also been shown during the addition of some probiotics, particularly *Bacillus* (Verschuere et al., 2000). This is due to Gram positive *Bacillus* being more effective in converting

organic matter to carbon dioxide than Gram negative bacteria (Kesarcodi-Watson et al., 2008). Some activities of probiotics have growth enhancing effects on aquatic organisms, by involvement in nutrient assimilation and providing nutrients and vitamins. Probiotics can also improve the growth of aquatic organisms (Kesarcodi-Watson et al., 2008).

Mud crabs belonging to the genus *Scylla* spp., commonly inhabit mangrove areas and are found in coastal waters of 5 - 30 parts per thousand (ppt.) salinity (Co-Thach, 2009). They are also commercially important crustaceans as there is a large demand in Malaysia and in many other Southeast Asian countries (Keenan, 1999). Aquaculture of *Scylla* in Malaysia alone was at 41.11 tonnes (t), with a wholesale value of RM 639000, retailing at RM 938540 in 2012 (Department of Fisheries Malaysia, 2013). Currently, *Scylla* aquaculture is an upcoming industry in Malaysia. In 2013, the average price for *Scylla* crabs selling at a typical farm in Malaysia was RM 13.00 – 51.00/kg for 200 - 500 g crabs (Kwong, 2013). Average wholesale price in 2010 was RM 16.73/kg, and retail price was RM 19.86/kg (Department of Fisheries Malaysia, 2013).

The genus *Scylla* includes the species *Scylla serrata, Scylla tranquebarica, Scylla olivacea,* and *Scylla paramamosain* (Quinitio & Parado-Estepa, 2008). The four species can be differentiated through their external morphology (Keenan et al., 1998). *Scylla serrata, S.tranquebarica,* and *S. olivacea* are common in the Philippines, whereas *S. paramamosain* is common in Vietnam, Indonesia, and Thailand. All species are suitable for aquaculture and are farmed commercially. In Malaysia, the most common species are *S. olivacea, S. tranquebarica,* and *S. paramamosain*, while on the Penang Island *S. olivacea* and *S. paramamosain* were dominant (Naim et al., 2012), with *S. tranquebarica* being the least commonly caught species there. There have been reports of wild *S. serrata*

occurring in the Matang Mangrove Forest Reserve, Perak, and Kota Marudu (Sabah), within Malaysia (Ikhwanuddin et al., 2014).

Mud crabs of the genus *Scylla* has shown tremendous potential as an aquaculture species in Southeast Asia. However, the farming has mostly been carried out on small scales by local fishermen, based mainly on the culture of wild crablets (C) to marketable sizes. In Vietnam, there were trials done where survivals of larval culture has been reported low and inconsistent (Nghia, 2005). Currently, the insufficient supply of *Scylla* juveniles from wild stocks is one of the main reasons for the decline in it's aquaculture in Malaysia. Production declined from 600 t in 1999 to 29.53 t in 2010 (Department of Fisheries Malaysia, 2013; Food and Agriculture Organization, 2013). In order to encourage it's aquaculture, hatchery raised juveniles are required.

Bacterial infection is an important factor contributing to high mortalities seen at the early zoeal (Z) stages throughout the industry (Nghia et al., 2007). Microbiological studies at Bribie Island Aquaculture Research Centre (BIARC, Australia) have noted that bacterial disease agents can be transmitted via the rotifers and Artemia added as feed, newly hatched larvae moved from the hatching tank, seawater, and even the air within the hatchery (Mann et al., 1998).

Luminescent bacterial disease is a serious and financially significant bacterial disease caused by certain members of the genus *Vibrio* and other associated genera (Shanmuga-Priya, 2008). Vibriosis affects a wide range of marine and brackish water shellfish species and is commonly secondary to other primary causes such as poor water quality, stress, and poor nutrition (Jithendran et al., 2010). *Vibrio harveyi* often infect crab larvae cultured in hatcheries (Jithendran et al., 2010). They multiply quickly and poor hatchery conditions exacerbate their pathogenicity (Jithendran et al., 2010). The affected larvae appear

glowing in dark conditions, resulting in lowered feeding and in serious circumstances high mortalities (Jithendran et al., 2010). Boer et al. (1993) reported that *Scylla* Zoea (Z) are sensitive to luminous bacteria with *V. harveyi* said to be a main cause of disease among a number of species. Parenrengi et al. (1993) isolated several species of *Vibrio*, including *V. harveyi* and *V. parahaemolyticus*, and studied their pathogenicity to Z of *Scylla*. Results suggested that all species were potentially pathogenic to Z; however their pathogenic ability was relatively low compared to *V. harveyi* (Parenrengi et al., 1993). Especially scarce in the literature is the benefits of probiotics in *Scylla* culture.

Antibiotics are used in aquaculture as prophylactics and therapeutics. Their unmanaged applications have caused immunity in pathogenic bacteria (Balcázar et al., 2006). Oxytetracycline (OTC) is the usual antibiotic used in larval culture to deter *Vibrio* infections, but it induces bacterial tolerance ten days after administration (Uno et al., 2010). *Vibrio* has been implicated as the main bacterial pathogens of *Scylla*. Antibiotics such as OTC, chloramphenicol, furazolidone, and streptomycine have been tested to reduce *Vibrios* in crustacean culture, but their effectiveness is now low. When antibiotics are used to control bacteria, some will remain alive as they possess the deoxyribonucleic acid (DNA) for tolerance. The surviving strains will multiply quickly as their competitors have been eliminated (Moriarty, 1999).

Currently, one of the main factors preventing the commercial viability of *Scylla* larval culture is the scarcity of crablet supply, which is directly affected by the control of pathogenic bacteria in high density hatchery culture situations. Antibiotics are currently used extensively in the *Scylla* hatchery industry to control pathogenic bacterial levels. However, this uncontrolled used of antibiotics has led to the development of resistance in bacteria to antibiotics, higher levels of virulence, and detection of restricted chemicals in

the flesh of farmed animals. Manipulation of the bacterial community through the application of probiotics can provide a solution.

The main aims of this study were:

1. To compare the effectiveness of probiotics [multispecies *Bacillus, Lactobacillus casei* (Shirota), and *L. plantarum*] at different concentrations on the survival rates of *Scylla paramamosain* larvae.
2. To characterise, identify, and quantify *Bacillus, Lactobacillus,* and *Vibrios* of *S. paramamosain* larval culture waters added with different concentrations of probiotics (multispecies *Bacillus, L. casei,* and *L. plantarum*).
3. To determine the effects of using probiotics (multispecies *Bacillus, L. casei,* and *L. plantarum*) on water qualities and Larval Stage Indexes (LSI) of *S. paramamosain* larval cultures.

CHAPTER 2

LITERATURE REVIEW

2.1 The Use of Probiotics in Aquaculture

The focus on probiotics for use as environmentally safe options are improving and their use is based on practical experience and research (Cruz et al., 2012). Worldwide demand for probiotic components, supplements, and feeds, reached USD 15900 million in 2008 and was around USD 19600 million by 2013, giving a yearly increase of 4.30 % (Soccol et al., 2010). There are now many commercially probiotics which includes single or multiple species, aimed at enhancing the culture of aquaculture organisms (Cruz et al., 2012). The continual search for aquaculture goods and a concurrently greater need for options other than antibiotics are ever present, and the field of probiotics for aquaculture is now attracting considerable attention. Some researchers have worked out the methods of infections by fatal pathogens, and the results of their studies shows improved survival of hosts challenged with bacterial pathogens, when probiotics were used (Taoka et al., 2006).

Many authors have published on the use of probiotics in aquaculture systems to control water quality, especially ammonia levels. Prabhu et al. (1999) tested probiotics in shrimp culture to determine their ability to regulate water quality. The outcome of their trial showed that water qualities were ideal in treated ponds, in contrast with the control. Douilett (1998) admistered probiotics as supplements which comprised of a mixture of probiotics in solution and particle form, within high density aquaculture tanks. The

mixture enhanced the water quality in crustacean cultures by reducing the concentration of organic materials and ammonia. This procedure was accomplished through a series of enzymatic processes carried out in succession by various strains present in the probiotic mixture.

Certain indigenously isolated *B. subtilis* have been used for culture. Some were isolated from shrimp environments, and these environments were found to have lowered ammonia compared to without probiotic application (Hu & Yang, 2006). There are now studies which indicate that adding *Bacillus* to culture environments can enhance the water quality (Hong et al., 2005).

Attachment of probiotic bacteria to the host's intestinal surfaces has been hypothesized to be associated with establishment of residence, stimulation of the immune system, and inhibitory activities towards enteropathogens (Gopal et al., 2001).

Probiotics are usually supplemented as feed and culture water additives (Avella et al., 2010; Suzer et al., 2008). Using probiotics results in modification of the bacterial communities in tank water, which can improve the cultivation of larval crustaceans and bivalves (Gómez et al., 2008; Guo et al., 2009; Riquelme et al., 2001). When the host or it's environment already carries an established and stable bacterial community, the probiotic will likely have to be supplied on a regular basis to achieve and maintain its artificial dominance (Verschuere et al., 2000). Thus, it is feasible to alter the make up of bacterial flora in culture through the supplementation of probiotics. This is important if a sustained influence is needed for the beneficial effect to occur (Verschuere et al., 2000).

Probiotics has been shown to improve the digestibility of energy resulting in higher growth rates of the cultured species (Avella et al., 2012). Preinoculation with probiotics can prolong the dominance of the desired bacteria in the culture environment (Verschuere

et al., 2000). This could lower the mortality due to the improved capacity of larvae to survive infections from pathogenic bacteria (Talpur et al., 2012).

2.2 Types of Probiotics

Various types of probiotics have been tested for crustacean culture, including Gram negative and positive bacteria, bacteriophage, yeast (*Saccharomyces*), and phytoplankton (Ajitha et al., 2004; Balcázar et al., 2006; Irianto & Austin, 2002). The use of *Bacillus* and *Lactobacillus* has shown tremendous potential, and they are known to synthesize proteases and other enzynes which aid in the digestion of the aquacultured organisms (Ziaei-Nejad et al., 2006). *Pseudomonas*, *Nitrosomonas*, *Nitrobacter*, *Acinetobacter*, and *Cellulomonas*, have been recorded to aid the petrification of liquid containing organics and lowering the build up of organics (Shariff et al., 2001). Many authors have published promising outcomes on the use of probiotics in aquaculture (Merrifield et al., 2010; Swain et al., 2009; Wang et al., 2008).

2.2.1 *Bacillus* as Probiotics

Bacillus is the prominent probiotic used in aquaculture to promote livestock health and growth rates through the optimisation of host's immune and digestive system, and exertion of antibacterial activities against many pathogenic microorganisms (Liu et al., 2009; Liu et al., 2010; Tseng et al., 2009). Members of this genus are capable of producing endospores which are resistant to unfavorable environmental conditions (Claus & Berkeley, 1986). *Bacillus* is known to inhibit pathogens in the culture water, and have

been used to enhance the water quality in aquaculture (Farzanfar, 2006; Irianto & Austin, 2002). *Bacillus* can synthesize polypeptides which are effective against a wide variety of bacteria, thus elucidating their inhibitory effects on pathogenic *Vibrios* (Perez et al., 1993). The inhibitory effects of *Bacillus* are attributed to the synthesis of antibacterial proteins, antibiotics, competition for vital nutrients, and surface area (Hu et al., 2010). Some researchers have recommended using more than three *Bacillus* species in aquaculture feed rather than a single strain (Arığ et al., 2013).

Isolation and description of antibiotic compounds in *Bacillus* will improve our understanding of their functions in the bacteria. Relatively few bacterial inhibiting toxins of *Bacillus* have been characterised, even though they have tremendous benefits in pathogen exclusion. Zokaeifar et al. (2012) isolated colonies of *Bacillus* from fermented cucumbers, acetic acid, *Allium* plants, and *Brassica oleracea capitata* plants. The colonies were analysed for their bacterial inhibiting reactions towards *V. harveyi*, and the two strains produced high levels of bacteriacidal effects towards *V. harveyi* and *V. parahaemolyticus*. Following the passage through the digestive tract, probiotics can attach and proliferate in the gut while being used by carbohydrates, which enhances the host's digestive enzyme activities and larval development (El-Haroun et al., 2006).

There are several commonly used *Bacillus* species for aquaculture purposes. *Bacillus subtilis* is a Gram positive, catalase positive, spore forming (toughness of spores allows for digestive tract colonisation), non pathogenic bacteria, which can inhabit the bottom of sea soils (Moriarty, 1999). It can be differentiated with other *Bacillus* by various biochemical reactions. *Bacillus subtilis* has been known to survive in temperatures between 11 - 52°C, NaCl concentrations of 0.00 – 9.00 %, and pH values of 5.00 – 10.00

(Zokaeifar et al., 2012). It can produce enzymes and subtilisin, with the latter being toxic to certain bacteria.

Bacillus subtilis has been reported to thrive in the gut of crustaceans, and synthesizes a range of antibacterials on agar plates (Katz & Demain, 1977; Korzybski et al., 1978). It is non pathogenic to aquacultured species, is used to improve the survival rates of cultured organims, enhancing water quality, acting as an ingested probiotic treatment, and prophylactic supplement for digestive issues (Mohapatra et al., 2012; Wen-Ying et al., 2010). *Bacillus subtilis* also synthesizes gut proteases and other enzymes which aid in the digestive processes of cultured animals, while acting as a source of micro and macro feed compounds themselves (Jafaryan et al., 2011; Ziaei-Nejad et al., 2006). *Bacillus megaterium* is used for the production of several α and β amylases. Neutral proteases are also produced and secreted by *B. megaterium* (Meinhardt et al., 1994).

Bacillus megaterium can synthesise vitamin B12 aerobically and anaerobically (Raux et al., 1998). It is non pathogenic to aquaculture organisms, is the major aerobic source for vitamin B12, which is synthesized from the haem biosynthetic pathway. Several other enzymes can be secreted by *B. megaterium*, including P-amylase, P-glucanase, and megacins.

2.2.2 *Lactobacillus* as Probiotics

Lactobacillus belongs to the lactic acid bacteria (LAB) group, and is Gram positive, non motile, non sporulating, produces lactic acid, have a wide range of physiology, and are catalase negative (MacFaddin, 2000). They produce antibacterials and bacteriocins which inhibits pathogens, preventing intoxication and infections (Hirano et al., 2003).

Lactobacillus synthesizes protease and other enzymes which enhance the innate digestive system of the aquacultured species (Ziaei-Nejad et al., 2006).

Lactobacillus can contribute to a well balanced bacterial flora in the digestive tract of it's host, minimizing disease occurences. They are sometimes found in sea water and can make up a portion of the bacterial assemblage of certain marine organisms (Hovda et al., 2007). Although it is not dominant in the normal intestinal microbiota of crustaceans, some strains can colonize the gut. Several researchers have noted that it was feasible to maintain artificial *Lactobacillus* colonies in the host's digestive tract through consistant supplementation (Ringø & Gatesoupe, 1998). *Lactobacillus* is not commonly isolated from aquaculture larvae, as water temperature, incubation period, and environmental glucose levels, can affect their presence and densities (Farzanfar, 2006). The last two factors are important for certain *Lactobacillus* species, as their growth is relatively slow and they require certain nutrients, such as sugar, a supply of carbon, nucleotides, fatty acids (FA), amino acids (AA), and vitamins (Ringø & Gatesoupe, 1998).

Feed poly unsaturated fatty acid (PUFA) levels, competition for nutrients, Chromic oxide (Cr_2O_3), salinity, and stress, are factors which can influence *Lactobacillus* colonisation in the gut (Ringø & Gatesoupe, 1998). Feed is the main supply source for proliferation of certain *Lactobacillus* in the gut (Farzanfar, 2006). Detection of *Lactobacillus* colonies in the culture water and larvae following probiotic supplementations, illustrated its ability for retention in aquaculture conditions (Talpur et al., 2012).

2.3 Use of Probiotics in Crab Culture

The use of probiotics to control pathogenic bacteria has been gaining attention in crab culture. Various species have been tested with promising results. Talpur et al. (2012) isolated *Lactobacillus* from the digestive tract of blue swimmer crab (*Portunus pelagicus*) and used the probiotic in the larval culture water to study it's influence on survival rate, water quality, and digestive enzyme activity. The combination of *Lactobacillus* species was inoculated into the culture water once a day at concentrations of 1.00×10^2 cfu/ml, 5.00×10^2 cfu/ml, and 1.00×10^3 cfu/ml of culture water. The use of *Lactobacillus* caused significantly higher survival rates for the larvae compared to the control. The mean survival rates for the probiotic treatments were 10.30 %, 11.20 %, and 11.00 %, and the *Lactobacillus* mix contains *L. plantarum*, *L. salivarius*, and *L. rhamnosus*. The probiotics reduced the pH levels and caused a higher digestive enzyme activity compared to the control (Talpur et al., 2012). Talpur et al. (2012) also analysed enzyme assays in *P. pelagicus* larvae, including protease and amylase. Enzyme activities in the treated groups were higher compared to the controls. This was due to *L. plantarum*'s effectiveness in improving the activity of the digestive enzymes (Talpur et al., 2012).

Probiotics can provide supplemental digestive enzymes, improve larval development, and FER; and reduce gut diseases (Verschuere et al., 2000). Throughout the larval developmental phase, probiotics can grow in the digestive tract of larvae, while utilising carbohydrates, and synthesizing amylase, protease, and lipase (El-Haroun et al., 2006). Talpur et al. (2012) showed that a mixture of *Lactobacillus* species gave additional protection during unfavourable conditions and invasion by pathogenic bacteria in *P. pelagicus* larvae. Improved defences of larvae when several species of probiotics were

used together may be due to each species providing a specific advantage, resulting in a wider range of beneficial effects (Talpur et al., 2012).

Talpur et al. (2012) showed that the highest enzyme activity in *P. pelagicus* was in treatments with mixtures of three *Lactobacillus* species at concentrations of 1.00×10^3 cfu/ml followed by 5.00×10^2 cfu/ml. They fed *P. pelagicus* larvae live feed, and suggested that the zooplankton prey had ingested probiotics from the treated culture water, improving the immunity and digestion of the larvae. Digestion in larval marine crabs is activated during the early growth phase, during which *Lactobacillus* can secrete a wide variety of enzymes (Moriarty, 1998). In a study by Talpur et al. (2012), activities of larval protease and amylase were significantly different in all experimental groups, of which treatments were different to the control. It was likely that probiotics influenced the digestive processes by enhancing good bacterial populations, enzyme activity, intestinal bacterial balance, digestibility, and absorption, resulting in improved survival rates due to the enhanced capability of larvae to cope with pathogens. The enzyme activities in the probiotic treated groups increased significantly over the control (Talpur et al., 2012).

Lactobacillus used in aquaculture is known to synthesize proteases and amylases (Suzer et al., 2008). In *P. pelagicus*, the increase in activities of digestive enzymes in the probiotic treatments was due to reductions of *Vibrio* in the gut of larvae and culture water, providing favourable conditions (Talpur et al., 2012). This contributed to the improved larval survival and enzyme activities in their study (Talpur et al., 2012). The authors also found that supplementation of *Lactobacillus* had an influence on the swimming behaviour of larvae, especially during the initial days post hatching (DPH) (Talpur et al., 2012). *Thalassobacter utilis* (strain PM4) has been used to control *V. harveyi* and improve survival of larval swimming crab, *Portunus trituberculatus* (Nogami et al., 1997).

The culture of *Scylla* still relies mainly on wild crablets. Hatchery techniques for this genus are approaching the commercial phase, but more studies are needed to achieve consistent results. *Scylla* commands good prices, but their survival during the hatchery phase are inconsistent and sometimes low, posing an impediment to the expansion of commercial farming. Larval mortalities are attributed mainly to *V. harveyi*, passed through broodstock's faeces and excreta in the hatching tanks, ending up in the larvae as they ingest the culture water (Talpur et al., 2011). *Vibrio alginolyticus* has been reported as environmental probiotic and to protect against disease in *Scylla* (Liessman, 2005). The strain *V. alginolyticus* LLB2 has been isolated and tested against a control in larval *S. serrata*, both challenged with *V. harveyii* (LLD1, pathogenic strain), with the treatment group giving a higher survival rate (Liessman, 2005). A second pathway for pathogenic bacteria to enter the culture environment of *Scylla* Z is through the plankton feed, as bacteria can enter the digestive tract of larvae orally (Hansen & Olafsen, 1999). Early *Scylla* Z are feeble, have an under developed immune system, and are vulnerable to pathogenic bacteria. The immune system of *Scylla* relies on the phenol oxidase enzymes (Gonzalez-Santoyo & Co´rdoba-Aguilar, 2012).

2.4 Pathogenic *Vibrios* and their Effects on Cultured Crab Larvae

Vibrio are one of the most important pathogens for cultured aquatic crustaceans (Lightner, 1993). Some of the *Vibrios* have been implicated principally with disease outbreaks in crustacean larviculture facilities (Lavilla-Pitogo et al., 1990). Among the *Vibrio, V. parahaemolyticus* has affected juvenile and adult crustaceans (Roque et al., 1998). Parenrengi et al. (1993) isolated several species of *Vibrio*, including *V.*

parahaemolyticus, and studied their pathogenicity to Z of *Scylla*. Results suggested that all species were potentially pathogenic to Z (Parenrengi et al., 1993). Furthermore, the bacterial ecology of *Scylla* larval cultures was also found to be unstable both between and within culture cycles, including changes in sucrose negative and positive *Vibrio* ratios (Mann et al., 1998). Constant high numbers of sucrose negative *Vibrio* (green colonies on TCBS agar) is regarded as a sign that high numbers of mortalities will be seen (Mann et al., 1998). The TCBS medium is an ideal agar to isolate *Vibrio* species, due to the high concentrations of Thiosulfate Citrate, bile and a high alkaline pH. The strains which cannot degradate sucrose are green in colony color, while the strains which ferment sucarose are yellow in colony color as they produce acid from the sugar. This is due to the color change of the pH indicator (Bromthymol Blue and Thymol Blue) which turns yellow under acidic conditions. The TCBS agar is selective for *Vibrios*, and differential due to the presence of sucrose and dyes. Sucrose fermentation produces acid, which converts the colour of bromothymol blue or thymol blue. Two dyes cause the medium to produce an array of yellow, green, or blue, making differentiating among *Vibrio* species possible. Therefore it is important to identify the *Vibrio* species in *Scylla* larval cultures, and to quantify their numbers (colony counts).

Vibrio harveyi and *V. parahaemolyticus*, have been known to be highly infective towards Z of *Scylla* and *P. pelagicus* (Parenrengi et al., 1993; Talpur et al., 2011), both are portunid crabs. In a study by Lavilla-Pitogo et al. (2002), luminescent *V. harveyi* (CLM3) were pathogenic to Z1 stage of *S. serrata*, inducing mortality of 63 – 64 % with an initial dose of 10^2 and 10^3 cfu/ml. In comparison, 40 % mortality was recorded in the control for their study. Although no bacteria was inoculated in the control, a mixed bacterial population of up to 10^4 cfu/ml was enumerated after 24 h. Scanning electron

microscopy (SEM) showed that infected larvae had plagues of bacteria on the mouth and feeding apparatus implying an oral route of entry for the pathogen. The sources of luminescent *V. harveyi* were determined from different hatchery components by Lavilla-Pitogo et al. (1992), with results showing that apart from near shore sea waters used for larval culture, broodstock's mid gut bacterial flora which contained 16 to 17 % luminescent *Vibrio* populations, were significant sources of luminescent *Vibrio*. Broodstock crabs have been observed to release large amounts of fecal material during spawning, thus facilitating bacterial colonization of newly spawned eggs (Lavilla-Pitogo, 1995).

Vibrio harveyi is the main species which causes luminescent Vibriosis, a frequent disorder in *Scylla* larval culture (Candelaria et al., 2010).

2.5 *Scylla* Larval Culture

Plate 2.1 shows a diagram of the different larval stages, and how to differentiate between them (Srinivasagam et al., 2000). Zoea 1 are stocked at a densities of 20 – 400 individuals per liter (Ngoc-Hai, 2011; Quinitio et al., 2001).

Hatcheries for *Scylla* larval culture can be built specifically for crabs or can be incorporated into hatcheries of other species of crustaceans (Ngoc-Hai, 2011). In the early 2000's, some shrimp hatcheries in the tropical and sub tropical regions were lying unused for substantial parts of the year (Srinivasagam et al., 2000). To reduce the high capital for setting up a new *Scylla* hatchery, some of the shrimp hatcheries were used, with appropriate alterations, for larval culture of *Scylla* (Srinivasagam et al., 2000). Culture systems can be in indoor hatcheries, with or without a transparent roof (Jamari, 1991).

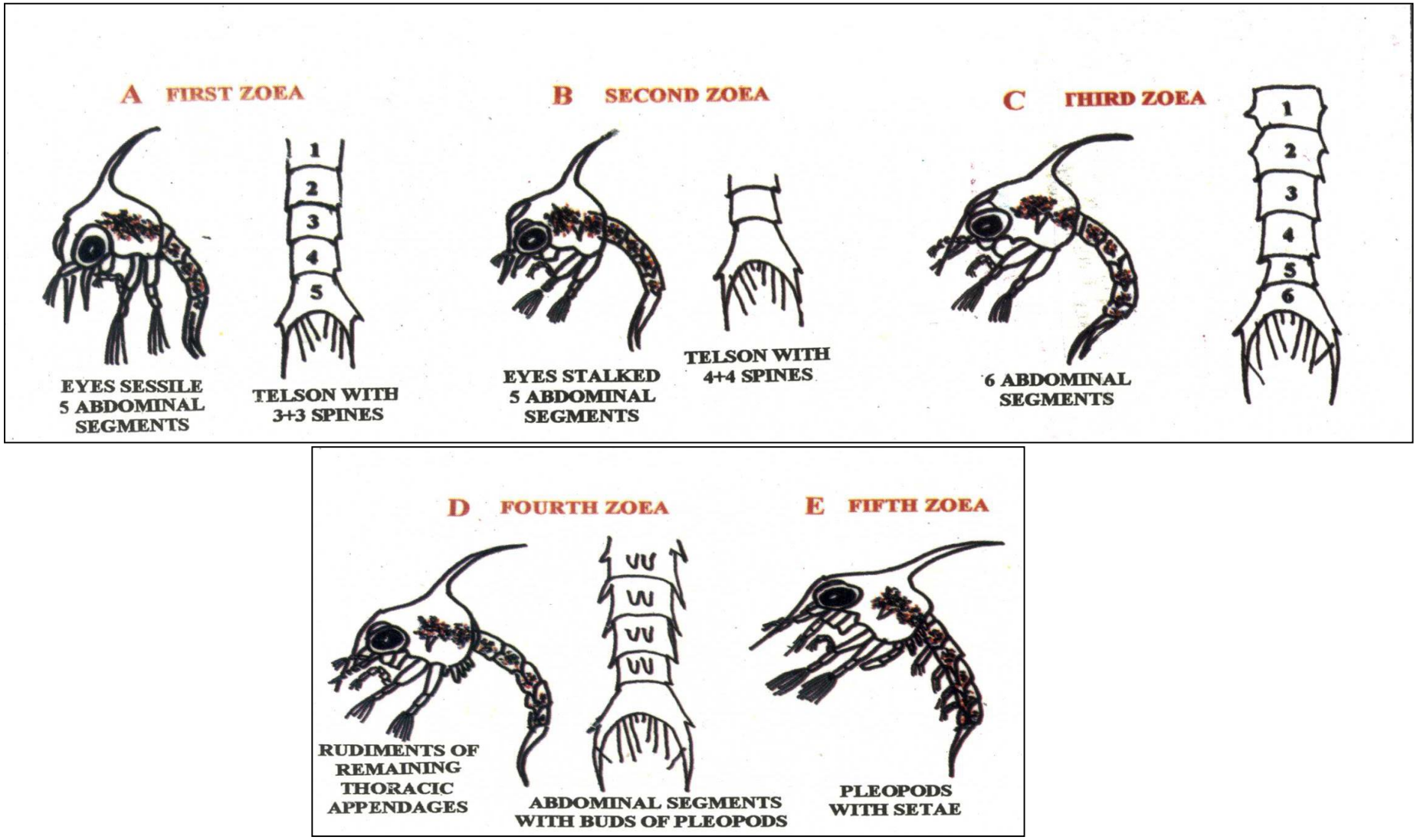


Plate 2.1. Larval stages of *Scylla*, showing their distinguishing features (Srinivasagam et al., 2000).

Tanks for larval culture can be round concrete tanks of 4.00 m diameter × 1.00 m height, and two and 10 t capacity, with conical or sloping bottoms (Jamari, 1991; Quinitio et al., 2001). Large cylindroconical, fiberglass tanks can be used for investigating zootechnical aspects or for nutritional studies (Nghia, 2005). Under pilot and commercial scale situations, larvae are cultured in plastic, fiberglass or reinforced concrete tanks with capacities of 1 to 200 m^3 (Hamasaki et al., 2002; Quinitio et al., 2001).

In Vietnam, composite 0.50 – 1.00 m^3 tanks with cone bases are employed (Ngoc-Hai, 2011). In Japan, a mesocosm system is used to culture larvae in large tanks (10 m^3 and above) (Nghia, 2005). The tanks were filled with clean seawater during the course of the Z2 – Z3 stages, and during the Z4 and M stages water was exchanged on flow through basis (Hamasaki et al., 2002). Filtration of the water included bag, sand, cartridge, and carbon filters (Ngoc-Hai, 2011). The Z are cultured at salinities of 29.00 – 36.00 ppt., water temperatures of 26.00 – 32.00ºC, and photoperiods of 11 - 13 h light to 11 - 13 h dark or 24 h light (Ngoc-Hai, 2011; Quinitio et al., 2002). A stable water temperature is recommended by culturists and researchers (Ngoc-Hai, 2011; Shelly & Lovatelli, 2011). Light intensity during the day is maintained at 5000 lux (Ngoc-Hai, 2011), pH range of 8.00 – 8.70 (Jamari, 1991), whereas optimum total ammonia and nitrite levels are less than 1.00 mg/L and 0.10 mg/L respectively (Ngoc-Hai, 2011). Dissolved oxygen (D. O.) levels can be up to 8.00 mg/L with no detrimental effects on the larvae (Jamari, 1991).

For conditioning the culture water, diatom *Skeletonema costatum* is introduced in the culture tanks at 5,000 – 8,000 cells/ml (Jamari, 1991). Alternatively, *Isochrysis* can be used at similar densities (Jamari, 1991). *Chlorella*, *Nannochloropsis*, *Isochrysis*, and Spirulina (*Arthrospira*), have also been used with positive results (Jamari, 1991; Ting et al., 1980). The culture water is replaced daily at 25 – 30 % starting on day one to three,

and increasing to 30 - 80 % as larvae get larger and when luminescent bacteria are seen in the culture water and on the larvae (Quinitio et al., 2002). The initial Z phase are given *Brachionus rotundiformis* or *Brachionus plicatilis* rotifers at a rate of 10 - 60 ind./ml, based on the concentration of larvae in the water.

Early *Scylla* stages are zooplanktons which uses chance encounters to obtain feed (Heasman & Fielder, 1983). *In vitro* trials have shown that larval survival increases with higher rotifer concentrations (30 – 80/ml; Suprayudi et al., 2002; Zeng & Li, 1999). In a commercial hatchery setting though, practical factors such as maintaining the water quality and rotifer production volumes can necessitate that reduced concentrations be utilized (10 – 20 ind./ml) (Baylon et al., 2001; Quinitio et al., 2001). Phytoplankton such as *Nannochloropsis* and *Chlorella* are supplemented in the culture water (50000 cells/ ml) to enhance the nutritional profile of rotifers (Quinitio et al., 2002). *Artemia* nauplii are provided to Z3 and higher stages (0.50 – 3.00 ind./ml), and densities can be adjusted to account for culture conditions, larval density, and other factors (Quinitio et al., 2002). The concentration of *Artemia* used in the literature are 0.50 – 10.00 ind./ml (Mann et al., 2001; Quinitio et al., 2001), but when Z densities are high (greater than 100 ind./L), *Artemia* concentrations of 20 ind./ml are sometimes used (Nghia et al., 2001). However, *Artemia* concentrations of more than 5 ind./ml are currently unrealistic outside of laboratory situations owing to the high costs (Quinitio et al., 2002). Concentration of *Artemia* is therefore based on practical factors including *Scylla* densities, culture methods, and costs (Nghia, 2005). A variety of feeding regimens are used by hatchery managers for larval cultures (Table 2.1). Larvae can be fed twice to four times daily (Jamari, 1991). One of the major research efforts on *Scylla* larval nutrition is focused on the balance of FA in the diet of *Scylla* larvae. According to Suprayudi et al. (2004), eicosapentaenoic acid (EPA,

20: 5n-3) and docosahexaenoic acid (DHA, 22: 6n-3) content should be adjusted to 0.71 – 0.87 % and 0.49 – 0.72 % respectively to produce high survival rates, shortening the intermoult time, and giving a larger carapace width in the C1 stage. During rotifer feeding, *Scylla* larvae need 1.00 % EPA (Suprayudi et al., 2004). Furthermore, enriching rotifers with DHA can lower the need for n-3 highly unsaturated fatty acids (HUFA) to the level of 0.80 % (0.30 % EPA and 0.10 % DHA). Too much FA in the feed and an imbalance of DHA and EPA has been known to reduce the growth performance of crustaceans (Suprayudi et al., 2004).

Treflan at concentrations of 0.70 – 10.00 mg/L is used to control fungal growth (Jamari, 1991). Antibiotics are used at 2 mg/L Sodium nifurstyrenate (Hamasaki et al., 2002) or as a bath (100 mg/L OTC) for Z1 larvae before being stocked into pilot scale systems (Baylon et al., 2001). Alternatives to antibiotics such as formalin and probiotic preparations have been applied (Nghia, 2005; Ngoc-Hai, 2011). Shelly and Lovatelli (2011) stated that factors essential to successful larval culture of *Scylla* were stable water temperatures, low organic levels in the culture water, low bacterial levels in the culture tanks, excellent hygiene practices, correct salinity, maintaining the Z and M in suspension, and consistent high quality food.

Table 2.1
Feeding schedule used by various large scale culturists of larval *Scylla*, from the Z1 – Z5 stages.

Author	**Z1** (ind./ml)	**Z2** (ind./ml)	**Z3** (ind./ml)	**Z4** (ind./ml)	**Z5** (ind./ml or % BW/day)
Jamari (1991)	5 – 10 R + AF1	5 – 10 R + A + AF1	10 – 15 R + 10 A + AF2	20 – 30 R + 15 A + AF2	15 A + AF2
Ngoc-Hai (2011)	20 R + 5 A	20 R + 5 A	5 – 10 A + AF3	5 – 10 A + AF3	5 – 10 A + AF4
Quinitio (2004)	10 – 15 R	10 – 15 R + 0.5 – 3 A	0.5 – 3 A	0.5 – 3 A	0.5 – 3 A + 20 MM
Quinitio et al. (2001)	10 - 15 R + AF4	10 - 15 R + AF4	1 - 3 A	1 - 3 A	1 - 3 A
Quinitio et al. (2002)	10 - 15 R	10 - 15 R	0.5 - 3 A	0.5 - 3 A	0.5 - 3 A
Ronquillo et al. (1998)	20 – 40 R + 2 – 10 A	20 – 40 R + 2 – 10 A	20 – 40 R + 2 – 10 A	20 – 40 R + 2 – 10 A	20 – 40 R + 2 – 10 A

R: rotifer, A: *Artemia*, AF1: BMC, feed for shrimp larvae imported from Japan (protein 49.00 %, fat 31.00 %, ash 5.50 %, and moisture 4.30 %), at 3.00 – 5.00 g/ml. AF2: SUTIMAL, feed for giant freshwater prawn (protein 55.00 %, fat 8.00 %, ash 7.00 %, and moisture 5.50 %), at 7.00 – 12.00 g/ml. AF3: Frippak, Lansy, 150 – 500 µm, 1.00 – 5.00 g/m^3 daily. AF4: Quinitio et al. (1999). MM: marine meat, brown mussel meat (*Modiolus metcalfei*), or fish.

CHAPTER 3

MATERIALS AND METHODS

3.1 Flow Chart

Figure 3.1 shows a flow chart for the overall framework for this study to investigate the effects of *Bacillus* and *Lactobacillus* as probiotics on *Scylla paramamosain* larval culture performance.

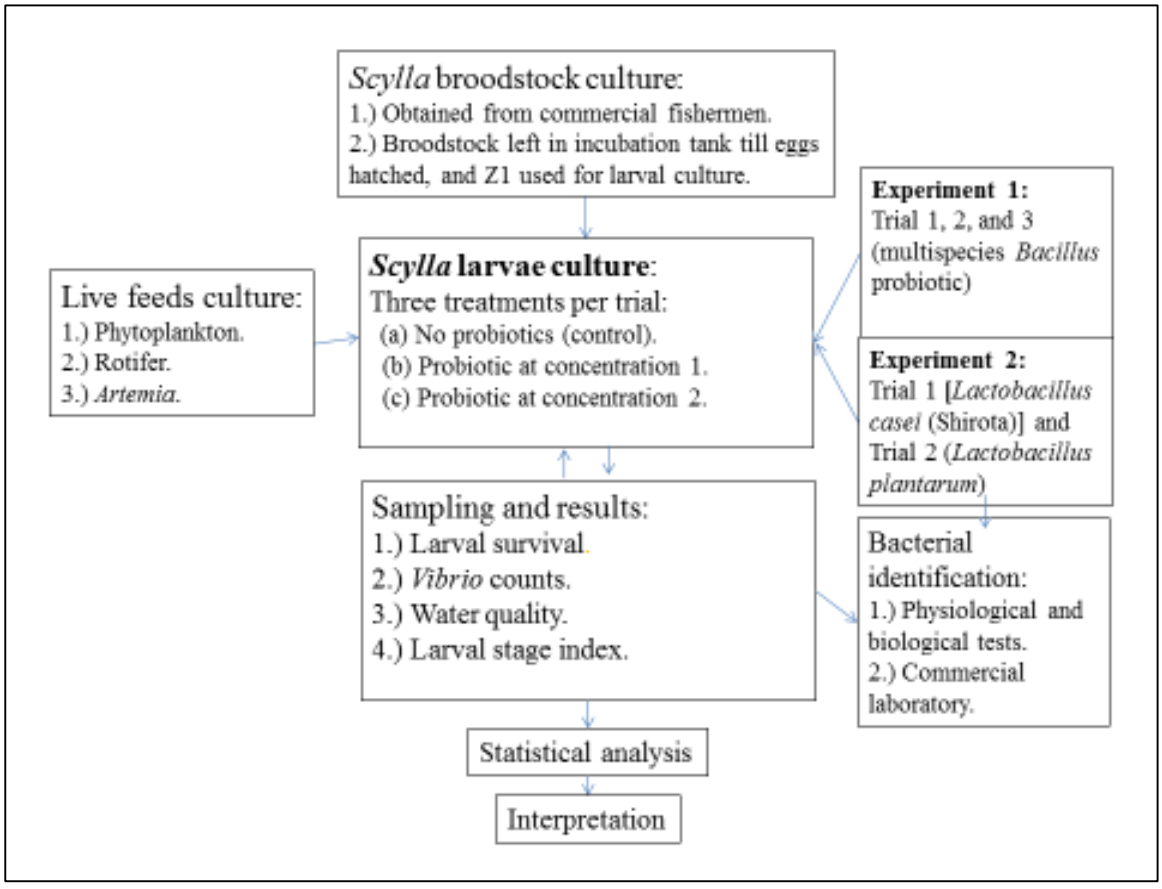
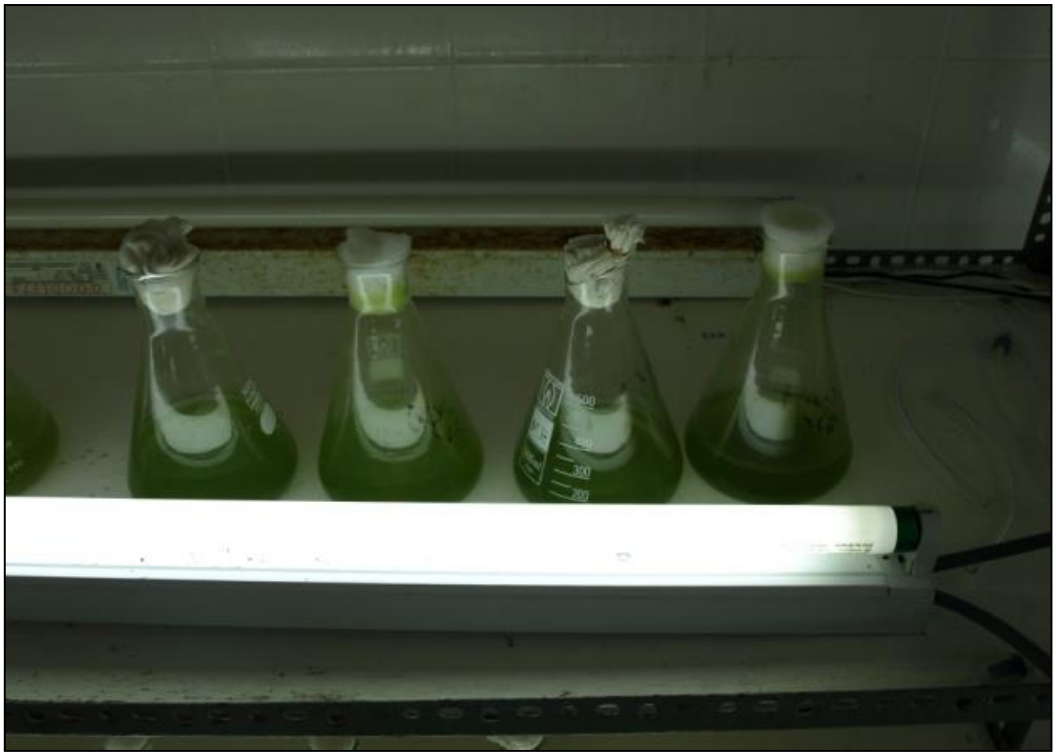


Figure 3.1. Framework for this study.

3.2 Live Feed Culture

Live feed culture was done as part of the project for larval culture of *Scylla*. Phytoplankton was produced at the Centre for Marine and Coastal Studies (CEMACS), Universiti Sains Malaysia's (USM) Phytoplankton Laboratory and Hatchery to support rotifer and *Scylla* larval culture. Phytoplankton (*Nannochloropsis oculata*) were cultured in carbuoys (20 L each) in doors at the phyplankton culture area (CEMACS, USM) by providing artificial fluorescent lights. Each carbouy was inoculated with 20 L of bag filtered and chlorinated sea water (around 30 ppt.; treated), 2.00 L of pure *Nannochloropsis* culture (6.38×10^7 cells/ml), and 1.00 L of Conway media on the first day of culture, following the method of Quinitio and Parado-Estepa (2008). Rotifer (*Brachionus rotundiformus*) culture at CEMACS was performed according to the batch culture method of Lavens and Sorgeloos (1996). *Artemia franciscana* (Kellogg 1906) nauplii (Golden Dolphin® Premium Plus Red, vacuum packed cysts) were hatched as described by the manufacturer and Van Stappen (1996).



(A)

(B)

Plate 3.1. (A) Pure phytoplankton (*Nannochloropsis oculata*) cultures and (B) harvested paste in bottles, held in the Plankton Laboratory (CEMACS).